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10th Central European Congress on Food

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Preface

The collection of papers from the 10th Central European Congress on Food (CEFood) contains selected best-quality papers presented at this Congress. The Congress took place in the period June 10–11, 2021, in online format, due to the COVID-19 pandemic, in Sarajevo, Bosnia, and Herzegovina, organized by the Faculty of Agriculture and Food, University of Sarajevo.

The first CEFood Congress was held in 2002 in Ljubljana, Slovenia, with the idea to bring together representatives of universities and research institutions but also food producers and distributors in order to promote research, development, innovation, and education in food science and technology. Since then, the organizers of CEFood Congresses were Budapest—Hungary (2004), Sofia—Bulgaria (2006), Cavtat—Croatia (2008), Bratislava—Slovakia (2010), Novi Sad—Serbia (2012), Ohrid—North Macedonia (2014), Kyiv—Ukraine (2016), and Sibiu—Romania (2018). CEFood Congress was held under the sponsorship of The European Federation of Food Science and Technology (EFFoST).

At the 10th CEFood Congress, the total number of presented papers was above 100 with over 200 participants coming from 16 countries: Bosnia and Herzegovina, Serbia, Croatia, North Macedonia, Albania, Kosovo, Monte Negro, Slovenia, Germany, Austria, Poland, Spain, Portugal, Turkey, Romania, and Slovakia. The program was organized in the following sections: Food Analysis, COVID-19 Challenges, Food Energy Systems, Food Trends and Competitiveness, Food and Feed Chain Management and Modern Challenges.

For publishing in the Book of Proceedings, only the highest quality of paper with positive reviews and approved by the Congress Scientific Committee were accepted regardless of which section they belonged to. However, papers from every section could be found in this Book of Proceedings. It is expected that results presented at 10th CEFood Congress and published in this Book of Proceedings will highlight some of new approaches and achievements in food science.

Editors

Contents

Determination of Quality Parameters of Dehydrated Carbohydrate Based Baby Food

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Abstract. Commercially produced baby food has to satisfy all nutritional needs and safety requirements. Also, the physical properties of such powdered products are also very important due to their possibility of preparation before consuming and manipulation in transport and storage.

The aim of this paper is to investigate different quality aspects of powdered carbohydrate based baby food. Five samples of commercial cereal based baby food for infants aged from 4 to 12 months were collected from a Sarajevo market and analysed for different parameters: 1) chemical (moisture, water activity, fat, ash, chlorides); 2) nutritional (vitamin C and energy value); and 3) physical properties (bulk density, swelling power, water solubility index, density of prepared sample, dynamic and kinematic viscosity).

Result showed that differences in analysed parameters occurred as result of differences in sample composition and infant age. All samples had moisture under required limit of 5%. Vitamin C content varied between 20.76 mg% and 74.84 mg%. Baby food sample for the age of over 12 months had significantly ($p \leq 0.05$) lower values for fat, ash, chlorides and vitamin C and significantly ($p \leq 0{,}05$) higher viscosity. Highest viscosity (72.8 mPas) was noted in the sample with honey which is recommended for older infants and young children over 12 months, and the lowest in sample based on rice flour (30.5 mPas) which is recommended for younger infants over 4 months. The best sensorial properties had sample with cookie powder and the worst in sample with fruit powder.

Keywords: Baby food · Infants · Physico-chemical properties · Sensorial properties

1 Introduction

The highest intensity of infant growth and development is in the first months after birth. After six months infant weight should be doubled, and after 12 months should be triple in comparison to weight on birth. After six months breast milk is not enough to satisfy all nutritional needs for normal growth and development. Carbohydrate or cereal based baby food is recommended to supply infant nutrition from 4 months [1], because it satisfies and supplies additional energy value needed for growth, and also this kind of food helps

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infant nutrient absorption and transition to normal adult nutrition. Commercial cereal based baby food should satisfy all requirements of Codex Alimentarius [2]. According to Codex standards [2–4] cereal based baby food can be prepared from one or more milled cereal products, such as wheat, rice, barley, oats, rye, maize, millet, sorghum and buckwheat. They may also contain legumes (pulses), starchy roots or starchy stems or seed oils in smaller proportions. The main carbohydrate sources in this kind of food are maltose, sucrose, dextrose and modified starch. Fat content in cereal based baby food should not exceed limits of 4.5 g/100 kcal (baby food that already contains milk powder) and 3.3 g/100 kcal (without milk powder). Products containing honey or maple syrup should be processed by techniques which would destroy spores of *Clostridium botulinum*, if present. All ingredients are required to be clean, safe, suitable and of good quality. Processing and drying should be carried out in a manner that minimizes loss of nutritive value [4].

In addition to nutritional value, safety properties and physical properties (as consistency, swelling power, solubility and wettability) are very important, because this kind of product must have good instant properties, easy preparation and reconstruction. When prepared according to the directions for use, processed cereal-based foods should have a texture appropriate for the spoon feeding of infants or young children of the age for which the product is intended [4].

The aim of this study was to investigate physical, chemical and sensorial quality parameters of commercial cereal based baby food, and to compare baby food samples recommended to different infant age.

Table 1. List of the samples.

Code	Commercial name	Description	Infant age
BF1	Rižolino	Powdered porridge based on rice semolina and powdered banana	≥4 months
BF2	Keksolino	Powdered and flaked porridge based on milled wheat flour cookie with addition of butter, wheat semolina and whole milk powder	≥6 months
BF3	Frutolino	Powdered porridge based on wheat semolina and dried fruit (apple, peach, pear, apricot) with added whole milk powder	≥6 months
BF4	Čokolino	Wheat based flaked porridge with cocoa and chocolate powder without milk	≥8 months
BF5	Medolino	Wheat based flaked porridge with honey and without milk	≥12 months

2 Material and Methods

Analyses were performed on five samples of commercial powdered baby food produced by Podravka (Koprivnica, Croatia) obtained from a local market in Sarajevo. All samples

were cereal based with presence of other ingredients (fruit, honey or chocolate powder) and were aimed for infants aged 6–12 months (Table 1, Fig. 1).

Fig. 1. Baby food samples

2.1 Chemical Properties

The following methods were used for analysing chemical properties:

1. moisture by drying at 105 °C in a drying oven until a constant weight has been reached [5];
2. water activity (aw) using aw meter (Lab Swift aw meter Nowasina, Switzerland);
3. pH value measured with a pH meter (Mettler Toledo);
4. ash content by burning of samples in a muffle furnace at 550 °C for 8 h [6];
5. fat content by Soxhlet extraction using diethyl ether [7]
6. chloride content by Mohr titration [7, 8]
7. vitamin C by iodine titration considering that 1 ml of 0.001 M KJO_3 corresponds to 0.088 mg of vitamin C [7, 9].

Energy value (kCal/100 g) of the baby food samples was calculated using the following equation [1]:

$$Energy\ value = (Protein + carbohydrates) \times 4.1 + Fat \times 9.3 = \left[kcal/100\,\mathrm{g}\right] \quad (1)$$

The sum of total proteins and carbohydrates was calculated by subtracting the sum of the values of moisture, ash, and fat from 100 (per 100 g) [10]:

$$Sum\ of\ proteins\ and\ carbohydrates\ (\%) = 100 - (\%moisture + \%fat + \%ash) \quad (2)$$

2.2 Physical Properties

Bulk density was calculated as ratio of weight and volume of powder. 50 g of the sample was put into a 100 ml graduated measuring cylinder. The volume of sample was recorded and bulk density was calculated as ratio of sample weight and volume [11].

For determination of viscosity and density of prepared samples, samples were prepared according to instruction from packaging. 30 g of baby food powder was mixed with 200 ml of water. Density of prepared sample was determined at 25 °C by measuring the weight of prepared sample in a measuring cylinder and liquid density was calculated as ratio of weight and volume.

Dynamic viscosity was measured using a rotational rheometer (Myr, VR 3000) at 30 °C and rotation speed of 60 and 100 rev/min. Kinematic viscosity was calculated as the ratio of dynamic viscosity and density of reconstructed samples (liquid density). Kinematic viscosity was calculated as a ratio of dynamic viscosity and density.

Swelling power (SP) was determined using the method described by Imtiaz et al. [11]. 1 g of sample was mixed with 10 ml of distilled water, heated at 80 °C for 30 min, and centrifuged at 100x g for 15 min. The swelling power was calculated as ratio of paste weight and volume of dried powder.

For water solubility index (WSI) 2.5 g of sample was mixed with 25 ml of distilled water, stirred for 30 min, rinsed in a centrifuge tube with addition of 32 ml of water and centrifuged at 3000x g for 10 min. After decanting, the supernatant was dried to a constant weight, and dried matter was determined. The WSI was calculated using the following formula [11]:

$$WSI(\%) = (mass\ of\ solid\ in\ supernatant / mass\ of\ initial\ powder)100 \qquad (3)$$

Wettability was determined using the method described by Fernades et al. [12] with some modifications. Amount of 0.5 g of baby food powder was put over the calm surface of 200 ml of distilled water at 20 °C without agitation. A stopwatch was turned on and the time was recorded when all powder particles had been wetted.

2.3 Sensorial Evaluation

Sensorial evaluation was done using hedonic scale according to a method described by different literature sources [13–16] with some modifications. The panellists (n = 26) were semi-trained students of the Faculty of Agriculture and Food Sciences University of Sarajevo, who attended the course Technology of baby food. A five-point hedonic scale was used with following points and descriptions: 1 = extremely dislike (extremely bad), 2 = moderately dislike (bad), 3 = neither like nor dislike (may be good or bad), 4 = moderately like (good) and 5 = extremely like (excellent).

2.4 Statistical Analysis

All analyses were done in triplicate and results are shown as mean value with standard deviation. Statistical analysis was performed using one-way ANOVA and post-hoc Tukey test ($p \leq 0.05$) using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

3 Results and Discussion

3.1 Chemical Properties

The results of main chemical properties are given in Table 2.

Table 2. Chemical properties of baby food samples (mean ± SD)

	BF1	BF2	BF3	BF4	BF5
pH	6.31 ± 0.02^{ab}	$5.60 \pm 0{,}01^{c}$	$6{,}25 \pm 0{,}02^{b}$	$6{,}45 \pm 0{,}05^{a}$	$5{,}34 \pm 0{,}00^{d}$
aw	0.252 ± 0.00^{a}	$0.246 \pm 0{,}00^{b}$	$0{,}202 \pm 0{,}07^{c}$	$0{,}230 \pm 0{,}00^{d}$	$0{,}226 \pm 0{,}00^{e}$
Moisture (%)	3.91 ± 0.04^{a}	$3.55 \pm 0{,}10^{b}$	4.17 ± 0.03^{a}	3.19 ± 0.04^{c}	$3.31 \pm 0.01b^{c}$
Fat (%)	2.97 ± 0.03^{b}	3.23 ± 0.00^{a}	1.33 ± 0.01^{d}	2.35 ± 0.13^{c}	0.42 ± 0.01^{e}
Energy (kCal/100g)	401.76 ± 0.18	402.70 ± 1.37	384.33 ± 0.01	403.29 ± 0.65	393.16 ± 0.13
Ash (%)	1.87 ± 0.02^{bc}	2.33 ± 0.24^{b}	3.78 ± 0.04^{a}	1.42 ± 0.05^{c}	1.33 ± 0.03^{d}
Chlorides (mg/100g)	257.37 ± 8.88^{b}	405.23 ± 3.02^{a}	416.06 ± 7.82^{a}	97.41 ± 3.04^{c}	59.77 ± 8.82^{c}
Vitamin C (mg/100g)	23.73 ± 0.40^{d}	20.76 ± 0.41^{d}	74.84 ± 0.42^{a}	49.83 ± 0.20^{b}	42.40 ± 1.41^{c}

*Values with different letters in the same row differ significantly ($p \leq 0.05$)

pH value varied between 5.34 (BF5 – sample with honey powder recommended to ages ≥ 12 months) and 6.45 (BF4 – sample with cocoa powder). ANOVA showed significant influence ($p \leq 0.05$) of sample composition on pH value. These results are in agreement with literature data [11]. Many literature data like FAO [17], Imtiaz et al. [11] and Amankwah et al. [13] reported that pH value in cereal based baby food ranged between pH 5.3 and 6.06, which is very similar to our results.

Low water activity (0.202–0.252) in baby food samples could indicate good microbiological stability (aw < 0.6) and inhibition of enzymatic activities (aw < 0.75) [18]. Results obtained in our study were very close to reported data [19, 20] for aw in infant formula and also in other similar powdered products, like wheat and corn breakfast flakes [21]. Sample type had significant ($p \leq 0.05$) influence on water activity value and all differences were significant.

Moisture content varied in the range 3.19–4.17% and depended on the sample type. The highest was in sample BF3 with fruit powder and the lowest in sample with chocolate powder BF4. Moisture content between samples differed significantly ($p \leq 0.05$). All samples had moisture content bellow value of 5%, which is in agreement with FAO/WHO requirements [17]. According to literature data [11, 22–27], the moisture content in different powdered baby food can vary in a wide range between 2.43–7.33%, but mostly for cereal based baby food moisture ranged between 2.43–5.04%.

The highest fat content (3.23%) was in cookie based baby food sample (BF2) which is aimed towards infants ages ≥ 6 months and the lowest (0.42%) in sample with honey recommended for the ages ≥ 12 months (BF5). All differences were significant ($p \leq 0.05$). These results are in agreement with the infant nutrition recommendation prescribed by Codex Alimentarius [2, 28] and other literary data for baby food. In dependence of sample type, fat content in dried cereal based baby food and infant formula could vary in a wide range 0.95 – 13.7% [11, 22, 26, 29], but in the most cases fat content was in the range

1.23–2.47% [8, 26]. According to Commission Directive [4] and Codex standard [3] the maximal amount of fat in cereal based baby food should be 3.30 g/100 kcal. From Table 2, it can be seen that all samples had fat values lower than 3.30 g/100 kcal. Amounts of fat calculated and expressed in g per kCal of baby food powders for particular samples were 0.74 g/100 kcal (BF1), 0.80 g/100 kcal (BF2), 0.35 g/100 kcal (BF3), 0.58 g/100 kcal (BF4) and 0.11 g/100 kcal (BF5).

Energy value ranged from 384.33 to 403.29 kcal/100 g. Differences were not significant ($p \leq 0.05$). The highest energy value was in sample with chocolate powder (BF5), and the lowest in the sample with fruit powder (BF3). Results for energy value were in agreement with literary data. Different authors [1, 11, 13, 16, 30, 31] reported that energy value in similar baby food varied from 376.6 to 450 kcal/100 g. The most similar values were reported by Imtiaz et al. [11] which ranged 376.6–377.82 kcal/100 g, Amankwah et al. [13] which ranged 395–404 kcal/100 g and El Gindy [30] which ranged 398.11–405.88 kcal/100 g. According to Codex Standard [3] energy value of cereal baby food should not be less 80 kcal/100 g. All samples had energy values in required limits [3].

The highest ash content was in sample with fruit powder (BF3), and the lowest in sample with honey (BF5). Results for ash were in agreement to data reported in literature [11, 24, 26, 29, 32], ash content in cereal based baby food mostly ranged between 2.4 and 4.7%. All samples had total ash content in recommended limit $\leq 5\%$ [24].

Content of chlorides was 59.77–416.06 mg/100 g, which is in agreement with recommendations and requirements. Average content of chlorides in human breast milk is 400 mg/kg [33] and in infant formula 485 mg/kg [34]. The recommended intake of chlorides is about 300 mg/kg for infants 7–11 months old [33]. Mariam [35] reported that prepared baby food meals contained chlorides in range 4.17–234 mg/100 g. After calculation of chloride content in prepared meal, analysed samples had 7.79–54.34 mg in 100 g of ready meal. In comparison to literature [35], the differences could be explained by different ingredients in these two studies.

The highest content of vitamin C was in BF3 (sample with fruit powder) and the lowest in BF5 (sample with cookie powder). All differences in vitamin C content between baby food samples were significant ($p \leq 0.05$). Recommended total daily intake of vitamin C for infants is 40 mg. Considering our results (presented as mg/100 of sample powder), and after calculations per powder amount need for one meal of baby food (30 g of powder + water), it could be seen that amount of vitamin C in one meal varied between 6.23–22.45 mg. According to literature [36] recommended daily requirements of vitamin C for infants are 40 mg for 0–6 and 50 mg for 7–12 month old infants. Obtained values of vitamin C in prepared meals can satisfy about 15.58–56.13% of daily needs. Amounts of satisfaction of infant daily needs for vitamin C by consuming of meals prepared from baby food samples BF1- BF5 were 17.80%, 15.58%, 56.13%, 29.90 and 25.44% respectively. These results are in agreement with literary data [14, 27, 29] where reported vitamin C content was in the range 18.16–74.66 mg/100g, depending on sample composition. According to Commission directive [4], the amount of vitamin C in cereal based baby food should not exceed 12.5 mg/100 kcal and 25 mg/100 kcal for iron fortified products.

3.2 Physical Properties

Results of physical properties are given in Table 3.

Bulk density ranged between 312.50 and 635.00 kg/m^3. Sample with fruit powder (BF3) has the highest, and sample with chocolate (BF5) the lowest bulk density. Sample type had significant ($p \leq 0.05$) influence on bulk density. These results are in agreement with literary data for similar powdered samples, where bulk density ranged between 310 and 700 kg/m^3 [1, 11, 37–40]. Bulk density is an important parameter for packing, transport and reconstruction of powdered samples. Also, certain data suggest that baby food with lower bulk density has better digestibility [40].

Table 3. Physical properties of baby food samples (Mean ± SD)

	BF1	BF2	BF3	BF4	BF5
BD (kg/m^3)	312.50 ± 0.10^{d}	434.70 ± 0.10^{b}	635.00 ± 16.46^{a}	263.00 ± 1.00^{e}	357.10 ± 0.10^{c}
LD (kg/m^3)	1076.80 ± 32.42	1046.24 ± 47.68	1037.40 ± 26.67	1022.22 ± 29.40	1031.02 ± 26.89
DV (mPas)	30.50 ± 0.50^{c}	42.20 ± 1.40^{b}	46.48 ± 2.00^{b}	40.55 ± 0.85^{b}	72.80 ± 2.20^{a}
KV (cm^2/s)	0.28 ± 0.01^{d}	0.40 ± 0.02^{c}	0.45 ± 0.01^{b}	0.40 ± 0.01^{c}	0.71 ± 0.03^{a}
SP (g/ml)	6.54 ± 0.09^{a}	5.31 ± 2.00^{abc}	4.47 ± 0.42^{c}	5.77 ± 0.06^{ab}	6.38 ± 0.29^{a}
WSI (%)	17.76 ± 0.17^{b}	15.60 ± 0.55^{c}	4.50 ± 0.19^{d}	24.06 ± 0.15^{a}	5.23 ± 0.06^{d}
Wettability (s)	264.76 ± 88.32^{a}	178.34 ± 63.80^{ab}	166.90 ± 41.12^{ab}	83.23 ± 12.42^{bc}	50.41 ± 6,27^{c}

*BD – bulk density, LD – liquid density of reconstructed sample, DV – dynamic viscosity, KV-kinematic viscosity, SP – swelling power, WSI – water solubility index

**Values with different letters in same row differ significantly ($p \leq 0.05$)

Density of prepared samples varied from 1022.22 kg/m^3 in BF4 (sample with chocolate) to 1076.80 kg/m^3 in BF1 (sample based on rice semolina). Differences between samples were not significant ($p \leq 0.05$). The similar results were reported by literature Alvarez et al. [41], where the highest density was in rice based baby food (1080 kg/m^3) and the lowest in sample with addition of cocoa.

The ($p \leq 0.05$) highest viscosity was noticed in sample produced with honey (BF5) which is recommended for ages over 12 months. The lowest viscosity (thinnest consistency) in sample BF1 (based on rice semolina) that is recommended for infants in ages ≥4 months. These results were expected, because it is well-known that younger babies in the first months of their lives need more liquid and thin food. Parvin et al. [1] reported that wheat based baby food with powdered mango had viscosity of 34.5 mPas, while Usman et al. [42] reported that sorghum and soy bean based baby food had viscosity values 11.90–33.61 mPas at 40 °C. Slightly lower values reported in literature in comparison to our results could be explained by higher measurement temperature (40 °C vs. 30 °C), and it is well-known that viscosity of liquid increases when temperature decreases.

Values for swelling power ranged from 4.47 g/ml in BF3 and 6.54 g/ml in BF1 (Table 3). Sample with fruit powder (BF3) had the lowest swelling power and solubility. According to available literary data, the swelling power of cereal based baby food can vary between 3.4 and 10.9 g/ml, depending on sample composition and measuring temperature [30, 38, 43–45]. Baby food samples in our study had swelling power similar to

values between 3.4 and 7.43 g/ml reported by El Gindy [30], Ikpeme et al. [38] and Asare et al. [43]. James et al. [45] found swelling power between 6.52 and 10.92 g/ml in millet based baby food at 60 °C. As increase of temperature influences the increase of swelling power, it is expected that our results obtained at 20 °C had lower values. Another explanation could be high amylose content. According to literature, high amylose content is in negative correlation with swelling whereas starches with lower amylose content have better swelling properties. Generally, in comparison to rice, wheat has a higher amylose content and lower swelling power [46, 47]. As the main swelling ingredient is starch, it is expected that dried fruit should have lower swelling power. This could be the reason why baby food with fruit has significantly ($p \leq 0.05$) lower swelling in comparison to other samples.

Water solubility index (WSI) of all samples differed significantly ($p \leq 0.05$), and highest solubility was in sample with chocolate powder (BF4) and the lowest in sample with fruit powder (BF3). Differences between WSI were probably the consequence of drying technology, and could rather be explained by differences in physical structure and particle size and shapes, then the differences in sample composition. Samples with flake-shaped particles (BF4 and BF2) had the better solubility in comparison to powder-like samples (BF3 and BF5) (Fig. 1). These results are in agreement with literature [16, 30, 43, 48] for WSI in baby food (2.90–28.40%). Mostly, the results of analysed samples are similar to results reported by Borby et al. [48], Asare et al. [43] and Temesgen et al. [16]. Borbi et al. [48] reported that water solubility for rice and banana based baby food powder had value of 17%, which is very close for BF1.

Wettability varied in range 50.4–264.8 s. There is lack of available literature data related to wettability of cereal baby food. In comparison to analysed samples (B1-B5), Anosike et al. [49] and Msheliza et al. [50] reported much lower values (10.50–46.5 s) for wettability, which could be explained by differences in determination method and some modification in used methodology (like sample weight, ratio powder: water, water temperature).

3.3 Sensorial Evaluation

The results of sensorial evaluation are given in Fig. 2. All samples were assessed as acceptable with scores for overall acceptability near or over 4 points. Significant differences ($p \leq 0.05$) occurred only in consistency, taste and overall acceptability. BF3 sample (rice based powder) has significantly the lowest acceptability in comparison to other samples. This sample had significantly the lowest scores for consistency and taste. The bad consistency of BF3 probably could be explained by the lowest values of swelling power and solubility index (Table 3) and the thinnest consistency in comparison to other samples. This sample is recommended for young infants aged over 4 months, and because of that it had the thinnest consistency. The thinner consistency could be a reason for the lowest scores for consistency (2.63 ± 1.50) and overall acceptability (3.27 ± 1.23). Although this sample had the lowest scores in comparison to other samples, it was not rated as unacceptable or bad. The overall acceptability for BF3 was rated with scores 3.27 ± 1.30, what could be described as “neither like nor dislike” or “may be good or may be bad”. Also very high standard deviation indicate that panellists did not have uniform opinion about sensorial acceptability of this sample. From Fig. 2 it can

be seen that BF3 sample had the highest value of standard deviation for all sensorial properties in comparison to other samples.

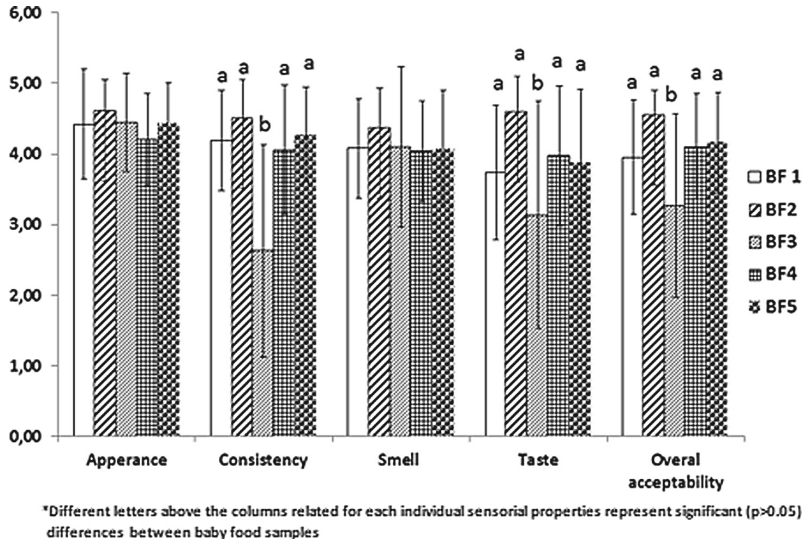

Fig. 2. Results of sensorial evaluation (n = 26; mean ± SD)

The highest scores for all sensorial properties were noticed in BF2 sample (porridge with cookie powder), but significant differences occurred only between BF2 and BF3. In comparison to other samples of baby food, there were not significant differences in comparison to BF1, BF4 and BF5. BF2 sample was rated with 4.55 ± 0.35 for overall acceptability, 4.59 ± 0.46 for the taste, 4.38 ± 0.55 for the smell, 4.51 ± 0.55 for the consistency and 4.62 ± 0.44 for appearance. Although BF2 had the highest scores for taste, consistency and overall acceptability, it differed significantly only in comparison to BF3. High scores for consistency could be explained with good solubility index and good swelling power (Table 3).

There were no sample which was rated as excellent with scores near to maximum scores (5.00), and also there was not a sample which was rated as unacceptable or extremely bad. Results for sensorial evaluation are in accordance to literature [13], while other authors reported lower scores for experimentally prepared baby food based on sorghum [15], sweet potato and soy bean [51]. Generally, in accordance with many other studies [15, 16, 31, 51], commercial baby food was better rated for all sensorial properties in comparison to homemade or experimentally produced. From Fig. 2 it can be seen that taste and consistency had the highest effect on overall acceptability. This observation is in agreement with Kiin-Kabari et al. [52].

4 Conclusion

Baby food samples had values of physico-chemical properties in the ranges characteristic for this type of product. This study has shown that differences in analysed parameters

occurred as a result of differences in sample composition and recommendation for infant age. All values for chemical composition were in allowed ranges required by Codex Alimentarius [2]. Low water activity values (0.202–0.252) indicated good microbiological stability and ability to be stored at room temperature. The highest fat content was in baby food with cookie powder recommended for infants over 6 months. Sample with fruit powder (BF3) had significantly highest amount of total ash and vitamin C. Baby food for infants over 12 months had significantly ($p \leq 0.05$) lower values for fat, ash, chlorides and vitamin C and significantly ($p \leq 0{,}05$) highest viscosity. All samples had total ash content in recommended amount $\leq 5\%$. The highest viscosity and the thickest consistency were noticed in sample with honey which is recommended for infants over 12 months. Significantly lowest viscosity was in sample based on rice flour for infant ≥ 4 months. The lowest swelling power, solubility and sensorial evaluation scores were noticed in sample with fruit powder. Samples with higher moisture content had higher aw, vitamin C, bulk density and wettability, while samples with higher fat content had lower content of vitamin C and wettability. Sample with highest viscosity (BF 5 with honey powder) had high value of swelling power, low value of water solubility index and the lowest wettability time. On the other hand, sample BF1 had the lowest viscosity and bulk density, highest liquid density, high value of water solubility index and the highest wettability. All analysed samples had good sensorial acceptability. Sample with cookie powder had the best sensorial evaluation for all sensorial properties, while the lowest scores were in sample with fruit powder. Significantly lowest ($p \leq 0.05$) overall acceptability was in sample with fruit powder, which had significantly lowest scores for taste and consistency in comparison to other samples, and also the lowest solubility index and swelling power. It could be considered that low solubility and low swelling power had effect on bed sensorial properties. All results were in agreement with literary data.

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Physical Properties of Vegetable Food Seasoning Powders

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Abstract. The aim of this study is to investigate different physico-chemical properties of commercial vegetable based food seasoning powders obtained from local producers and to assess the cohesive and flowability properties of food seasoning samples.

The samples were analysed for composition (content of dried vegetables, moisture, water activity, ash and NaCl content) and physical properties (electrical conductivity, wettability, bulk density, tapped density, dispersibility, granulation and angle of repose). Moisture, NaCl content and dried vegetable content were in agreement with national regulations, and varied in following ranges: 1.29–2.69% (moisture); 41.89–59.43% (NaCl) and 11.50–21.50% (dried vegetables). Aw values were 0.265–0.365 that indicated good microbiological stability and ability to be stored at room temperature.

All samples had the largest content of small particles with diameter < 0.5 mm (68.11–84.04%). Physical properties varied in the range 0.90–1.07 g/ml (bulk density), 1.15 and 1.23 g/ml (tapped density), 78–85% (dispersibility). Values of Carr index showed that cohesiveness of seasoning powder was very low to intermediate, while values of Hausner ratio showed that flowability was passable to excellent. Samples with higher amount of smaller particles and moisture had higher angle of repose and higher cohesiveness, while samples with higher amount of large and very large particles and lower moisture content had lower angle of repose and better flowability.

Keywords: Seasoning powder · Flowability · Cohesiveness · Physical properties

1 Introduction

Seasoning powders can be defined as mixtures of different compounds whose main role is to enhance taste and flavor of ready to eat food to which they are added. According to European Spice Association ESA [1], seasoning is a blend of permitted food ingredients added as necessary to achieve the purpose for which it is designed (e.g. to improve the taste, eating quality and/or functionality of a food). Seasoning typically contains one or more herbs or spices and other flavor enhancing ingredients. Besides flavor and

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taste enhancing purpose, there is the seasoning category with functional properties, for example thickening, emulsifying, preserving, tenderizing and coloring [1].

Spice blends and food seasoning powder make for a better taste of food and meals. Unlike fresh spices which are added to a dish at the end of cooking, generally commercial powdered seasonings are added before or during cooking. Rarely, the seasonings may also be added to finished dishes before serving, like garnishes for salads, soups, cooked rice, and noodles. Some specific and traditional commercial spice blends allow consumers to create meals that are authentic and tasty with shorter preparation time. Commercial spice blends or seasonings consist of spices, vegetables and other ingredients, including flavorings, salt, sugar, dextrose, corn syrup solids, starches, maltodextrin, yeast extracts or hydrolysates, hydrolysed proteins, monosodium glutamate, or nucleotides. These ingredients are added with spices to enhance or intensify the overall flavor. Industrial production of food seasonings can provide uniform flavor, color, taste or texture required in food service and industrial kitchens, and also in home consumption [2].

Nowadays, it is possible to find plenty of different seasoning and spice blends, which differ in taste, aroma, form (liquid or powder) and application. Commercial spice blends and seasoning formulations can be divided into different groups depending on their composition, usage in specific kinds of dish (e.g. for poultry, meat, barbecue, sauces, marinades, salads, soups, dips, snacks and condiments), or in some specific or traditional cuisines (like Mediterranean, Turkish, Chinese, Indian, Brazilian cuisine). In the production of commercial seasoning blends, spices and other ingredients are first plated on some sort of carrier (e.g. salt, sugar, dextrose, or maltodextrin), before being mixed with other spices or ingredients. Anti-caking agents are usually added in required amounts below 2% [2].

Vegetable based seasoning powders can be described as mixtures of dried vegetable, table salt, taste enhancers and other compounds (like starch, maltodextrin, sugar, colors like beta carotene or curcumin). The main carrier materials in these seasoning blends is table salt. Quality of food seasonings is prescribed by national Regulation standards for soups, sauces, their concentrates and food seasonings [3] relevant in Bosnia and Herzegovina. According to these standards, powdered vegetable based food seasonings must satisfy following requirements: moisture $\leq 5\%$, NaCl $\leq 60\%$, monosodium glutamate $\leq 33\%$ and dried vegetables $\geq 8\%$ [3].

Dried spices and seasonings generally have no affinity for spoiling, because of low moisture, aw and high salt content. But during long keeping periods they can lose aroma and change color. Spice blends and seasonings last about 1 or 2 years depending on moisture content [2].

Besides their taste, aroma and composition, seasoning powders also need to have certain physical and functional properties, which are very important during processing, packaging, handle, manipulation, storage, and application in the kitchen. Many physical properties are very important for packing and storage. Water activity is important for microbiological stability during storage. The most important physical properties of food seasoning powders are bulk density, granulation, wettability, dispersibility and flowability. Bulk density is a ratio of weight of powdered material and volume which that certain powder weight occupies. It depends on granulation, particle size and shape. Particles with irregular shape have lower bulk density. Bulk density is important for packaging

and storage space. Dispersibility is important for uniform mixing after addition to liquid food medium. Flowability is important for transport operations during processing and good flowability without caking is required.

There is a lack of literature data related to quality of seasoning powders and that is the reason of this study.

The aim of this study is to investigate physical properties of commercial seasoning powders and to find relationships between chemical composition and physical properties as well as between physical properties themselves.

2 Material and Methods

Research was done on seven samples of commercial vegetable food seasonings (Table 1, Fig. 1) purchased from a local market in Sarajevo, Bosnia and Herzegovina.

Table 1. List of samples

Code	Commercial name	Producer
SP1	Master	Vispak, Visoko, Bosnia nad Herzegovina
SP2	Kolmix	Kolmix, Velika Kladuša, Bosnia and Herzegovina
SP3	Vegamix	Vegamix Živinice, Bosnia and Herzegovina
SP4	Boni	Bonito, Prnjavor; Bosnia and Herzegovina
SP5	Vegeta	Podravka, Koprivnica, Croatia
SP6	Začin C	Nestle Adriatic, Belgrade, Republic of Serbia
SP7	Kulinat Classic	Aleva, Novi Kneževac, Republic of Serbia

2.1 Chemical Properties

Moisture content was determined by drying at 105 °C until a constant weight is reached [4]. Total ash content was determined by burning at 550 °C for 8 h [5]. Mohr titration with $AgNO_3$ using potassium chromate as an indicator was used for determination of NaCl content [6].

2.2 Amount of Dried Vegetable

Amount of dried vegetables was determined using the method described by Kostić [7]. 10 g of seasoning powder was mixed with 100 ml of distilled water in 250 ml laboratory glass. The mixture was boiled and left for about 10–15 min to let the vegetables swell. Then the mixture was filtered through a quantitative filter paper until all liquid passed. Filter paper together with the sludge was dried carefully at 90–100 °C until a constant weight was reached. Amount of dried vegetable was calculated using equation:

$$\%\ dried\ vegetable(\%) = (A - B)100/m \tag{1}$$

where A is the mass of the filter paper with sludge after filtration and drying, B is the mass of the filter paper before filtration and m is total sample mass.

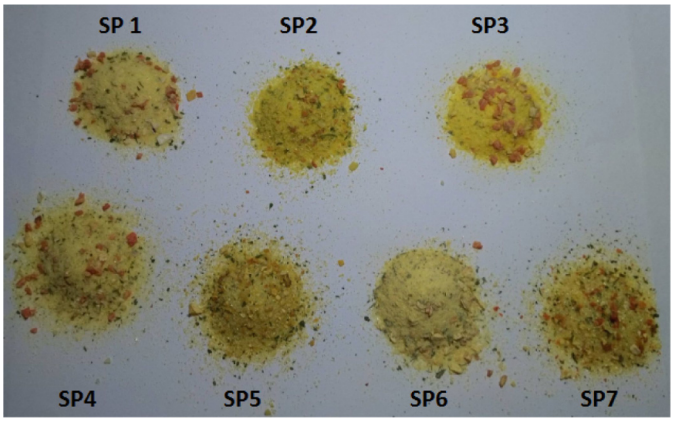

Fig. 1. Food seasoning samples

2.3 Electrical Conductivity, pH Value and Water Activity (aw)

Electrical conductivity and pH value were determined in dissolved samples according to producer recommendation. 3 g of seasoning powder were mixed with 250 ml of distilled water, and left to stay five minutes. After five minutes the pH value was measured using laboratory pH-meter (Mettler Toledo) and electrical conductivity was measured using conductometer (Nahita 908/5). Aw of seasoning powders was measured using laboratory aw-meter (Lab Swift-aw Novasina Switzerland).

2.4 Granulation

For determination of granulation, the whole amount of the packed sample was sieved through four sieves (Prufsieb ISO 3310-1) with hole diameters 2 mm, 1 mm, 0,5 mm and 0,25 mm, and five fractions were obtained ($\geq$2 mm, $\geq$1 mm, $\geq$0,5 mm, $\geq$0,25 mm and $<$0,25 mm).

2.5 Dispersibility

5 g of seasoning powder were put in a 50 ml measuring cylinder and distilled water (t = 20 °C) was added to 50 ml. The mixture of powder and water was vigorously stirred and left aside for 3 h without agitation for the solid particles to settle. The volume of the liquid phase was measured. Dispersibility was calculated as the ratio of liquid phase volume V_{liquid} and total volume V_{total} (50 ml) [8–10]:

$$Dispersibility(\%) = \left(V_{liquid}/V_{total}\right)100 \quad (2)$$

2.6 Wettability

Wettability was determined using the method described by Fernandes et al. [11] with some modifications. 1 g of seasoning powder was sprinkled carefully on the calm surface of 100 ml of distilled water temperature 20 °C. The time was recorded when all powder particles became wetted.

2.7 Bulk Density and Tapped Density

Bulk density (BD) and tapped bulk density were determined by method described by Fernandes et al. [11] and Imtiaz et al. [12] with some modifications. 30 g of powder was put in a 50 ml graduated measuring cylinder and the volume was recorded. Bulk density was calculated as a ratio of sample weight and occupied volume.

Tapped density (TD) was determined after determination of bulk density. 30 g of sample in measuring cylinder was tapped 100 times on a plain surface from a height of about 10–15 cm. After tapping, the new tapped volume was recorded, and tapped density was calculated as ratio of sample weight and tapped volume.

2.8 Carr Index, Bulk Porosity and Hausner Ratio

Carr index (or compressibility index) was used as a measure of powder compressibility. Carr index and porosity were calculated from measured bulk density (BD) and tapped density (TD) using following formulas [13]:

$$Carr\ index(\%) = (TD - BD)100/TD \tag{3}$$

$$Bulk\ porosity(\%) = (TD - BD)100/BD \tag{4}$$

Hausner ratio (HR) was calculated as ratio of bulk density (BD) and tapped density (TD) using the formula [13]:

$$\text{HR} = \text{bulk density/tapped bulk density} \tag{5}$$

2.9 Angle of Repose

The angle of repose is the angle formed by the horizontal base of the bench surface and the edge of a cone-like pile (heap) of powder. The height of powder heap above the surface and the diameter of the heap at its base were measured and the angle of repose (α) was calculated. 50 ml of each powder was carefully delivered through a funnel with a diameter of 1 cm on a plain surface base. Angle of repose α was calculated using the formula [14]:

$$tg\ \alpha = 2\ h/D \tag{6}$$

where are α - angle of repose, h – a height of pile and D – a diameter of pile base.

2.10 Estimation of Powder Flowability and Cohesiveness

Flowability and cohesiveness were estimated through values of Hausner ratio, Carr index of compressibility and angle of repose [14–16]. Angle of repose, Carr index and Hausner ratio will increase when cohesion increases. Lower values of these parameters indicate lower cohesiveness and better flowability. Higher values of cohesiveness indicate lower and harder flowability. Referent values of Carr index, Hausner ratio, angle of repose and related description of flowability and cohesiveness are given in Tables 2 and 3.

Table 2. Referent values of Carr index, Hausner ratio and angle of repose for flowability estimation [14, 15]

Carr index	Hausner ratio	Angle of repose (degrees)	Flowability
≤10	≤1.11	≤30	Excellent, easy flow
11–15	1.12–1.18	31–35	Good
16–20	1.19–1.25	36–40	Passable
21–25	1.26–1.34	41–55	Poor
26–31	1.35–1.45	46–55	Poor
26–31	1.35–1.45	46–55	Poor
32–37	1.46–1.59	56–65	Very poor, limited
>38	>1.60	>66	Very, very poor

Table 3. Referent values of Hausner ratio and angle of repose for estimation of powder cohesiveness [15, 16]

Hausner ratio	Cohesiveness	Angle of repose (degrees)	Cohesiveness
≤1.11	Very low or negligible	≤30	Non cohesive or negligible
1.12–1.20	Low	30–45	Low, some cohesiveness
1.2–1.40	Intermediate	45–55	Cohesive, true cohesiveness
>1.40	High	>55	Very high cohesive, sluggish

2.11 Statistical Analysis

Statistical analysis was performed by one-way ANOVA and Tukey test ($p \leq 0.05$) using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). All analysis was done in triplicate and results were shown as mean value ± standard deviation (SD).

3 Results and Discussion

The results related to composition of seasoning powders are given in Table 4. Significant differences ($p \leq 0.05$) between samples are found for moisture, NaCl and dried vegetable, while ash content in samples did not differ significantly.

Table 4. Composition of seasoning powder samples (Mean ± SD)

Sample	Moisture (%)	NaCl (%)	Ash (%)	Dried vegetable (%)
SP1	2.28 ± 0.20^{ab}	54.03 ± 1.66^{bc}	62.29 ± 0.35	11.50 ± 0.71^{b}
SP2	1.43 ± 0.15^{b}	58.98 ± 0.86^{ab}	67.24 ± 0.29	15.43 ± 3.43^{ab}
SP3	2.69 ± 0.04^{a}	53.84 ± 1.39^{c}	59.04 ± 3.80	17.00 ± 1.41^{ab}
SP4	1.96 ± 0.59^{ab}	41.89 ± 2.25^{d}	61.25 ± 3.33	17.61 ± 1.16^{ab}
SP5	1.54 ± 0.13^{b}	43.96 ± 0.47^{d}	61.89 ± 2.23	17.18 ± 0.95^{ab}
SP6	1.29 ± 0.07^{b}	59.43 ± 0.93^{a}	63.79 ± 1.00	13.83 ± 1.17^{b}
SP7	1.32 ± 0.00^{b}	56.84 ± 0.71^{abc}	58.77 ± 2.18	21.50 ± 2.12^{a}

*Different letters in columns represent statistically significant differences ($p \leq 0.05$) between samples

It can be observed that NaCl was the main ingredient and carrier compound with high amounts between 41.89 and 59.43%. Amount of NaCl for analyzed samples was under the allowed limit (60%) prescribed by national Regulation standards [3]. The highest amount of NaCl was in SP6 and the lowest in SP4. Samples SP3 and SP4 had significantly ($p \leq 0.05$) lower NaCl content in comparison to other samples. Besides that, these results are in agreement with literary data for similar food seasonings. According to literature, NaCl content in seasoning powders can be in a pretty wide range 14.2–67% [2, 17–22]. Literary data suggest that the most common salt content in commercial seasoning powder is about 50% [22] and 57.90% [17], which is very similar to results obtained in this study. Although other authors report mostly lower NaCl content (14–38%), these data are similar to other kinds of seasoning powder, which contain larger amounts of other taste-enhancing ingredients and compounds. Salt content in other seasoning mixtures was: 1–6% in reduced NaCl seasoning powder [20], 8.5% in Moroccan seasoning [2], 14.30–20% in snack seasoning blend [19], 25% in Jamaican seasoning mixture, 20.70% in red curry mixture and 38% in Brazilian seasoning mixture [2].

Ash content varied between 58.77 and 67.27% without significant differences, and values of ash suggested that NaCl was the main compound in the ash.

Moisture content varied between 1.43–2.69%. According to national Regulation standard for seasoning powder [3] moisture content has to be lower than 5%. All samples had moisture in allowed amounts. Food grade salt should have less than 3% of moisture required by Codex Alimentarius [23]. Considering that salt is the dominant ingredient, the obtained results for moisture in seasoning powders are expected.

Content of dried vegetables varied between 11.50–21.5%. For all samples dried vegetable content was higher than 8%, which is the minimum required by national

Regulation standard [3] for this kind of product. According to literature, [17] seasoning powder can contain about 21% of dried spices and vegetables and that is very similar to dried vegetable value observed in sample SP7. Sample SP7 with highest vegetable content differs significantly ($p \leq 0.05$) from samples SP1 and SP6 with the lowest vegetable content. Other differences were not significant.

Table 5. Water activity (aw), pH and electrical conductivity of seasoning samples (mean ± SD)

Sample	aw	pH	Electrical conductivity (mS)
SP1	0.365 ± 0.01^{a}	7.06 ± 0.07^{a}	8.30 ± 1.13
SP2	0.299 ± 0.02^{b}	6.63 ± 0.04^{bc}	10.50 ± 0.28
SP3	0.297 ± 0.01^{b}	6.63 ± 0.06^{bc}	6.50 ± 2.12
SP4	0.265 ± 0.00^{c}	6.68 ± 0.04^{b}	7.09 ± 2.43
SP5	0.292 ± 0.01^{bc}	6.55 ± 0.06^{bc}	5.65 ± 0.07
SP6	0.280 ± 0.00^{bc}	6.42 ± 0.02^{c}	9.50 ± 0.99
SP7	0.293 ± 0.00^{bc}	6.66 ± 0.08^{b}	8.90 ± 1.27

*Different letters in column represent statistically significant differences ($p \leq 0.05$) between samples

Results of aw, pH and electrical conductivity are given in Table 5. Values for aw obtained in our study ranged between 0.265 and 0.365. Water activity (aw) is an important indicator of microbiological stability. If aw value is lower than 0.6 the product is stable, and there are no conditions for microbe growth. Many dried products, like dried spices, spice mixtures and vegetables have aw < 0.6 [24, 25]. Many literature data for dried vegetables and seasoning reported low aw values and relation between moisture content and aw. Air dried and vacuum dried carrot had aw values 0.49 and 0.28 [26]. Onion powder and garlic salt seasoning with 6% of moisture had aw 0.351 and 0.413 respectively [27], parsley had aw between 0.16 and 0.30 depending on the drying conditions [28] and different powdered food with moisture content lower than 5% had aw under 0.42 [29]. For dehydrated powdered flavourings and seasonings typical aw value is 0.17–0.26 [30]. According to Modi et al. [18] aw value of spice mixture with 7–10% of moisture varied between 0.52–0.58. Sample SP1 had significantly ($p \leq 0.05$) higher water activity value in comparison to other samples. Values for aw obtained in our study ranged between 0.265 and 0.365. These results are in agreement with literary data [26–28]. Modi et al. [18] obtained higher moisture content and consequently higher aw values in spice seasoning mixture.

Significantly ($p \leq 0.05$) higher pH value was noticed in sample SP1 (Table 5), which had highest aw and the lowest vegetable content. pH value could be related to chemical composition, presence of acids and alkali compounds. Nitrogen based substances can increase pH value, salts are neutral (pH = 7), while many vegetable species are slightly acidic with pH under 7. For example pH values for some kinds of vegetables are: carrot: 5.88–6.40, onion: 5.3–5.9 and celery: 5.7–6.0 [31]. Probably, because of this fact sample with the lowest vegetable content had the highest pH value. On the other hand, taste

enhancers as monosodium glutamate have pH 6.7–7.2 [32], higher in comparison to vegetables. Considering that this type of food seasoning powders always contain taste enhancers and monosodium glutamate (pH 6.7–7.2) in allowed amount under 33% [3] and obtained results for dried vegetable in amount over 11.50% (Table 5), these results for pH were expected.

Results for electrical conductivity did not differ significantly ($p \leq 0.05$). It can also be noticed that samples with higher amount of ash and NaCl had higher electrical conductivity.

Table 6. Granualtion of seasoning powders (%) (Mean ± SD)

Sample	Very large perticles ≥2 mm	Large particles ≥1 mm	Medium particles ≥0.50 mm	Small particles ≥0.25 mm	Very small particles <0.25 mm
SP1	8.04 ± 0.49^{bc}	4.15 ± 0.42^{d}	5.02 ± 0.20^{d}	74.07 ± 0.86^{a}	8.46 ± 0.45^{d}
SP2	5.43 ± 1.15^{c}	5.07 ± 0.27^{cd}	4.84 ± 0.44^{d}	71.46 ± 0.29^{a}	12.58 ± 0.45^{c}
SP3	12.11 ± 0.69^{a}	5.76 ± 0.27^{c}	7.96 ± 0.20^{c}	59.23 ± 0.94^{b}	14.55 ± 0.64^{b}
SP4	9.51 ± 0.53^{ab}	5.12 ± 0.86^{cd}	3.00 ± 0.30^{e}	64.53 ± 4.80^{b}	15.60 ± 0.46^{b}
SP5	7.71 ± 1.56^{bc}	10.82 ± 0.85^{a}	13.10 ± 0.85^{a}	64.41 ± 0.89^{b}	3.96 ± 0.14^{e}
SP6	5.90 ± 0.64^{c}	5.34 ± 0.38^{cd}	5.49 ± 0.85^{d}	64.64 ± 0.86^{b}	18.35 ± 0.13^{a}
SP7	4.18 ± 0.25^{c}	8.43 ± 0.20^{b}	11.41 ± 0.29^{b}	63.44 ± 0.52^{b}	7.58 ± 0.10^{d}

*Different letters in column represent statistically significant differences ($p \leq 0.05$) between samples

Results of granulation analysis are given in Table 6. It can be seen that all samples had the largest amount of small particles with diameter under 0.5 mm. The total amounts of small particles with diameter <0.50 mm (<0.25 mm + ≥0.25 mm) were 82.87, 84.04, 73.73, 82.82, 68.11, 83.27 and 76.15% for samples SP1, SP2, SP3, SP4, SP5, SP6 and SP7 respectively. It is also seen that samples differ significantly in the each fraction. Sample SP3 had significantly highest amount of largest particles (≥2 mm) and significantly highest amount of particles between 1 and 2 mm was in sample SP5. SP5 sample had lowest amount of particles bellow 0.25 mm. The most represented fraction for all samples were particles between 0.25 and 0.50 mm. Amount of this fraction ranged between 59.23 and 71.07%. SP1 and SP2 had significantly higher amount of this fraction in comparison to other samples. Significantly largest amount of smallest particles (<0.25 mm) were found in sample SP6 (18.35%) and the smallest in sample SP5. Sample SP5 has the lowest amount of smallest particles < 0.5 mm and significantly highest amount of middle fraction (≥0.5 mm and ≥1 mm).

After observation of each particular fraction, it can be seen that larger fractions are mostly or completely comprised of dried vegetable pieces with dominant carrot content. Next fraction with diameter ≥ 1 mm mostly consisted of leafy vegetables (with larger leaves) and smaller pieces of carrot and other root vegetables. On the other hand, finer fraction (<1 mm and <0.5 mm) were mostly comprised of salt crystals, and other

powdered ingredients with smaller amounts of small vegetable leaves (see Fig. 2). The highest amount of NaCl was in sample SP6 which had the highest amount of very small particles < 0.25 mm (18%).

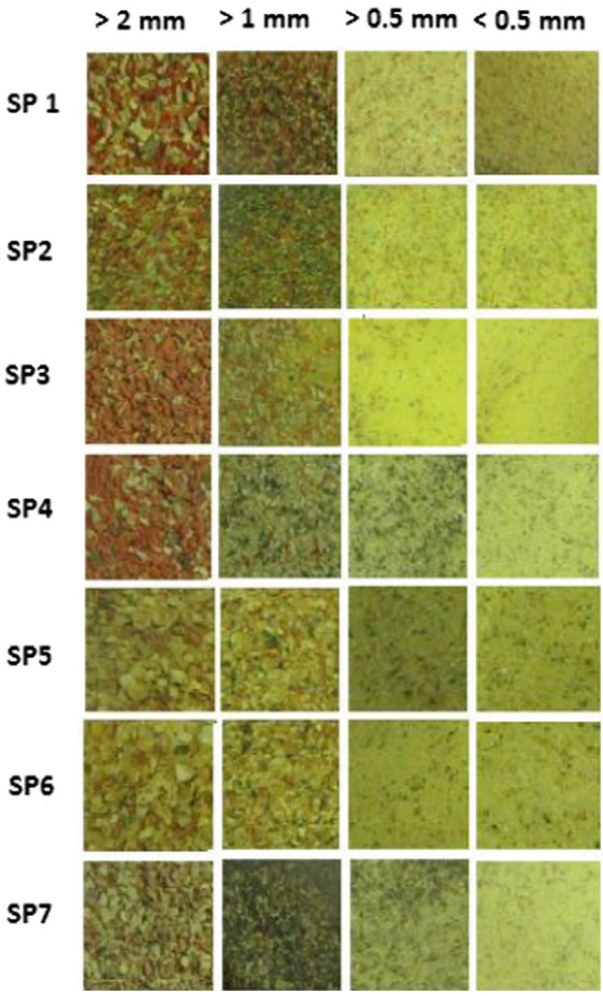

Fig. 2. Granulation of food seasoning samples – image of fractions

Results for bulk density, tapped density, bulk porosity, dispersibility and wettability are given in Table 7.

Bulk density (Table 7) of seasoning powder varied between 0.90 and 1.07 g/ml. The lowest bulk density was in sample SP1 and the highest in sample SP3. Sample with the lowest bulk density had the lowest amount of dried vegetables and spices. These results could be explained by the fact that bulk density depends on chemical composition, particle size and particle shape. Smaller particles with regular spherical

shape can better fill the space, which results in higher bulk density. Besides, bulk density depends on the amount of each chemical compound and their density. Because of that, bulk density of samples mostly depended on the amount of dried vegetables, NaCl and granulation, particle size and shape. Irregularity of particle shape probably decreased bulk density. Samples with higher amount of large irregularly-shaped particles had lower bulk density. In this case the fraction of large irregularly-shaped particles consists of dried vegetables, mostly carrot. And according to that fact, it could be clear why samples with the largest amount of fraction with particles < 0.5 mm had the highest bulk density, and the sample with higher amount of particles > 2 mm had the lowest bulk density. It is well known that minerals and salt have the highest particle densities. Because of complex composition of food seasoning powders, different literary data could serve to explain obtained results. The dominant constituent is NaCl (Table 4) with values 41.89–59.83%, and dried vegetables also contribute with significant amount (11–21%). Because of that, these two compounds have great influence on bulk density of seasoning powders. For example the density of table salt is about 2.16–2.17 g/ml and bulk density is 1.22–1.38 g/ml [33, 34]. Particle densities of other food constituents are: starch 1.55 g/ml, cellulose 1.27–1.61 g/ml, proteins 1.25–1.4 g/ml, fat 0.89–0.95 g/ml and ash 2.42 g/ml [35]. According to FAO [33] bulk density of spice blend without salt was 0.58 g/ml. Results for bulk density obtained in our study are almost two times higher in comparison to FAO's data. This could be explained with high NaCl content in the analysed samples, which increased bulk density. On the other hand, presence of dried vegetables and spices decreased bulk density of seasonings, because of their rough irregular shape and lower densities of vegetables in comparison to density of NaCl. For example, according to Donsi et al. [36] bulk density of dried carrot with 10% of moisture is 0.3 g/ml.

Tapped density varied between 1.15 and 1.23 g/ml, but differences between samples were not significant (Table 7). All samples had higher tapped density in comparison to loose bulk density. The highest increase of bulk density after tapping was noticed in sample SP6. This sample had the highest level of NaCl (59.43%), highest amount of very small fraction < 0.25 mm (18%) and also pretty low amount of very large particles with d > 2 mm (5.90%). This could be explained by fact that small particle had ability to be better filled in the pores of empty regions between powder particles after tapping. If we compare bulk density and tapped density, the increasing between these two values (difference between tapped and bulk density) ranged between 0.12 g/ml and 0.28 g/ml or expressed in percentages 11.5–31.11%. The highest increasing (the largest differences) was noticed in SP1 (31.11%) and SP2 (26.10%). Both samples had pretty large amount (82.87 and 84.04%) of small and very small particles with sizes <0.5 mm. It is also very interesting that sample SP1 with pretty high moisture content (2.28%) (Table 4) had the highest increasing of tapped density and largest difference between tapped and bulk density. The reason for this is probably related to the fact that presence of moisture helps particles to adhere, and that high amount of moisture could also cause undesirable caking. However, sample SP3 with the highest moisture content (2.69%) did not have the highest difference between tapped and bulk density. This leads to the conclusion that moisture is not the main factor for increasing of bulk density after tapping. The combination of different factors can cause the increase of tapped density. In this case (for SP3) low difference between bulk and tapped density could be explained by

granulation. SP3 sample had significantly higher amount of very large particles > 2 mm (12%), lowest amount of small fraction with sizes between 0.25 and 0.50 mm. Also total amount of particles bellow 0.50 mm was pretty low (73.73%) in comparison to many other samples. Results for bulk porosity were following the results of tapped density. Values of bulk porosity actually presented the percentage of difference between tapped and bulk density. Highest porosity had sample with lowest vegetable content (SP1) and the lowest porosity was in SP7 which had the highest value of dried vegetables. Bulk porosity between these two samples differs significantly.

Table 7. Other physical properties of powdered seasoning samples (Mean ± SD)

Sample	Bulk density (g/ml)	Tapped density (g/ml)	Bulk porosity (%)	Dispersibility (%)	Wettability time (s)
SP1	0.90 ± 0.04^{b}	1.18 ± 0.01	31.73 ± 4.87^{a}	82.67 ± 4.04	0.84 ± 0.13^{b}
SP2	0.92 ± 0.01^{b}	1.16 ± 0.00	26.59 ± 2.18^{ab}	83.00 ± 8.19	0.52 ± 0.06^{b}
SP3	1.07 ± 0.00^{a}	1.19 ± 0.02	12.31 ± 1.80^{ab}	78.00 ± 5.29	1.08 ± 0.06^{b}
SP4	1.00 ± 0.03^{ab}	1.15 ± 0.02	15.03 ± 5.59^{ab}	80.67 ± 4.04	1.66 ± 0.80^{b}
SP5	1.01 ± 0.01^{ab}	1.16 ± 0.04	15.04 ± 5.60^{ab}	82.67 ± 1.53	0.80 ± 0.09^{b}
SP6	1.06 ± 0.00^{a}	1.23 ± 0.09	15.80 ± 8.61^{ab}	85.00 ± 1.00	7.67 ± 1.29^{a}
SP7	1.04 ± 0.06^{a}	1.16 ± 0.10	11.16 ± 3.47^{b}	79.33 ± 1.53	1.31 ± 0.08^{b}

*Different letters in column represent statistically significant differences ($p \leq 0.05$) between samples

All samples had pretty good dispersibility (Table 7) with values 78–85%. Differences between dispersibility were not significant. Sample SP6 with the highest NaCl content, highest level of smallest particles and pretty low vegetable content had the highest dispersibility. The lowest dispersibility values are noticed in SP3 and SP7. It could be explained by the fact that SP3 had the highest amount of very large particles over 2 mm and SP7 had the highest amount of dried vegetables. The conclusion would be that dried vegetables disperse hardly while NaCl is easily dispersible.

Wettability ranged between 0.52 and 7.67 s (Table 7). Significantly ($p \leq 0.05$) highest wettability had sample SP6, probably because of largest amount of very small particle < 0.25 mm. Because of their small size, these particles stayed longer on the water surface and needed more time to be wetted or fall through water surface.

Hausner ratio and Carr index of compressibility are indicators of flowability and cohesiveness. Lower values of these two properties indicate better flowability and lower cohesiveness. Results of Carr index and Hausner ratio are given in Table 8. Husner ratio varied between 1.11 and 1.32. These values correspond to very low to intermediate cohesiveness and passable to excellent flowability. According to obtained values, there were no samples that could be considered as cohesive. Also, values for Hausner ratio showed that there no sample with low or limited flowability. Highest value of Hausner ratio and Carr index was in SP1 and SP2. These two samples were the most cohesive samples and had the lowest flowability. Flowability of these samples was marked as

passable, while other samples had good or excellent flowability. The excellent flowability was only in sample SP7. If these values are compared with values for other physic-chemical properties, it could be shown that granulation, especially amount of large particle, moisture and dried vegetable content effected flowability and cohesiveness. Sample SP7 with excellent flowability had the following characteristics: low moisture content (1.32%), significantly higher content of dried vegetables (21.50%), the lowest amount of small particles with sizes 0.25–0.50 mm (63,44%) and the lowest amount of particles > 2 mm (4.18%). On the other hand, sample SP1, which was the most cohesive and had the worst flowability, had the highest moisture content, the lowest dried vegetable content, highest bulk porosity and highest amount of small particles with sizes between 0.25 and 0.5 mm. It can be concluded that factors like high presence of small particles and high moisture content could increase seasoning powder cohesiveness, which could result in decreased flowability. Presence of dried vegetables can increase flowability, and this can be explained by larger particle sizes of dried vegetables in comparison to other compounds such as small NaCl crystals. These results are in agreement with literary data for similar powdered products. Chinwan et al. [37] reported that increasing of moisture content increased Carr index and cohesiveness. Polyherbal powders with moisture content ranged 3.50–5.50%, had Hausner ratio 1.23 and Carr index 23.50 [38], which is very similar to results obtained in this study. Microspheres with encapsulated sized 0.62–0.78 mm had Carr index in range 12.59–19.55 and Hausner ratio 1.15–1.23, which was also very similar to results of this study. According to other literary data, spice blend powders had higher values of Hausner ratio (1.28–1.79) and Carr index (22.15–40.55) [39], but this could be explained by different composition and moisture content.

Table 8. Carr index and Hausner ratio as indicators of flowability and cohesiveness of seasoning samples (Mean ± SD)

Sample	Carr index	Cohesiveness	Hausner ratio	Flowability
SP1	24.03 ± 2.81^{a}	Intermediate	1.32 ± 0.05^{a}	Passable
SP2	20.99 ± 1.36^{a}	Intermediate	1.27 ± 0.02^{a}	Passable
SP3	10.95 ± 1.43^{bc}	Low	1.12 ± 0.02^{ab}	Good
SP4	12.96 ± 4.23^{b}	Low	1.15 ± 0.06^{ab}	Good
SP5	12.97 ± 4.24^{b}	Low	1.15 ± 0.06^{ab}	Good
SP6	13.41 ± 6.43^{b}	Low	1.16 ± 0.09^{ab}	Good
SP7	9.99 ± 2.81^{c}	Very low	1.11 ± 0.03^{b}	Excellent

*Different letters in column represent statistically significant differences ($p \leq 0.05$) between samples

Besides Carr index and Hausner ratio, angle of repose is another important indicator of flowability. Values of repose angle ranged between 24.18° and 32.13° (Table 9) and there wasn't any significant difference. According to referent values of angle of repose all samples had good or excellent flowability with low or no cohesiveness. The lowest value

of angle of repose was noticed in SP7 and the highest in SP1, what is in accordance to results of Carr index and Hausner ratio. Results indicated that angle of repose was related to moisture content, bulk porosity, granulation and dried vegetable amount (Tables 4, 5, 6 and 7). Sample SP7 with the lowest value of repose angle had high bulk porosity, low moisture content, the lowest content of small particles with sizes between 0.25 and 0.50 mm and the highest amount of dried vegetable. Presence of higher amount of larger particles of dried vegetable influenced that spilled pile of seasoning powder was better spread out with a bigger diameter and lower height, which resulted in a lower angle. On the other hand, sample SP1 had the highest amount of small particles with sizes between 0.25 and 0.50 mm, the highest moisture content and the lowest bulk porosity and dried vegetable content. Because of the high amount of small particles this sample made a pile with the highest repose angle. Another sample with a high repose angle was SP6. Although this sample had second high repose angel, the values of Carr index and Hausner ratio were not such high. High value of repose angle for SP6 sample could be explained by other physic-chemical properties. Although the moisture content in this sample was not high, the main characteristics of SP6 samples were: the highest amount (18.35%) of very small particles under 0.25 mm, the highest NaCl content and pretty low dried vegetable content. This sample also had pretty high total content of small and very small particles bellow 0.50 mm (83.87%), pretty low total content of all particles larger than 1 mm (11.24%).

Table 9. Angle of repose as indicator of flowability and cohesiveness of food seasoning samples (mean ± SD)

Sample	Angle of repose (degrees)	Flowability	Cohesiveness
SP1	32.13 ± 4.41	Good	Low cohesiveness
SP2	29.80 ± 1.43	Excellent	No cohesiveness
SP3	25.90 ± 4.43	Excellent	No cohesiveness
SP4	24.80 ± 1.70	Excellent	No cohesiveness
SP5	29.03 ± 2.39	Excellent	No cohesiveness
SP6	31.25 ± 1.29	Good	Low cohesiveness
SP7	24.18 ± 3.38	Excellent	No cohesiveness

*Different letters in column represent statistically significant differences ($p \leq 0.05$) between samples

Angle of repose varied between 24.18°and 32.13° without significant differences. The highest value was noticed in SP1 sample and the lowest in SP7. The similar relations between samples were obtained in Hausner ratio and Carr index. All these properties are related to flowability estimation. According to repose angle, the best flowability was in SP7 with lowest angle of repose value. This sample (SP7) had pretty high porosity, low moisture and the highest dried vegetable content. On the contrary, SP1 had the lowest dried vegetable content and the highest amount of small particles with sizes between 0.25 and 0.50 mm.

Taking into consideration the values of repose angle, it can be seen that flowability of two samples (SP1 and SP6) was estimated as good, while other samples had excellent flowability. The cohesiveness of same two samples (SP1 and SP6) was estimated as low, while other samples were without cohesiveness. It could be supposed that the high values of repose angle in SP6 could be influenced by the highest values of very small particle bellow 0.25 mm, tapped density and NaCl content. High values of NaCl content could be related to high values of small or very small particles, due to the presence of small fine particles of salt.

According to obtained results, this study can suggest that Carr index and Hausner ratio gave more precise results for estimation of flowability and cohesiveness (Tables 8 and 9). Using the values of repose angle, flowability for almost all samples (except SP1 and SP6) was estimated as excellent, while using Carr index and Hausner ratio only one sample had excellent flowability. Flowability of SP2 sample was estimated as excellent using angle of repose and as passable using Carr index. Also the cohesiveness of SP2 sample was estimated as intermediate by Hausner ratio, while using angle of repose this sample was marked as non-cohesive. These differences can be explained by pretty high value of angle of repose (29.80°), which is very close to limit reference value of ($\leq$30°) between low cohesive and non-cohesive powders. Because of that, SP7 sample can be rather classified as low (intermediate) than non-cohesive powder. For the same reason, the flowability for SP7 can be rather assessed as "passable to good" then excellent.

These results are in agreement with literary data reported for similar products. Angle of repose for seasoning powder samples mostly depended on moisture content, composition and granulation. Seasoning powder samples with higher NaCl content had higher angle of repose. According to Salt institute [40] angle of repose of food grade salt is 32°. Another literature source [41] reported repose angle values of 31° for iodized salt and 37.5° for sea salt. Dried vegetables, spices, flavorings and seasonings can have different values of repose angle depending on degree of particle size reduction, type and composition. According to Johanson et al. [41] angle of repose for different spices had values between 33.5° and 37, and for dried and crunched dill repose angle was 35.5°. According to Andersson [42] different kinds of food seasoning powders had angle of repose between 22.5° and 37°. Wheat flour flavoring had values 33°–54° [43]. The lowest angle was found in barbecue seasoning and Italian salad seasoning (28–30°), while the highest in Fajita (30 and Taco (33–37°) [43].

Angle of repose of wheat semolina and flour ranged between 28.07° and 48.58° depending on particle size. The highest repose angle was found in soft flour with the finest particles with average size of 0.098 mm, and lowest in semolina with mean particle size of 0.519 mm [44]. Angle of repose will decrease if particle size increases [41]. On the other hand, higher moisture content in powdered samples will cause increasing of repose angle [43, 45, 46].

Obtained results for repose angle are in agreement with literary data for similar products and the differences could be explained by different moisture content or different particle sizes. In the many cases, it is well known that angle of repose depends on moisture content and granulation. Values of angle of repose rise in the cases of smaller particle sizes [41] and higher moisture content [46, 47].

4 Conclusion

Vegetable food seasoning samples were investigated in terms of their physical properties and influence of main ingredients on physical properties. All samples had values of characteristic for that kind of products according to literature data and in agreement with national regulation standard for soups, sauces, their concentrates and food seasonings [3]. According to this standard [3] vegetable food seasoning samples are required to have ≤5% of moisture, ≤60% of NaCl and ≥8% of dried vegetable.

This study has shown that differences in obtained values occurred because of differences in composition. The amount of main compounds in food seasoning samples had following ranges: moisture 1.29–2.69%, NaCl: 41.89–59.43% and dried vegetable: 11.50–21.50%. Aw values (0.265–0.365) indicated good microbiological stability and ability to be stored at room temperature.

Analysis of granulation showed that all samples had the highest amount (59.23–74.02%) of small particles between 0.25 and 0.50 mm and amount of all particles smaller than 0.50 mm (68.11 and 84.04%). Bulk density varied between 0.90 and 1.07 g/ml, and tapped density between 1.15 and 1.23 g/ml, while dispersibility was pretty good and ranged between 78 and 58%.

Higher amount of NaCl and moisture content influenced higher values of bulk density, dispersibility and lower flowability and bulk porosity. On the other hand, higher amount of dried vegetables and lower amount of moisture influenced higher amount of large particles, higher values of bulk porosity and better flowability.

Flowability of seasoning samples depended on their composition and granulation and varied from passable to excellent. Values of Carr index showed that cohesiveness of seasoning powders was very low to intermediate. Samples with higher amount of smaller particles and moisture content had higher angle of repose, lower flowability and higher cohesiveness, while samples with higher amount of large and very large particles and lower moisture content had lower angle of repose, lower cohesiveness and better flowability. Accordingly, the best flowability was in sample SP7, which had low moisture content and the highest dried vegetable content. The lowest flowability was in sample SP1, which had the highest amount of small particles, the lowest dried vegetable content and high moisture content. Higher amount of dried vegetable influenced better flowability and lower cohesiveness, because of significant presence of larger sizes particles.

For estimation of flowability and cohesiveness, Carr index and Hasner ratio gave more precise results in comparison to angle of repose. This study could suggest application of Carr index and Hausner ratio as acceptable indicators of flowability and cohesiveness.

Statistical analysis has shown that significant differences ($p \leq 0.05$) occurred in all properties except ash content, electrical conductivity, dispersibility, tapped density and angle of repose.

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The Influence of Essential Oils on the Quality and Stability of Olive Oil

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Abstract. Vegetable oils, especially unrefined oils, are subject to oxidation and hydrolytic changes under the influence of elevated temperature, light, and moisture. The degree of deterioration of the oil and fat can be affected by the type of feedstock, the chemical composition, and the conditions of processing and storage. In addition to changes in the organoleptic properties of oils and fats, their nutritional value also changes. In addition, essential oils and various herb extracts such as rosemary, sage, oregano, thyme, mint, basil, etc. have an impact on the sustainability of oils and fats. They have antioxidant activity and are very stable at high temperatures. Many studies have shown that the increased stability of vegetable oils is influenced by natural antioxidants. The study aimed to determine the quality and oxidative stability of extra virgin olive oil obtained by the cold pressing process. Olive oil was used as base oil, treated with the addition of different types and concentrations of essential oils: rosemary, mint, and basil with 0.5%, 1.0%, and 2.0%. The methods used to determine the viability of the oil samples were the Schaal Oven test for 24 h, in an oven at 63 ± 2 °C, and the UV lamp treatment. Changes in the analyzed oils were monitored by determining the acidity of the oil samples (% of free fatty acids) and the peroxide number. A statistically significant difference in peroxide number values was observed using the Schaal Oven test, at a concentration of added essential oil of 2.0%, compared to those of 0.5 and 1.0%; as in the oil with the addition of mint essential oil, compared to the addition of rosemary and basil essential oil. With UV treatments, with the addition of different types of essential oils at the concentration of 1.0%, statistically, significant differences in peroxide values were observed, compared to the added concentrations of 0.5 and 2.0%; as well as between all types of essential oils except between the essential oil of mint and basil. Applying the Schaal Oven test and UV treatment, the essential oil concentration of 2.0% was significantly different from the essential oil concentrations of 0.5 and 1.0% when determining the free fatty acid content. Moreover, a statistically significant difference was found in the content of free fatty acids for all three types of added essential oil.

Keywords: Essential oil · Oil stability · Auto-oxidation of oils · Antioxidants

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1 Introduction

Throughout history, vegetable oils and medicinal herbs have been used for a long period of time in Bosnia and Herzegovina. Olive oil is characterized by a high content of essential fatty acids such as oleic, stearic and palmitic acid; many minerals, enzymes, etc. Vitamin E and oleic acid are well-known and powerful antioxidants. Due to the short shelf life, undesirable changes in the composition of oils and fats are also possible, which are manifested by a decrease in nutritional value and changes in organoleptic properties. This occurs by spoiling oils and fats, thus losing essential fatty acids, vitamins, and provitamins. The most common types of deterioration are oxidative and hydrolytic deterioration, caused by the action of high temperature, humidity, and light. Regardless of the type of spoilage, the consequences are the same: the formation of decomposition products of particularly volatile carbonyl compounds that give an unpleasant odor and taste to oils, harmful to human health [1–3]. The process of oxidation of vegetable oils is inevitable, and the duration of the process depends on the composition of the oil and the presence of factors that accelerate or slow down the oxidation. By adding essential oils which function as natural antioxidants, the aim is to increase the oxidative stability of vegetable oils. Essential oils are plant products and are obtained from various plant parts by the processes of distillation, squeezing or extraction. The antioxidant properties of essential oils depend on the ability of components, especially phenols, to stop or delay the aerobic oxidation of organic matter. Scientific research has proven a wide range of pharmacological activities of essential oils, on which their use in pharmacy and medicine is based.

2 Materials and Methods

2.1 Materials

Extra virgin olive oil in the original packaging, stored in a dark green glass bottle with a volume of 1500 ml, originating from Italy, was taken for testing. For the distillation of essential oils, samples of dried herbs were taken, namely: rosemary, mint, and basil, packed in bulk, weighing 1 kg each, by the individual producer “BOLETUS” d.o.o. Sarajevo.

2.2 Methods

The extraction of essential oils was performed by hydrodistillation using an adapted Neo Clevenger apparatus, and the essential oil content was expressed in ml/100 g of plant sample. To test the oxidative stability of the oil, analytical determination methods were applied, namely the Schaal Oven test [4] in an oven at a temperature of 63 ± 2 °C in contact with air, without the influence of light. This is a dynamic test, in which oxidative changes occur in the oil due to the action of heat without the influence of light. The second applied method is based on the action of UV light, with the use of a “Hanau” lamp, 365 nm wavelength.

Oxidation of polyunsaturated fatty acids leads to the formation of conjugated fatty bonds, which have absorption maximum in the ultraviolet range of 230–375 nm. Standard titrimetric method was used for determining the peroxide value (expressed as meq O_2/kg) and free fatty acid content (expressed as % of oleic acid present), respectively [5, 6]. The peroxide number and content of free fatty acids were tested in all samples, before treatment. At the end of the treatment, the peroxide number was determined in all samples, and the content of free fatty acids only in the samples after 24 h of treatment. Parameter tests were performed 8, 12, and 24 h after the treatment. Results were expressed as mean and standard deviation. To determine statistically significant differences in the values of peroxide number and free fatty acids depending on the type of added essential oil and its concentration, two-factor analysis of variance was used, using the statistical program SPSS 20.0 [7] and to determine statistically significant differences within groups (post-hoc) the Tukey-test was used (significance level $\alpha = 0.05$).

3 Results and Discussion

Oxidative decomposition processes can depend on several factors: the raw material from which the oil or fat is produced, the composition of the oil, storage conditions, and ingredients that can speed up or slow down the reaction [8, 9]. Olive oil was used as the control oil, subsequently treated with the addition of various types and concentrations of essential oils, namely: rosemary, mint, and basil with concentrations of 0.5%, 1.0%, and 2.0%. Essential oils of herbs are natural substances that can be used to improve the sustainability of oils since they have significant antimicrobial, antioxidant, and other biological activities [10]. The addition of the mentioned essential oils in the stated concentrations provides thermal stability and high resistance to oxidative degradation of the product [11]. By examining the initial values of peroxide content and free fatty acids of extra virgin olive oil (without the addition of essential oils), which was used as a control, it was found that the values of peroxide number of 2.55 ± 0.07 meq O_2/kg, and the content of free fatty acids of $0.17 \pm 0.00\%$, which correspond to the provisions of the Ordinance [12, 13], which stated the values of the peroxide number for cold-pressed oils should be 10 meq O_2/kg and 7 meq O_2/kg, and free fatty acids up to 2% and 3% (as % oleic acid). Virgin olive oil is characterized by very high antioxidant stability. High stability can be correlated with antioxidant molecules and their activity (phenolic compounds, carotenoids, pigments) and the high content of monounsaturated fatty acids in triacylglycerol molecules (about 70% oleic acid) [14]. According to the research [15], the values of the peroxide number for cold-pressed olive oil are from 1.00 to 3.00 meq O_2/kg, and according to [16] from 1.53 to 3.84 meq O_2/kg. The stated values are in accordance with the obtained results in our research.

3.1 Results of the Peroxide Number Test Using the Schaal Oven Test and Treatment Under a UV Lamp

Observing the initial value of peroxide number of the control sample, in relation to the initial values of the olive oil samples to which different concentrations of rosemary and basil essential oils were added, their antioxidant effect is observed because the peroxide

number decreased. This was observed with the addition of both types of oil, at all concentrations, except for the sample with added basil of 2.0%, which can be explained by the fact that the concentration of 2.0% has the opposite effect on oil stability, which can be attributed to oversaturation, after which the peroxide value begins to decline. The addition of mint essential oil, in all concentrations, did not show significant antioxidant activity, due to the intensive increase in the value of the peroxide number. Peroxide number values, for all tested samples before treatment, ranged from 2.18 ± 0.25 meq O_2/kg for sample B-0.5% (olive oil with 0.5% basil essential oil) up to a maximum value of 12.50 ± 0.70 for the oil sample with the addition of essential mint oil 2.0%. Observing the stated average values of the peroxide number in all samples with the addition of basil essential oil, an increase in the same can be observed in proportion to the treatment time (8, 12, and 24 h). A significant increase in the value of the peroxide number is manifested after 24 h, after the treatment in the dryer, by 50%. However, it can be observed that the values of the peroxide number, at a concentration of basil essential oil of 2.0%, after 8 and 12 h decreased compared to the initial analysis and that after 24 h, the value of the peroxide number increases again (4.35 meq O_2/kg) and has an identical value of the base oil sample (4.23 meq O_2/kg). If the concentration of added basil essential oil is observed during the treatment in the dryer after 24 h, the value of the peroxide number is on average 4.45 meq O_2/kg, which corresponds to the values of the Ordinance [12, 13]. Comparing the values of the peroxide number of olive oil without additives, and samples of olive oil with the addition of mint in different concentrations, more intense oxidative changes can be observed, given the increase in the value of the same. Observing the influence of the concentration of added mint essential oil, in the treatment in the dryer, differences in the values of peroxide number with the addition of 0.5% and 1.0% in relation to the added concentration of mint essential oil 2.0% were observed. In addition, after 12 h, there is a decrease in the value of the peroxide number (by adding 1.0% and 2.0%), when at a concentration of 1.0%, the peroxide number continues to decrease after 24 h, and when adding a concentration of 2.0%, it increases again. The consequence of such changes is the occurrence of oversaturation with the addition of higher concentrations of essential oil. When the values of the peroxide number are observed, in all samples with the addition of rosemary essential oil, in the treatment in the dryer, they stagnate after 12 h and the oil samples show marked stability. During the same treatment, after 24 h, the value of the peroxide number increases [12]. An exception was observed in the oil sample with added 2.0% of rosemary essential oil, when the value of peroxide number slightly increases, which leads to the conclusion that a higher concentration of essential oil (up to 1.0%) improves the oxidative stability of olive oil. Rosemary extract has strong antioxidant properties due to its phenolic hydroxyl groups [17]. The antioxidant properties of rosemary are mainly attributed to its main ingredients diterpenes, carnosol, and carnosic acid, as well as the components of the essential oil [18]. Due to its antioxidant and antimicrobial activity, rosemary essential oil can prolong the shelf life of food products and maintain their quality during storage [19, 20].

Observing the type of treatment, more intense changes in peroxide count values can be observed in samples after treatment under a UV lamp, compared to the Schaal Oven test. This can be explained by the influence of light and oxygen, since the analysis

was performed "outdoors", in contrast to the treatment in the dryer, where the influence of light and oxygen is minimized. If the concentration of added basil essential oil is observed, when treated under a UV lamp, after 24 h of treatment, the value of the peroxide number averaged 12.30 meq O_2/kg, which does not correspond to any of the mentioned provisions of the Ordinance [12, 13]. Such oil is designated as the oil in which the oxidative deterioration has occurred. The concentration of basil essential oil of 0.5% proved to be the most effective, followed by supersaturation (observed in the initial oil samples, at a concentration of 1.0% and 2.0%, and after 24 h of treatment). Also, it was noticed that with the addition of mint essential oil, after 12 h there is a decrease in the value of the peroxide number with the addition of a concentration of 1.0% and 2.0% when at a concentration of mint essential oil 1.0%, the peroxide number continues to decrease after 24 h and increase again at an added concentration of 2.0%. So, the value of the peroxide number increases with the increase of temperature and treatment time, after that decreases, so that with a longer processing time, small values of the peroxide number are obtained again. The reason for this is the decomposition of peroxide and hydroperoxide during the action of temperature, which results in a decrease in the value of this parameter. The decrease in the peroxide number is the result of the formation of secondary oxidation products. In this case, reducing the value of this parameter does not mean that the oil returns to its original properties, on the contrary, it means that the quality of the oil decreases. In the treatment under a UV lamp, the peroxide number, after 24 h, at all concentrations of mint essential oil, after 24 h, at a concentration of 2.0%, exceeded the values specified in the Ordinances [12, 13]. The values of the peroxide number, with the addition of rosemary essential oil, ranged on average up to 9.65 meq O_2/kg, which is close to the values of the Ordinances [12]. Peroxide number values, when treated under a UV lamp, with the addition of rosemary essential oil, after 24 h, for all concentrations are generally approximate, averaging 9 meq O_2/kg, with more intense changes after 24 h.

During the application of the Schaal Oven test (in an oven at 63 ± 2 °C) and treatment under a UV lamp, the influence of the concentration of added essential oil, as well as its type, on the value of the peroxide number was investigated (Table 1).

Table 1. Results of determination of peroxide number (meq O_2/kg) when applying Schaal Oven test and treatment under UV lamp

Control sample	2,55 ± 0,07		
Essential oil concentration	Type of essential oil	Schaal Oven test	Treatment under UV lamp
Concentration 0,5%	Basil	4.58 ± 0.52^{Bb}	10.29 ± 0.41^{Ba}
	Mint	6.83 ± 0.04^{Ba}	12.38 ± 0.34^{Ba}
	Rosemary	4.46 ± 0.06^{Bb}	8.42 ± 0.12^{Bb}

(*continued*)

Table 1. (*continued*)

Control sample	2,55 ± 0,07		
Essential oil concentration	Type of essential oil	Schaal Oven test	Treatment under UV lamp
Concentration 1%	Basil	4.39 ± 0.03^{Bb}	14.39 ± 0.04^{Aa}
	Mint	5.18 ± 1.08^{Ba}	13.80 ± 0.09^{Aa}
	Rosemary	4.90 ± 0.00^{Bb}	9.66 ± 0.35^{Ab}
Concentration 2%	Basil	4.36 ± 0.15^{Ab}	12.00 ± 1.41^{Ba}
	Mint	10.70 ± 0.42^{Aa}	11.36 ± 0.42^{Ba}
	Rosemary	2.99 ± 0.02^{Ab}	9.21 ± 0.29^{Bb}

***mean values marked with different lower case letters (a–c) differ significantly at all concentrations, in terms of the type of essential oil added
***mean values marked with different capital letters (A–B) differ significantly in all species, in terms of the concentration of added essential oil

The average values of the peroxide number, using the Schaal Oven test, ranged from 2.99 ± 0.02 (addition of rosemary 2.0%) to 10.70 ± 0.42 (addition of mint 2.0%). According to the table above, the oil with the addition of 2.0% of rosemary essential oil has the best oxidative stability and the worst has oil with the addition of 2.0% of mint essential oil. In this case, mint oil acted as a prooxidant. The Tukey test showed that the concentration of essential oil of 2.0% was significantly different (for the level of significance $\alpha = 0.05$) from the concentrations of 0.5% and 1.0%. Also, a statistically significant difference (for the level of significance $\alpha = 0.05$) was found in the value of the peroxide number in the oil with the addition of mint essential oil, compared to the addition of rosemary and basil essential oil. Mint essential oil differed significantly compared to the other two types of added essential oils [21, 22], which examined the effect of the addition of rosemary and oregano essential oil, as well as sweet and hot red spicy peppers, on the oxidative stability of virgin olive oil, state the influence of natural additives on antioxidant properties and sensory quality of virgin olive oil. The aim of the study was to determine the induction period of olive oil with and without the addition of oregano and rosemary extract stored at room temperature in a dark room after one year. The authors tested the addition of 2.0% oregano, rosemary, and garlic extract, monitoring changes in the stability of olive oil during storage at 37 °C until the peroxide number reached 70 meq O_2/kg. Based on the presented results, it can be seen that the samples enriched with natural additives show a longer induction period compared to pure olive oil, which confirms the positive effect of the additives on the sustainability of the oil. A longer induction period indicates better durability and stability of the oil. At the moment when the control sample reached the value of peroxide number 70 meq O_2/kg of oil, in the samples with the addition of rosemary extract and oregano the value of peroxide number was 2 to 4 times lower, which proves their efficiency in increasing oil stability. In the initial oxidation phase, oregano and rosemary with the addition of 2.0%, showed approximately the same antioxidant stability. However, it can be noticed

that after a long storage time, rosemary extract (2.0%) showed a higher efficiency. This is probably due to the higher content of non-volatile components in rosemary extract.

This explains why rosemary essential oil at a concentration of 2.0%, showed very good results in terms of stability of cold-pressed olive oil [23] examined the viability of cold-pressed hazelnut oil with the addition of various antioxidants. According to the results of Schaal Oven test, it can be seen that rosemary extract compared to other added antioxidants in different concentrations (green tea, pomegranate, olive leaf extract, and essential oil of oregano, sage) provided significantly better stability and sustainability of the oil, which manifested in a low peroxide value of 0.98 meq O_2/kg. Determination of the oxidative stability of hazelnut oil was performed by a four-day accelerated oxidation test, Schaal Oven test at 63 °C, and the oxidation result was expressed by the value of the peroxide number. The addition of natural antioxidants in concentrations of 0.1 and 0.3% rosemary extract (powder) best protects hazelnut oil from spoilage. The reason for this is that rosemary extract contains a large proportion of carnosic acid, which has an extremely high antioxidant activity, higher even than some synthetic antioxidants. It was also concluded that the addition of tested natural antioxidants at a concentration of 0.3% has a better effect on the stability of the oil against oxidative spoilage than the concentration of natural antioxidants 0.1%. This proves that a higher concentration of added antioxidant (rosemary essential oil) in most cases has a better effect on the stability of the oil.

Peroxide number values in all samples, using the treatment under a UV lamp, ranged from 8.42 ± 0.12 (oil sample with the addition of 0.5% rosemary essential oil) to 14.39 ± 0.04 (oil sample) with the addition of 1.0% basil. Observing the concentration factor, it was found that the concentration of essential oil of 1.0% was significantly (for the level of significance $\alpha = 0.05$) different from the concentrations of 0.5% and 2.0% of added essential oil, and the values of peroxide number in these concentrations are significantly different from the values at concentrations of 0.5% and 2.0%. Also, a statistically significant difference (for the level of significance $\alpha = 0.05$) was found in the values of the peroxide number between all types of essential oils except between the essential oil of mint and basil. The best stability of the oil sample was observed with the addition of rosemary essential oil at a concentration of 0.5%.

3.2 Results of Testing the Content of Free Fatty Acids Using the Schaal Oven Test and Treatment Under a UV Lamp

Observing the initial values of free fatty acid content, in relation to the initial values of oil samples to which different concentrations of rosemary and basil essential oils were added, the antioxidant effect of the same can be concluded, since a decrease in free fatty acid content was observed as an indicator of hydrolytic spoilage. This was observed with the addition of both types of oil, at all concentrations, except for the sample with added basil of 2.0%, which can be explained by the fact that the concentration of 2.0% has the opposite effect on oil stability, oversaturation, after which the value of free fatty acid content begins to decline. The addition of mint, in all concentrations, did not show significant antioxidant activity, due to the intense increase in the value of the content of free fatty acids. Free fatty acids in samples treated with the Schaal Oven test were analyzed in all initial samples before treatment and after 24 h of treatment. When the

values of free fatty acids, measured at the beginning and at the end of the treatment range from 0.06 ± 0.00 (initial analysis on a sample of olive oil with the addition of 0.5% basil essential oil) to 0.20 ± 0.04 (analysis after 24 h with the addition of 2.0% rosemary essential oil). The results show that no sudden increase in free fatty acid values was observed. It is interesting to note that the hydrolytic changes were reduced during the dryer treatment after 24 h (reduced or the same values of free fatty acid content). It can be concluded that the concentration of mint essential oil does not affect the value of free fatty acid content. The value of free fatty acids in all samples treated with the Schaal Oven test corresponds to the provisions of both, previously mentioned Ordinance [12, 13]. If the value of free fatty acids in the sample of olive oil without additives is observed, and after the addition of different concentrations of rosemary essential oil, a decrease in the value of free fatty acids is observed, and it can be said that the rosemary essential oil showed good antioxidant properties. As for the content of free fatty acids, the lowest values were obtained during the treatment in the dryer, after 24 h, with the addition of rosemary essential oil of 0.5 and 1.0%. According to the research [15], the content of free fatty acids, expressed as % oleic acid, ranges from 0.40 to 0.80%. [16] states the value of the acid number for samples of cold-pressed oils from 0.8 to 4.75 mg KOH/kg.

The content of free fatty acids, in the tested samples treated under a UV lamp, ranged from 0.06 ± 0.00 (initial oil sample with the addition of 0.5% basil) to 0.28 ± 0.00 (oil sample with the addition of 2.0% basil essential oil and oil with the addition of 1.0% mint essential oil, after 24 h of treatment). In general, there was no sudden increase in the value of free fatty acids, although it is more intense in the treatment under a UV lamp. Observing the initial values of free fatty acids in olive oil without additives, a decrease in the values of free fatty acids was observed, after the addition of different concentrations of mint essential oil. Hydrolytic changes are more intense during treatment under a UV lamp (increase in the value of free fatty acid content, after 24 h on average by 50%). It can be concluded that the concentration of mint essential oil does not affect the value of the content of free fatty acids, unlike the treatment (treatment under a UV lamp has a greater impact). The addition of 0.5% basil essential oil had a very positive effect on the stability of the oil in terms of hydrolytic spoilage. Mint essential oil, added in various concentrations and treatments acts as both a prooxidant and an antioxidant. The addition of rosemary essential oil at a concentration of 1.0% also preserved the stability of the oil samples. The value of free fatty acids, in all samples, corresponds to the provisions of both previously mentioned Ordinances [12, 13].

During the application of the Schaal Oven test (in an oven at 63 ± 2 °C) and treatment under a UV lamp, the influence of the concentration of added essential oil, as well as its type, on the value of free fatty acid content was examined (Table 2).

In summary, the value of free fatty acid content ranged from 0.11 ± 0.00, for most of the tested samples with the addition of different concentrations, up to 0.20 ± 0.04 for the oil sample with the addition of 2.0% of essential rosemary oil. The Tukey test showed that the concentration of essential oil of 2.0% was significantly different (for the level of significance $\alpha = 0.05$) from the concentrations of 0.5% and 1.0% of added essential oil. The values of free fatty acid content at a concentration of 2.0% differed significantly from the values at concentrations of 0.5% and 1.0%.

Table 2. Results of determination of free fatty acid content (%) when applying Schaal Oven test and treatment under UV lamp

Control sample	0,17 ± 0,00		
Essential oil concentration	Type of essential oil	Schaal Oven test	Treatment under UV lamp
Concentration 0,5%	Basil	0.11 ± 0.00^{Aa}	0.09 ± 0.04^{Aa}
	Mint	0.11 ± 0.00^{Ab}	0.26 ± 0.04^{Ab}
	Rosemary	0.17 ± 0.00^{Ac}	0.22 ± 0.00^{Ac}
Concentration 1%	Basil	0.11 ± 0.00^{Aa}	0.11 ± 0.00^{Aa}
	Mint	0.11 ± 0.00^{Ab}	0.11 ± 0.00^{Ab}
	Rosemary	0.17 ± 0.00^{Ac}	0.17 ± 0.00^{Ac}
Concentration 2%	Basil	0.17 ± 0.00^{Ba}	0.17 ± 0.00^{Ba}
	Mint	0.11 ± 0.00^{Bb}	0.11 ± 0.00^{Bb}
	Rosemary	0.20 ± 0.04^{Bc}	0.20 ± 0.04^{Bc}

***mean values marked with different lower case letters (a–c) differ significantly at all concentrations, in terms of the type of essential oil added
***mean values marked with different capital letters (A–B) differ significantly in all species, in terms of the concentration of added essential oil

Also, a statistically significant difference (for the level of significance $\alpha = 0.05$) was found in the value of free fatty acid content between all three types of added essential oil (rosemary, basil, mint) [24] examined the viability of vegetable oil with the addition of natural antioxidants using the Schaal Oven test. The largest change and the highest values of acid grade and acid number are shown by samples with the addition of antioxidant mint and refined sunflower oil. After every 24 h, the amount of free fatty acids in the sample with mint was further increased by 0.25 to 0.29%. It was concluded that the added antioxidant has a positive effect on oxidative changes in the first 72 h, after which accelerated oxidation occurs. When it comes to testing hydrolytic changes, by monitoring the parameters of free fatty acids expressed as % oleic acid, it was concluded that the value of free fatty acids is more intense with the addition of mint essential oil, as opposed to basil and rosemary essential oil, as evidenced by previous work. It is important to note that refined sunflower oil does not have such good stability, unlike cold-pressed oil, especially olive oil. Extra virgin olive oil is known for its high content of oleic acid, polyphenols, and vitamin E [25]. It is known that essential oils are composed of numerous lipophilic and highly volatile constituents obtained from a large number of different chemical classes, which are subject to translation and degradation reactions. Oxidation and polymerization processes can result in a loss of quality of essential oils. There are different degradation pathways of essential oils exposed to external parameters. In particular, it is recognized that temperature, light, and oxygen availability have a crucial impact on the integrity of the essential oil [26]. This explains the fact of the action of mint essential oil as a prooxidant, at certain concentrations and treatments.

In the UV lamp treatment, the lowest value of free fatty acid content was in the oil sample with the addition of 0.5% basil essential oil (0.09 ± 0.04), and the highest in the sample with the addition of 2.0% rosemary essential oil (0.20 ± 0.04). Tukey test showed that the concentration of essential oil of 2.0% was significantly different (for the level of significance $\alpha = 0.05$) from the concentrations of 0.5% and 1.0% of added essential oil, and the values of free fatty acids at the indicated concentration, was significantly different from the values at concentrations of 0.5% and 1.0%. Also, a statistically significant difference (for the level of significance $\alpha = 0.05$) was found in the values of free fatty acid content for all three types of added essential oil (rosemary, basil, mint). According to [27], small variations in free fatty acid content were observed in all treated samples (extra virgin olive oil with the addition of 0.1 and 0.4% essential oil of mint, rosemary, and basil). The values of the content of free fatty acids in the oil samples with the addition of aromatic herbs and olive leaf extract showed a significant increase after a 10-min-treatment in the microwave oven, which was caused by the appearance of hydrolytic enzymes.

4 Conclusions

The tested parameters (peroxide number and free fatty acids) are very important for the oxidative stability of the oil, and the obtained initial values for determining these two parameters must be within the limits prescribed by law for this type of oil. The results of the initial analysis have corresponding values, and the amounts for the peroxide number in cold-pressed olive oil are 2.55 meq O_2/kg, and for free fatty acids 0.17%.

The addition of rosemary, mint, and basil essential oils was aimed to improve the stability of the oil, acting as antioxidants. Statistical analysis of the data showed the influence of the concentration and type of added essential oil on the values of peroxide number and the content of free fatty acids. The addition of rosemary and basil essential oil in various concentrations leads to a decrease in the content of peroxide number and the content of free fatty acids. A concentration of 2.0% of basil essential oil, has the opposite effect on the stability of the oil and is considered unacceptable, it leads to oversaturation. Mint essential oil, in different concentrations and different treatments, acts both as a prooxidant and as an antioxidant. The effects of elevated temperature (temperature 63 ± 2 °C) and UV radiation do not have an intense effect on hydrolytic, unlike oxidative oil spoilage. The most favorable antioxidant effect was shown by the addition of rosemary essential oil, in all added concentrations. The addition of different essential types of oils and different concentrations, in extra virgin olive oil and many other cold-pressed vegetable oils, produced by an identical technological process, by cold squeezing and/or pressing, give the possibility of longer storage in appropriate packaging and storage conditions from the envisaged period of 12 months. On the other hand, essential oils, in this case, the base, olive oil give appropriate antioxidant properties and sustainability, as well as acceptability from the aspect of its sensory properties. Today, there is growing consumer interest in flavored olive oil and many other flavored vegetable oils, which have significant application not only from the aspect of nutritional values but also from a health aspect, which primarily refers to the antioxidant properties of the oil.

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Biosynthesis of ZnO Nanoparticles and Their Application in Food Production as an Antimicrobial Reagent

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Abstract. There are many ways of ZnO nanoparticles synthesis that are based on chemical and physical methods. Such methods usually require non-ecological chemicals, high pressures and temperatures and high costs. These days, attention is drawn to biosynthesis, which utilises natural plant extracts (various parts of plants, fruit, seeds) and/or microorganisms. The aim is to prevent the use of toxic chemicals and reduce energy consumption, and biosythesis as a natural approach can be described as a simple, quick and safe method for obtaining nanoparticles. Today, special attention is focused on the incorporation of nanoparticles with antimicrobial action in packaging. The main goal was to biosynthesise ZnO nanoparticles from pineapple and orange, and to confirm their antimicrobial activity against Gram-negative *Escherichia coli* and *Salmonella spp.* The synthesis of ZnO nanoparticles was carried out using naturally occuring reducing agents from the fruit extract (pineapple, orange). Zinc chloride was used as a source of Zn ions. Biosynthesized ZnO nanoparticles are characterized by UV-Vis spectroscopy. On the obtained ZnO nanoparticles, the antimicrobial activity towards pathogenic organisms was examined using the disc diffusion method. Biosynthetic ZnO nanoparticles have shown antibacterial action against *Salmonella* compared to *Escherichia coli* with an average inhibition zone of 26 mm. The conclusion is that nanoparticles of ZnO were successfully biosynthesized using these fruit extracts and they has also been proven to show antibacterial action against the *Salmonella* strain, but this is not the same case when it comes to *E. Coli.*

Keywords: ZnO nanoparticles · *Ananas comosus* · Orange · Extract · UV-vis · Antibacterial action

1 Introduction

Nanomaterials in comparison to the same materials in macro and micro dimensions have different catalytic properties, thermal conductivity, nonlinear optical properties and chemical stability due to the large ratio of surface to volume. [1] Zinc oxide nanoparticles are the focus of research due to their wide application, unique properties, permeability

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and high binding energy of exitons [2]. An exiton is a neutral quasi-particle composed of one electron and one cavity connected by Coulomb force. It is known that they exhibit potential antimicrobial, antifungal, antidiabetic and antioxidant properties. The peculiarity of zinc oxide nanoparticles is morphological diversity, which is the basis of their diverse use [3]. Zinc oxide nanoparticles have a potential application in many fields such as biosensors, nanomedicines and bionanotechnology, and use in solar energy conversion, chemical sensors, transparent coatings, light photocatalysis, etc.

The methods used for nanoparticle synthesis are: physical and chemical methods and green synthesis/biosynthesis. Along with the research of metal nanoparticles, a large number of chemical and physical methods for their synthesis have been developed. The choice of method depends on the material, the necessary physical and chemical properties of the nanoparticles (surface modification and charge), as well as their size and shape. There are two methods for the synthesis of nanoparticles, the top-down based on reducing the size of the macroscopic crystal to micron or nanometer dimensions and the bottom-up which involves methods such as chemical or electrochemical reduction of metal precursor salts to metal nanoparticles and represents the aggregation of metal atoms to nanometer-sized particles while controlling the synthesis conditions [4].

Various plant metabolites including terpenoids, polyphenols, sugars, alkaloids, phenolic acids and proteins, play an important role in the bioreduction of metal ions, creating nanoparticles [5]. Due to the phytochemicals produced by plants they are used for the synthesis of zinc oxide nanoparticles and are considered to be the best reagents for obtaining various metal nanoparticles in the field of green synthesis [6]. Such phytochemicals are also present in pineapple and orange fruits. Green synthesis involves three main steps, namely: the selection of an acceptable solvent, an environmentally harmless reducing agent, and the selection of non-toxic substances for nanoparticle stabilization [7, 8]. Phytochemicals, which contain natural materials, play the most important role because they act as reducing agents and as stabilizing agents. Zinc ions in a solution of a natural extract create a complex with polyphenols (or other phytochemicals) in the form of Zn2+. This is followed by the formation of zinc hydroxide (Zn(OH)2) by hydrolysis and finally after calcination, the complex decays, favoring the formation of ZnO nanoparticles [9].

The mechanism of metal nanoparticle synthesis from plants and plant extracts includes three main phases:

1. the activation phase during which the metal ions are reduced and the metal reduced atoms are nucleated
2. the growth phase in which small adjacent nanoparticles spontaneously merge into larger particles (direct creation of nanoparticles by heterogeneous nucleation and growth, and further reduction of metal ions; a process called Ostwald maturation), which is accompanied by an increase in thermodynamic stability of nanoparticles and
3. the process completion phase that determines the final shape of the nanoparticles [5].

In this research orange and pineapple extracts have been used as a cheap and readily available potential source of necessary phytochemicals.

2 Material and Methods

2.1 Materials

All the chemicals were acquired from commercial sources and used without further purification.

The pineapples and oranges were purchased at a local market in Sarajevo, Bosnia and Herzegovina. A zinc chloride solution (0.25 M) was used as a source of zinc ions and distilled water was used for solution and extract preparation.

2.2 Extract Preparation

Chunks of peeled pineapple fruit were washed in distilled water and homogenized using a pestle and mortar. The juice was separated using gravitational filtration. Two samples of 50 mL were used for further synthesis. The sample volume was doubled with distilled water. The extracts were then incubated for 10 min at 40 °C. Clear extracts were obtained after filtering through a Whatman white ribbon filter paper and stored at 4 °C until further use.

Orange extracts were obtained by separating the squeezed juice from the pulp through plain filter paper. After setting the pH to 4–5 the extract was pasteurized at 80 °C for 10 min. Two 50 mL samples were doubled in volume with distilled water and heated to 40 °C. These were further used for nanoparticle synthesis.

2.3 ZnO Nanoparticle Biosynthesis

The zinc chloride solution was mixed with the fruit extract in a 1:3 ratio. In the case of orange extracts, special attention should be paid to the pH level of the reaction mixture. pH levels lower than 6 can delay the process of nanoparticle formation, and higher values have been found to lead to unwanted metalation.

The reaction mixtures were heated until a white precipitate or cloudiness could be seen, which are attributed to the presence of ZnO nanoparticles.

3 Results and Discussion

3.1 Nanoparticle Characterization

UV-Vis spectroscopic analysis was carried out using the Cary/1E/ spectrometer. The spectra were acquired in the 800–280 nm range, using the juice extracts as blank probes.

The nanoparticles obtained using pineapple extracts displayed absorption maxima at 361 and 358 nm respectively, while ZnO nanoparticles obtained from two orange extract samples showed an absorption maximum at 360 nm (Fig. 1).

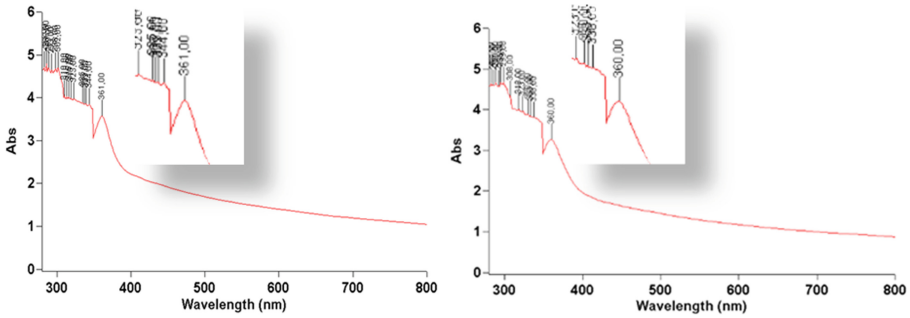

Fig. 1. The results of UV-Vis spectroscopy for ZnO nanoparticles obtained from pineapple (left) and orange (right)

3.2 Antimicrobial Activity

One of the objectives of the study was to determine the antimicrobial potential of nanoparticles from pineapple and orange extract for the growth of G-bacteria from the family *Enterobacteriaceae*, with a comparative analysis of nanoparticles. A comparative analysis of the obtained results with standard values of antibiotics Gentamicin for the bacteria *Escherichia coli* and *Salmonella spp.* Nanoparticles produced from both extracts have higher antibacterial activity towards Salmonella, which may be due to slower reproduction compared to *E. Coli.* The bacterium *E.coli* showed marked stability, in which case the nanoparticles did not show any antibacterial activity against this bacterium but indicated effective action against the strain *Salmonella spp.*.

Interpretation of results was performed according to EUCAST standards where the action of antibiotics/nanoparticles is classified into 3 categories:

- S – sensitive - the likelihood of success of therapy is high after the use of normal doses of antibiotics, given in the usual way;
- I - intermediate (moderately) sensitive-may success of therapy if the antibiotic is given in maximum concentrations and in a parenteral way;

Table 1. Antibiogram for Escherichia coli and Salmonella spp.

The diameter of the inhibition zone Escherichia coli			
Category	S	I	R
Gentamicin	≥15	13–14	≤12
The diameter of the inhibition zone Salmonella spp.			
Category	S	I	R
Gentamicin	≥17	13–16	≤14

- R – resistant - never used in therapy; regardless of the dose, therapy is very likely unsuccessful (Table 1)

Table 2. Measured diameters of inhibition zones for *E.coli* and *Salmonella spp.* from pineapple and orange extract

The diameter of zone of inhibition (mm)	Pineapple nanoparticles *Salmonella spp.*	Orange nanoparticles *Salmonella spp.*	Pineapple and orange nanoparticles *Escherichia coli*
Disk 1	27	24	/
Disk 2	28	25	/
Disk 3	26	18	/
Disk 4	25	25	/
Average value	**26.5**	**23**	/

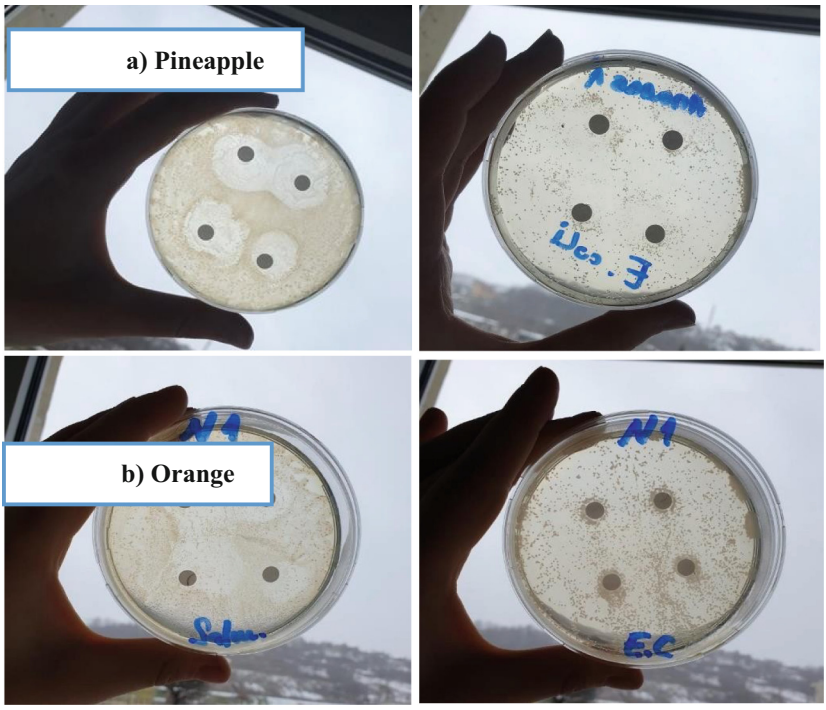

Fig. 2. Results of the action of nanoparticle suspensions on Salmonella spp. and E.Coli

Based on these results, we can conclude that nanoparticles obtained from pineapple and orange extracts have a greater antibacterial effect on *Salmonella spp.* with an

average inhibition zone diameter of 26.5 mm for nanoparticles obtained from pineapple and 23 mm for nanoparticles obtained from orange, while the same nanoparticles from pineapple and orange extract did not show antimicrobial activity towards *E.coli* because no inhibition zone has formed. When we compare the stated average values with the standard values of the antibiotic Gentamycin, which can be seen in Table 2, it can be observed that nanoparticles have an inhibitory effect only on *Salmonella spp.*, we classify these tested strains as S-sensitive, which means that the probability of success with therapy is high, when using the usual doses of nanoparticle extract (Figs. 2 and 3).

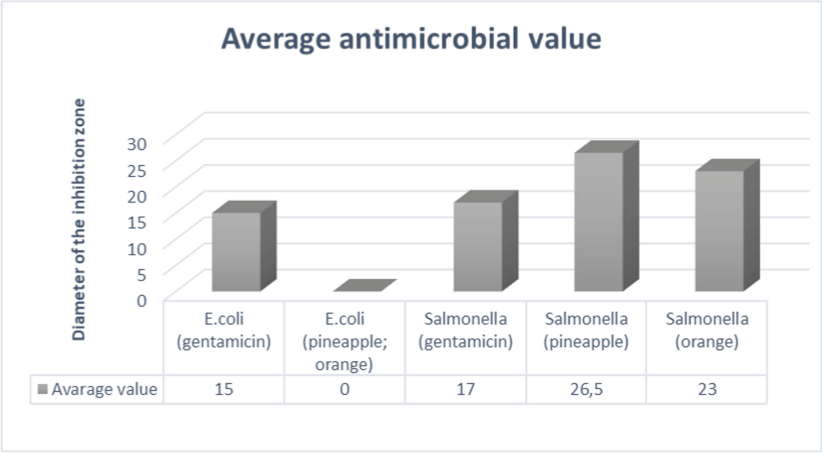

Fig. 3. Antibacterial activity synthesized nanoparticles compared with the antibiotic Gentamicin

4 Conclusion

In this study, ZnO nanoparticles were successfully prepared by the method of green synthesis, using pineapple and orange extracts that showed interesting properties. Prepared ZnO nanoparticles showed high UV absorption. The disc diffusion method test confirmed that nanoparticles obtained by pineapple and orange biosynthesis act as inhibitors to *Salmonella* growth, with an average inhibition zone value of 26 mm for pineapple and 23 nm for orange. In contrast, *E. Coli* was more resistant and did not show sensitivity to ZnO nanoparticles, and based on this, no inhibition zone was formed. By comparing the average diameter of the inhibition zone with the standard values of the antibiotics Gentamycin for *E.coli* and *Salmonella spp.*, ZnO nanoparticles showed stronger inhibitory activity, when it comes to Salmonella, than Gentamicin itself. Gram-negative bacterium that showed strong sensitivity (*Salmonella spp.*) to ZnO nanoparticles and as such is classified in the S-sensitive category. The results of this research have shown that biosynthesized ZnO nanoparticles have a promising potential in medical use, food packaging and industrial application as well as alternatives to chemical compounds.

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Biosynthesis of ZnO Nanoparticles from Basil Extract and Their Antimicrobial Activity

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Abstract. In recent years, new ways to synthesise ZnO nanoparticles have been studied. The goal is to improve upon usual chemical and physical methods which require non-ecological chemicals, high pressures and temperatures as well as high costs. Nanoparticles synthesized via biological methods using microorganisms, enzymes, and plant extracts have been suggested as a more eco-friendly alternative to usual industrial methods. The aim is to prevent the use of toxic chemicals and reduce energy consumption, and biosythesis as a natural approach can be described as a simple, quick and safe method for obtaining nanoparticles. Zinc Oxide is an inorganic metal oxide and has the property of exhibiting a wide range of nanostructures. The photo oxidizing and photocatalytic properties are used against biological and chemical species, which are used to characterize bio-synthesized metal oxides. The nanostructured and highly stable zinc oxide nanoparticles are produced using zinc acetate and *Ocimum Basilicum* leaf extract. For this paper, the environmentally benign leaf extract of *Ocimum Basilicum* was chosen as a source of phytochemicals, primarily due to its numerous medicinal properties. Nanoparticle formation has been confirmed via UV/Vis spectroscopy, while the antimicrobial activity has been characterised using the disc diffusion method.

Keywords: ZnO nanoparticles · *Ocimum Basilicum* · UV-vis · Antimicrobial activity

1 Introduction

Nanotechnology is the art and science of manipulating nanoscale materials to create new and unique materials and products with great potential that can have a significant impact on society [1, 2]. Nanoparticles have a wide range of applications. Nanotechnology is the molecular production or creation of things and objects at the atomic level, grouping individual atoms and molecules, which even more simply means precisely "placing atoms in the right place" [3].

The constant advancement of technology has encouraged the development and application of new products in the food industry, which bring new challenges for food safety. As the demands on the use of chemicals become louder, due to their harmfulness to

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human health, alternatives are being sought to avoid the use of chemicals, them mainly being natural materials. For this reason, the growing interest in green chemistry, which is based on minimizing waste, avoiding the use of hazardous substances, has led to the development of a method that is environmentally friendly, and that is green synthesis. Nanotechnology based on the synthesis of nanoparticles with the help of plants, i.e. plant extracts is gaining increasing importance and interest among researchers. Nanoparticle biosynthesis from plant extracts is an excellent, cheap, simple, harmless, but also environmentally friendly alternative to common methods that require the use of chemicals, most of which are dangerous and harmful to human, animal or plant health.

Chemical and physical methods result in clean and well-formed nanoparticles, however the chemicals used in the synthesis are toxic, energy consuming, expensive and unsuitable for biological use [4]. Compared to conventional synthesis methods, green synthesis has shown many advantages such as simplicity, speed, economy and most importantly, safety. Green synthesis, i.e. biosynthesis includes three main steps that are in accordance with the principles of green chemistry, and they are:

1. selection of an acceptable, harmless solvent,
2. selection of biodegradable agents for metal ion reduction and
3. selection of non-toxic substances that will prevent the so-called adhesion of nanoparticles and thus ensure their stabilization.

Medicinal and spice plants contain various phytochemicals that are suitable for the synthesis of nanoparticles of metal compounds. Phytochemicals from plants act on the formation of metal oxides from metals, and also by their action metal ions reach the phase of growth and stabilization. Phytochemicals are antioxidants, so they can act as a reducing and stabilizing agent [5]. Nanoparticles synthesized in a green way show better antibacterial effects due to functional groups that come from phytochemicals [6].

Recent research has shown the importance of green synthesis of metal oxide nanoparticles where metal oxides of zinc, gold, silver, copper, etc. are isolated [7–9]. Of the various metal oxides, zinc oxide (ZnO) nanoparticles show high electron mobility, high exciton binding energy, wide bandwidth, and high optical permeability [3, 10]. These optical properties of zinc oxide are used in the manufacture of sensors [11–13]. Due to the increasing resistance of microorganisms to existing antimicrobial agents or antibiotics, as well as due to the development of increasingly resistant cultures, a large number of scientific research is focused on finding new antimicrobial materials. Research on metal nanoparticles and their oxides has intensified in recent years, which has contributed to the application of these materials for antimicrobial agents in medicine, agriculture, industry, wastewater treatment, and in the food industry. The main connection of nanotechnology with agriculture and food industry is the improvement of food processing and safety, the ability of plants to absorb nutrients, the detection of pathogens in food, food functionality, environmental protection [14]. Protecting food from microbiological contamination is the most important step in food packaging, and ZnO nanoparticles have become the subject of research and are increasingly being used as an antibacterial agent in the food packaging process. ZnO nanoparticles are included in a number of food coatings in packaging to avoid food spoilage [15]. Nanotechnology can have a strong

positive effect on the food industry by providing it with stronger food packaging materials with high barriers to microorganisms, stronger antimicrobial agents and sensors that can detect trace contaminants, gases or microorganisms in packaged food, and it is also possible to monitor food during storage and transport. Active food packaging is a new approach that replaces conventional ways of food packaging, creates an effective antimicrobial effect on food and thus prevents spoilage. The release of nanoparticles that act bacteriostatically or as bactericidal agents on the surface of food where bacteria reside, stops growth and thus prevents food spoilage [16]. This type of active packaging is also called antimicrobial packaging, where there is a direct interaction between products and nanoparticles, which leads to death or inhibition of bacterial growth on the food surface [17].

2 Materials and Methods

2.1 Materials

All the chemicals were acquired from commercial sources and used without further purification.

Dried basil was purchased at a local market. As a source of zinc ions, a 0.2 M solution of zinc acetate was used. The solution was prepared using deionized water.

2.2 Extract Preparation

The plant extract was prepared by heating 10,02 g of dried basil in 100 ml of deionized water for 2 h while the temperature was maintained at 80 °C. The extract was then cooled to room temperature. The extract was first filtered through a qualitative filter paper to remove the big particles of plant material. The filtrate was then re-filtered through a Whatman Blue Ribbon filter paper to obtain a clear extract.

2.3 ZnO Nanoparticle Biosynthesis

To obtain nanoparticles, the plant extract was mixed with a solution of zinc acetate at room temperature. 50 ml of basil plant extract and 115 ml of the zinc acetate solution were used. By adding the zinc acetate solution to the plant extract, it was immediately observed that zinc oxide nanoparticles appeared. The color of the mixed plant extract and solution remained brown, no discoloration occurred, but white zinc oxide nanoparticles were immediately observed.

3 Results and Discussion

3.1 Nanoparticle Characterization

UV-Vis spectroscopic analysis was carried out using the Cary/1E/ spectrometer. The spectra were acquired in the 800–280 nm range, using the plant extract as a blank probe (Fig. 1).

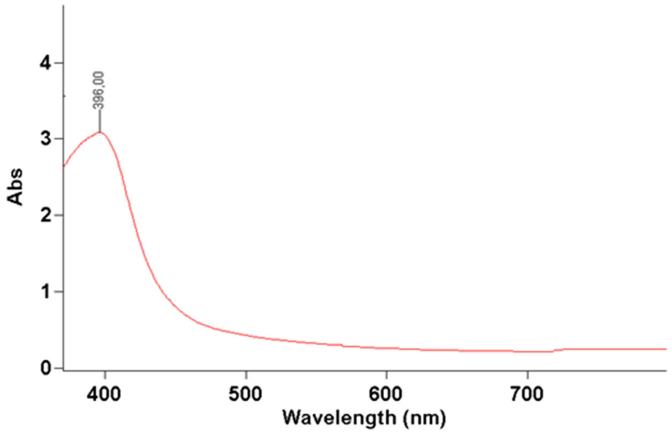

Fig. 1. The results of UV-Vis spectroscopy for ZnO nanoparticles obtained from the basil extract

The peak absorption of zinc oxide nanoparticles from basil extract was at 396 nm which was taken as a confirmation of ZnO nanoparticle synthesis.

3.2 Antimicrobial Activity

The antibacterial activity of zinc oxide nanoparticles in this study was examined on *Enterobacteriaceae* (*Esherichia coli* and *Salmonella spp.*). In order to examine and analyze the antimicrobial activity of the obtained zinc oxide nanoparticles, the diffusion method or the method of testing the diffusion antibiogram was used. The disk diffusion method implies the principle of placing antimicrobial impregnated paper discs on the surface of the nutrient agar which has previously been sown with the bacteria being tested. Examination of the antimicrobial effect of basil extract obtained nanoparticles on the growth of Gram-negative bacteria was performed on a Mueller-Hinton nutrient medium.

The paper discs are soaked in the nanoparticle suspension and placed on the surface of a nutrient medium previously inoculated with bacterial culture. The discs are sterilized and prepared, and with the help of sterile tweezers they are immersed in the nanoparticle suspention and properly arranged in Petri dishes, 6 discs at a distance of 15 to 24 cm from each other. Petri dishes were incubated for 24 h at 37 °C (Table 1 and Fig. 2).

Table 1. Measured diameters of inhibition zones for *E.coli* and *Salmonella spp.* for ZnO nanoparticles obtained from the basil extract

Inhibition zone radius (mm)	*Esherichia coli*	*Salmonella spp.*
Disc 1	11	28
Disc 2	15	26

(*continued*)

Table 1. (*continued*)

Inhibition zone radius (mm)	*Eshericha coli*	*Salmonella spp.*
Disc 3	10	30
Disc 4	22	26
Disc 5	25	25
Disc 6	15	23
Average radius	16.33	26.33

Fig. 2. Results of the action of the nanoparticle suspension on Salmonella ssp. and E.Coli

There is a lack of data on the results of the antimicrobial action of ZnO nanoparticles obtained by biosynthesis from basil extract on pure cultures, and especially not those performed by diffusion test. In a study by Wahab et al. [18], it was shown that increasing the concentration of ZnO nanoparticles in pits or discs increases the inhibition of microorganism growth. They showed in the study that the effectiveness of the inhibition zone of ZnO nanoparticles largely depends on the selected concentration, size and shape. In addition, we concluded that the size of the inhibition zone is influenced by various factors such as bacterial type, size, and concentration of ZnO nanoparticles. The antibacterial activity of ZnO powders and nanoparticles has been effectively studied against some multidrug-resistant pathogens such as *Staphylococcus aureus* and *Escherichia coli* [16, 19]. In a study by Liu et al. [2] found that ZnO nanoparticles have antibacterial activity against *E. Coli* 0157: H7. They also state that inhibitory effects increase with increasing zinc oxide (ZnO NP) nanoparticles. The results indicate that ZnO NP can distort and damage the bacterial cell membrane, resulting in leakage of intracellular contents and ultimately bacterial cell death.

4 Conclusion

In this study, ZnO nanoparticles were successfully prepared by the method of green synthesis, i.e. biosynthesis using basil extract. The prepared ZnO nanoparticles showed high UV absorption. The antimicrobial effect of ZnO nanoparticles was confirmed by the disk diffusion method or the diffusion method test. ZnO nanoparticles obtained by biosynthesis from basil extract showed a larger zone of inhibition, ie a stronger antimicrobial effect on bacteria of the genus *Salmonella spp*, compared to the bacterial species *E. Coli.* The diffusion test was used to determine the average zone of inhibition for *Salmonella spp.* which was 26.33 mm, and for Echerichia colli 16.33 mm. From this it can be concluded that the synthesis of target nanoparticles, in this case the biosynthesis of zinc oxide nanoparticles has a significant potential and a number of significant advantages over traditional methods of nanoparticle synthesis.

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BOOST BY PANDEMIC: Family Farms on the Path of Innovations

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Abstract. The COVID-19 pandemic has caused adverse changes in the operations of the family farms. As the primary actors of the agricultural sector, they met several challenges such as closure of business cooperation with the HoReCa distribution chains, labour shortages, closure of primary sales channels, and inadequate storage of fast-perishable products. One of the main strategies for survival and stability, family farmers has introduced innovations in their family business operations. The results of the study carried out on the sample of 16 family farmers in the first wave of pandemic, showed that the technological and social innovations, followed by institutional innovations were the primary survival strategies implemented by the family farms to tackle the obstacles imposed by the pandemic.

Keywords: Pandemic · Family farms · Innovations · Strategies

1 Family Farms on the Hit of Pandemic

Family farms are the largest part of the world's food industry being the main actor of the food supply chain and a significant investor in the agriculture sector (FAO, IFAD 2019). COVID-19 pandemic caused an immense impact on the operation of family farms in terms of loss of primary distribution channels (open air market), due to introduction of lockdown measures, termination contracts with commercial sales channels (schools, kindergartens and public sector), due to social distancing measures and lack of adequate storage facilities for fast-perishable products such as fruit, vegetables, meat and milk products (Brady et al. 2020) as a consequence of transportation delays. On the other hand, pandemic have resulted in improvements in food processing and product marketing according to environmental and social characteristics, such as lower production units, switch towards organic production and short supply chain development (Cavalli et al. 2020). Developed countries have turned focus towards local producers, social media became foundations for building new producer-customer relationships, farmers have discovered advantage of the direct marketing followed by newly introduced delivery services while unexpected shortage of seasonal labour motivated the local population to provide voluntary support to farmers in need (Darnhofer 2020).

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In Croatia, primary holders of agricultural production are family farms where jobs are performed mainly by family members (Ministry of Agriculture 2019). According to the Paying Agency for Agriculture, Fisheries and Rural Development (2020) out of 162 966 registered farmers in 2019, there are 96,8% of family farms, 60% from which are crop producers. However, more prominent impact of the pandemic was observed on financial structure of those family farms whose distribution is dependent on tourism sector, those with exclusively direct sales distribution, and those engaged in the production of more luxurious products (artisanal cheese, olive oil, marmalades, wine). As a result, these family farms tried to sustain on the newly emerging market by introducing survival strategies from which innovations were the most evident one.

Family farms' innovations are crucial factors of technological and institutional transformations, which could affect production, sustainability, and poverty reduction (Röling 2009; Dogliotti et al. 2014) and moreover, could direct the changes of market models, through niches and new opportunities in crisis such as COVID-19 pandemic. Innovation strategies are also one of the primary tactics in building resilience in time of unpredictability that arise from capacity to mitigate the crisis, to adjust to crisis and from the ability to change (Béné et al. 2012; Davoudi et al. 2013; Keck and Skadapolrak 2013; Darnhofer 2014). Based on French et al. (2014) there are three main types of agricultural innovation - institutional innovation, technological innovation, and social innovation. In the time of uncertainty, such as COVID-19 pandemic showed, farmers were pushed to find, implement, and adjust innovation strategies into their traditional business operations to preserve their economic stability and market competitiveness. The research objective was to determine which innovation strategies family farms implemented in their business operations as a respond to the COVID-19 pandemic.

2 Methodology

The data for this qualitative research were collected by using a semi-structured interview on a deliberate non-probabilistic sample comprised of 16 family farm owners. In the overall number of participants, majority were males (69 percent). Regarding the age and educational structure, our participants were somewhat younger and more educated than the average farmer in Croatia. There were only 13% of young farmers (under 40) and 40% of those older than 65 on the national level comparing to 19% of young and 19% of older than 65 in our sample. More than half of the participants finished high school education, while around 37.5% of them possess university degree, comparing with 7% of farmers who obtained university degree based on national data set. For 66.7% of the participants, agricultural production is the primary source of their income. The sampling strategy was threefold, to include farmers focused on direct sales channels, to include heterogenous farmers due to gender, age, agricultural production and geographic location, and also to include some of the most agile Croatian family farms from pre-pandemic time, in order to show how some of the progressive farmers coped with the pandemic-driven need to adapt and which innovations they manage to implement. The interviews with farmers were conducted in October of 2020 and their focus was on the impact of the first wave of pandemic on their family farms' operations.

3 Type of Innovation as a Respond to Pandemic

Family farmers have made improvements in the various fields of their business operations and thereby adapted their production strategies in order to remain on the market under new pandemic circumstances. The variety of changes implemented is seen as a diversification of agriculture that is not limited to the introduction of processing operations or new cultures but also in the implementation of new services and marketing activities as a sign of a multi-functionality of agriculture. The adjustments to lockdown conditions introduced by the family farms have been effective, ensuring continual agricultural productivity and attracting new or retaining old customers. The findings depicting family farms' response on the pandemic conditions and the innovation strategies they adopted while coping with pandemic uncertainty will be discussed below.

3.1 Institutional Help to Farmers

Institutional innovations usually include the change of policy, norms, legislation, procedures, agreements, models, organizational strategies, administrative practices, and interactions with other organizations, in order to develop a more diverse atmosphere and enable an organization to enhance its efficiency and make it more collaborative and competitive (French et al. 2014). To alleviate the negative economic consequences caused by pandemic, Croatian government introduced a new institutional policy and organizational strategy at the national and local level. Several measures were implemented from Croatian government to protect employment and liquidity such as "COVID-19 loans" and "Rural development micro-loans" for small-economy operators in agricultural, processing and forestry sectors (Government of the Republic of Croatia 2020). For producers of the dairy sector, government has implemented the exceptional aid measure for the withdrawal, purchase of dairy products and free distribution to organizations in need such as homeless shelter and humanitarian associations (Ministry of Agriculture 2020a). Special assistance measures have also been introduced for the livestock producers (Ministry of Agriculture 2020b), the producers in fishing sector (Ministry of Agriculture 2020c), and for viticulture and winemaking producers (Ministry of Agriculture 2020d). Government created the informational web platform for an enquiry about cooperation problems, exceptional allocations and measures as same as for production and distribution of the products that farmers faced during pandemic (Ministry of Agriculture 2020e). One of the most meaningful innovations was the implementation of online platform "Tržnica.hr" which provided free access to family farmers all over Croatia with a benefit of free marketing and distribution of products (Ministry of Agriculture 2020f). At the local level, it is noted that the various counties and smaller towns organized local family farms on the online platforms with an aim of free promotion, sales and distribution of products. Many towns have introduced the local delivery services of family farms products with an aim of reaching the fragile parts of local community such as elderly and people with serious health conditions. One of the noteworthy innovations was mobile application which gathered local family farm producers and provided a new way of distribution as a useful alternative in times of closure of the open-air markets and farmers' fairs.

3.2 Diversification Through Technological Innovations

The product improvements and increase in efficiency of processes are usually related to technological innovations. They include growth, manufacture and sale, reorganization, or improvement of production processes as well as significant improvement of the operation through innovations, technical knowledge and/or technical practices (French et al. 2014). Our findings have shown that almost all family farms have expanded their product range as the result of the intensified reviews and feedbacks from the consumers. For instance, the introductions of sheep yogurt and chocolate berries have proved to be very successful business innovations that received a large supporting feedback from customers. Another farmer's idea was introduction of the new crops such as beans that arose from the decrease in demand for the luxury products such as blackberry wine, being farmer's main source of income in the pre-pandemic period. The other family farm started to observe the consumers' trends and offered products that are in increased demand outside of the local season production (e. g. strawberries). It was evident that some family farms introduced the changes in the packaging as consumers adjust their consumption habits towards more diverse and smaller amounts of their orders. Plentiful technological innovations were introduced together with the implementation of new promotion instruments (educational workshops about organic honey inhalations, free goat milk cosmetics samples) thus showing the creative potential of diversification. Significant technological innovation appeared in the case of the farmer who started a mobile store, and thus changed his business operations in the longer term. Most of the participants launched sales and introduced the communication with consumers via online platforms. More than half of them stated that the pandemic has motivated them to devote more time to the advertising of products. To engage in social networks, farmers needed to master skills for frequent online communication. Although they expressed that they were overwhelmed by "computer-time" at the beginning, they *"became more comfortable with usage of the modern language"*. Most participants engaged in sponsorships of different actions, rewarded their consumers, or invested effort in organizing entertaining content such as interactive quizzes.

Our findings have also shown that some family farms started to invest more effort in the attractive appearance of products, noticing that type of packaging played an important role in consumers' choices. Finally, almost all participants introduced door-to-door delivery to their customers and thus widen their services corresponding to pandemic context. At the same time, those deliveries could be considered as not only technical but also as social innovation.

3.3 Toward Changing Social Relations

Social innovations are considered as an enhancement of strategies, concepts, organizations, commodities, or services with a purpose of creating meaningful changes to address or meet social needs. They are established with an ambition of strengthening life quality by creating jobs, consumption, and participation (French et al. 2014). In our research we found several elements in farmers' approach that imply novelty in social arrangements, and even though it could be discussed whether it is a social innovation per se, we argue that those arrangements present a social novelty in those farmers' businesses which are

provoked by pandemic circumstances, and as such should be justifiably considered as, at least, a step towards higher quality of social relations and social innovation way of thinking.

Our findings have shown an improved relationship quality of producers and consumers, as well as those of employers and employees. Some of the family farms involved new employees from the local rural community; others collaborated with media, the local tourist boards, or local government. There has been a considerable increase of the new partnerships, local as well as international ones. Farmers offered information and services, rewarded faithful customers with free products and largely adapted to the individual needs of customers, resulting in a better relationship quality and trust between farmers and buyers. Some of the participants indicated an improvement of the relationship between and with the employees in the form of increased mutual understanding and assistance, while the travel restriction between counties has encouraged farmers to look for (and hire) local rural women. One significant aspect of the social innovation was the support and solidarity in local communities especially on isolated islands faced with absence of animal feed, which manifested through sharing the feed among farmers. The

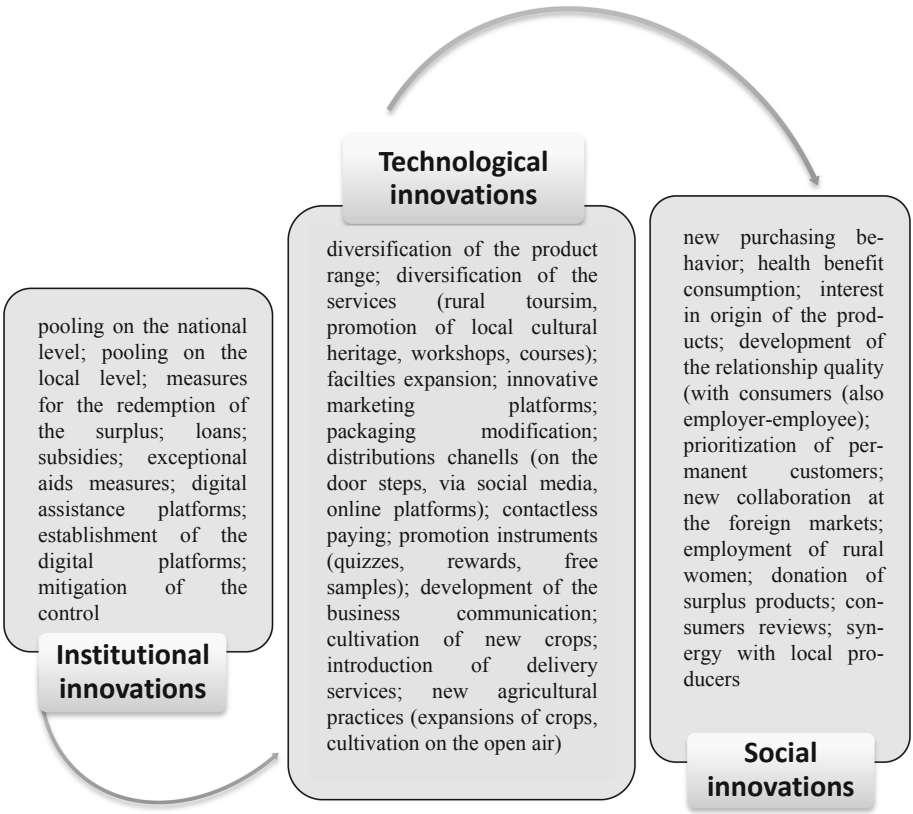

Fig. 1. Innovations of family farms as a response to pandemic (modified by authors from French et al. 2014)

other example of solidarity was the farmer who donated surplus olive oil soaps to the local hospital. Furthermore, a disturbance and termination of pre-pandemic partnerships motivated family farmers to develop new ones, both on local and international market. Other participants developed co-operation on Austrian, Irish, Chinese, and Japanese (and potentially German and Swedish) market. The case of the farmer who diversified its services by providing workshops and seminars which represented the local culture brought a co-operation with the National Museum and thus engagement in rural tourism showed as a ground for different stakeholders' participation (Fig. 1).

Our findings imply that social innovations could become an important aspect of the family farms' new way of business operations. Being the main family farms' strategy in tackling negative pandemic effects, innovations have a potential of enhancing family farms toward more adaptive and responsive farming and marketing. Furthermore, the synergy and effective collaboration of family farms with other local community actors such as local rural labour, tourism boards, local government, local television and consumers may boost the multifunctionality of local agriculture.

4 Discussion

As Darnhofer (2020) indicated and our findings confirmed, farmers seized the opportunity of unexpected and uncertain circumstances to try new practices, to learn new skills and to make new collaborations. Regarding the sample characteristics, as it included younger and more educated farmers than the average Croatian farmer is, it was expected that their coping with the pandemic would be relatively well maintained. However, the scopes of their new ideas, skills, and collaborations, as well as agile and creative approach have not been anticipated. It shows that innovations and ability to change, as our farmers have done by 'overnight' switching to online setting, indeed were the primary strategy to alleviate the crisis and to contribute to their own resilience as well as to the resilience of local communities, as many scholars noted before and during the COVID crisis (Béné et al. 2012; Davoudi et al. 2013; Darnhofer 2020). Additionally, we tried to identify whether farmers' adaptation only settled the crisis, or it turned out to be a good direction for changing market models, exploiting niches, and new opportunities. The findings indicate that changes which farmers introduced, especially online marketing and communication, are likely to stay, as they undoubtedly note that they are, despite the challenges, much more encouraged and motivated to adopt changes as a result of customers' direct feedback.

The Fig. 2 presents the coding scheme which comprises all descriptive codes that we extracted by thematic analysis from the interviews with farmers. The codes are arranged in three groups, according to the type of innovation which are introduced in the first wave of pandemic in 2020. Some of the relevant connections between the codes are also indicated in the scheme. For example, one of them is quality of farmer-customer relationship (B7) which became better due to changes which farmers adopted, namely, innovative marketing platforms (C3), introduction of delivery (C9), new promotion instruments (C7) as well as customers' reviews (B1) and customers' interest in the origin of the products (B8). Above that, the customers' reviews made an impact on the extension of product range (C12). Those relations indicate that direct communication between

farmers and customers can easily make farmers to bring more agile decisions and faster changes according to customers' needs. It raises the whole new set of questions, some of them being what the possible consequences of these new relations are, will it change the social and economic status of the farmer or his/hers negotiation position towards more independent one, will it change economics toward less dependence on global trading system, will it boost environmentally sound agriculture, or will it bring some other, perhaps not so desirable outcomes.

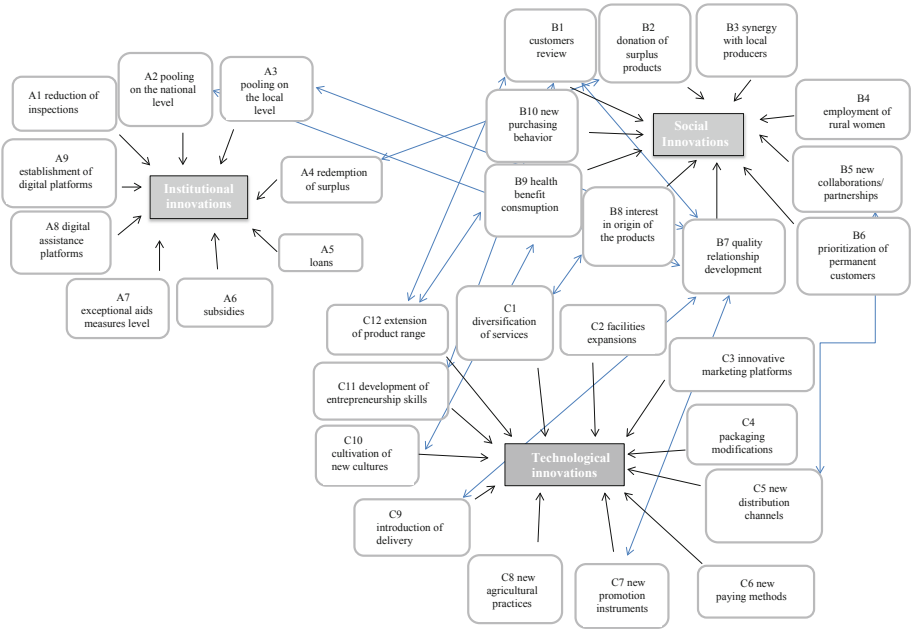

Fig. 2. Code schema of the introduced innovations, made by authors

5 Conclusion

In this paper we presented the innovation strategies implemented by family farms as a respond to COVID-19 crisis. As much as the pandemic has burdened the entire society in the whole range of aspects, it has perhaps also accelerated a range of innovations (other than vaccines) and new practices which helped mitigate the adverse pandemic outcomes. At the same time, the *new normal* is comprised of some favourable changes towards developing more local economy, more responsive, creative, and resilient farmers, perhaps even more responsible farming, and shopping. Farmers who are more prone to changes in skills and practices, and buyers who act responsibly to local producers thus tackling contemporary economic and environmental challenges, are surely adequate actors for coping with today's and future economic and climate uncertainties.

Farmers' newly developed skills and innovations changed their farm and market activities and not only allowed the survival of their farms, but those innovations are likely to endure and change the farmers-customers relations, with possible long-term beneficial effect on the local economies.

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Characterization of the Fermentation Process and Aroma Profile of Carob Brandy

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Abstract. The possibility of producing carob brandy was investigated, focusing on the fermentation of carob and the preliminary characterization of the volatile components in the obtained distillates. Fermentations were carried out in carob mash with or without added nutrients by five *Saccharomyces cerevisiae* strains at three different temperatures. The obtained wine was subjected to fractional distillation in a copper still to produce carob spirit. Analysis of sugars and fermentation products was performed by high performance liquid chromatography. Gas chromatography and gas chromatography coupled with MS detection was used to analyze the volatile components of carob wine and brandy. Carob flour and the strains used can be efficiently used for the fermentation process to produce carob wine with ethanol content ranging from 46.4 to 50.5 gL^{-1} and corresponding yield coefficients ranging from 0.45 to 0.49 gg^{-1}. More than ninety compounds detected in carob spirit; ethyl 2-methyl butanoate, ethyl 2-methyl propanoate, ethyl cinnamate, ethyl hexanoate, beta-ionone, ethyl butanoate and ethyl octanoate, largely contribute to the bouquet of the spirit. Thus, a novel volatile spirit may be an additional product in the carob processing chain and represent a new potential, especially for small carob growers.

Keywords: Fermentation · Yeast · Carob · Carob brandy · Distillate · Fruit spirit

1 Introduction

Fruit brandy is a strong alcoholic beverage made exclusively by the alcoholic fermentation of fleshy fruits or must from such fruits, with or without stones, and distilled at less than 86% vol. so that the distillate has an aroma and flavor derived from the raw materials distilled [1]. Depending on the availability in different countries, different fruits are used for spirit production. Although common fruits such as plums, apples, pears or cherries are usually used, unconventional fruits such as those of wild service tree (*Sorbus*

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domestica), black currants, blueberries, figs, melons, bananas and citrus fruits are also fermented [2, 3]. One of the most important constituents of fruits that makes them a suitable raw material for alcoholic fermentation is free sugars. Carob is an evergreen Mediterranean tree whose pods are used for human consumption and as animal feed. Ripe carob pods are characterized by a high sugar content [4] and a pleasant aroma associated with chocolaty flavors, and therefore can be an interesting raw material for alcoholic fermentation. The total sugar content in carob ranged from 40 to 55%. Sucrose is the main carbohydrate in carob, fructose and glucose are also present [5].

Carob (*Ceratonia siliqua* L.) is a perennial evergreen tree native and widely distributed in the Mediterranean region [6] and is an important component of vegetation for economic and ecological reasons. It is known as a food for both animals (livestock) and humans (carob powder in biscuits, cakes and other sweets). In the food industry, carob seeds are mainly used for the extraction of carob bean gum from carob seeds [7]. This leaves a pulp rich in sugar, a perfect source for use in fermentation processes. Recently, other ways of using carob in the food industry have been explored [5]. In our previous research, the possibility of using carob flour in the development of new products such as instant beverage powder based on carob or pastry fillings was tested. For this purpose, the physical properties of carob flour with and without seeds, such as particle size, moisture content, cohesion and clumping, as well as its chemical properties (total polyphenols and carbohydrate composition) and antioxidant capacity were studied [8, 9]. Due to its high sugar content (mainly sucrose, fructose and glucose), carob is recognized as a new raw material for various biotechnological processes. Yatmaz and Turhan [10] summarized all laboratory-scale studies in which carob pod extracts were used to produce bioethanol, organic acids, single-cell oil, proteins, enzymes, and other value-added products by yeasts, algae, or fungi. However, information on the production of carob brandy is lacking in the literature [11].

The first step in making fruit distillates is to prepare the mash for fermentation. In contrast to fresh fruit, the first step in carob distillates is water extraction of the sugar. Turhan et al. [12] reported that the optimum conditions for sugar extraction from carob are at 80 °C for 2 h at a dilution ratio of 1:4 (fruit: water ratio), yielding about 115.3 g L^{-1} sugar. Sánchez et al. [13] studied carob pods as an economic source for bioethanol production. They showed that almost complete aqueous extraction of sugars from carob pods was achieved in a short time (less than 30 min) at room temperature. Accordingly, aqueous extraction of sugars from carob pods can be considered as easy for industrial application.

In Croatia, as part of the Mediterranean region where carob is grown, only carob liquor is produced, mostly as a domestic product. In liquor production, carob pods are macerated for a certain time in the hydroalcoholic base with the addition of sugar, according to traditional recipes [14]. In contrast, pure spirit production by alcoholic fermentation and distillation of carob is very rare and the compounds responsible for the characteristic bouquet of carob liquor are unknown. Therefore, the aim of this study was to evaluate the potential of carob as a raw material (source of sugars, nutrients and yeast inhibitors) in the production of carob spirit using different commercial yeast strains. In addition, volatile compounds were identified in the distillate obtained and their contribution to odor and aroma was considered.

2 Materials and Methods

2.1 Fermentation Media Preparation

Commercial carob flour obtained by grinding dried carob pods was used. The particle size of carob flour was measured using Mastersizer 2000 analyzer [15]. Before fermentation, we used a modified procedure of Turhan et al. to extract the fermentable sugar was used [12]. Carob flour was mashed with water at a ratio of 1: 4 and heated (50 °C/2 h, [12, 13]). After cooling the composition and properties of the fermentation media were carried out repeatedly (n = 8) and the complete carob mash was used as the fermentation medium.

2.2 Alcoholic Fermentation

The potential of four commercially available yeasts (Y-1 and Y-3 *Saccharomyces cerevisiae* var. *bayanus*; Y-2 *Saccharomyces cerevisiae* var. *burgunder*; Y-B *Saccharomyces cerevisiae* distillery yeasts) and one laboratory *Saccharomyces cerevisiae* strain (Y-A, Laboratory of Fermentation and Yeast Technology, Faculty of Food Technology and Biotechnology, University of Zagreb) for fermentation of prepared carob mash was tested. The mash was inoculated with rehydrated yeast as recommended by the manufacturer (0.33 g dry yeast L^{-1} of mash; water sterilized to 10 times its weight and left for 30 min). Fermentations were performed in duplicate at temperatures of 20 and 30 °C in 500-mL conical flasks in laboratory thermostats with temperature control. To test whether carob contained sufficient nutrients for yeast growth and fermentation, fermentation of carob mash was performed with 0.5 gL^{-1} nutrient supplement (NS, containing diammonium phosphate and vitamin B1) and without nutrient supplement. After inoculation, the flasks were sealed with fermentation caps and left to ferment. The fermentation profile of the yeasts was monitored by CO_2 evolution and weight loss as described in the literature [16]. The flasks were weighed at 12–24 h intervals using a digital balance (Gorenje, Slovenia, range 1–5000 g). Additional Erlenmeyer flask that contained water served as a control to correct for water evaporation loss, which was included in the mass balance. The end of fermentation is defined as stable flask weight for two days. Analysis of sugars and volatiles in the samples was performed by high-performance liquid chromatography and gas chromatography, respectively. The fermentation were performed in duplicate.

2.3 Distillation

To obtain enough fermented liquid for distillation, fermentations were continued in a larger volume (5L flask containing 4.2 L of carob mash) at room temperature (25 ± 2 °C) using *S. cerevisiae* var. *burgunder* (Y-2), a yeast selected for carob fermentation based on flask microfermentation. Fermentations were carried out at room temperature, as in traditional family farm spirit production. The fermentations were performed repeatedly (n = 4), simultaneously and under identical conditions. Immediately after alcoholic fermentation, the fermented liquids were distilled in a copper laboratory still (5 L volume, "Fresl", Croatia) by two-stage distillation (Fig. 1). During the second distillation, three fractions were collected: heads, hearts and tails.

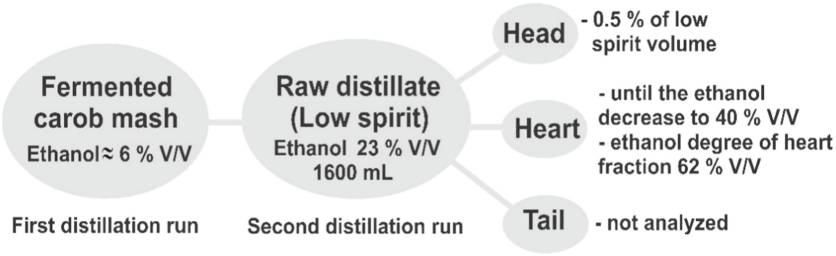

Fig. 1. Double distillations of fermented carob mash in a copper pot still with fractionation technique applied.

2.4 Analytical Procedure

Analysis of Sugars and Ethanol in Carob Mash Before and After Fermentation
Analysis of sugars and fermentation products was performed by high performance liquid chromatography [14, 17]. Before analysis, Carrez reagents (Carrez solution No.1: 3.6 g potassium ferrocyanide dissolved in 100 ml water; Carrez solution No.2: 7.2 g zinc sulphate dissolved in 100 ml water) were added to the sample and the precipitated proteins were removed by filtration. The filtrate obtained was filtered through a 0.22-mm nylon syringe philtre (LAB Logistics Group GmbH, USA) and used for HPLC analysis with a chromatograph (CLASS -VP LC -10A VP; Shimadzu, Kyoto, Japan) equipped with a SupelcogelTM C-610 H ion exchange column and a SupelcogelTM H guard column and a refractive index detector. Separation and elution were performed using phosphoric acid (0.1% w/w) as the mobile phase at a flow rate of 0.5 mL min^{-1}. The column and refractive index detector were maintained at 30 °C and the sample injection volume was 20 μL. The qualitative and quantitative determination was based on the injection of sucrose, fructose, glucose and ethanol standards.

Analysis of Phenols in Prepared and Fermented Carob Mash
The amount of total phenolic compounds was determined spectrophotometrically using the Folin-Ciocalteu method [18]. The absorbance of the samples was measured at 760 nm using a UV/Vis spectrophotometer (Heλios β, Unicam). Total phenolic content was expressed as mg gallic acid equivalents (GAE) per g carob mash.

GC-FID Analysis of Volatile Compounds
All samples used for HS -GC- FID analysis was diluted to an alcohol content of 5% v/v and transferred to a 50-mL volumetric flask containing 5 μL of internal standard (butan-1-ol). Ten millilitres of the sample was added to a 20 mL headspace vial (Macherey-Nagel GmbH & Co. KG, Germany) and sealed with PTFE/silicone septa (Macherey-Nagel GmbH & Co. KG, Germany). The column ZB-5MS 60 m × 0.25 mm I.D. × 0.50 μm df (Zebron, Phenomenex, USA) was used for the determination of volatile compounds in fermented carob must. The required conditions for the headspace sampler HS 40XL (Perkin Elmer) and gas chromatograph PE Autosystem XL (Perkin Elmer) are listed in the Appendix (Appendix 1). Unfermented flour suspended in water and spiked with

6%v/v ethanol, which is the concentration in carob wine after fermentation, served as a blank. Data for validation of the GC method are given in the Appendix (Appendix 2). Analyzes were performed repeatedly (n = 4).

Headspace (HS) Solid Phase Microextraction (SPME) and GC/MS Analysis of Volatile Compounds

An aliquot of 8 mL of the diluted sample was pipetted into a 20 mL glass vial, mixed with 2 g NaCl, and sealed with a silicone septum. Manual sampling was performed using a 50/30 μm DVB/CAR/PDMS (divinylbenzene/carboxene/polydimethylsiloxane) 1 cm StableFlex fiber (Supelco, Bellefonte, PA, USA) recommended for aroma compounds (volatiles and semi-volatiles). Prior to use, the fiber was conditioned according to the manufacturer's instructions. After stabilizing the sample for 10 min, the fiber was exposed to the sample headspace for 40 min at 60 °C with continuous magnetic stirring [19]. The SPME fiber was thermally desorbed in the programmed temperature evaporator injector at 250 °C for 5 min in splitless mode. A GC instrument (GC 6890) with a MS detector (5973 Inert Mass Selective Detector) (Agilent Technologies Network, Santa Clara, CA, SAD) was used for volatile analysis. Separations were performed using a 20 m × 0.18 mm (i.d.) capillary column coated with a 0.18 μm film of Rxi-5 ms (5%- Phenyl-Arylene −95%-dimethylpolysiloxane) stationary phase. Helium was used as the carrier gas with a column flow rate of 0.78 mL min-1 and a total flow rate of 3.8 mL min^{-1}. Analyzes of the SPME samples were performed by GC/MS under the following conditions: The temperature of the oven program used was: 5 min at 40 °C, 40–160 °C at a rate of °C min-1, and 160–240 °C at a rate of 10 °C min-1, with a final temperature hold for 3 min, resulting in a total run of 46 min. The interface temperature for GC-MS was 260 °C. The ion source temperature was 250 °C. Mass spectra were acquired in electron impact mode (70 eV) using full scan with a mass acquisition range of 30–450 m/z. Identification of volatiles was based on mass spectra libraries (NIST 47, NIST 147 and Wiley 175) and from literature data. All analyzes were performed in triplicate. The contribution of each volatile compound to the carob brandy bouquet was evaluated by using total peak area of each compound in the brandy sample and odor threshold value of the compound in water/ethanol solution [20]. Odor threshold values were obtained from the literature [21].

Statistical Analyses

The results obtained were analysed using XLstat (Addinsoft). The statistical significance of the difference between the main volatiles in carob wines produced by *S. cerevisiae* var. *bugunder* with (Y-2- NS) and without (Y-2-0) nutrient addition was evaluated using t-test. Statistical differences were considered significant at $p < 0.05$. Principal component analysis (PCA) of the main volatiles detected in carob wines was also used to group the samples based on yeast strains, fermentation temperature and nutrient addition.

3 Results

3.1 Carob Fermentation Medium and Fermentation Process

The composition and characteristics of the fermentation media obtained by mashing of carob flour with water is shown in Table 1. The applied mashing procedure yielded about

10% (w/v) of total sugar which is optimal sugar concentration for the yeast fermentation and the broth pH value was in the favorable range for yeast activity [22]. Sucrose was dominant sugar in the prepared carob mash, making 65% of total sugar. Glucose and fructose were also present, making 22.2 and 12.7% of total sugar, respectively. It is important to point out that carob flour particle size is an important factor which affects fermentation. Coarser particle size like semolina and 90% of the particles has dimensions up to 0.45 mm was used in this study (Table 1). If carob particles are too small like flour (0.15–0.30 mm), the carob layer is compressed, which makes fer-mentation difficult (own experience).

Monitoring of fermentation profiles of carob mash was conducted indirectly, by measuring liberated CO_2 which is stoichiometrically related with the consumed sugar and produced ethanol (Fig. 2, Fig. 3). The results showed that commercially available yeast strains can ferment carob mash. Lag phase, fermentation time and fermentation rate varied between yeast strains, depending on the yeast strain and its characteristics. Yeast Y-1 had the highest fermentation rate but also a very long lag phase, regardless of the fermentation temperature. Commercial yeasts need some time to adapt, and under slightly stressful conditions this may take some time. As expected, fermentation temperature significantly affected the fermentation length. The tumultuous phase of must fermentations at 30 °C lasted three days for all yeasts, while the fermentation at 20 °C lasted much longer, especially for yeasts Y-1 and Y-3 (Fig. 2). In all fermented samples (with and without nutrient supplement), fermentation was brought to completion and the nutrient did seems to have an effect by completing fermentation faster (Fig. 3).

Table 1. Features of the carob mash prior to fermentation obtained by mashing carob in water at 1: 4 dilution ratio and extraction at 50 °C for 2 h (n = 8).

Total sugar (g L^{-1})	Sucrose (g L^{-1})	Glucose (g L^{-1})	Fructose (g L^{-1})	Total phenols (g L^{-1})
103.30±0.74	67.4±1.2	22.7±0.5	13.1±0.5	1.6±0.0
pH	Carob flour particle size used in mash preparation (μm)			
4.6±0.0	d(0.1) 141.2	d(0.5) 276.2	d(0.9) 454. 9	

d(0.1); d(0.5); d(0.9) – 10; 50 and 90 % of the particles has dimensions up to the stated value (μm)

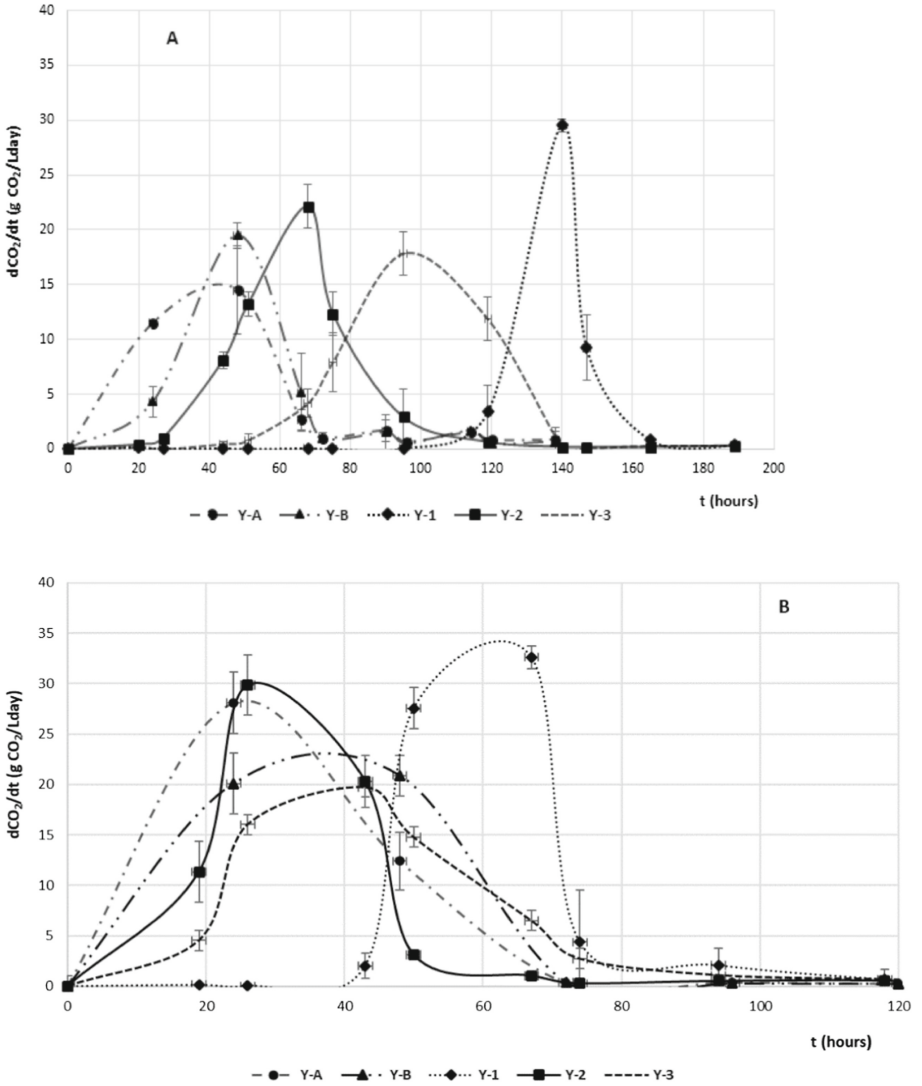

Fig. 2. CO_2 production rate of five yeast strains during carob mash fermentation without nutrition supplement at 20 °C (A, n = 2) and 30 °C (B, n = 2), respectively; ($dCO_2/dt = CO_2$ production rate).

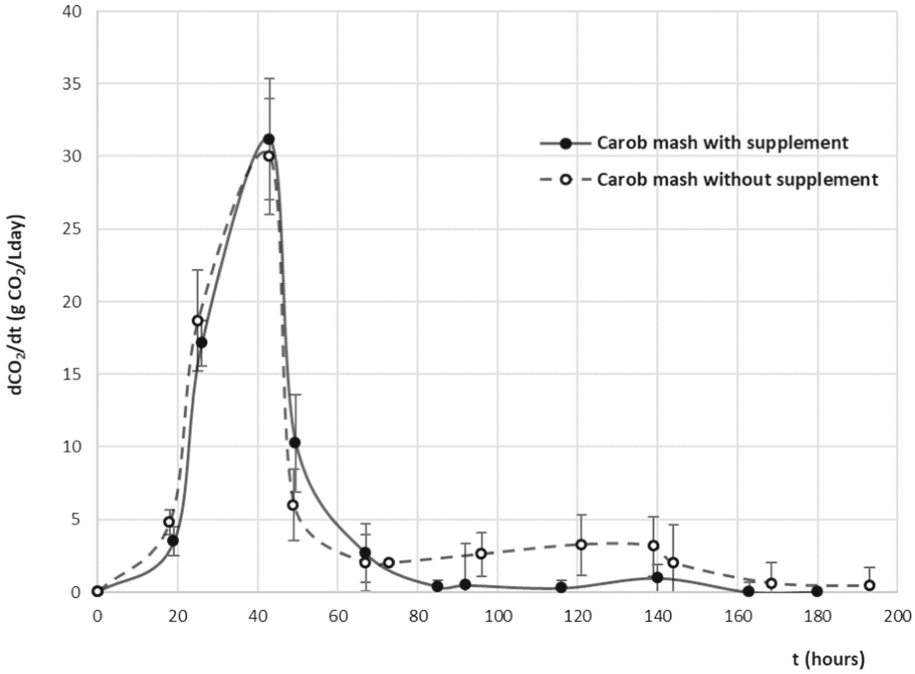

Fig. 3. CO_2 production rate (dCO_2/dt) of yeast *S. cerevisiae* var. *burgunder* during carob mash fermentation with (Y-2-NS) and without (Y-2–0) nutrient supplementation at room temperature C (n = 4).

3.2 Analysis of Fermented Carob Wine

Results of the chromatographic analysis of the fermented carob wines are summarized in Table 2. The alcohols detected were ethanol, propan-1-ol, 2-methylpropan-1-ol, 2-methylbutan-1-ol and 3-methylbutan-1-ol. Esters detected in the samples were ethyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate and ethyl octanoate. Concentration of ethyl octanoate was below limit of quantification. PCA analysis of major detected compounds indicated that the strain is the dominant factor explaining variation in the PCA, closely followed by cultivation temperature (Fig. 4, dotted line). The PCA also confirmed that the addition of extra nutrients had no significant impact on the composition of volatiles. Variables 1 and 2 together explain 76.5% of the total variance. Table 3 shows the bioprocess efficiency of small-scale carob mash fermentation with yeast *S. cerevisiae* var. *burgunder* (Y-2). Tested strain can be efficiently used for the fermentation process to produce carob wine with ethanol content ranging from 46.4 to 50.5 gL^{-1} and corresponding yield coefficients ranging from 0.45 to 0.49 gg^{-1}.

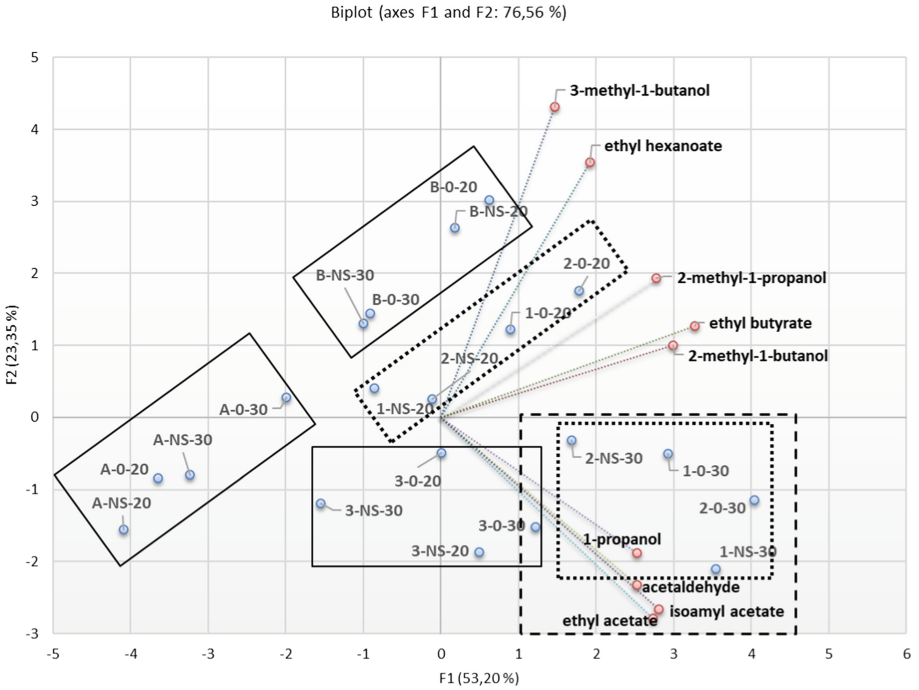

Fig. 4. PCA analysis of major volatiles detected in carob wines produced by five different yeast strains (Y-1; Y-2; Y-3; Y-A and Y-B) with (-NS) and without (-0) nutrition supplement in carob mash at 20 °C and 30 °C.

Table 2. The main volatiles in the carob wines produced by *S. cerevisiae* var. *burgunder* with (Y-2-NS) and without (Y-2–0) nutrient supplementation at room temperature (n = 4).

	Samples		
γ (mgL^{-1})	**Y-2-0**	**Y-2-NS**	**T-test ($p < 0,05$)**
Propan-1-ol	36.1±6.5	33.5±5.8	0.23
Ethyl acetate	12.1±3.5	19.7±2.5	**0.04**
2-Methyl propan-1-ol	97.9±23.7	81.2±25.8	0.49
3-Methyl butan-1-ol	74.8±9.1	68.8±9.9	0.29
2-Methyl butan-1-ol	38.2±5.2	31.5±5.8	0.17
Ethyl butanoate	3.3±0.4	2.9±0.9	0.54
Isoamyl acetate	0.06±0.0	0.05±0.0	0.18
Ethyl hexanoate	3.1±0.3	4.3±1.1	**0.05**

Table 3. Certain bioprocess efficiency parameters of the small-scale carob mash fermentation performed by *S. cerevisiae* var. *burgunder* with (Y-2-NS) and without (Y-2-0) nutrient supplementation at room temperature (n = 4).

Samples	Initial total sugar ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	$Y_{P/S}$ (Ethanol yield) ($g\ g^{-1}$)
Y-2-0	103.3 ± 0.7	46.3±1.4	0.45±0.0
Y-2-NS		50.5±3.1	0.49±0.0
Recovery			
Carob 1 kg		Brandy (EtOH 40% v/v) 1 bottle - 0.7 L	

3.3 Profile of Carob Brandy Volatiles

Volatile compounds identified in the carob distilled spirit obtained from carob wine produced by yeast *S. cerevisiae* var. *burgunder* (Y-2) without nutrient supplementation at room temperature shown in Fig. 5, 6, 7 and 8. Ninety-two volatile compounds were identified by HS-SPME-GC- MS. The volatile compounds profile included 37 esters, 13 ketones, 11 alcohols, 3 aldehydes and 4 acids. In addition, 24 unidentified compounds (hydrocarbons, ethers) were also present. The esters contributed to almost 70% of the total detected volatiles. The most abundant were fatty acid ethyl es-ters: ethyl octanoate, ethyl hexanoate, ethyl decanoate, ethyl benzoate, ethyl (2E)-3-phenylprop-2-enoate, ethyl nonanoate and ethyl 2-methylpropanoate (Fig. 5). (Z)-Non-6-en-1-ol, isoamyl alcohol, nonan-2-ol and nonan-1-ol were the most abundant alcohols (Fig. 6), while undecan-2-one, nonan-2-one, tridecan-2-one and nonanal, non-2-enal and benzaldehyde were the most abundant among ketones and aldehydes (Fig. 7). Dodecanoic, octanoic, hexadecanoic and tetradeca-noic acid were also detected (Fig. 8). It is known that the aroma is not closely correlated with the content of individual compounds. Aroma com-bines effective volatiles and precursor's content, sensory threshold and competing effects among aroma compounds in the mixture. Therefore, volatile compound contribution was calculated (Table 4) considering the corresponding peak area for assessment of relative importance of each compound and literature odor threshold. Ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, ethyl cinnamate, ethyl hexanoate, beta ionone, ethyl butanoate and ethyl octanoate mostly con-tribute to the fruity and floral carob brandy odour. Furthermore, some unpleasant odour components like non-2-enal with greasy and grassy odour were also present.

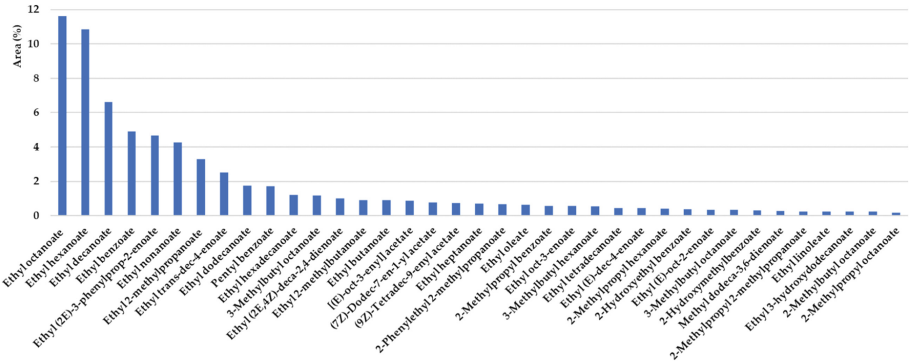

Fig. 5. Esters content in carob brandy produced by *S. cerevisiae* var. *burgunder* without nutrient supplementation at room temperature and copper alembic double pot still distillation (fractional distillation) obtained by SPEME – GC- MS analysis (n = 3).

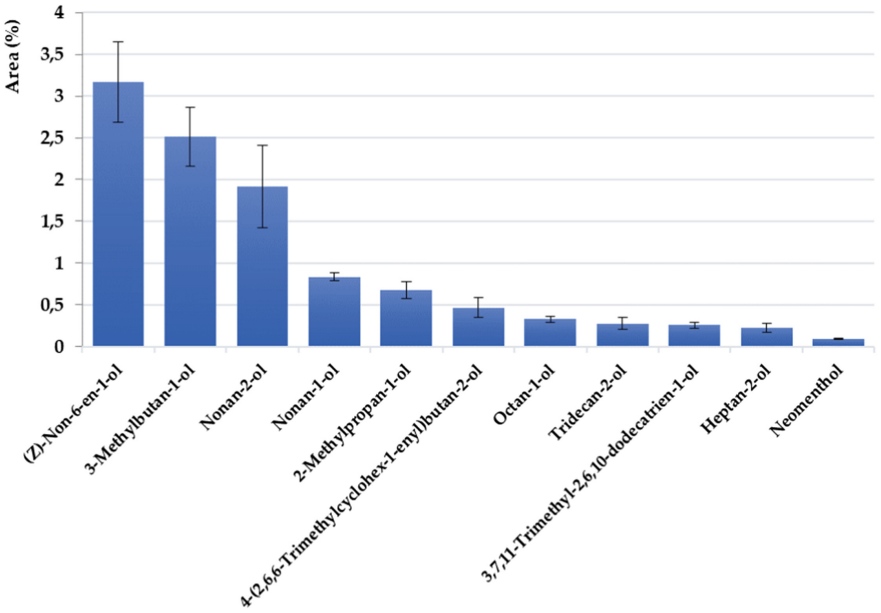

Fig. 6. Alcohols content in carob brandy produced by *S. cerevisiae* var. *burgunder* without nutrient supplementation at room temperature (n = 3) and copper alembic double pot still distillation (fractional distillation)

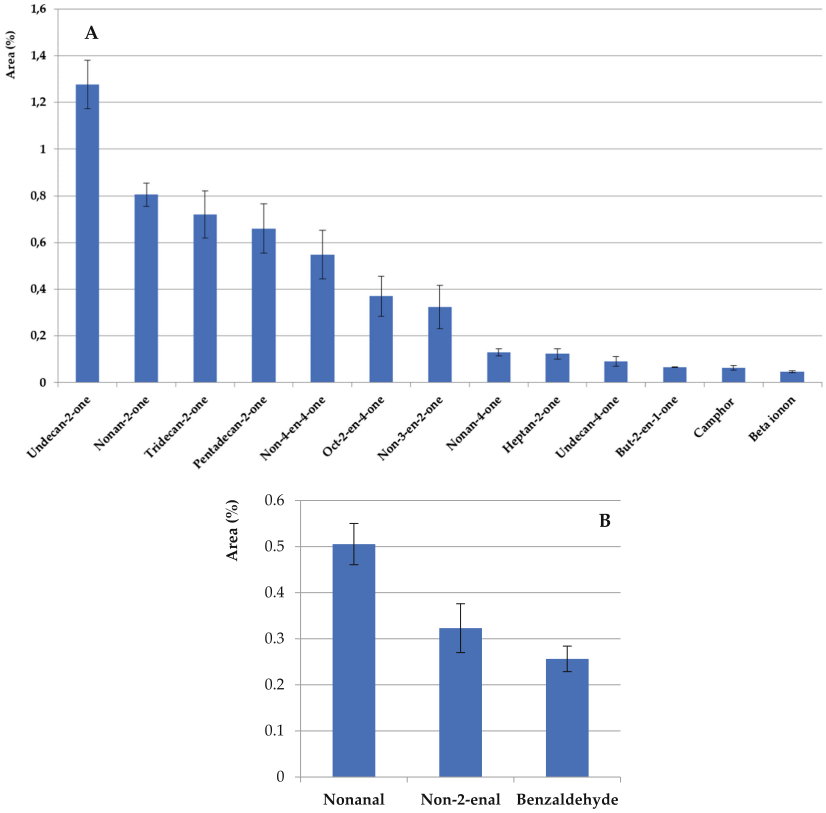

Fig. 7. Aldehydes (A) and ketones (B) content in carob brandy produced by *S. cerevisiae* var. *burgunder* without nutrient supplementation at room temperature (n = 3) and copper alembic double pot still distillation (fractional distillation)

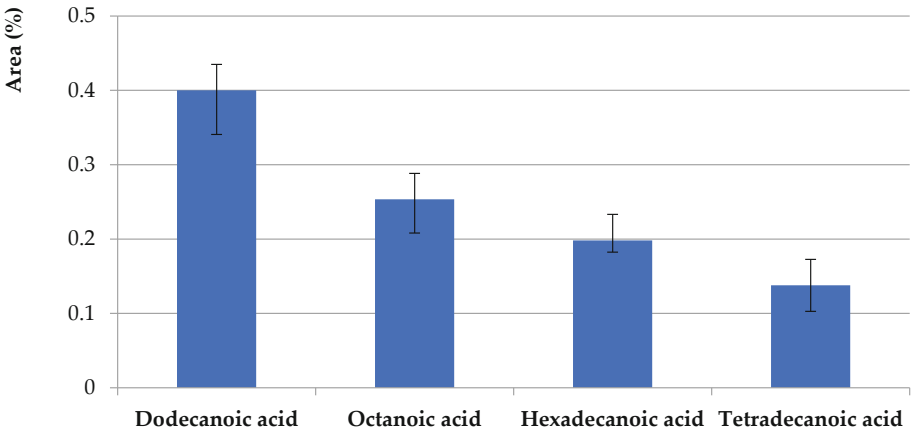

Fig. 8. Acids content in carob brandy produced by *S. cerevisiae* var. *burgunder* without nutrient supplementation at room temperature (n = 3) and copper alembic double pot still distillation (fractional distillation)

Table 4. Active volatile compounds identified in carob brandy produced by *S. cerevisiae* var. *burgunder* without nutrient supplementation at room temperature (n = 3) and copper alembic double pot still distillation (fractional distillation).

Number[a]	Odourant name[b] (IUPAC/trivial)	Odour description[c] [21]	Rt/QM	Area	Odour threshold[c] ($\mu g\ L^{-1}$) [21]	Volatile compound contribution[d]
1	Ethyl 2-methylbutanoate/Ethyl 2-methylbutyrate	Fruity	5.47/94	0.90 ± 0.17	0.2	45.2
2	Ethyl 2-methylpropanoate/ Ethyl isobutanoate/Ethyl isobutyrate	Fruity	3.01/83	3.28 ± 0.18	4.5	7.3
3	(*E*)-non-2-enal /2-nonenal	Greasy, grassy	22.92/89	0.32 ± 0.05	0.6	5.4
4	Ethyl (2E)-3-phenylprop-2-enoate/Ethyl cinnamate	Fruity	31.40/96	4.67 ± 0.57	9.7	4.8
5	Ethyl hexanoate	Fruity	12.30/93	10.85 ± 0.98	30	3.6
6	4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-3-en-2-one/ Beta ionone	Flowery, violet-like, raspberry, strawberry	30.61/96	0.05 ± 0.00	0.2	2.3
7	Ethyl butanoate/ Ethyl butyrate	Fruity, sweet	4.10/95	0.90 ± 0.05	9.5	0.9
8	Ethyl octanoate	Fruity	19.88/97	11.62 ± 0.33	147	0.8
9	Ethyl benzoate	Sweet, wintergreen, fruity, cherry, grape	20.84/91	4.90 ± 1.01	150	0.3
10	Nonanal	Astringent, bitter, aldehyde	16.72/96	0.50 ± 0.04	18	0.3
11	Nonan-2-ol	Coconut	15.86/83	1.91 ± 0.49	75	0.2
12	Nonan-1-ol	Coconut, walnut, oily, citrus	18.94/90	0.84 ± 0.05	80	0.1

[a]Odorants were consecutively numbered according to their volatile compound contributiond; [b]Odorants were identified by their mass spectra obtained by GC-MS; [c]odor threshold (40% ethanol, if available); [d]Volatile compound contribution = peak area divided by odor threshold; Rt - retention time; QM – match factor of MS spectrum

4 Discussion

To prepare carob mash for fermentation we used a modified procedure of Turhan et al. [12]. To avoid high heating temperatures prior to fermentation, we decided to use a moderate extraction temperature of 50 °C and a dilution ratio of 1:4. The results in Table 1 and literature data indicate the rich nutrient composition of carob [5, 6]. Therefore, carob mash can be considered as a good medium for microbial growth.

To ensure good undisturbed fermentation, total phenolics in carob mash medium were also determined (Table 1). According to literature, many phenolic compounds are found in carob which may negatively affect yeast activity [23]. Carob pods contain 448 mg/kg of extractable polyphenols consisting of gallic acid, hydrolysable and condensed tannins, flavonol glycosides and traces of isoflavonoids [5]. Carob fruit is one of the richest sources of gallic acid. Tannins represent the most characteristic group of polyphenols in carob fruit and contribute to its astringency [5]. After fermentation, the concentration of total phenols did not change (data not shown). The completed fermentation without residual sugar shows that the carob mash did not contain inhibitors that would affect alcoholic fermentation, including the phenols present.

In addition to the major and minor components of the medium, yeast strain, temperature, size of the inoculum, fermentation pH and fermentation technique are important parameters affecting alcoholic fermentation [22]. Five different yeast strains intended to produce fruit brandies and wines were used for the fermentation of carob mash at two different temperatures (Fig. 2). Their fermentation activities as well as the composition of the carob wines produced were compared. To investigate whether the carob contains sufficient nitrogen sources and vitamins, the influence of nutrient additions on alcoholic fermentation was also studied. The maximum CO_2 production rate of 30–35 g CO_2 L^{-1} day^{-1} was obtained by yeast Y-1, which was slightly higher than that obtained by yeasts Y-2 and Y-A (approximately 25–30 g CO2 L^{-1} day^{-1}). The CO_2 production rates agreed with literature data for blackberry wine production using some commercial yeasts [16] but were significantly slower than the fermentations of carob described by Sánchez et al. [13]. They fermented sterilized carob peel extracts containing 197.5 g L^{-1} total sugars and 61.36 g L^{-1} reducing sugars with three different *S. cerevisiae* yeasts from different commercial suppliers. Only 30 h of incubation was required to reach a maximum ethanol content of 12% v/v. Possible reasons for these differences may be high initial inoculum concentration (15 g L^{-1} versus 0.33 g L^{-1} in our experiment), higher fermentation temperature (35 °C), and removal of insoluble solids before fermentation. Indeed, the separation of the liquid part is not common in the production of fruit distillates.

Nitrogen concentration regulates the formation of fermentation byproducts: fatty acids, higher alcohols and esters, which affect the chemical and sensory properties of the fermented product and distillate [22]. Limited nitrogen content has been recognized as one of the factors leading to stuck and sluggish fermentations [22]. To test the possible influence of limited nitrogen on our carob fermentation, supplementation with diammonium phosphate and vitamin B1 was carried out in addition to the medium without any supplementation. The results shown in Fig. 3 indicated that the nutrient did indeed seem to have an effect, completing the silent fermentation faster. In addition, a slightly higher concentration of ethanol was obtained with the addition of nutrients

(Table 3). These results agree with those of Turhan et al. [12], who studied the effect of five different nitrogen sources on the kinetic parameters of carob fermentation. The kinetic parameters for carob pod extract without any nutrient addition remained at the lowest values and all maximum values of kinetic parameters were obtained with yeast extract addition to carob mash. A significant difference in the higher content of ethyl acetate and ethyl hexanoate was observed in fermentation with the addition of nutrients to carob mash by yeast Y-2. The results obtained agree with those described by Saerens et al. [24]. In a series of fermentations with constant carbon content and increasing nitrogen content, they found that the concentration of ethyl hexanoate showed little variation with increasing nitrogen content. Ethyl octanoate and ethyl decanoate are constant when the free amino nitrogen content is about 150 mg L^{-1}, which is a standard free amino nitrogen content in regular fermentation medium. As with acetate esters, both ethyl and isoamyl acetate concentrations increase when more nitrogen is present in the fermentation medium. The same authors also studied the effect of temperature on the synthesis of the volatile compounds. The results show that the production of ethyl octanoate and decanoate increased with increasing temperature, while the production of ethyl hexanoate decreased. In contrast, acetate ester production gradually increased with increasing fermentation temperature [24]. In our experiments, the carob mash fermented with yeasts Y-1, Y-2 and Y-B followed the same trend (Fig. 4, dashed line).

Table 3 shows the bioprocess efficiency of small-scale carob mash fermentation with yeast *S. cerevisiae* var. *burgunder* (Y-2). One bottle (0.7 L) of carob brandy (40% v/v) can be obtained from 1 kg of carob. These data agree with those published for carob by Sanchez et al. [13], who reported a yield of 0.32 L absolute alcohol kg^{-1} from carob.

The volatile compounds in the distillate originate from the raw material of the starting fruit and the yeast metabolism, distillation and aging processes. These processes influence the formation of aroma compounds in spirits, which in turn affects their quality. The fermentation phase consists of, among other things, the appropriate selection of yeast strains and the choice of fermentation parameters, and the distillation process depends on the type of equipment used. Apart from the process, the presence and concentration of volatile compounds depend on other variables such as fruit variety, soil type and climatic conditions. Farag and El-Kersh [25] discovered short chain fatty acids such as pentanoic acid (15–25%) and hexanoic acid (20%) as major components in the volatiles of carob pods. Several other less abundant acids were detected including pyruvic acid, isobutyric acid, butyric acid, heptanoic acid, octanoic acid and benzoic acid. Krokou et al. [26] also demonstrated that acids are the most dominant volatile organic compounds of carob fruit. Acids and esters were responsible for 96% of the emitted volatile organic compounds in carob. The acids detected include acetic acid, 2-methylpropanoic acid, butanoic acid and hexanoic acid, while from the class of esters, 2-methylpropanoic acid methyl ester, butanoic acid methyl ester, hexanoic acid methyl ester and 2-methylbutyl-2-methylpropanoic acid ester were detected. Among them, 2-methylpropanoic acid is the most abundant. Hanousek Čiča et al. [14] identified the ethyl esters of these acids as the main volatile components of carob liquor. There are two main categories of flavor-active esters in fermented beverages: acetate esters of higher alcohols, mostly 3-methylbutyl acetate (isoamyl acetate), and medium-chain fatty acid ethyl esters (ethyl butanoate, ethyl hexanoate and ethyl octanoate).

Low molecular weight ethyl esters: ethyl hexanoate, ethyl 2-methylpropanoate, ethyl octanoate, ethyl benzoate, ethyl butanoate and ethyl cinnamate are the most abundant volatiles in carob liquor [14]. Their content depends on the maceration conditions and the content of ethyl hexanoate and ethyl 2-methylpropanoate is about 50% of the total volatiles. Ethyl octanoate, ethyl hexanoate and ethyl decanoate were the most abundant compounds in carob spirit (Fig. 5). However, due to different thresholds, 2-methyl butanoate, 2-methyl propanoate, cinnamic acid ethyl ester and hexanoic acid ethyl ester were responsible for the bouquet of carob spirit (Table 4). The esters generally have a pleasant aroma and a very intense odor and are important beverage flavor components. These compounds contribute positively to the overall quality of the spirit and are responsible for the "fruity" and "floral" smell (Table 4). Ethyl hexanoate exhibits a tropical fruit aroma, while ethyl octanoate is associated with banana, pineapple and brandy-like aromas. Like many volatile esters, ethyl 2-methylpropanoate has a fruity, aromatic odor. High levels of these esters may be associated with yeast metabolism, but also with high levels of carboxylic acids. Like Krokou et al. [26], Cantalejo [27] also found that carob contains an exceptionally high proportion of acids (72.6%), particularly methylpropanoic acid, hexanoic acid, 2-methylbutanoic acid and butanoic acid. In addition to acids, carob also contains a high alcohol content, particularly 3-methylbutan-1-ol (isoamyl alcohol) and 2-methylpropan-1-ol (isobutyl alcohol) [27]. These alcohols are the most abundant in carob spirit (Fig. 6) but cannot be exclusively associated with carob as a raw material, as they are also synthesized during fermentation. Among the aldehydes, benzaldehyde plays the main role in the raw carob distillate, but nonanal and non-2-enal were also detected in the distillate (Fig. 7). Non-2-enal, like beta-ionone, has a low threshold and contributes significantly to the bouquet of carob distillate (Table 4). In contrast to beta-ionone with floral, violet odor description and all detected nine-chain alcohols and aldehydes with coconut, walnut, oily, citrus odor, non-2-enal contributes to the greasy and grassy aroma of the dis-tilled spirit.

5 Conclusions

Carob brandy is a rare product. Unlike other fruit raw materials, which are usually fermented, carob has long been considered a forest tree and the use of carob flour in food products is common (cakes, liqueurs, extracts…). More recently, however, the suitability of carob as a raw material for fermentation has been explored to find new, cheaper substrates for biotechnological processes. The results of this work showed that carob flour can be used for the economically justified production of distilled spirits rich in pleasant volatile components. Aqueous extraction of sugars from carob pods can be considered as easy for industrial application. Yeast strains selected for wine and fruit fermentation can also be used for carob fermentation and no inhibitory effect of carob on yeast growth has been observed. Carob itself contains nutrients needed for fermentation, but the addition of nutrients (nitrogen sources) makes the silent fermentation faster. More accurate information on the initial nutrient content of the carob mash and detailed studies needs to be conducted to see how nutrient supplementation affects volatile synthesis and distillate flavor.

The production of carob brandy could be a way to add value to the carob production chain. The production of this spirit as a high added value product could be justified from an economic point of view. Integrated processes could be considered where high value products (seeds, phenolic compounds) would be separated from the flour before fermentation of the carob tree for brandy and the rest of the sugar and nutrients would be used for fermentation. This could increase the economics of using the raw material, but it should be investigated whether such processing reduces the fermentation activity of the yeast and reduces the flavor quality of the brandy. Furthermore, the pods used for the production of carob distillates are mostly those that are below the shape standards, i.e. small pods, damaged pods and pods with irregular shape. These pods are mostly ground into a whole meal and used as an additive to feed mixes. But as we know, the whole meal can be ground into flour by another milling process. On the other hand, due to the positive effects of carob liquor on human health, the production of carob brandy and liquor means added value, as these pods, i.e. the pods below the standard, can also be utilized.

Appendix: Appendix 1: Conditions of head space gas chromatography with flame-ionization detection (HS and GC-FID), **Appendix 2**: Validation parameters for analysis of VOC's by HS-GS-FID method.

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Appendix 1. Conditions of Head Space Gas Chromatography with Flame-ionization Detection (HS and GC-FID)

HS conditions	GC conditions
O/N/T 80/100/110 °C	Injector temperature: 110 °C
Thermostating time: 20 min	Detector temperature: .0 °C
Pressurization time: 0.2 min	Oven program:
Injection time: 0.05 min	35 °C, 5 min
Withdrawal time: 0.1 min	10 °C/min 60 °C
Carrier: He	60 °C, 2 min
Pressure: 25 psgi	10 °C/min, 180 °C
	180 °C, 7 min

Appendix 2. Validation Parameters for Analysis of VOC's by HS-GS-FID Method

Compound	Linearity range (ppm)	R^2 over calibration curve range	LOD (ppm)	LOQ (ppm)	mean ± sd	A (%)	CV (%)
Propan-1-ol	3–100	0.9976	2.564	8.548	2.960 ± 0.001	−1.33	0.04
					98.260 ± 1.77	−1.74	1.77
Butan-2-ol	1–100	0.9968	0.508	1.692	1.070 ± 0,001	+7.00	0.09
					96.446 ± 0.627	−3.55	0.65
Ethyl acetate	1–100	0.9953	0.171	0.572	1.007 ± 0.013	+0.70	1.29
					97.387 ± 1.477	−2.61	1.52
2-methylpropan-1-ol	1–100	0.9976	0.474	1.581	1.009 ± 0.044	+0.90	4.36
					96.606 ± 0.511	−3.39	0.53
3-methylbutan-1-ol	0,5–100	0.9986	0.472	1.574	0.534 ± 0.037	−10.40	6.80
					97.103 ± 1.587	−2.897	1.63
2-methylbutan-1-ol	1–100	0.9978	0.387	1.290	1.085 ± 0.040	+8.5	3.69
					95.047 ± 1.515	−4.95	1.59
Ethyl-butanoate	0,5–80	0.9972	0.063	0.208	0.389 ± 0.085	−22.20	21.85
					77.497 ± 0.202	−3.13	0.269
Iso-amyl-acetate	0,05–80	0.9960	0.005	0.014	0.051 ± 0.009	+2.00	17.64
					79.980 ± 0.584	−0.03	0.73
Ethyl-hexanoate	1–80	0.9919	0.032	0.106	1.164 ± 0.063	+16.40	5.41
					77.011 ± 0.415	−3.74	0.54
Ethyl-octanoate	0,5–100	0.9904	0.060	0.200	0.492 ± 0.073	−1.60	14.84
					96.436 ± 0.568	−0.36	0.59

Data on GC Method Validation

Method linearity was determined by evaluating the regression curve and it is indicated by the square correlation coefficient (R^2). Linearity was achieved with a minimal R^2 of 0.990.

Detection limits were determined by replicate HS-GC-FID analysis with the lowest concentration for all tested compounds (Table 1). The limit of detection (LOD) and quantification (LOQ) were calculated using the following equations:

LOD = 3 * S/N ratio * lowest concentration of linear sample

LOQ = 10 * S/N ratio * lowest concentration of linear sample

S/N ratio was determined using TotalChrom GC software (Perkin-Elmer).

Precision was expressed as the coefficient of variation (%CV) of HS-GC-FID method and it was determined in five replicates in concentrations pointed out in Table S4.

Accuracy (A) was calculated as the percentage relative error of the method:

A = (mean calculated concentration – nominal concentration)/nominal concentration * 100%.

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A Multi-criteria Decision Making Approach for Prioritization of the Common Market Organization Policy Measures in North Macedonia

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Abstract. Introduction of market regulation policy measures should be conducted gradually in countries that are in the process of approximation towards the European Union Common Agricultural Policy. Such policies are often implemented in complex socio-economic settings, where numerous stakeholders hold different and potentially conflicting values. Therefore, an effective implementation of Common Market Organization (CMO) policies in aspiring countries requires prioritization of the different available policy instruments. The aim of this study is to determine the farmers' preferences in context of introduction of key CMO policy measures in North Macedonia. This paper presents an Analytical Hierarchy Process based modelling framework, as a multi-criteria decision making technique, for prioritization of the CMO interventions for fresh fruits and vegetables. The results suggest that four policy measures are prioritized, among which the measures supporting the establishment of producers' organizations and for products withdrawal are most prominent (45% of vegetable producers and 57% of fruit growers). Another priority is further given to the policy instruments supporting the introduction and the implementation of the marketing standards for fresh fruits and vegetables, followed by the introduction of the schools' schemes. Analyses of this kind, considering the bottom-up approach in valuing the farmers' opinions and needs for introduction of certain policy measures are scarce in practice, but are very important in tailoring the agricultural policy to the real needs of its beneficiaries. Therefore, the presented methodology might be beneficial for introduction and implementation of other policy instruments, but also in other countries aspiring to become a part of the European Union.

Keywords: Analytical Hierarchy Process · CMO Policy · Farmers' Preferences

1 Introduction

As a key component of the Common Agricultural Policy (CAP), the Common Markets Organization (CMO) offers the framework for market support schemes in several agricultural sectors. The internal aspect of the regulation refers to the market interventions

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and rules on marketing and producer organizations, while externally it regulates the trade with third countries (import and export certificates, import duties, administration of tariff quotas, export refunds, etc.). The CMO programs for promoting the consumption of fruits and vegetables at school have been extended, while the provisions on producer organizations, associations of producer organizations, and inter-branch organizations have been foreseen to all sectors in order to strengthen farmers' bargaining power [3].

In regards to North Macedonia and the situation concerning the Common Market Organization policy, the country has incorporated market regulation measures in its national policy, and the system for monitoring the markets and improving its certainty was established with the Law on Agriculture and Rural Development [6]. In order to ensure stability of the markets and to maintain the incomes of the agricultural producers, a system of direct price support has been introduced in form of additional payments per volume of delivered agricultural products to processors or traders (so-called premium payments).

Concerning the marketing and quality of agricultural products, the Law on quality of agricultural products, along with the respective by-laws regulate the marketing quality standards for certain agricultural products, their labelling and information sharing.

The regulation on designation of origin with geographical or traditional features exists but its implementation has started even in 2017 with one product (Ohrid Cherry) being registered. Within the same group of measures, the recognition and operation of Producers Organizations are regulated in the respective Law on Agriculture and Rural Development, but the official establishment of Producers Organizations has not been done yet. Interbranch organizations are mentioned in one article of Law without overall recognition and support properly elaborated. In addition, set of measures to support producers'/exporters' associations in the promotion of the agri-food industry abroad have been introduced and are functional.

Worth mentioning is also that the National Strategy for Agriculture and Rural Development 2014–2020 considers the issue on Common Markets Organization, by identifying the priorities such as: i) implementation of minimum quality standards for agricultural products regulated in the Law on quality of agricultural products, ii) strengthening of the capacity of producers' associations, iii) organization of the food-processing chain, and iv) the protection and promotion of the products quality.

Considering that the introduction and implementation of different policy measures should be conducted gradually in transition countries that are in the process of approximation towards the EU CAP, the prioritization of the policy measures is strongly recommended in order proper planning of specific policy instruments. In this context, the application of Analytical Hierarchy Process (AHP) as a Multi-Criteria Decision Making (MCDM) method confirmed to be an appropriate operational research technique. MCDM has been widely used in various fields of agricultural research, proving to be extraordinarily elegant for solving alternative problems with multiple conflicting criteria [10, 11], however when it comes to policy decision making the policy-makers tend to use different criteria in order to determine the strategic variables and factors influencing agricultural development [7]. Yet, because of the complexity of agricultural systems, the ability of researchers and policy-makers to prioritize variables is often limited, and

consequently, the majority of previous studies have dealt with this subject from a limited point of view [1].

The study is not intended to be uncritically used for immediate policy decision-making, but rather to show the importance of such a prioritization approach in participatory policy applications. Having this in mind, the aim of this study is to illustrate how considering the farmers' preferences could contribute to the research-based policy decision making and in the case of this research to the introduction and implementation of Common Market Organization policy measures in North Macedonia.

2 Materials and Methods

Primary data was obtained in October 2019 through direct interviews with fruit and vegetable growers in order to gain a better knowledge of the farmers' preferences in connection to the introduction of CMO policy measures. The questionnaire used for the prioritization exercise was divided into two parts: the first part contains general socioeconomic data and the second part contributes to the Analytical Hierarchy Process modelling pairwise comparison survey. For the qualitative comparisons, all respondents supplied verbal judgments ranging from equal to extreme importance, which were then converted into numerical values using an integer scale from 1 to 9 [8].

In this case, 35 farmers (15 vegetable growers and 20 fruit growers) were analysed using the purposive sampling methodology as a nonprobability and non-random type of sample [2]. Unlike statistical approaches, which require a large sample size, mathematical modelling allows for a smaller sample population [5], like in this study.

The data was then processed employing Goepel's AHP template [4], which was created expressly for doing the prioritization analysis in MS Excel. The Analytical Hierarchy Process is a structured multi-criteria decision-making technique which helps the decision-makers in setting priorities and making the best possible decision. In order to simplify complex decisions, AHP involves a series of paired comparisons that take into account both the subjective and objective components of a choice [1].

The AHP hierarchy is organized in a descending order, starting with the overall goal and progressing via criteria, sub-criteria, and alternatives [8]. The relative importance of the criteria and alternatives is estimated using the pairwise comparison scale introduced by Saaty [9], which involves pairwise comparisons of all elements.

The hierarchy might have numerous levels depending on the goal's complexity [5]; however, in this study, a three-level decision hierarchy was constructed.

In terms of structuring the decision-making problem in a hierarchy, this mathematical technique is an appropriate tool if each category includes at least four but no more than seven to ten sub-categories, because more than that would require over 45 pairwise comparisons, resulting in a complex and confusing decision-making [4]. Considering that only three criteria with eight possible alternatives are taken into account in this study, the applied operational research method provides plausible results. The top level of the hierarchy denotes the overall goal (G), which is to prioritize Common Market Organization policy measures for regulating the market of fruits and vegetables, while the second level refers to the decision-making criteria (C), which are the benefits of introducing and implementing CMO instruments in the sub-sectors under consideration.

Consequently, the benefits include increased farmer competitiveness, reduced income volatility during times of crisis, and higher consumption of fruits and vegetables. The possible CMO policy measures that are projected to contribute to the achievement of the given criteria are represented as alternatives (AL) at the third level of the hierarchy. Figure 1 depicts the illustrative presentation of the conceptual framework employed in this study.

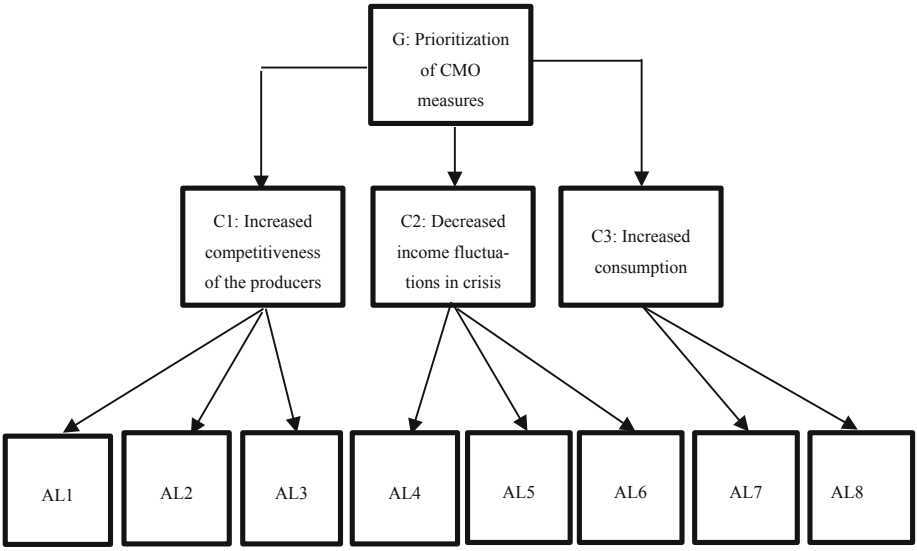

Fig. 1. AHP decision hierarchy for prioritization of the Common Market Organization policy measures Explanation of variables: AL 1: Producers' organizations, AL 2: Interbranch organizations, AL 3: Marketing standards, AL 4: Market interventions with product withdrawal, AL 5: Green/Non harvesting, AL 6: Harvest insurance, AL 7: School schemes, AL 8: Import/Export rules

3 Results and Discussion

3.1 Description of the Sample Farms

The prioritization exercise includes 35 respondents (15 vegetable growers and 20 fruit growers). The majority of the vegetable farmers are from the Southeast region (80%), which is the most representative of the country's vegetable production, while only a few are from the Vardar and the Northeast region. When it comes to fruit growers, the majority of them (74%) are coming from the Southwest region, which is one of the most well-known regions for fruit production, particularly for apple production (Table 1). The gender distribution was nearly equal among the vegetable growers, while the distribution of male fruit producers dominates, with 71% of all respondents being male farmers.

In terms of education, the majority of the vegetable and fruit representatives (47% and 45% respectively) have a high school education, while 30% of the vegetable growers

and 32% of the fruit growers have a higher education. Aside from the main sample characteristics, it's worth noting that 50% of all vegetable producers and 42% of all fruit producers are not members of any group of producers, whereas around 30% belong to a cooperative, and a small share to a specific agricultural association (Table 1).

For their production, all respondents use various marketing channels, with the majority selling to buy-out centers, some selling straight from the farm, and others selling at green markets.

Table 1. Characteristics of the sample farms.

	Vegetable producers	Fruit producers
Region (in %)		
Southeast	80	:
Southwest	:	74
Northeast	7	:
Vardar	13	:
Skopje	:	26
Education (in %)		
Primary education	23	23
Secondary education	47	45
Higher education	30	32
Membership in group of producers (in %)		
Not a member	50	42
Cooperative	35	33
Agricultural Association	15	25

3.2 Farmers' Preferences Toward the Introduction of Common Market Organization Policy Measures

To better understand the farmers' priorities for introducing and implementing the Common Market Organization policy measures in North Macedonia, the study included purposely selected fruit and vegetable farmers in order to determine the priority of one element over another in the hierarchy decision-making tree based on their personal preferences. Due to the specific characteristics of fruit and vegetable production both groups of farmers might have distinct preferences, and therefore two separate AHP models were used to estimate the priorities for both fruit and vegetable growers.

Table 2 shows the aggregated priorities and the consistency ratio (CR) for each criterion the observed farmers will benefit from as a result of the CMO policy initiatives

being implemented in the country, using the eigenvector approach (EVM). The eigenvalue (lambda) of 3.003 in the case of vegetable producers and of 3.013 in the case of fruit growers, which is almost the same as the matrix size, confirms sample consistency and allows for further prioritization. In terms of the consistency ratio (CR = 0.3% for vegetable farmers and CR = 1.14% for fruit growers), the acceptable threshold of less than 10% was examined, and the results demonstrate that the participants' judgements are perfectly consistent. The group consensus indicator of 46.6% and 58.9% in both cases, on the other hand, suggests a rather low level of consensus among the vegetable and fruit growers included in the study.

Table 2. Prioritization of the benefits from the CMO policy measures introduction.

Criteria Weights	Vegetable producers Weights	Fruit producers
Increased competitiveness of farmers	35.1%	26.3%
Decreased income fluctuations in crisis	41.8%	21.0%
Increased consumption of fruit and vegetable products	23.1%	52.7%
Eigenvalue (Lambda):	3.003	3.013
Consistency Ratio (CR)	0.3%	1.14%
Group Consensus Indicator	46.6%	58.9%

Figure 2 depicts the relative importance (weights) of the benefits (criteria) from the implementation of Common Market Organization policy instruments using the AHP approach. According to the estimated weighted average of the decision matrix elements based on the individual preferences, vegetable producers mostly need an introduction of CMO policy measures that will contribute to the decreased income fluctuations during times of crisis (41.8%), followed by increased competitiveness of the vegetable farmers (35.10%). On the other side, due to the specific market situation for the fruit production in the country, the fruit growers reveal different a perspective, i.e. most of them prefer an introduction of the CMO measures that will lead to the increased consumption of fruit products (52.7%). Additionally, the increased competitiveness of the fruit growers is chosen as a second priority with a relative importance of 26.30%.

Considering that the introduction and implementation of different policy measures should be conducted gradually in countries that are in the process of approximation towards the EU Common Agricultural Policy (CAP), prioritization of policy measures is strongly recommended in order to properly plan and organize the introduction of specific policy instruments. In this regard, the implementation of CMO policy measures in North Macedonia should take into account the priorities set by the relevant stakeholders. Therefore, the analysis further focuses on determining the farmers' preferences in relation to the specific Common Market Organization policy instruments available for the fruit and vegetable producers that will lead towards the accomplishment of specific

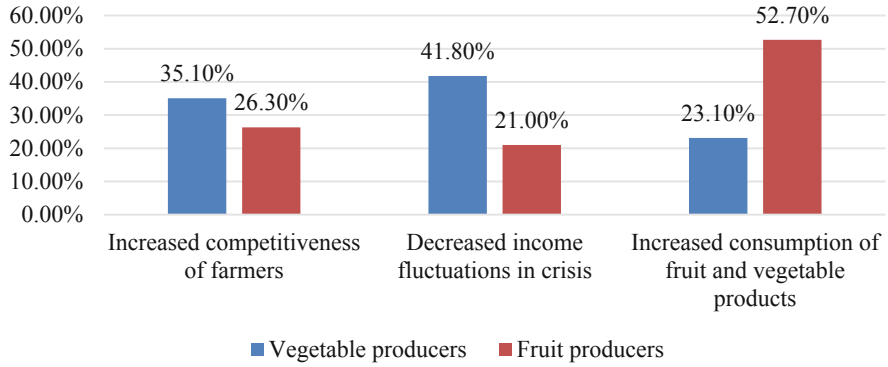

Fig. 2. Relative importance of benefits from CMO policy measures introduction

benefits from their introduction in North Macedonia. Herewith, eight policy measures are included in the analysis, as described under the conceptual framework of the study.

The pairwise comparison between the available alternatives, i.e. the specific policy instruments under each criterion resulted in equality of the eigenvalue with the matrix size, indicative of sample consistency (Table 3). The consistency ratio for each benefit from the introduction of these measures in North Macedonia also reveals perfect consistency. However, the group consensus indicator indicates a low consensus reached in the prioritization of the available policy measures for the vegetable sub-sector and moderate consensus among fruit growers.

When the relative importance of policy instruments contributing to an increase of the competitiveness of the observed sub-sectors is considered, the results reveal that farmers prefer support for the establishment of producers' organizations in both cases, which is especially prominent for the fruit growers with a relative importance of 57%. The introduction of policy support for ensuring specific marketing standards for each production is ranked as the second priority for both the vegetable farmers and the fruit growers with 31.6% and 25.2% respectively. The vertical integration among the primary producers, traders and processors is still undervalued in both sectors, thus support for the establishment of interbranch organizations ranks third in terms of its implementation in the country (Fig. 3).

In regard to the farmers' preferences for introduction of policy support that will lead toward decreasing of the farmers' income fluctuations (Fig. 4), providing support for products withdrawal in times of crisis is the highest priority for both groups of respondents, with 44.3% for the vegetable producers and 57% for the fruit growers. Harvest insurance as policy instruments is ranked second in both cases (32.8% for vegetable production and 25.2% for fruit production), especially considering the fact that harvesting, if being untimely or inappropriate, causes losses and decreases quality of the products. Additionally, the lack of post-harvest practices and standards is a particular issue. The green harvesting or non-harvesting is given lowest priority by both groups of farmers.

The third group of Common Market Organization policy instruments is expected to contribute to the increased consumption of the fruit and vegetable products in the

Table 3. Prioritization results of the pairwise comparisons of the policy instruments under each benefit from the CMO policy introduction.

	Indicators	Increased competitiveness of the sector	Decreased income fluctuations in crisis	Increased consumption
Vegetable producers	Eigenvalue (Lambda)	3.010	3.000	2.000
	Consistency Ratio (CR)	1.00%	0.00%	0.00%
	Group Consensus Indicator	54.70%	56.80%	44.40%
Fruits producers	Eigenvalue (Lambda)	3.025	3.025	2.000
	Consistency Ratio (CR)	2.60%	2.60%	0.00%
	Group Consensus Indicator	67.10%	67.10%	54.30%

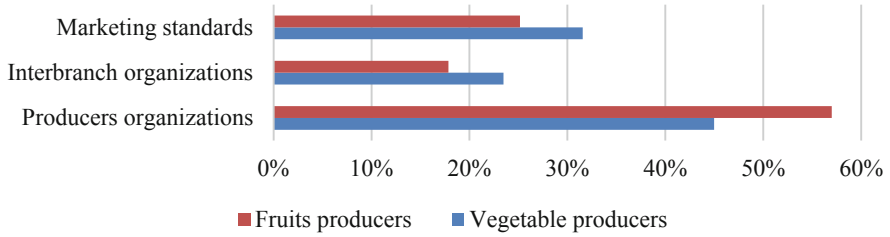

Fig. 3. Relative importance of the CMO policy measures for increasing the competitiveness

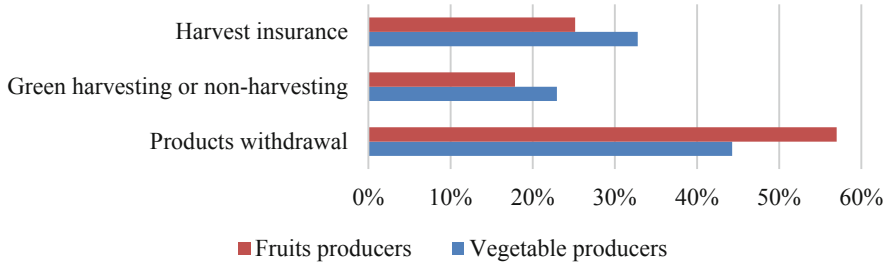

Fig. 4. Relative importance of the CMO policy measures for decreasing of income fluctuations

country (Fig. 5). North Macedonia has always been an important fruits and vegetables supplier in the region, nevertheless its market position has slowly deteriorated on the regional markets and is especially rapidly changing in the last few years, especially the competition from neighboring countries is becoming increasingly present on the domestic market. Considering these circumstances, both vegetable producers and fruit growers give highest priority to the introduction of import and export rules for fruit and vegetable products with a relative importance higher than 50% in both cases. Although a second priority, the introduction of school schemes is also valued with almost equal relative importance by the sample respondents.

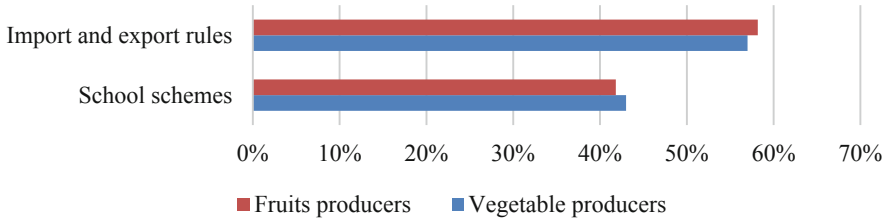

Fig. 5. Relative importance of the CMO policy measures for increasing consumption

4 Conclusion

The use of the analytical hierarchy process as a multi-criteria decision-making methodology allowed the farmers' priorities to be determined when choosing Common Market Organization policy support. In reality, this is the country's first study of its kind, attempting to consider the opinions and needs of farmers in regard to introduction of specific policy measures. As a result, this study adds to the literature by emphasizing the need of taking a bottom-up approach when considering farmer priorities, which is especially relevant when developing agricultural policy and national support programs and measures.

Considering the different circumstances in which the farmers operate, the findings from this study suggest that the vegetable producers mostly need policy support for decreasing the farmers' income volatility in crisis through product withdrawal and harvest insurance, while the fruit growers mostly prefer introduction of CMO policy measures that will lead to an increased competitiveness of the fruit sub-sector in the country. For that purpose, the fruit producers point to the need for the establishment of a producers' organization. Both groups of respondents consider that there is a need for introduction of import and export rules, and look at the school schemes as possible solutions for increasing the consumption of fruit and vegetable products.

Although applied on a small sample, the employed method might be implemented on a larger sample population since the developed methodological framework revealed a well suitability in complex policy decision making. The method is relatively simple, the consistency tests confirm the consistency in the individual judgments, thus leading to plausible results. Analyses of this kind, considering the bottom-up approach in valuing

the farmers' opinions and needs for introduction of certain policy measures are scarce in practice, but are very important in tailoring the agricultural policy to the real needs of its beneficiaries. Therefore, the presented methodology might be beneficial for introduction and implementation of other policy instruments, but also in other countries aspiring to become part of European Union.

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Anti-adhesion Activity of Phenolic Compounds Against *Campylobacter jejuni* and *Listeria monocytogenes* Evaluated with PCR-Based Methods

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Abstract. *Campylobacter jejuni* and *Listeria monocytogenes* are food-borne zoonotic bacteria with the highest notification rate and the highest death rate in EU, respectively. Both bacteria form biofilms on surfaces with the mechanisms which are not well understood. In our research we initially focused on the optimization of molecular methods for the quantification of adhered cells of *C. jejuni* and *L. monocytogenes*. With digital PCR (dPCR) we established a standard calibration curve, and with real-time PCR (qPCR) we determined the number of amplified target DNA, which represented the proportional amount of adhered bacteria to polystyrene. Secondly, we used an optimized method to evaluate the anti-adhesion activity of phenolic compounds: carvacrol, thymol, α-pinene and epigallocatechin gallate (EGCG) at a subinhibitory concentration of 0.1 mg/ml. The absolute quantification of *C. jejuni* and *L. monocytogenes* by dPCR and the generation of a standard curve with qPCR allowed the absolute quantification of the target DNA. The combination of dPCR and qPCR methods thus allowed an accurate, reproducible and rapid quantification of bacterial cells adhered to polystyrene. In *C. jejuni,* carvacrol, thymol and EGCG showed anti-adhesion activity at a concentration of 0.1 mg/ml with 96%, 97% and 91% reduction, respectively. All tested compounds – carvacrol, thymol, α-pinene and EGCG – showed anti-adhesion activity also for *L. monocytogenes* with 84%, 81%, 83% and 69% reduction, respectively. They represent an alternative strategy to prevent the adhesion of *C. jejuni* and *L. monocytogenes* and thus biofilm formation on abiotic surfaces.

Keywords: Anti-adhesion · Quantification based by PCR · Pathogenic bacteria

1 Introduction

According to the World Health Organisation (WHO), food-borne illnesses represent a public health challenge. Food contamination can occur at any stage in the farm-to-consumer continuum, from environmental, animal or human sources [1]. The increasing

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resistance of pathogenic bacteria to antibiotics and the resistance of biofilms on abiotic surfaces in the food industry is a major challenge for ensuring safe food. *Campylobacter jejuni* and *Listeria monocytogenes* are food-borne zoonotic bacteria with the highest notification rate and the highest death rate in EU, respectively. This causes a high economic burden in treating patients with campylobacteriosis and recalls of food contaminated with *L. monocytogenes*. For more than a decade, Gram negative *C. jejuni* has been the leading global cause of bacterial gastroenteritis in the developed world. The incidence and prevalence of campylobacteriosis is associated with consumption of undercooked poultry meat products, with outbreaks also arising from contaminated water [1, 2]. The foodborne disease listeriosis is an important public health problem as it is associated with high hospitalization and mortality rates and it is caused by Gram positive *L. monocytogenes*. These bacteria are ubiquitous in soil and water, and can colonize mammalian hosts [3].

Both bacteria form biofilms on surfaces with the mechanisms which are not well understood. One of the critical factors that ensure the protection of *Campylobacter* and *Listeria* against harsh conditions is the formation of biofilms. To combat the increasing occurrence of resistance to antimicrobials in bacteria, detailed understanding of biofilm formation is crucial. Adhesion of bacteria to an inert or biotic surface represents a key step in environmental persistence in form of biofilms and in pathogenesis as one of the initial stages of host infection [4]. Research is focusing on the substances to prevent bacterial adhesion to abiotic surfaces and to elucidate the mechanisms of this effect on bacterial cells. Studies of the processes involved in the different stages of biofilm formation will provide new concepts for development of novel antibacterial strategies.

In *Campylobacter* and *Listeria* biofilm research, new methods that enable reproducible detection and quantification of low numbers of bacteria are needed, due to the low adhered biomass in the first steps of biofilm formation. However, both current and new methods have limitations, such as limited accuracy and precision, high detection limits, high cost, long duration, and high workload. Adhesion and biofilm assays include direct and indirect methods, whereby the latter require the detachment of the microorganisms from the surface prior to their counting. Several methods are introduced which include the culture-based plate counting method, biomass staining methods, e.g. with crystal violet and safranin red, DNA staining methods, e.g. Syto 9, use of chromogenic or fluorogenic metabolic substrates for detection of live bacteria, e.g. tetrazolium salts, resazurin, transformed bacteria with a plasmid for inducible expression of NanoLuc luciferase for bioluminescence signal measuring and also qPCR and digital droplet PCR for quantification of bacterial DNA [4–8].

The principle of molecular methods is changing and improving very fast and thus we aim to introduce a novel approach with digital PCR (dPCR) to establish a standard calibration curve, and with quantification PCR (qPCR) we determined the copy number of amplified target DNA, which represented the proportional amount of adhered bacteria to polystyrene. The model organisms for molecular quantification were adhered cells of Gram negative *C. jejuni* NCTC 11168 and Gram positive *L. monocytogenes* serotype 4b. Therefore, we aim to introduce the accurate, reproducible and rapid quantification of bacterial cells adhered to polystyrene by combination of dPCR and qPCR methods. Further we used them to determine the anti-adhesion activity of phenolic compounds

carvacrol, thymol, α-pinene and epigallocatechin gallate (EGCG) at a subinhibitory concentration of 0.1 mg/ml.

2 Materials and Methods

2.1 Flow Chart

Flow chart of experimental work is described in the Fig. 1.

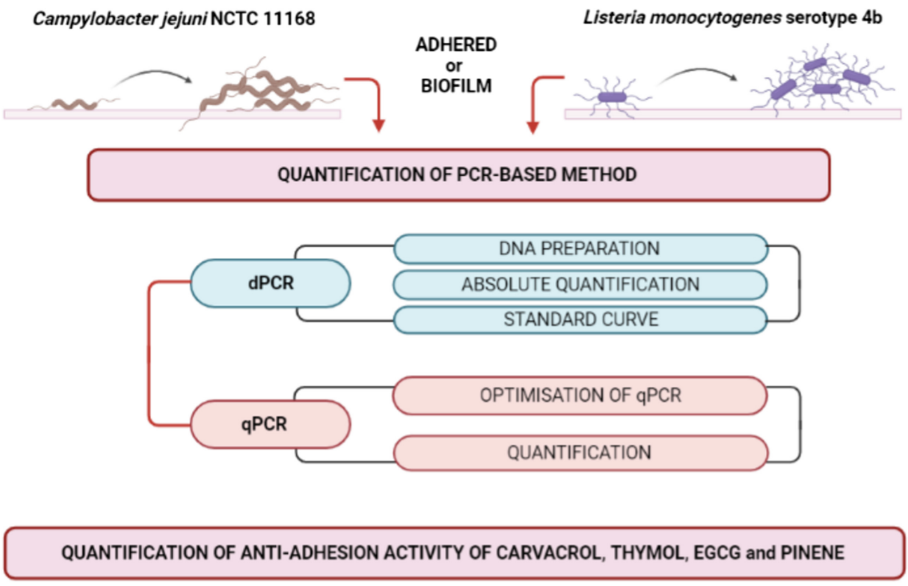

Fig. 1. The scheme of experimental work.

2.2 Bacterial Strains and Growth Conditions

For base sample construction of the standard curves for qPCR, *C. jejuni* 81–176 and *C. jejuni* NCTC 11168 as reference strains; *C. jejuni* 58429 B862, 57360 B861, 57359 B860, and 122/08 B847 as chicken meat and faeces isolates; *C. jejuni* 9711 B879, 9090 B871, 9581 B877 as human strains; *C. jejuni* 211/08 B855 as bovine faeces isolates; *C. jejuni* 1271/08 B849, 654/08 B907 as turkey isolates; and *C. jejuni* B886 strain isolated from water. We also used *L. monocytogenes* ŽM58 (serotype 4b) as reference strain from Institute of Hygiene and Microbiology, Wuerzburg, Germany) all from the collection of the Laboratory for Food Microbiology at the Biotechnical Faculty, University of Ljubljana, Slovenia (strain designation: ŽM). All *C. jejuni* strains were maintained in a Mueller–Hinton broth (MHB; CM0405B; Oxoid, Hampshire, England) and *L. monocytogenes* strains in a Tryptone soya broth (CM029; Oxoid) containing 20% glycerol (v/v)

(Kemika, Zagreb, Croatia) and stored at − 80 °C. *C. jejuni* strains were grown microaerobically (5% O_2, 10% CO_2, 85% N_2) at 42 °C on Mueller–Hinton agar (CM0337B; Oxoid), and *L. monocytogenes* aerobically at 37 °C on Tryptone soy agar (CM0131; Oxoid).

2.3 Phenolic Compounds and Adhesion Assay

The adhesion of *C. jejuni* and *L. monocytogenes* was analysed for treatment with subinhibitory concentrations (0.1 mg/ml) of carvacrol, thymol, (−)α-pinene and epigallocatechin gallate (EGCG) (Sigma Aldrich, Germany). Stock solutions were prepared in DMSO (Merck, Germany) and stored at −20 °C. Adhesion was examined as previously described [9]. Briefly, polystyrene microplates (Nunc, Denmark) were inoculated with *C. jejuni* and *L. monocytogenes* planktonic cells to a final concentration of 10^6 CFU/ml and incubated for 24 h in an appropriate atmosphere for each bacterium. Furthermore, for each of these adhesion conditions the supernatant with free-floating cells was removed from each well and the plates were rinsed three times with PBS.

2.4 Extraction of DNA for Preparation of Standard Curves and DNA from Adhered Cells

The bacterial DNA pool for the dPCR was prepared from adhered strains as described above. The DNA was isolated using the PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific, USA) directly from the adhered cells in the microplates, as previously reported [5, 6]. The extracted DNA was stored at 4 °C prior to use.

2.5 Digital PCR (dPCR) and Quantitative Real-Time PCR (qPCR)

DNA was precisely quantified by dPCR (QuantStudio™ 3D Digital PCR Instrument; Thermo Fisher Scientific, brand Applied Biosystems, USA) with purpose to create the standard curve of the serial dilutions using qPCR. DNA was loaded onto the QuantStudio™ 3D Digital PCR 20K Chip v2 using a QuantStudio™ 3D Digital PCR 20K Chip loader, in a mixture of 2 × QS 3D QuantStudio™ 3D Digital PCR Master Mix v2, 900 nM forward and reverse primers, 200 nM TaqMan MGB probe, and 1.5 µl of three dilutions of the DNA (10,000-fold, 50,000-fold, 100,000-fold). The chips were sealed and loaded onto a ProFlex™ PCR System. After cycling, the end-point fluorescence of the partitions on the chips was measured by transferring the chips to the measurement unit of the Applied Biosystems QuantStudio™ 3D Digital PCR Instrument (all from Thermo Fischer Scientific).

Quantification of the adhered cells was performed by qPCR (ABI Prism 7500 real-time PCR system; Thermo Fisher Scientific, brand Applied Biosystems, USA). The PCR assays detected specific sequences of *ccoN* for *C. jejuni* [10] and of listeriolysine O *hlyA* for *L. monocytogenes* [5]. The cycle conditions used were an initial cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The fluorescence was analysed using the Sequence Detection Software (SDS software 1.3, Thermo Fisher Scientific). The threshold line was fixed at 0.20. The Ct values obtained were calculated for the DNA copy number, using the equation obtained from the standard curve.

2.6 Standard Curves from the Ct Values and the Corresponding DNA with qPCR

Standard curves were prepared from Ct values obtained with the qPCR from diluted DNA (100 to 10^{-8}) from both bacteria. Ct value of less than 40 cycles were considered. The sensitivity of the method is expressed by the Limit of Detection (LOD) and the Limit of Quantification (LOQ). The detection window (for the linear range of detection) was determined using linear regression, and was used to calculate the slope (S) of the standard curve, the amplification efficiency (E), and correlation coefficient (R^2). The efficiency of amplification was calculated as E (%) [11, 12].

$$E(\%) = ((10(^{1/-S})) - 1) \times 100 \quad (1)$$

2.7 Statistical Analysis

The data are expressed as mean DNA copy numbers ± standard deviation (SD) within all of the sample groups. The means and SD were calculated using analysis of variance. Statistical calculations were analysed using paired t tests from the IBM SPSS Statistics Version 20 statistical software (Statsoft Inc., Tulsa, OK, USA), based on a confidence level of $\geq 95\%$ (i.e., level of significance, 0.05).

3 Results

3.1 Standard Curves

A standard curve was constructed by combining absolute quantification of the base sample with dPCR and quantification of the dilution series with qPCR. The dPCR method was used to measure the absolute copy number in the base sample, which was 1.78 x 10^7 copies/µl for *C. jejuni* (Fig. 2A) and 4.85 × 10^5 copies/µl for *L. monocytogenes* (Fig. 2B). The qPCR method was used to quantify a series of seven dilutions, where the suspension of cell lysates was diluted to a dilution level of 10^{-7} for DNA of *C. jejuni* and 10^{-5} for DNA of *L. monocytogenes*. In constructing the standard curve, all positive samples with a Ct value of less than 40 cycles were considered. By combining the dPCR and qPCR results, a standard curve could be constructed (Fig. 2).

As presented in Fig. 2A the standard curve with the equation $y = -3.57 * x + 41.62$ and $R^2 = 0.99$ is suitable for further use and quantification of *C. jejuni* samples according to the requirements. R^2 represents the linear relationship of the two variables and must take the values 1 R2 0.98. The value of k, which in our case is −3.57, represents the slope of the line. Under optimal multiplication conditions, i.e. at 100% multiplication efficiency, the slope of the curve is k = −3.32. According to the authors [13, 14], the range of good and acceptable response efficiency is between 90–110%, which corresponds to a slope of the curve between k = −3.58 and −3.10. The response efficiency of the standard curve is E = 90.7%. Thus, the resulting curve corresponds to further absolute quantification of qPCR results from samples containing DNA from the *C. jejuni* species. By constructing a standard curve, the sensitivity of the method was also determined by calculating the parameters LOD and LOQ. LOD represents 1 ± 1 copy of DNA. The

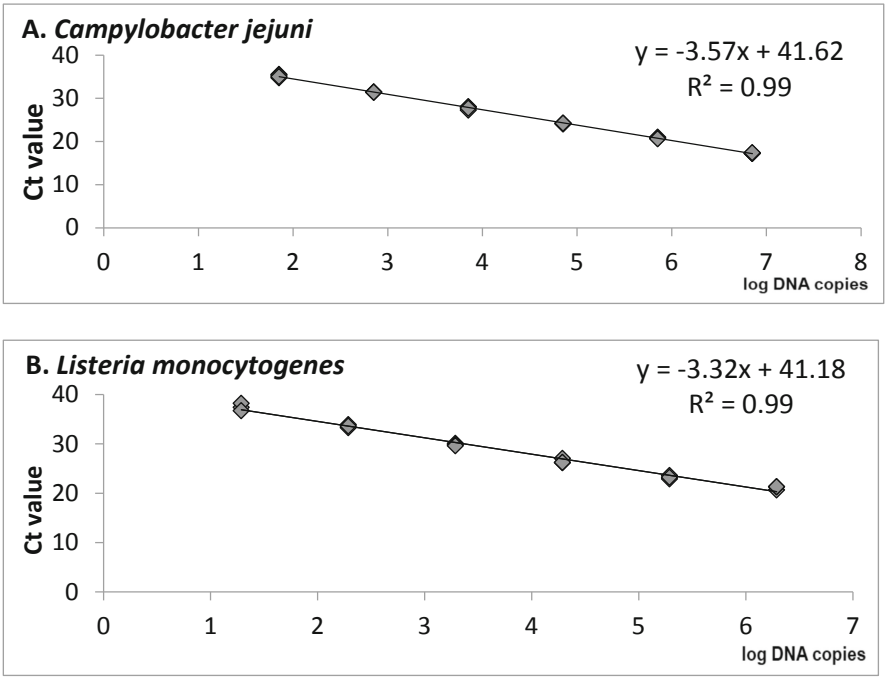

Fig. 2. Standard curve for absolute quantification with qPCR of A. *C. jejuni* and B. *L. monocytogenes.*

lower and upper limits of quantification (LOQ) given represent the range of quantification of the number of copies of the target DNA between 4.2×10^2 and 2.2×10^8.

As presented in Fig. 2B the standard curve with equation $y = -3.32x + 41.18$ and $R^2 = 0.99$ is suitable for further use and quantification of *L. monocytogenes* samples. LOD represents 8 ± 10 copies of DNA. LoQ represents the range of quantification of the number of copies of the target DNA between 4.14×10^3 and 6.70×10^7 copies.

3.2 Quantification of Anti-adhesion Activity of Phenolic Compounds on *C. Jejuni* Using qPCR

A standard curve was constructed by combining absolute quantification of the base *C. jejuni* were treated by the addition of pure phenolic compounds at a subinhibitory concentration of 0.1 mg/ml. Ct values obtained by the qPCR method and DNA copy number values obtained by combining the dPCR method and qPCR are collected. The positive control represents adhesion under the given conditions. The qPCR results are expressed as Ct $\pm$ SD values and the anti-adhesive efficacy as % reduction in Δ DNA copy number relative to the positive control (Table 1).

Table 1. Anti-adhesive efficacy of phenolic compounds on *C. jejuni* at a subinhibitory concentration of 0.1 mg/ml. The results represent Δ DNA copy number relative to the positive control. Results are statistically significantly different relative to control (* p 0.05) or statistically significantly different from all (^{x}p 0.05).

Phenolic compound	Ct	SD	DNA copies	SD	DNA copies	Anti-adhesion (%)
Carvacrol	31.82	0.41	2.59×10^4	1.1×10^4	1.71×10^6	98.5 *
Thymol	31.54	0.07	3.26×10^4	3.37×10^3	1.70×10^6	98.1 *
α-pinene	25.91	0.06	2.74×10^6	1.57×10^5	1.01×10^6	0 *,^{x}
EGCG	30.12	0.21	1.24×10^5	2.4×10^4	1.61×10^6	92.9 *
Control	26.62	0.003	1.74×10^6	3.68×10^3	/	/

The results show the best anti-adhesive effect of carvacrol (98.5%, DNA copy number $= 2.59 \times 10^4$) and thymol (98.1%; DNA copy number $= 3.26 \times 10^4$), as their DNA copy number is lower than that of the positive control (DNA copy number $= 1.74 \times 10^6$) by up to 2 log. The addition of EGCG at the same concentration reduced the number of DNA copies compared to the control sample, but only by one log (1.24×10^5). Thus, EGCG reduced the number of adherent cells by 92.9%. The addition of α-pinene at the subinhibitory concentration of 0.1 mg/ml had no anti-adhesive effect, but increased the number of attached cells by up to 58.1%.

3.3 Quantification of Anti-adhesion Activity on *L. Monocytogenes* Using qPCR

L. monocytogenes were treated by the addition of pure phenolic compounds at a subinhibitory concentration of 0.1 mg/ml. The positive control represents complete adhesion under the given conditions. The qPCR results are expressed as Ct ± SD values and the anti-adhesive efficacy as % reduction in DNA copy number relative to the positive control (Table 2).

Table 2. Anti-adhesive efficacy of phenolic compounds on *L. monocytogenes* at a subinhibitory concentration of 0.1 mg/ml. The results represent Δ DNA copy number relative to the positive control. Results are statistically significantly different relative to control (* p 0.05) or statistically significantly different from all (^{x}p 0.05).

Phenolic compound	Ct	SD	DNA copies	SD	DNA copies	Anti-adhesion (%)
Carvacrol	25.53	0.06	5.39×10^6	2.46×10^5	2.05×10^7	79.1 *
Thymol	25.31	0.08	6.23×10^6	3.76×10^5	1.96×10^7	75.9 *
α-pinene	25.45	0.14	5.69×10^6	6.12×10^5	2.02×10^7	78.0 *
EGCG	24.61	0.10	9.58×10^6	6.81×10^5	1.63×10^7	63.0 *, ^{x}
Control	22.88	0.10	2.59×10^7	1.74×10^6	/	/

The results showed the strongest anti-adhesive activity (79.2%) compared to other components when *L. monocytogenes* was treated with the active ingredient carvacrol. α -pinene with 78.0% and thymol with 75.9% anti-adhesive activity also showed good effect. All the components have anti-adhesive activity with 50.0% activity at sub-inhibitory concentration (Table 2).

The results show the best anti-adhesive effect of carvacrol (98.5%, DNA copy number $= 2.59 \times 10^4$) and thymol (98.1%; DNA copy number $= 3.26 \times 10^4$), as their DNA copy number is lower than that of the positive control (DNA copy number $= 1.74 \times 10^6$) by up to 2 log. The addition of EGCG at the same concentration reduced the number of DNA copies compared to the control sample, but only by one log (1.24×10^5). Thus, EGCG reduced the number of adherent cells by 92.9%. The addition of α-pinene at the subinhibitory concentration of 0.1 mg/ml had no anti-adhesive effect, but increased the number of attached cells by up to 58.1%.

3.4 The Comparison of Anti-adhesion Activity on *C. jejuni* and on *L. monocytogenes*

We also compared the antiadhesive activities of phenolic compounds against *C. jejuni* and *L. monocytogenes* (Fig. 3).

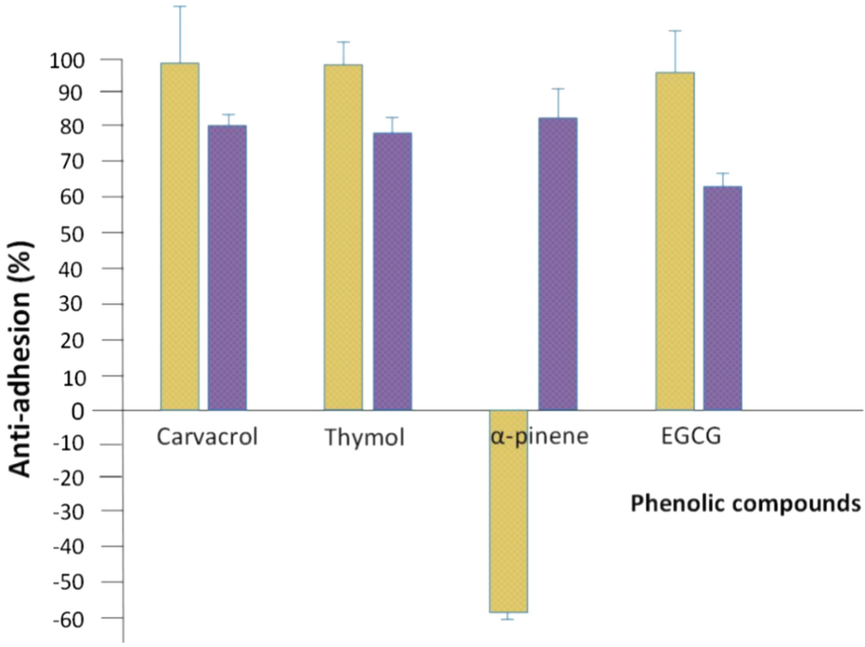

Fig. 3. Comparison of the anti-adhesion (%) activity of phenolic compounds on Gram negative *C. jejuni* and Gram positive *L. monocytogenes.*

Results on Fig. 3 showed generally better anti-adhesive activity of the natural agents on *C. jejuni* than *L. monocytogenes*. A special case is the effect of α-pinene, which caused

a 58.1% adherence stimulation of *C. jejuni,* but it had strong anti-adherence activity on *L. monocytogenes.*

4 Discussion

qPCR has long been established as a suitable method for the quantification of bacterial adhesion because the method is rapid, accurate, sensitive, and suitable for the study of bacterial adhesion [15]. Nevertheless, the method has some limitations, the most important being its high cost [16].

Despite the successful development of methods to quantify adherent bacterial cells, successful studies and methods are still lacking. Molecular methods have been cited as the most suitable methods for determining bacterial cell adhesion [17]. Among the most promising are qPCR-based methods. In our study, the quantification of adherent cells was based on the previous findings [5, 10, 17]. At the same time, it is also necessary to point out the lack of studies in this field. Using a validated method of combining dPCR and qPCR [5, 10], we used dPCR to absolutely quantify the copy of target DNA to create a standard curve and generated it using the qPCR method. By using sequence-specific probes and oligonucleotide primers, we ensured high sensitivity and specificity of the method and confirmed the suitability of the method we used [5, 10, 17]. The multiplication efficiency in the construction of the standard curve for *C. jejuni* was 90.7% and in the case of *L. monocytogenes* 100.1%, the slopes of the curves were in the optimal range k = −3.58 and −3.10 and the correlation coefficient between 1 R2 0.98. The qPCR results also showed the extreme sensitivity of the method with a detection limit of 1 ± 1 (*C. jejuni*) and 8 ± 10 (*L. monocytogenes*) DNA replicates. The obtained properties of the standard curves are in accordance with the requirements for the implementation and validation of qPCR methods [13, 14] and are comparable to the experimental ones we followed [5, 10, 17]. Thus, the combination of the dPCR and qPCR methods to generate a standard curve and absolute quantification of DNA samples proved to be a method with good efficacy, sensitivity, and specificity. Direct sampling from a microtiter plate and derivation of the dPCR and qPCR method in a few hours allows rapid quantification of adherent cells. Obtaining results with low standard deviations testifies to the good repeatability of the method, and the combination of dPCR and qPCR methods allows us to absolutely quantify the attached bacteria while excluding the influence of the methods.

The addition of phenolic compounds at subinhibitory concentration has already been shown to be an effective approach to combat adhesion and biofilm formation of *C. jejuni* and *L. monocytogenes* [5, 9, 18–20]. In our study, the anti-adhesive activity of carvacrol, thymol and EGCG against *C. jejuni* and *L. monocytogenes* was determined or confirmed by adding these agents at subinhibitory concentration (0.1 mg/ml). Carvacrol showed the best antimicrobial activity in both bacteria with 98.5% (*C. jejuni*) and 79.2% (*L. monocytogenes*). The carvacrol isomer thymol had similar efficacy in both bacteria (98.1% and 75.9%), confirming the influence of the properties and structure of the molecule on the specific effect. We hypothesize that carvacrol and thymol act specifically on whips [19]. Despite its extraordinary antimicrobial activity [21], the phenol EGCG showed lower anti-adhesive activity against *L. monocytogenes* at subinhibitory concentration, reaching only 63.0%. Interesting results were obtained by terpene α-pinene, which has

a very specific effect depending on the type of bacteria. According to [22], α-pinene has weak antimicrobial activity, while at subinhibitory concentrations it acts as an inhibitor of *C. jejuni* efflux pumps. In our case, α-pinene at subinhibitory concentrations showed good anti-adherent activity on *L. monocytogenes* (78.01%), while it had the opposite effect on *C. jejuni*, increasing adherent biomass by up to 58.1% compared to the control. Thus, we have shown that α-pinene at low concentrations (0.1 mg/ml) and after 24 h of incubation has a stimulatory effect on the adhesion of *C. jejuni*, while it has an inhibitory effect on the adhesion of *L. monocytogenes*.

Gram negative bacteria (*C. jejuni*) are generally more resistant to antimicrobial factors than Gram positive bacteria (*L. monocytogenes*). In particular, phenolic compounds show greater antimicrobial activity against Gram positive bacteria because their cell wall structure allows them to enter the cell interior more easily [23]. However, since we only wanted to determine the effect on adhesion and not on growth or viability by applying natural compounds at subinhibitory concentrations, we are interested in their effect on adhesion mechanisms and not on the ability to enter the cell. The results of poorer anti-adhesive activity against *L. monocytogenes* can be attributed to several factors. Gram positive bacteria contain teichoic acid in the cell wall, which is covalently bound to glycolipids of the cytoplasmic membrane. This increases the negative charge of the cell wall of Gram positive bacteria and the intensity of adhesion [24]. Thus, faster and more intense adhesion of *L. monocytogenes* is possible. Based on the comparison of the control samples of *C. jejuni* and *L. monocytogenes*, we also observed a higher number of DNA in the control sample of *L. monocytogenes*, indicating a higher degree of adhesion of *L. monocytogenes*. A higher degree of adhesion of Gram positive than Gram negative bacteria was also found by [9]. The latter finding may also indicate the importance of the time frame for adhesion measurement. It is likely that more *L. monocytogenes* cells adhere than to *C. jejuni* within 24 h of incubation. Rapid adhesion also leads to a more rapid transition to further stages of the biofilm, against which agents at subinhibitory concentrations are no longer sufficiently effective, as biofilms are generally more resistant. It is also possible to act specifically on bacteria of the species *C. jejuni* and *L. monocytogenes* and not generally on Gram negative and Gram positive. The good anti-adhesion effect on both Gram positive and Gram negative bacteria encourages further research and the possibility of using these agents in the fight against biofilms in the food industry.

5 Conclusion

The attachment of pathogenic bacteria to abiotic surfaces, the formation of biofilms and the spread of the spectrum of antibiotic-resistant bacteria is one of the most important food safety issues in the food industry. Therefore, research in this area aims to understand the effects of agents on adhesion and biofilm formation, and to develop new, more efficient and industrially suitable methods for the detection and quantification of pathogenic bacteria. In this work, we focused on using qPCR and dPCR based method for absolute quantification of bacterial adhesion. At the same time, we aimed to test the anti-adhesive activity of the natural compounds carvacrol, thymol, α-pinene and EGCG, which are good and effective alternatives to chemical antimicrobials. Anti-adhesive efficacy was

determined based on absolute quantification of the standard by dPCR and quantification by qPCR. At the same time, we succeeded in optimizing the method for absolute quantification of adherent cells using the Applied Biosystems 7500 Real-Time PCR system and the QuantStudio 3D Digital PCR system. *C. jejuni*, representing Gram negative bacteria, and *L. monocytogenes*, representing Gram positive bacteria, were selected as test bacteria. In our study, we found good antiadhesive activity of carvacrol, thymol and EGCG at a subinhibitory concentration of 0.1 mg/ml, with approximately 90% anti-adhesive activity. The terpene α-pinene also showed interesting activity by exhibiting increased growth and better adhesion (58%) at the subinhibitory concentration. Thus, it confirmed its dual activity against *C. jejuni*, depending on the concentration range. All the tested compounds also showed good anti-adhesive activity against *L. monocytogenes.*

Our results suggest the possibility of using natural agents as alternative means to prevent bacterial cell adhesion to abiotic substrates. We also demonstrated the possibility of rapid, specific, accurate and reproducible absolute quantification of adherent cells by dPCR and qPCR methods.

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Effect of Hen Breed and Production System on the Egg Weight, Egg Components Percentage and Yolk to Albumen Ratio

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Abstract. The objective of this study was to compare egg and major egg components' weight, as well as yolk to albumen ratio, laid by hens of two commercial breeds (Hy-Line and ISA Brown) and a Croatian local breed, Hrvatica. Eggs were collected from different production systems: Hy-Line-enriched cage housing, ISA Brown-aviaries housing, ISA Brown-free range raising, Hrvatica hen-free range raising (organic production). The heaviest eggs were laid by the Hy-Line hens from enriched cage housing (70.06 g), while Hrvatica hens laid significantly the lightest eggs (51.01 g). The eggs from Hrvatica hens had significantly the lowest albumen percentage (53.30%) and highest yolk percentage (33.96%). Consequently, the yolk to albumen ratio was the highest for Hrvatica hen eggs (0.609), which is on average 44.6%, 52.6% and 36.8% higher in comparison to eggs from cage housed Hy-Line, aviaries housed ISA Brown and free-ranged ISA Brown hens, respectively.

Keywords: Laying hens · Production system · Egg components · Yolk to albumen ratio

1 Introduction

Eggs are one of nature's nearly perfect protein foods, offering nutrients of great biological value and are of considerable importance in feeding the population, as they are a relatively inexpensive and complex food source of very high biological value. The daily animal protein requirement for the human body can be covered with eggs in the cheapest way and with the lowest environmental impact [1, 2]. Consumers are more and more interested in egg quality, which means that ensuring high quality of poultry products takes on increasing significance [3]. The egg morphological characteristics, such as weight and percentage of main components and their correlations, are very important because they influence egg quality [4]. Egg component yields may have little importance to the consumer, but they are significant to the egg-processing industry, with yolk having a higher market value [5]. Egg quality is influenced by many internal and external factors, of which genotype and housing system are major importance [6]. Currently, the production of table eggs in the European Union is mainly based on the enriched cage

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housing system. This system is the most economical form of egg production because it allows the largest number of laying hens per unit area compared to other systems and increased profit per egg. In addition, cage housing system provides good hygienic conditions which are very important for obtaining quality eggs and also protects birds from predators and ensures controlled environmental conditions [7]. As animal welfare, as well as environmental and health awareness issues, become increasingly prominent in developed countries, consumer demand for eggs has also changed significantly in recent years, with an increasing number of consumers buying eggs produced in non-cage housing systems, including the organic system [2]. Organic eggs are expected to have better egg quality compared to conventional ones due to the hen's free access to outdoor life and organic feed consumption. By eating various grasses and herbs, free-range layers enrich their food and assimilate valuable natural nutrients that influence some egg quality traits. Therefore, consumers are willing to pay more for organically produced eggs compared to conventional ones [8]. In recent years, a relatively large number of consumers in Croatia have been interested in buying free-range or organic eggs.

The aim of the study was to compare the effect of different layers genotype kept in different housing systems on the egg quality characteristics such as weight and composition of eggs laid by hens of two commercial breeds and one Croatian domestic breed.

2 Materials and Methods

2.1 Sampling Protocol

Eggs were collected from four farms with different production systems located near Bjelovar, a small town 80 km north-east from Zagreb, the capitol of Croatia (latitude 45° 54′ N, longitude 16° 50′ E). Two commercial and one domestic breed were included in this study. Initial samples of eggs were collected at 38 weeks of age and a total sample of 100 eggs from each breed were used as samples. On the first farm Hy-Line hens were housed in enriched cages; on the second, ISA Brown hens were housed in aviaries; on the third, ISA Brown hens were raised free-range; and on the fourth Hrvatica hens were raised free-range. A Croatian local breed called Hrvatica was created in the first half of the 20th century in the area along the Drava river in north-west of Croatia by crossing domestic hens with Leghorn roosters, while the final characteristics of Hrvatica hens were obtained by a crossing with breed Wellsummer. The Hrvatica hens are characterized by good egg production (200–220 eggs per year) and tasty meat, and hen mass is 1.6–1.8 kg. Although they were almost eradicated after World War II, now their number is increasing, and Hrvatica hens are being bred on small farms in almost all of Croatia. The most applicable breeding system is free-range raising and hens are fed with organic feed to obtain organic eggs, which are sold at a better price on the market [9]. The Hy-Line and ISA Brown laying hens were fed ad libitum with a commercial feed for laying hens (11.68 MJ ME/kg, 179 g CP/kg, 39.3 g Ca/kg, 4.6 g P/kg). Hrvatica hens were fed only maize grits, free of chemical products and medicines, so this production can be considered as organic.

2.2 Analytical Methods

To evaluate the weight, eggs were weighed separately on a precision electronic balance reading to 0.01 g, then cracked, and the yolk was carefully separated from the albumen manually. The chalazae were carefully removed from the yolk and all yolks were also rolled several times on a paper towel to remove adhering albumen before weighing. The shells were carefully washed to remove albumen and dried at 21 °C for 48 h before weighing. Albumen weight was determined by subtracting yolk and dry shell weights from the total egg weight. Using the individual weight of each egg and its components, yolk percentage (yolk weight/egg weight × 100), albumen percentage (albumen weight/egg weight × 100), eggshell percentage (eggshell weight/egg weight × 100) and yolk to albumen ratio (yolk weight/albumen weight) were calculated [10].

2.3 Statistical Analysis

The obtained data were analysed applying the analysis of variance (ANOVA) using the general linear models procedure of SAS software [11]. When the ANOVA showed significant differences, the LSD test was used to compare the mean results. The differences were considered as significant if $p < 0.05$.

3 Results and Discussion

The total weight and components weight of eggs from different hen breeds and production systems are presented in Table 1. Significantly heavier eggs were laid by commercial breeds, among which were the heaviest eggs from cage housed Hy-Line hens (70.06 g), while the lightest were laid by Hrvatica hens from a free-range system (51.01 g). The Hrvatica hen eggs were on average 37.3%, 34.0% and 21.9% lighter in comparison to cage-housed Hy-Line, aviaries-housed ISA Brown and free-ranged ISA Brown eggs, respectively. The significantly lower egg weight in local breeds in comparison to commercial breeds was also reported by other authors [5, 12–15]. The significantly lower egg weight in local breeds is not surprising as commercial breeds have been submitted to important breeding pressure for egg weight improvement [16].

In accordance with the results obtained for total egg weight, albumen weights were also significantly lower for eggs from Hrvatica hens (27.71 g) in comparison to commercial breed hens. The heaviest albumen was observed in eggs laid by ISA Brown hens from an aviaries-housing system (42.80 g). The higher albumen weight of eggs from a commercial breed than from a local breed was also obtained by Tixier-Boichard et al. (2006) and Islam et al. (2017) [13, 15]. The positive correlation between total egg weight and albumen weight has been reported by Suk and Park (2001) and Hartmann et al. (2003) [12, 17].

Table 1. Total and components weight of eggs from different hen breed and production system.

		Egg weight (g)	Albumen weight (g)	Yolk weight (g)	Shell weight (g)	Y: A ratio
Hy-Linecage housing	Mean	70.06[a]	42.56[a]	17.78[a]	8.84[a]	0.4213[a]
	Max.	81.16	52.21	20.98	10.17	0.5259
	Min.	62.40	36.34	15.06	7.10	0.3212
	S_d	4.49	4.03	1.42	0.77	0.0513
ISA Brown aviaries	Mean	68.37[a]	42.80[a]	16.96[a]	8.61[a]	0.3993[a]
	Max.	80.09	49.67	19.77	10.95	0.5068
	Min.	61.61	36.74	14.72	7.56	0.2976
	S_d	4.25	3.70	1.32	0.73	0.0466
ISA Brown free-range	Mean	62.18[a]	37.56[a]	16.64[a]	7.92[a]	0.4454[a]
	Max.	65.17	44.95	19.06	10.52	0.5239
	Min.	59.58	31.40	11.66	5.16	0.2594
	S_d	1.66	2.61	1.52	1.05	0.0588
Hrvatica hen free-range	Mean	51.01[a]	27.71[a]	16.66[a]	6.64[a]	0.6092[a]
	Max.	61.10	34.52	22.82	9.89	0.9641
	Min.	41.29	19.87	10.80	4.71	0.3711
	S_d	3.99	2.93	2.31	0.94	0.1155

[a]Means in the same row with different superscripts differ significantly at $p < 0.05$

The highest yolk weight was observed for eggs laid by cage-housed Hy-Line hens (17.78 g), while no statistical difference of yolk weight was observed between the ISA Brown aviaries-housed (16.96 g), ISA Brown free-ranged (16.64 g) and Hrvatica hen free-ranged hens (16.66 g). Rizzi and Marangon (2012) [18] observed a significantly heavier yolk in the eggs from local Italian breed hens in comparison to commercial hybrids.

The heaviest shell was observed for eggs laid by cage-housed Hy-Line hens (8.84 g) and ISA Brown aviaries (8.61 g), while the lightest shell for eggs laid by Hrvatica hens (6.64 g). These results show a positive correlation between shell weight and total egg weight, and this is in accordance with results of Suk and Park (2001) and Rizzi and Marangon (2012) [12, 18], who reported heavier shells for eggs from commercial hybrids compared to eggs from local breeds.

The yolk to albumen (Y:A) ratio was the highest for Hrvatica hen eggs (0.609), which is on average 44.6%, 52.6% and 36.8% higher in comparison to eggs from cage-housed Hy-Line, aviaries-housed ISA Brown and free-ranged ISA Brown eggs, respectively. The significantly higher Y:A ratio for eggs from local breeds in comparison to commercial hybrids is also reported by Suk and Park (2001) and Rizzi and Marangon (2012.) [12, 18]. This is in accordance with Dottavio et al. (2005) [10], who found that smaller eggs from local breeds had higher Y:A ratios than larger eggs from commercial hybrids.

Figure 1 provides the percentage of egg components related to egg weight. The eggs from Hrvatica hens had a significantly lower albumen percentage (53.30%) and higher yolk percentage (33.96%) than eggs from commercial breed hens. The highest albumen percentage (62.53%) and the lowest yolk percentage (24.86%) were observed for eggs from aviaries-housed ISA Brown hens. Sun and Park (2001) [12], Tixier-Boichard et al. (2006) [13], Moula et al. (2010) [14], Rizzi and Marangon (2012) [18] and Lordelo et al. (2020) [5] reported that eggs from commercial hybrids have a greater proportion of albumen and lower proportion of yolk in comparison to local breed eggs. Kouba (2003) [19] obtained a higher proportion of yolk from organic eggs (35%) compared to conventional eggs (35% vs. 33.8%) and a lower albumen percentage (54.6% vs. 55.7%). The importance of yolk proportion is due to its large impact on yolk production and total egg dry matter, a trait of great importance for the egg processing industry. The yolk value can be twice that of the albumen, and a higher yolk yield can significantly affect the egg processing industry's profitability [17].

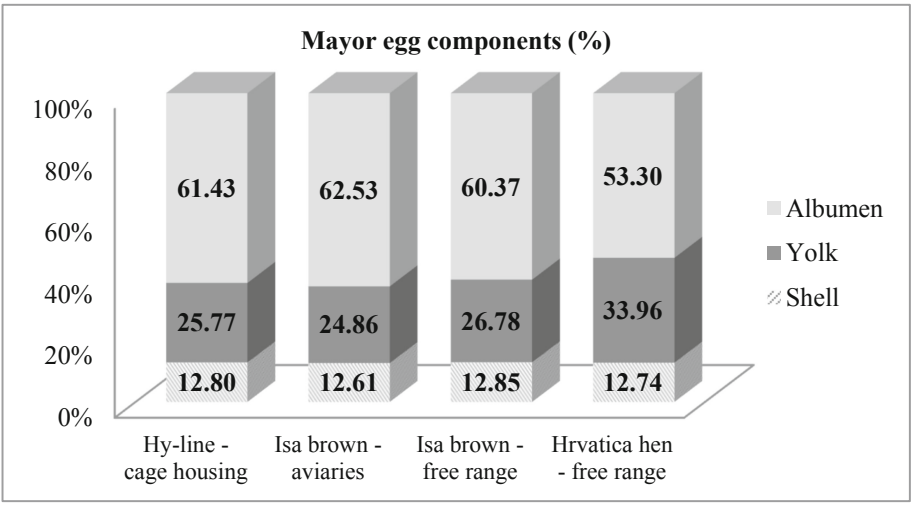

Fig. 1. Percentage of major components of eggs from different hen breeds and production systems.

In this study there were no significant differences in shell percentage of total egg weight between observed breeds. The highest shell percentage was observed for eggs from free-ranged ISA Brown hens (12.85%) and the lowest for eggs from aviaries-housed ISA Brown hens (12.61%). Lordelo et al. (2020) [5] reported that eggs from commercial hybrid have a higher shell percentage than eggs from local Portuguese breeds. On the other hand, Tixier-Boichard et al. (2006) [13] and Moula et al. (2010) [14] reporded a significantly higher shell percentage for eggs from local than eggs from commercial hybrids.

4 Conclusions

Eggs of a Croatian local breed, Hrvatica, from free-ranged organic production were significantly lighter in comparison to eggs of commercial breeds from three different production systems. Among these productions, the heaviest eggs were laid by enriched cage-housed Hy-Line hens. Hrvatica hen eggs had significantly lower albumen and higher yolk percentage than eggs of commercial breeds, while there were no significant differences in shell percentage between observed breeds. The yolk to albumen ratio was significantly higher for Hrvatica hen eggs in comparison to eggs from cage-housed Hy-Line hens, as well as aviaries and free-ranged ISA Brown hens.

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Investigation on Plant Distillation Products Addition on Biopolymer Film Properties

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Abstract. The goal of this study was to see how plant distillation products affected the characteristics of polysaccharide biopolymer films. It was chosen to test hydrolats and essential oils of two plant varieties: *Artemisia dracunculus* and *Artemisia absinthium*. During biopolymer synthesis essential oils were added in a percent of 0.1% and 0.5%, while hydrolats were added in a percent of 10% and 50%. Eight groups of samples were obtained while plain film (with 0% added component) was used as control. Mechanical (thickness, tensile strength and elongation at break), physico-chemical (moisture content, solubility and swelling), structural properties (FTIR) and antioxidative properties (DPPH) were tested. With the inclusion of active components, tensile strength reduced and elongation at break rose, according to the obtained data. The moisture content was slightly reduced by the addition of active components. Also, the obtained results show that the hydrophobic component addition reduced the biopolymer film based on polysaccharide ability to swell in water. Antioxidant activity was dose-dependent, more pronounced in samples to which essential oil was added compared to samples with added hydrolates. The difference between the applied varieties of plant distillation products was also reported. The results of this research represent the initial step of biopolymer film properties optimization with the final aim to be applied for food packaging.

Keywords: Biopolymer films · Essential oils · Hydrolats · *Artemisia dracunculus* · *Artemisia absinthium* · Properties

1 Introduction

Polymer materials are the most commonly used packaging materials, but, in addition to numerous of advantages and benefits of their application, they are accompanied by two major disadvantages: they are obtained from non-renewable raw materials, as well as they present serious burden on the environment after use and disposal (Lazić and Novaković 2010). There has been an increase in demand for packaging materials that

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are biodegradable, offer less environmental risk, and are produced from sustainable and renewable resources (Han et al. 2018). Creating unsustainable (due to environ-mental difficulties) synthetic plastics takes 65% more energy and produces 30% to 80% more greenhouse gases than producing bioplastics (Tajeddin and Arabkhedri 2020).

Biopolymer films are a modern alternative to the use of commercial packaging materials. Polysaccharides are a applicable substrate for biopolymer film synthesis because they have good mechanical, structural, and gas barrier properties (Falguera et al. 2011). Polysaccharide based biopolymer films have weak barrier qualities to water vapor, high values of solubility, and a high swelling degree, due to their hydrophilic nature, which limits their broad use.

Starch is one of the maximum tremendous polysaccharides in nature, which is obtained from renewable sources, whose price is low, so using starch-based packaging withinside the area of food packaging is a capability course for the improvement of packaging materials nowdays (Šuput 2016a). It is possible to easily form (by wet - "casting" or dry – extrusion process) packaging films from starch that are flavourless, odor-free and translucent, for that reason stopping any alternate withinside the taste, aroma and look of the packed food products (Chiumarelli and Hubinger 2012).

Starch is a heterogeneous polymer consisting of α-D-glucose units. Unites are linked by α-(1,4)- and α-(1,6)- bonds (Huijbrechts 2008). Starch granule dry matter (98–99%) is consisted of amylose (linear) and amylopectin (branched) (Hódsági 2011). The mechanical residences of starch biopolymer films are described through the macromolecular chain withinside the amorphous phase mobility, amylose and amylopectin ratio and plasticizers and water content quantity. Great gas barrier properties is one of the most important starch-based films application advantages but poor water vapor barrier properties makes their imperfection. This property can be improved by the addition of hydrophobic components, such as, for example, essential oils. Combining natural antimicrobial and antioxidant substances, such as e.g., essential oils, with starch films can give excellent results in the form of obtaining active packaging materials (Šuput et al. 2016b). The biopolymer film's active role is meditated withinside the food safety from oxidation and microbiological damage, which results in progressed quality and safety of packaged food products (Kim et al. 2012; Lee et al. 2012).

The object of this manuscript was to examine the effect of plant distillation products – essential oils and hydrolats of two plant varieties (*Artemisia dracunculus* and *Artemisia absinthium*) on starch biopolymer films properties regarding mechanical, physico-chemical, structural and antioxidative properties.

2 Materials and Methods

Materials

Modified corn starch was provided by Palco (Šabac, Serbia), while essential oils and hydrolats were kindly provided from Institute of field and vegetable crops (Novi Sad, Serbia). Glycerol (99,8%) was purchased from Laboratorija d.o.o. (Novi Sad, Sebia)

and 2, 2-difenil-1-pikrilhidrazil (DPPH˙) was acquired from Sigma-Aldrich Chemical Co. (Saint Louis, USA).

Methods

Aqueous modified corn starch solution (1.5 % (w/v)) was heated at 90 ˚C in a water bath (60 min). Glycerol (40% of the original starch mass) was added to the solution, that was maintained hot for 10 min more. Finally, essential oils and hydrolats were added according to the Table 1.

Table 1. Experimental design

Label	*Artemisia dracunculus*		*Artemisia absinthium*	
	Essential oil	Hydrolat	Essential oil	Hydrolat
C	0	0	0	0
Ad 0.1	0.1	0	0	0
Ad 0.5	0.5	0	0	0
Adh 10	0	10	0	0
Adh 50	0	50	0	0
Aa 0.1	0	0	0.1	0
Aa0.5	0	0	0.5	0
Aah 10	0	0	0	10
Aah 50	0	0	0	50

Essential oils and hydrolats were obtained by the method described in Aćimović (2021). The film-forming solution was homogenized (10000 rpm for 1 min) and then degassed beneath vacuum to cast off dissolved air. 50 g of film-forming solution was spill into Petri dishes and left to dry for 5 days at room temperature on a leveled area after which they were analyzed. Plain film (with 0% addition of any component) served as a control (blank shot).

Mechanical Properties

Film thickness was assessed by micrometer with 1 μm sensitivity. Ten replicates were carried out on each sample.

Tensile strength (TS) and elongation to break (EB) were quantified by using Instron Universal Testing Instrument Model No 4301 (Instron Engineering, Canton, Massachusetts, USA), in accordance with EN ISO 527-3:1995. Film samples had been cut (15 × 90 mm). The preliminary grip separation became set at 50 mm, and crosshead velocity became set at 50 mm/min. TS and EB measurements for every pattern had been repeated 8 times.

Physical Properties

Water content (*WC*) was calculated as the percentage of initial film weight loss during drying and reported on a wet basis, Eq. (1):

$$W_c = 100 \times \frac{w_1 - w_2}{w_1} \tag{1}$$

Film samples (1 × 1 cm) were weighed ($w1$), dried at constant temperature (105 ˚C) for 24 h and eventually weighed again ($w2$).

Swelling capacity became decided at the portions of films (1 × 2 cm), which have been weighed in air-dried conditions (W_d), after which they were dipped in deionized water (25°C) for two min. Samples were finally trampled with paper to dispose extra liquid and weighted again (W_w).

Swelling was assessed by changed technique defined through Bigi et al. (2001). The quantity of adsorbed water was evaluated along with Eq. (2) and expressed as percentage:

$$\text{SSSSSSSSSSSSS aaaaaaaaaaaaa} = 100 \times \frac{WW_{ww} - WW_{dd}}{WW_{dd}} \quad [\%] \tag{2}$$

where: w_w and w_d are the weights of the wet and air-dried samples.

The film solubility was determined by Kaewprachu et al. (2017) method. For total soluble matter quantification, small film portions (2 × 2 cm) have been oven dried (at 105 ˚C for 24 h) to acquire the preliminary film dry mass. Thereafter, films were lightly shaken in test tubes filled with 20 ml of deionized water at room temperature for 24 h. Residual film portions were oven dried at 105 °C for 24 h to acquire ultimate film dry mass. The percent of general soluble depend (% TSM) of the movies turned into calculated the usage of the Eq. (3).

$$TTSSTT\ [\%] = 100 \times \frac{WW_1 - WW_2}{WW_1} [\%] \tag{3}$$

where: w_1 and w_2 are initial dry mass before the test and final dry mass after the test.

Antioxidative Properties

DPPH radical scavenging activity

The DPPH radical scavenging activity of the active films was evaluated according to Morales and Jimenez-Perez (2001) method, with several modifications. An aliquot of 200 µl of the sample was added to 1 ml of a freshly prepared solution of DPPH (74 mg/l) in ethanol. The films were cut in pieces (10 × 10 mm), mixed with 2 ml of the DPPH ethanolic solution and left on a shaker for 24 h. The absorbance values were measured at 520 nm (T80 UV–Vis Spectrophotometer; PG Instruments, Lutterworth, UK) and the DPPH radical scavenging activity was calculated by the following equation (4):

$$\text{DPPH radi. scav. acti.} = \frac{\text{Ablank} - \text{Asample}}{\text{Ablank}} \times 100 \tag{4}$$

where Ablank is the absorbance value at 520 nm of the ethanolic solution of DPPH without the added sample and Asample is the absorbance value at 520 nm of the ethanolic solution of DPPH with the added sample.

Structural Properties

Fourier transform spectroscopy

FTIR analysis of the film samples was carried out using the IR spectrophotometer, Nicolet IS10, Thermo Scientific (Massachusetts, USA) and attenuation total reflection (ATR) extension. in the wave number range 4000 to 400 cm^{-1}, at a resolution of 4 cm^{-1}, Each sample was scanned 32 times, while background shot was taken before the analysis of each sample. Omnic 8.1. software (Thermo Fisher Scientific, MA, USA) was used to operate the FTIR spectrometer, collect and process all the data.

Statistical Analysis

MicroSoft Excel was used to run descriptive statistical analysis for computing the means and standard error (MicroSoft Office 2010). All obtained results were expressed as the mean ± standard deviation (SD).

3 Results and Discussion

Mechanical Properties

The resulting films were transparent, odourless, and easy to handle, according to ocular inspection. The films were not oily or sticky in any way. Films to which both essential oils were added had a mild odor of added oil, while those with hydrolats were neutral.

Film thickness was uniform and in the range 104.42 to 107.11 μm (Table 2). Film uniformity was demonstrated by very minimal standard deviation values, regardless of whether the films studied were biologically active compounds.

Table 2. Starch based edible films mechanical properties with different amount of added essential oil/hydrolat (mean ± SD)

	d (μm)	TS (N/15 mm)	EB (%)
C	104.42 ± 2.15	13.40 ± 0.25	23.88 ± 3.13
Ad 0.1	106.85 ± 3.16	6.25 ± 0.18	38.82 ± 1.75
Ad 0.5	106.31 ± 6.11	3.60 ± 0.09	43.97 ± 2.06
Adh 10	107.11 ± 2.85	3.73 ± 0.14	29.49 ± 1.05
Adh 50	106.96 ± 4.63	2.70 ± 0.09	57.97 ± 2.19
Aa 0.1	105.14 ± 5.38	8.80 ± 0.31	46.99 ± 1.86
Aa 0.5	105.95 ± 3.25	2.37 ± 0.11	55.93 ± 2.70
Aah 10	106.05 ± 3.62	5.14 ± 0.16	42.18 ± 1.45
Aah 50	105.21 ± 5.39	2.75 ± 0.06	60.12 ± 1.63

Results related to mechanical properties are also shown in Table 2. The mechanical properties are improved by the addition of essential oils and hydrolats, which have a plasticizer effect and thus attract water molecules. As a consequence of the presence of essential oil, interactions occur between essential oil molecules and starch molecules instead of "starch-starch" interactions. Table shows that the addition of both essential oils and both hydrolats decreases the tensile strength value, which indicates a loss of macromolecular mobility which is consistent with the research of Souza et al. (2013). Decrease in value was correlated with concentration of added oil/hydrolat. There was not observed significant difference related to the used plant species. Elongation at break values of the tested films increased, which is consistent with the results of other authors (Ghasemlou et al. 2013; Benavides et al. 2012). Same as tensile strength, decrease in value was correlated with concentration of added oil/hydrolat. There was not observed significant difference related to the used plant species.

The values of tensile strength decrease, and the values of elongation at break increase, which is a consequence of the addition of essential oils to biopolymer matrices because the cohesion of starch network forces is reduced, which reduces the resistance of the film to cracking. In order for biofilm to find wider application in industry, it must be resistant to the stresses that occur during shipping, handling, and application, preserving the packaging's integrity and the packaged food's qualities. Starch films have poor tensile strength and elongation at break when compared to synthetic polymers, yet these properties are sufficient for them to be used in the food industry (Souza et al. 2013).

Physical Properties

Physical properties related to water content and swelling are presented in Table 3.

Table 3. Starch based edible films water content (%), swelling (%) and solubility (%) with different amount of added essential oil/hydrolat (mean ± SD)

	Water content (%)	Swelling (%)	Solubility (%)
C	16.53 ± 1.21	206.58 ± 5.38	56.62 ± 1.83
Ad 0.1	13.20 ± 1.06	188.55 ± 6.09	52.97 ± 0.95
Ad 0.5	14.48 ± 0.85	184.66 ± 3.33	48.11 ± 2.22
Adh 10	9.73 ± 0.74	190.92 ± 5.66	55.25 ± 1.95
Adh 50	5.17 ± 0.36	195.60 ± 3.18	53.05 ± 1.05
Aa 0.1	15.02 ± 1.22	165.75 ± 7.14	46.67 ± 2.36
Aa 0.5	12.21 ± 1.13	157.09 ± 4.21	33.56 ± 0.75
Aah 10	5.85 ± 0.51	172.45 ± 3.15	50.33 ± 2.66
Aah 50	3.69 ± 0.33	179.05 ± 6.66	48.12 ± 2.21

The water content in the obtained films significantly affects the physical and barrier characteristics. There is a decrease in the value of moisture content as the concentration of added oil increases. This decrement is even more pronounced when applying hydrolates. The highest measured value of moisture content was 16.53% for the control film sample and the lowest (5.17% and 3.69%) in samples with 50% of *Arthemisia dracunculus* and *Arthemisia absinthium* hydrolats, respectively.

When selecting materials for a specific purpose, such as food packaging with a high moisture content, the tendency to swell in water is an undesired attribute. The values of water swelling capacity of prepared starch films with and without essential oils/hydrolats are shown in Table 3. It's far anticipated that hydrophilic compounds have to increase film's swelling ability, while hydrophobic compounds lower it (Kavoosi et al. 2013). Results proved that crucial oil addition reduced swelling degree. Obtained results proved that the films solubility observed the equal fashion as moisture content material that's according with other findings (Ghasemlou et al. 2013; Fabra et al. 2010).

The water solubility of prepared starch films with/without essential oils is presented in Table 3. The obtained results are in agreement with Song et al. (2018) who pointed to interplay among the essential oil components and film hydroxyl groups in addition to lower withinside the films' hydrophilic nature, which could lessen availability of hydroxyl groups for interplay with water molecules, therefore ensuing in an extra waterproof film.

3.1 Antioxidative Properties

DPPH free radical scavenging is one of the most commonly used tests to investigate the antioxidant potential of various natural components in vitro. This test shows that the analyzed starch films can "scavenge" free radicals that act as free hydrogen atoms or as electron donors. As expected, the lowest value of AA (%) was detected for the control film.

From the Fig. 1 it can be seen that the presence of oil compared to hydrolates contributed to significantly higher values of antioxidant activity. Between the two plant species used, slightly higher activity is observed in *Atremisia dracunculus* oil and hydrolate. Also, from the figure it can be seen that the values obtained for 0.1% of the essential oil applied corresponded to the values obtained for 50% of the applied hydrolate for both plant species.

It has been proven that antioxidant values are directly proportional to the added amount of essential oils (dose-dependent), which is in accordance with the findings of other authors (Oriani et al. 2014; Abdollahi et al. 2012, Norajit et al. 2010).

Structural Properties

All collected spectra are shown in Fig. 2. What is common to all spectra is an extremely characteristic wide peak occurs in all samples of the examined starch films between 3600 cm^{-1} and 3000 cm^{-1}, centered at about 3300 cm^{-1}. The peak corresponds to the stretching region of hydrogen bonds, which is caused by free, inter- and intramolecularly

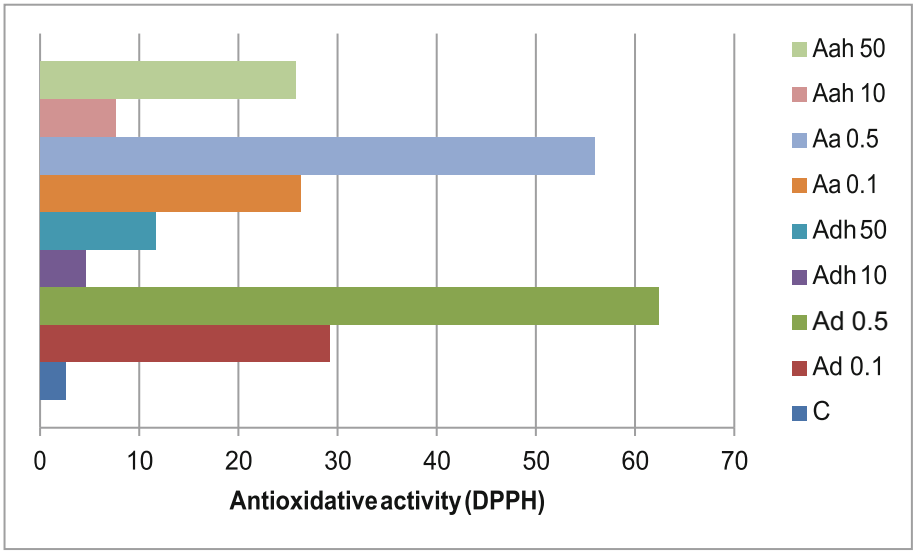
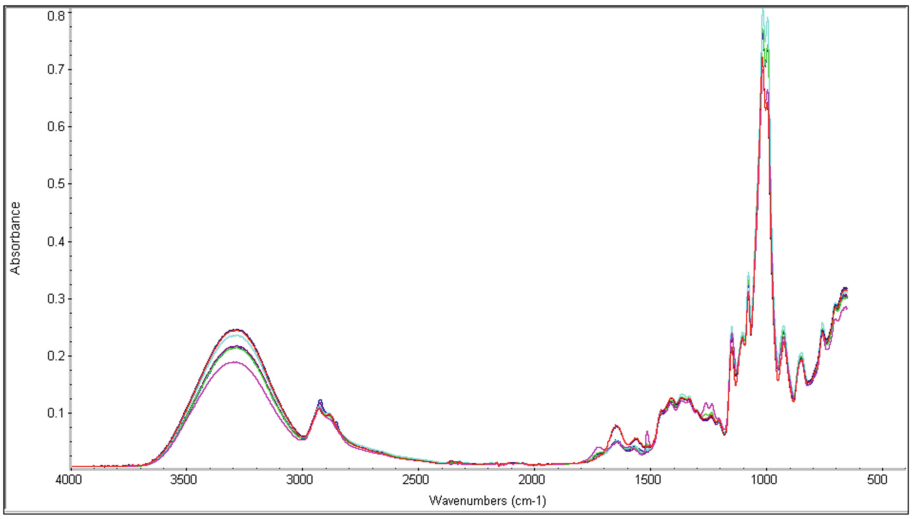


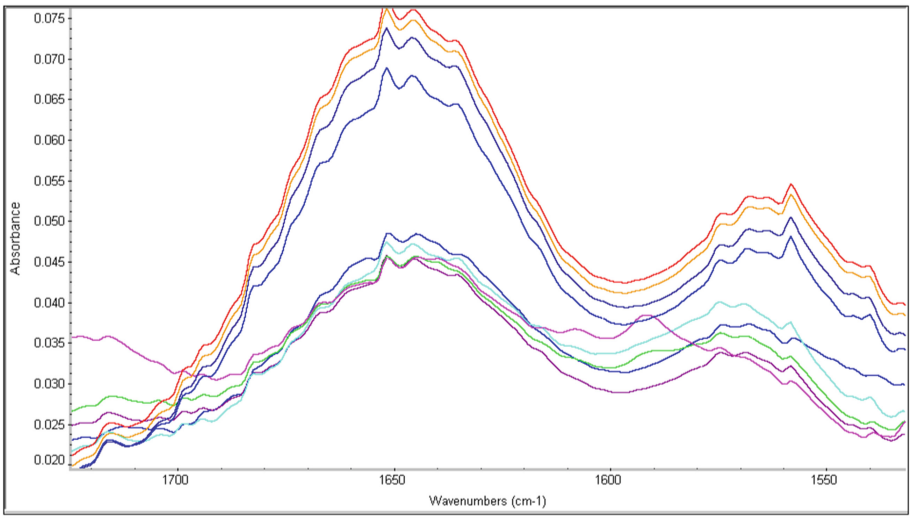
Fig. 1. Antioxidative activity (DPPH) of starch based edible films with different amount of added essential oils/hydrolats

bound hydroxyl groups that enter the starch structure, which is confirmed by the results of other authors (Xiong et al. 2008). According to Huang et al. (2006) the most intense absorption in the IR region covers the region of 1300 cm^{-1}–800 cm^{-1}, which is also a typical area of saccharide peaks. It became discovered that the absorbance value of the control film (without added active plant components) is highest at each wavelength, which means that the realized bonds are weaker in the samples in which they were added. The bonds are weak due to the presence of oil/hydrolate in the system, regardless of the plant species.

The area in which the absorbance value of the starch film samples with the addition of hydrolate is higher than the absorbance value of the control film and films with added essential oils is the area of 1550 cm^{-1}–1750 cm^{-1} (Fig. 2b). This spectral region is related to non-starch-derived structures, since native starch does not absorb in the 1800 cm^{-1}–1540 cm^{-1} region (Demiate et al. 2000). Absorption peaks in this region, centered at 1650 cm^{-1} are the result of stretching of the C = O bond, which proves the presence of aliphatic acetate esters.



(a)



(b)

Fig. 2. a. FTIR spectra of starch based edible films with different amount of added essential oils/hydrolats b. Spectral range 1550–1750 cm^{-1} of starch based edible films with different amount of added essential oils/hydrolats

4 Conclusion

The aim of this paper is characterization of starch biopolymer films to which active components obtained by distillation of plant material have been added: essential oils and hydrolates, namely *Artemisia dracunculus* and *Artemisia absinthium*. The positive effect of the added active components on the examined starch film properties, both

mechanical and physical, was proven, and the activity of the produced materials was confirmed by an antioxidant test. Further studies ought to target those active packaging materials application with accent on packed food products preservation.

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Galactogogue Herbs: Antioxidant Activity and Bioactive Compounds' Content Determined from Aqueous Extracts

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Abstract. According to the WHO (World Health Organization), infants should be exclusively breastfed during the first 6 months of life in order to have optimal growth, physical and psychomotor development and health. Women all over the world exhibit breastfeeding problems, so a proper nutrition and lifestyle are needed. To stimulate the lactation process, the consumption of a substance, a food or a plant called galactogogue should be taken into account. Even if galactogogue plants are well-known, the scientific literature is still in continuous development. This study aimed to determine the antioxidant activity and the content of bioactive compounds of such herbs, known for their ability to improve or to increase lactation. Four different extraction methods, such as maceration, infusion, decoction and microwave assisted extraction (MAE) were used to obtain aqueous extracts from eight different galactogogue plants. Because the use of plants is desireable especially for their galactogenic properties, the organic solvents were limited, due to safety reasons and so water extraction methods were the most appropriate. The extracts were characterized by their antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) cation (ABTS) assays and also for their total phenolic content (TPC) and total flavonoid content (TFC). The optimization of the extraction processes was modelled by using Artificial Neural Network (ANN) simulation. Three of eight plants, anise (*Pimpinella anisum* L.), lemon balm (*Melissa officinalis* L.) and thyme (*Thymus serpyllum* L.) showed significant antioxidant activity with DPPH inhibition values between 79.68% ± 0.0025 and 86.72% ± 0.0065. The provided information will allow health care professionals to choose the proper galactogogue plant or combination of plants to sustain the breastfeeding act.

Keywords: Antioxidant · Aqueous extract · Flavonoids · Galactogogue · Polyphenols

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1 Introduction

Breastfeeding is the most important way of supplying nutrients for infants, with strong impact on their health and development: it reduces risk of infectious diseases, diabetes, cancer, asthma, and obesity [1]. Even if the beneficial consequences of breastfeeding for both children and mothers have been intensively studied and the international recommendations include infants to be exclusively breastfed for the first six months of life, followed by continued breastfeeding combined with food for two years or beyond [2], the breastfeeding concerns are still topical in most countries [3, 4]. Studies show that one of the most frequent reasons for breastfeeding cessation is the insufficient amount of milk [5, 6]. In order to augment the milk production, synthetic (metoclopramide, oxytocin, chlorpromazine, sulpiride and domperidone) or herbal galactogogues can be used. The latter are preferred due to safety reasons [6]. Numerous plants showed galactogogue potential. Among them, fennel (*Foeniculum vulgare L.*) is one of the most important. It possesses antioxidant and antimicrobial properties [7], given by the high content in trans-anethole, estragole, fencone and α- phelandrene [8]. The efficiency of fennel utilization as a galactogogue has been reported by many studies [1, 6]. Anise (*Pimpinella anisum L.*) also contains anethole and estragole, compounds that may augment lactation [9]. At the same time, it is safe to be used during pregnancy and breastfeeding, due to its compounds' capacity of being excreted via kidneys and lungs, as studies showed [10]. Cumin (*Cuminum cyminum L.*) fruits are used in traditional Iranian medicine against dyspepsia, cramps, flatulence, diarrhea and also for increasing breastmilk production [11]. Star anise (Illicium verum L.) is used in traditional Chinese medicine and also as a spice, with multiple benefits such as antimicrobial, antioxidant and insecticidal properties [12]. Like the most galactogogues, the main compound in star anise is anethole, but special attention should be paid to the other compounds, which may present a certain degree of toxicity [13]. Seeds of fenugreek (*Trigonella foenum-graecum L.*) and milk thistle (*Silybum marianum L.*) were reported to stimulate lactation [14–17]. Lemon balm (*Melissa officinalis L.*) is an edible and medicinal plant, originating in Central Europe, with beneficial effects such as sedative, antipyretic, antispasmodic, antihypertensive, anti-Alzheimer's and antiseptic [18, 19]. Thyme (*Thymus serpyllum L.*) is used in traditional and complementary medicine against nausea and vomiting, against the common cold, heartburn and constipation in pregnant women [20].

The potential galactogogue compounds from plants have different and complex chemical structures. In such a situation a general extraction technique cannot be recommended for all types of compounds [21]. In the case of using plant extracts for their galactogogue properties, the organic solvents are limited, due to safety reasons. Water extraction methods like maceration, infusion, decoction and microwave assisted extraction (MAE) are more appropriate. These methods differ in point of water temperature, contact time between solvent and vegetal material or the way of in which heat is provided. For maceration, the vegetal material is placed into water, at room temperature and held in the dark for hours, while for decoction the plants are boiled into water for minutes [22]. In the case of infusion, the vegetal material is soaked in boiling water and left to extract for a determined time.

The aim of the present work is to determine the antioxidant activity and the content of bioactive compounds in herbs known for their ability to stimulate or increase lactation.

These plants were chosen after reading over 170 reviews and scientific articles presenting galactogogue plants and their benefits, but the main reason of selecting these 8 herbs was their availability and application in Romania. At the same time, the study proposes to find the best extraction method, processing the results via Artificial Neural Network (ANN) and Principal Component Analysis (PCA).

2 Materials and Methods

2.1 Chemicals and Reagents

The following chemicals and reagents were used to identify and quantify the bioactive compounds in the extracts: 2, 2-diphenyl-1-picryhydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox), $ABTS^{+\cdot}$ reagent, potassium persulfate ($K_2O_2S_8$), Folin-Ciocalteu reagent, gallic acid, sodium carbonate (Na_2CO_3) 20%, quercetin, sodium nitrite ($NaNO_2$) 5%, aluminium chloride ($AlCl_3$) 10%, sodium hydroxide (NaOH) 1M and methanol (HPLC grade), which were purchased from Sigma-Aldrich Steinheim, Germany.

2.2 Galactogogue herbs

For this study, fruits of fennel (*Foeniculum vulgare* L.), anise (*Pimpinella anisum* L.), cumin (*Cuminum cyminum* L.) and star anise (*Illicium verum* L.), seeds of fenugreek (*Trigonella foenum-graecum* L.) and milk thistle (*Silybum marianum* L.) and aerial parts of lemon balm (*Melissa officinalis* L.) and thyme (*Thymus serpyllum* L.) were purchased from an organic store in Galați, Romania, in October 2019. All plants were stored in a well-closed place, away from light and humidity until extraction.

Sample codification is presented as follows: An - anise (*Pimpinella anisum* L.), Fe - fennel (*Foeniculum vulgare* L.), Ci - thyme (*Thymus serpyllum* L.), Ar - milk thistle (*Silybum marianum* L.), Ro - lemon balm (*Melissa officinalis* L.), Sc - fenugreek (*Trigonella foenum-graecum* L.), As - star anise (*Illicium verum* L.), Ch - cumin (*Cuminum cyminum* L.).

2.3 Preparation of the Aqueous Extzracts of Galactogogue Herbs

Plant parts were grinded (Gorenje grinder SMK150B, Velenje, Republic of Slovenia) into fine particles and mixed with bidistilled water. After being extracted according to the methods described below, the samples were filtered using a Φ 150 mm/11 μm (particle retention) ashless filter paper purchased from Macherey-Nagel (Germany), cooled, and stored under refrigeration conditions (4 °C) for 24 h. The infusion technique was chosen after several attempts, in which the contact time varied. The maceration and decoction techniques were used according to the slightly modified method presented by [22], by changing the maceration time. Microwave assisted extraction process (MAE) was used according to the method described by [23], with the modification of the exposure time and power. All techniques were chosen because a harmless solvent, such as water, can be used.

Maceration Technique (M). 5 g of grinded sample were mixed with 125 mL of bidistilled water. The samples were stored in the dark for 24 h at room temperature, with frequent agitation, and then filtered and stored.

Infusion Technique (I). 5 g of grinded sample were mixed with 125 mL of bidistilled water, boiled at 100 °C. After 30 min of rest, the samples were filtered and stored.

Decoction Technique (D). In the decoction technique, 5 g of grinded sample were mixed with 125 mL of bidistilled water. The samples were boiled in a water bath for 30 min, then filtered, cooled and stored.

Microwave Assisted Extraction Process (MAE). MAE process was carried out in a microwave oven (Sharp Inverter Microwave R-98AO(ST)VM) at 735W. 5 g of the grinded sample were mixed with 125 mL of bidistilled water. The samples were subjected to microwave treatment for 30 s and 75 s, respectively. Subsequently, the samples were filtered, cooled and stored.

2.4 Evaluation of Antioxidant Activity

For testing the antioxidant activity, the aqueous extracts were further tested using two different assays. All tests were performed in triplicate.

DPPH^{-}Free Radical Scavenging Assay. To determine DPPH-free radical scavenging assay, a slightly modified method described by [24] was used. It involved the addition of 0.1 mL plant extract in a test tube, followed by 3.9 mL DPPH solution (0.1 M). For the control sample, the plant extract was replaced with 0.1 mL of methanol. The absorbance was determined spectrophotometrically at 515 nm with a UV–Vis spectrometer (Biochrom Libra S22, UK) after a 90-min incubation period at room temperature (22 °C), in the dark.

The variation of the antioxidant capacity corresponding to the different samples was studied by determining the inhibition for each sample to be analysed.

ABTS$^{+\cdot}$ Radical Cation Scavenging Assay. The antioxidant radical scavenging assay was determined using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) method, which is a procedure described by [25].

As described in the method, 150 μL/mL plant extract was mixed with 2.85 mL of $ABTS^{+}$ solution. The absorbance was measured at a wavelength of 734 nm with a UV–Vis spectrometer (Biochrom Libra S22, UK) after a 2-h rest in a dark place. Methanol replaced the plant extract in the control sample and the free radical scavenging assay was studied by determining the inhibition for each sample to be analysed.

2.5 Determination of Total Bioactive Compounds By Spectrophotometric Methods

Total Phenolic Content (TPC). The TPC was determined by Folin-Ciocalteau method, taking as reference the technique of [26]. Concisely, into a test tube, 0.20 mL of sample was mixed with 15.8 mL of distilled water and 1 mL of Folin-Ciocalteu reagent, before allowing to rest for 6 min in a dark place at room temperature. Then, 3 mL of 20% (w/v) solution prepared from sodium carbonate (Na_2CO_3) was added and the samples had a 60-min incubation period at room temperature, in the dark. Thereafter, the absorbance was measured at a wavelength of 765 nm using UV–Vis spectrophotometry (Biochrom Libra S22, UK). Methanol was used as blank versus the prepared sample. Results were expressed as mg of gallic acid equivalents/g sample ± SD (mg GAE/g).

All tests were performed in triplicate.

Total Flavonoid Content (TFC). The TFC was determined using the method described by [27]. A volume of 0.25 mL aqueous extract is mixed with 1.25 mL of distilled water and, subsequently, with 0.075 mL of 5% $NaNO_2$ solution. The mixture was let to react for 5 min, after which 0.15 mL of 10% $AlCl_3$ solution was added and allowed to react again for 6 min. Finally, 0.5 mL of 1M NaOH solution and 0.775 mL of distilled water were added. The absorbance of the resulting mixture was immediately read at a wavelength of 510 nm using UV–Vis spectrophotometry (Biochrom Libra S22, UK).

The TFC is determined using the standard quercetin curve and is expressed as mg quercetin equivalents/mL sample ± SD (mg EQ/mL).

All tests were performed in triplicate.

The TFC/TPC ratio was calculated using the method described by [28].

2.6 Confocal Laser Scanning Microscopy Analysis (CLSM)

The biologically active compounds from thyme and lemon balm extracts, obtained by decoction were analysed by confocal laser scanning microscopy, respectively with a Zeiss Confocal Laser scanning system (LSM 710). The system is equipped with a diode laser (405 nm), Ar-laser (458, 488, 514 nm), DPSS laser (diode pumped solid state–561 nm) and HeNe laser (633 nm). The images were obtained and analysed with the Black edition of the ZEN 2012 SP1 software. To observe the fluorescence, the samples were dyed with the Red Congo fluorophore (40 μM), in a ratio of 3:1.

The extracts were prepared in order to achieve the optimum concentration to identify the main compounds from the cellular level.

2.7 Fourier-Transform Infrared Spectroscopy (FT-IR)

The infrared spectra were collected using a Nicolet iS50 FT-IR spectrometer (Thermo Scientific) equipped with a built-in ATR accessory, DTGS detector and KBr beamsplitter. 32 scans were co-added over the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. Air was taken as the reference for the background spectrum before each sample. After

each spectrum, the ATR plate was cleaned with ethanol solution. In order to verify that no residue from the previous sample remained, a background spectrum was collected each time and compared to the previous background spectrum. The FT-IR spectrometer was sited in a room that was air conditioned with controlled temperature (21 °C).

2.8 Data Processing

Artificial Neural Networks Modelling. The Artificial Neural Network (ANN) is known as an artificial intelligence technique that mimics the human brain's biological neural network in a problem related to several processes. For this experiment, there was developed an artificial neural network model to predict and simulate which plant or extraction technique is the optimum one. The ANN was applied using the neural planning software EasyNN-plus, UK, which could process both logical and numerical data.

Five steps were followed to simulate the model: collecting data, pre-processing data, building the network, train and test the model performance. First of all, in order to operate in the EasyNN programme, the unicity of the data was determined by checking the coherence and uniqueness of the output values for a set of input data. All data were randomly divided into training (65%), testing (20%), and validation (15%) sets. After this, a neural network was built by using the generation module based on genetic algorithms. The data were modelled by using a feed-forward backpropagation model [29].

The architecture of the ANN model used to predict the data is presented in Fig. 1.

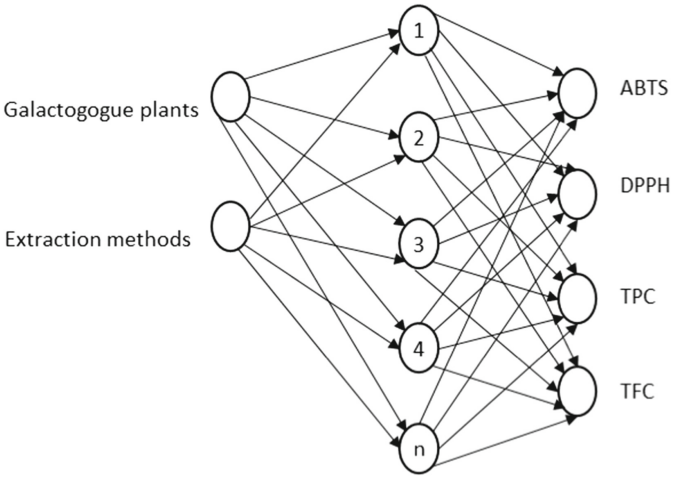


Fig. 1. Artificial Neural Network architecture with 2 inputs (the galactogogue plants and the extraction techniques) and 4 outputs (represented by the main analysis)

Principal Component Analysis. Data sets of ABTS radical assay, DPPH scavenging activities, total phenolic, and total flavonoid contents of extracts obtained from different galactogogue herbs were subjected to Principal Component Analysis (PCA) using Minitab 19 software (free trial).

Hierarchical Cluster Analysis. Hierarchical Cluster Analysis (HCA) was applied to see the similarities and relationships between the five extraction techniques using Minitab 19 statistical software (free trial).

2.9 Statistical Analysis

All analyses were performed in triplicate and data reported as mean ± standard deviation (SD). To identify significant differences, experimental data were subjected to one-way analysis of variance (ANOVA). The Tukey method with a 95% confidence interval was employed for post-hoc analysis; $p < 0.05$ was considered to be statistically significant. The statistical analysis was carried out using Minitab 19 statistical software (free trial).

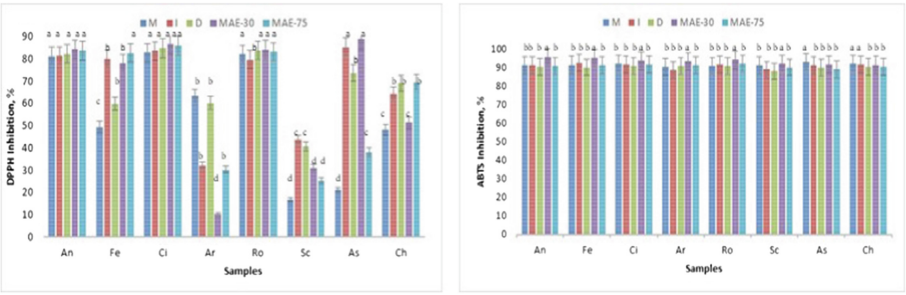
3 Results and Discussion

3.1 The Effect of Extraction Techniques on Antioxidant Capacity

There are many methods which can be used to assess the antioxidant activity, but each one has limitations. So, we tested the antioxidant activity of 8 galactogogue plant extracts, namely fennel, anise, cumin and star anise fruits, fenugreek and milk thistle seeds and the aerial parts of lemon balm and thyme by using the DPPH and ABTS free radical scavenging. DPPH and ABTS are two of the most popular methods used to evaluate the free radical scavenging ability of various compounds. Figure 1 exhibits the DPPH and ABTS inhibition property of galactogogue plant extracts. Current results of total antioxidant activity indicate that the inhibition (%) of DPPH and ABTS is good and very good, based on the inhibition values.

Values are represented as mean ± standard errors. A, b, c and d letters means significant differences between the antioxidant activity of galactogogue plants.

From Fig. 2(a) it could be observed that the highest value (88.78%) for the DPPH inhibition is exhibited by As sample, represented by the star anise aqueous extract, obtained by microwave-assisted extraction for 30″, followed by Ci sample (86.72%) represented by the thyme aqueous extract. The anise extract registered high values of DPPH inhibition in the range of 81.11–84.25%, independently of the extraction method. Differences in the antioxidant activity of the galactogogue plant extracts were significant ($p \leq 0.05$). Similar results were obtained for *Premna serratifolia* and *Premna serratifolia* leaves, for a hydro alcoholic extract of *Galega officinalis* and *Nigella sativa* mixture as well as for the ethanol extracts from different parts of *Silybum marianum* L. [30–32]. The lowest values for the DPPH inhibition are attributed to Ar sample 10.36%, registered for the microwave-assisted extraction for 30″, Sc also registered low values (25.49, 38.02%) for the microwave-assisted extraction at 75, respectively 30″. The data obtained by this assay corresponded to the overall flavonoids/phenols content found in the samples.



(a) (b)

Fig. 2. DPPH(a) and ABTS(b) inhibition property of galactogogue plant extracts An – Anise (*Pimpinella anisum* L.); Fe –Fennel (*Foeniculum vulgare* L.); Ci – Thyme (*Thymus serpyllum* L.); Ar – Milk thistle (Silybum *marianum* L.); Ro – Lemon balm (*Melissa officinalis* L.); Sc – Fenugreek (*Trigonella foenum-graecum* L.); As – Star Anise (*Illicium verum* L.); Ch – Cumin (*Cuminum cyminum* L); M- maceration; I-infusion; D – decoction; MAE-30 - Microwave assisted extraction process 30 s; MAE-75 - Microwave assisted extraction process 75 s.

The values for the ABTS inhibition are more uniform and the statistical differences between the samples and the extraction methods are insignificant ($p \geq 0.063$).

It seems that the extract of Sc was not able to scavenge the DPPH radical, but it was useful in the ABTS radical inhibition. The extraction methods do not influence the ABTS radical scavenging, all the methods revealed similar results. The values of the inhibition of ABTS are in the range of 90.5–94% for the lemon balm extract, which registered the highest values from the tested samples, while the lowest values (87.9–92%) were determined for the Sc sample. This assertion is correlated with the DPPH inhibition values for the same sample. Similar values were reported by [33] for both hot water and aqueous methanol (1:1) *Galega officinalis* extracts, ranging from 10.92% to 87.76% for the aqueous extract and 13.57% to 87.85% for the aqueous methanol one.

Even in the antioxidant activity of the studied plants, there are several expected variations induced by the biological material and the extraction methods, it seems that lemon balm Ro is the most stable and the values obtained for both assays are comparable. The values of percentage inhibition calculated for both methods (DPPH and ABTS) showed a higher inhibitory activity for quercetin than for Trolox. This may suggest that the antiradical activity of the galactogogue plants extracts is not based only on polyphenolic and flavonoids compounds. Thus, other bioactive compounds, which possess a strong antiradical activity, may contribute to the antioxidative potential of these extracts. Moreover, the synergism between the identified components and the unknown ones can also be assumed.

3.2 The Effect of Extraction Techniques on Total Bioactive Compounds

The total phenolics content of some galactogogue plants (Table 1) varied between 3.283 ± 0.011 mg GAE/mL and 34.315 ± 0.00 mg GAE/mL. Among all the galactogogue plants, the extract from leaves of Ro (*Melissa officinalis* L.) had the highest total phenolics content (34.315 ± 0.00 mg GAE/mL) obtained with decoction extraction technique,

Table 1. The influence of extraction techniques on total phenolic and total flavonoid content from galactogogue herb extracts

Extraction techniques	Galactogogue herbs							
	An	*Fe*	*Ci*	*Ar*	*Ro*	*Sc*	*As*	*Ch*
Means by extraction methods								
Total phenolic (mg GAE/mL)								
Maceration	6.649 ± 0.004C	5.151 ± 0.022C	12.504 ± 0.017B	3.515 ± 0.004C	24.40 ± 0.013A	3.374 ± 0.006C	4.535 ± 0.001C	3.958 ± 0.005C
Infusion	7.207 ± 0.05C	5.335 ± 0.01C	17.922 ± 0.03B	4.201 ± 0.02C	27.453 ± 0.04A	5.025 ± 0.01^{C}	8.266 ± 0.01^{C}	4.417 ± 0.00C
Decoction	6.972 ± 0.01C	5.033 ± 0.00C	18.452 ± 0.02B	6.638 ± 0.07C	34.315 ± 0.00A	6.183 ± 0.00C	6.128 ± 0.01C	4.872 ± 0.00^{C}
MAE – 30	7.058 ± 0.013C	5.465 ± 0.009C	11.515 ± 0.015B	3.283 ± 0.011C	28.822 ± 0.021A	4.735 ± 0.003C	12.335 ± 0.01C	4.272 ± 0.005C
MAE – 75	7.513 ± 0.005C	5.689 ± 0.003C	21.375 ± 0.017B	3.774 ± 0.004C	34.174 ± 0.024A	5.273 ± 0.003C	3.393 ± 0.003C	4.884 ± 0.004C
Total flavonoid (mg EQ/mL)								
Maceration	1.023 ± 0.01^{B}	0.774 ± 0.01^{B}	2.556 ± 0.04^{A,B}	0.534 ± 0.01^{B}	4.969 ± 0.08^{A}	0.389 ± 0.01^{B}	1.053 ± 0.02^{B}	0.752 ± 0.06^{B}
Infusion	1.067 ± 0.02^{B}	0.897 ± 0.018^{B}	2.739 ± 0.029^{A,B}	0.527 ± 0.012^{B}	3.670 ± 0.03^{A}	0.517 ± 0.011^{B}	1.223 ± 0.028^{B}	0.684 ± 0.004^{B}
Decoction	1.175 ± 0.011^{B}	0.820 ± 0.009^{B}	3.267 ± 0.016^{A,B}	1.122 ± 0.014^{B}	5.779 ± 0.014^{A}	0.905 ± 0.019^{B}	0.902 ± 0.013^{B}	1.207 ± 0.014^{B}
MAE – 30	3.062 ± 0.10^{B}	3.330 ± 0.29^{B}	3.095 ± 0.10^{A,B}	1.960 ± 0.10^{B}	5.304 ± 0.08^{A}	1.986 ± 0.08^{B}	3.517 ± 0.22^{B}	2.367 ± 0.07^{B}
MAE – 75	0.359 ± 0.01^{B}	0.366 ± 0.01^{B}	0.308 ± 0.00^{A,B}	0.181 ± 0.00^{B}	0.399 ± 0.01^{A}	0.145 ± 0.00^{B}	0.224 ± 0.00^{B}	0.371 ± 0.00^{B}
Means by galactogogue plants								
Ratio TFC/TPC								
Maceration	0.15	0.15	0.20	0.15	0.20	0.12	0.23	0.19
Infusion	0.15	0.17	0.15	0.13	0.13	0.10	0.15	0.15
Decoction	0.17	0.16	0.17	0.17	0.17	0.15	0.15	0.25
MAE – 30	0.43	0.61	0.27	0.60	0.18	0.42	0.29	0.55
MAE - 75	0.05	0.03	0.01	0.02	0.01	0.03	0.07	0.08

An – Anise (Pimpinella *anisum* L.); Fe –Fennel (*Foeniculum vulgare* L.); Ci – Thyme (*Thymus serpyllum* L.); Ar – Milk thistle (*Silybum marianum* L.); Ro – Lemon balm (*Melissa officinalis* L.); Sc – Fenugreek (*Trigonella foenum-graecum* L.); As – Star Anise (*Illicium verum* L.); Ch – Cumin (*Cuminum cyminum*L.);Mean of three determination ± SD (standard deviation); Values in the same row with different superscript (A-C) are significantly different.

whereas the seeds of Ar (*Silybum marianum* L.) had the lowest total phenolics content (3.283 ± 0.011 mg GAE/mL) obtained with MAE – 30 extraction technique. Similar results were reported by [34] for some plants used for medical and culinary purposes. As shown in Table 1, the highest total phenolic content was obtained with decoction extraction technique, followed by MAE – 30 > MAE – 75 > infusion > maceration. According to [35] this aspect can be explained by the fact that aqueous extracts can dissolve rapidly, based on their polarity, the carbonyl and organic acids. [36] observed similar results for water extract compared with MAE and ethanol extract in the case of Malaysia palm oil trunk epiphytes ferns while [37] for five plants used as Portuguese food spices. In the case of the studied galactogogue herbs the highest average of total phenolics content has been established for Ro followed by Ci > An > Fe > As > Ch > Ar > Sc. The presented results accentuated the importance of total phenolic compounds in the antioxidant behaviour of galactogogue plant extracts and indicated that the phenolic components significantly contributed to the values of the total antioxidant capacity.

Flavonoids are the dominant class of phenolic compounds found in almost all vegetables and plants that have antioxidant properties. The highest total flavonoid content (Table 1) of the studied galactogogue plant extracts was registered for the leaves of Ro (*Melissa officinalis* L.) and As (*Illicium verum* L.) (5.779 ± 0.014 and 3.517 ± 0.22 mg EQ/mL), obtained with two different extraction techniques (decoction and MAE - 30). However, the seeds or the leaves of Ar (*Silybum marianum* L.) and Sc (*Trigonella foenum-graecum* L.) showed the lowest total flavonoid content (0.181 ± 0.00 and 0.145 ± 0.00 mg EQ/mL). According to our results the aqueous extract with highest total flavonoid content is the one obtained by decoction. It seems that this aqueous extract is safe and non-toxic and can be used for direct consumption or application in food. A similar tendency was reported by [38] for some Malaysian wild edible plants.

The ratio of TFC/TPC of Fe (*Foeniculum vulgare* L.) extract was much higher (0.61) compared with the other galactogogue herb extracts and was followed by Ar > Ch > An > Sc > As > Ci > Ro. This could contribute to its total antioxidant capacity because flavonoid components are the most active antioxidant phenolic compounds.

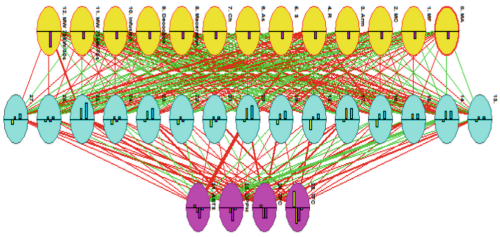
The different concentration of total phenolics and flavonoids in galactogogue herbs extract may be a result of several factors, like species, variety, the influence of vegetation season, as well as climatic, cultivation conditions and harvesting time [35, 39–41].

3.3 Artificial Neural Networks Models (ANN)

The empirical data were processed using the Artificial Neural Networks models. The input data are represented by the plant extracts and the extraction techniques, while the outputs are represented by the analysis (antioxidant activity determined by DPPH and ABTS assays, TPC and TFC).

Figure 3 presents the Artificial Neural Network for the galactogogue plant extracts, figure (a) represents the ANN architecture for the present study, while (b) is the performance of the resulted error values obtained from the training process.

From Fig. 3(a) it could be seen that the network includes only a hidden layer and a number of 32 neurons. After this step the network was trained using 192 learning cycles and the maximum error obtained was 0.01. The continuous decreasing of the error presented in the Fig. 3(b) indicates that the neural network is correctly built. After



Learning rate	0.60000
Momentum	0.800000
Accelarator	0.000000
Maximum error	0.009957
Average error	0.001009
Minimum error	0.000036
Target error	0.010000
Training examples	40

(a) (b)

Fig. 3. Artificial Neural Network for the galactogogue plant extracts (a) and the resulted error values (b)

obtaining the ANN, it could be used for the establishing of the maximum efficiency for every analysis. It seems that the most feasible combination between the plant and the associated extraction techniques is represented by the lemon balm extract obtained by microwave, maceration or infusion. For the TPC, DPPH and ABTS analysis, the best results were registered for lemon balm, star anise, extracted by maceration and microwave, while for TFC the optimum plants are lemon balm and cumin, respectively, extracted using a microvawe. Even if there are many combinations in which any analysis could be important or relevant, the extraction techniques and the plant types are decisive.

3.4 Principal Component Analysis Results (PCA)

In order to choose the extraction technique, which would ensure the highest efficiency of total phenolics extraction, total flavonoids extraction, DPPH scavenging activity and ABTS scavenging activity, the PCA (Fig. 4) was applied to evaluate correlations between TPC, TFC, DPPH or ABTS (loads) and 8 different galactogogue herbs (scores).

Figure 4 a and b shows the overall plot of total phenolic and total flavonoid content present in galactogogue herb extracts. Same tendencies were found when comparing quantities of total phenolic content presented in galactogogue herb extracts obtained with different extraction techniques, i.e. the highest TPC was found in the extract from leaves of Ro (*Melissa officinalis* L.), while the lowest TPC was obtained for the extract from seeds of Ar (*Silybum marianum* L.). For TPC it was found that some galactogogue herb extracts could be grouped together such as extracts from milk thistle (*Silybum marianum* L.), cumin (*Cuminum cyminum* L.), fenugreek (*Trigonella foenum-graecum* L.), fennel (*Foeniculum vulgare* L.) and anise (*Pimpinella anisum* L.).

Similar trends of TFC were found; in Fig. 4 b one may notice that some galactogogue herb extracts can be grouped together, such as fennel (*Foeniculum vulgare* L.), cumin (*Cuminum cyminum* L.) and anise (*Pimpinella anisum* L.). This suggests the similarity between the extraction techniques used to determine TPC and TPC in the case of the analysed samples. When the results of DPPH or ABTS scavenging activity were examined (Fig. 4 c and d) it was found that fennel, lemon balm, anise and thyme exhibit a high antioxidant activity and they are located diametrically opposite to cumin, fenugreek, milk

thistle and star anise which possess a low antioxidant activity. We can suggest that the contribution of TPC and TFC on antioxidant activity in the case of these galactogogue plants is the lowest. These results are in accordance with [42] who used PCA to classify different spices based on antioxidant activity and individual polyphenolic antioxidant compounds.

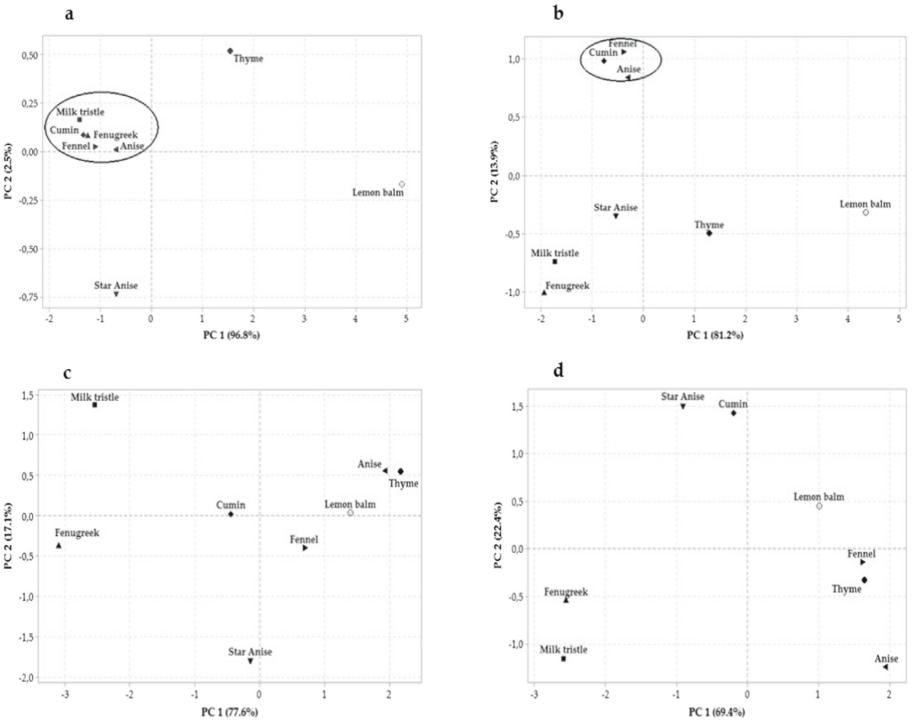
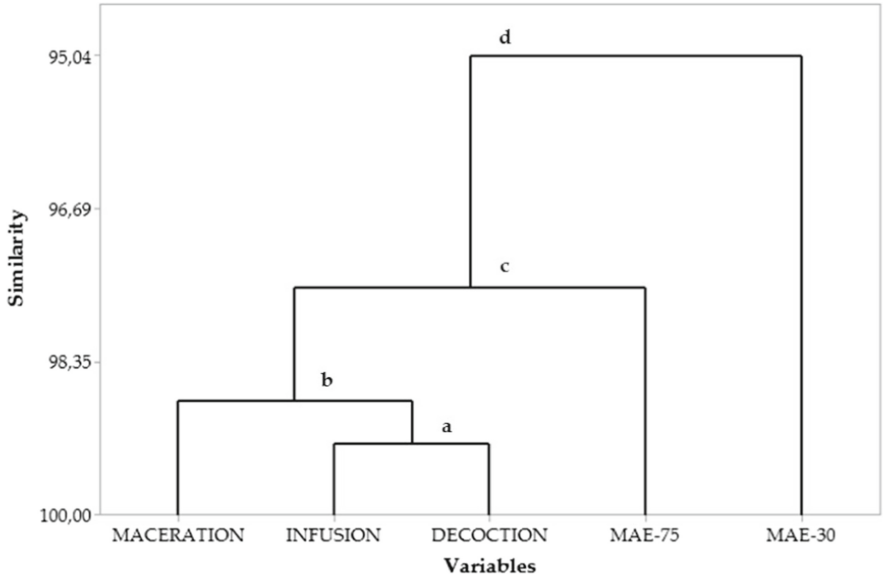


Fig. 4. Principal component analysis (PCA) plots a – TPC for galactogogue herb extracts; b – TFC for galactogogue herb extracts; c – DPPH in the galactogogue herb extracts; d – ABTS in the galactogogue herb extracts.

3.5 Hierarchical Cluster Analysis Results (HCA)

In our study, HCA (Fig. 5) was used to evaluate the similarities and the relationships between five extraction techniques used to establish the antioxidant activity (determined using the DPPH and ABTS assay) and for the separation of bioactive compounds (TPC and TFC). The obtained results following HCA, evidenced as a dendrogram, (Fig. 5), consist of four well-defined clusters. A group of extraction techniques (a) is clearly visible and is composed by infusion and decoction. These techniques are associated because both use bidistilled boiled water and need the same extraction time (30 min). A second cluster (b) consists of maceration alone, because this conventional extraction method is similar to infusion and decoction because, these techniques using water as

green solvent. A third cluster (labelled c) includes MAE – 75, while cluster D consists of MAE - 30; two electromagnetic force methods used for extraction. In the dendrogram the distance between cluster a, b and c is very tight, that means these extraction techniques present a great similarity.



Sample Clusters
a [Infusion, Decoction]; b [Maceration]; c [MAE – 75]; d [MAE – 30]

Fig. 5. Dendrogram of hierarchical cluster analysis of extraction techniques

Confocal laser scanning microscopy analysis results (CLSM)

The CLSM was used to determine the main bioactive compounds which are present in the extract's structure. This type of analysis could be useful to understand the phenomena and interactions between some compounds with health potential.

Due to the strongest antioxidant activity that was recorded for thyme and lemon balm extracts, obtained by decoction, these were the samples selected for the confocal microscopy analysis. The high content of biologically active compounds from the extracts of these plants, their diversity and complexity, determined a wide fluorescence emission range, between 550–650 nm. The thyme extract obtained by decoction (aqueous extract1:25), highlighted the largest spherosomes with dimensions between 10-30μm (Fig. 6 a1), while the thyme extract (aqueous 1:12.5), they were smaller and with a tendency to aggregate (Fig. 6 a2). The finest spherosomes (1–2 μm diameter) were obtained by decoction from *Melissa officinalis* (aqueous 1:25) (Fig. 6 b1), while in the second one (aqueous 1:12.5) favored the formation of large clusters of coacervates captured in a dense network with a predominantly red emission (620–650 nm). Point-by-point laser scanning images highlighted the biologically active compounds' richness that supports

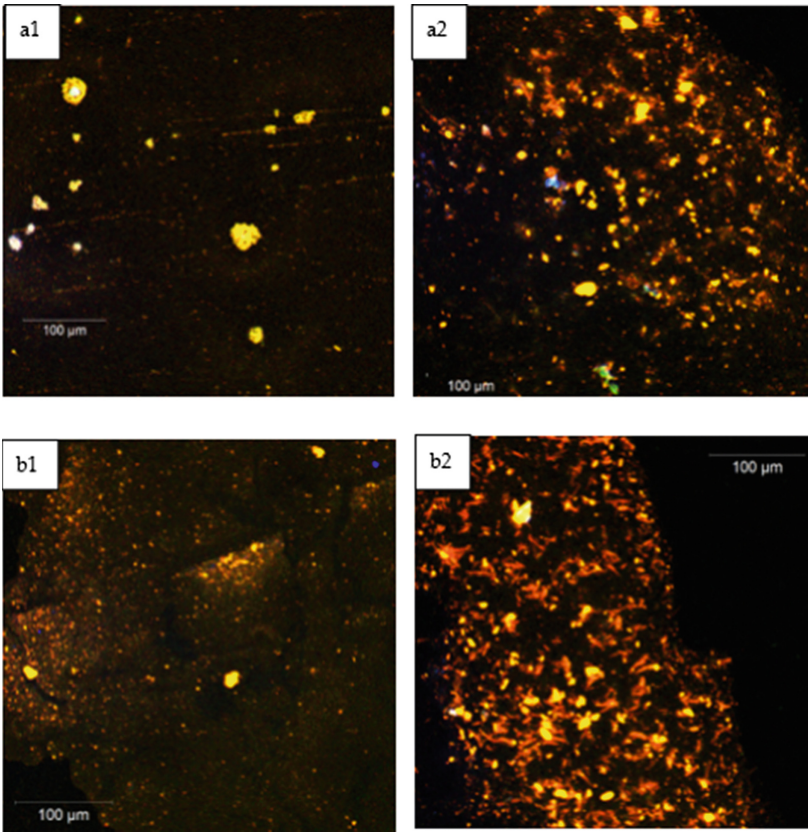


Fig. 6. Confocal Laser Scaning Macroscopy images of Ci (a1 and a2) and Ro samples (b1 and b2)

the intense antioxidant activity and justifies the use of the extracts in foods with a high nutritional value indicated in the diet of certain categories of people.

3.6 Fourier-Transform Infrared Spectroscopy Results (FT-IR)

The FT-IR analysis of aqueous plant extracts aimed to reveal the presence of some specific compounds or functional groups, which are involved in the health benefits mechanism (Fig. 7).

The FT-IR spectra for both extracts revealed the presence of the bands at 3288.6 cm^{-1} is attributed to –OH stretching vibration which could be attributed to phenols, esters or other compounds which are native in these extracts. The absorption bands in the range of 1700 − 1500 cm^{-1} region is defined by amide I and II groups responsible for the peptide linkages in proteins. The 1635 cm^{-1} indicates the C = O stretching) coupled with N–H in-plane bending [43] which are involved in maintaining the normal functions of the central nervous system. The similarity of both extracts of thyme and lemon balm induced the idea that some specific and common components are present in the samples.

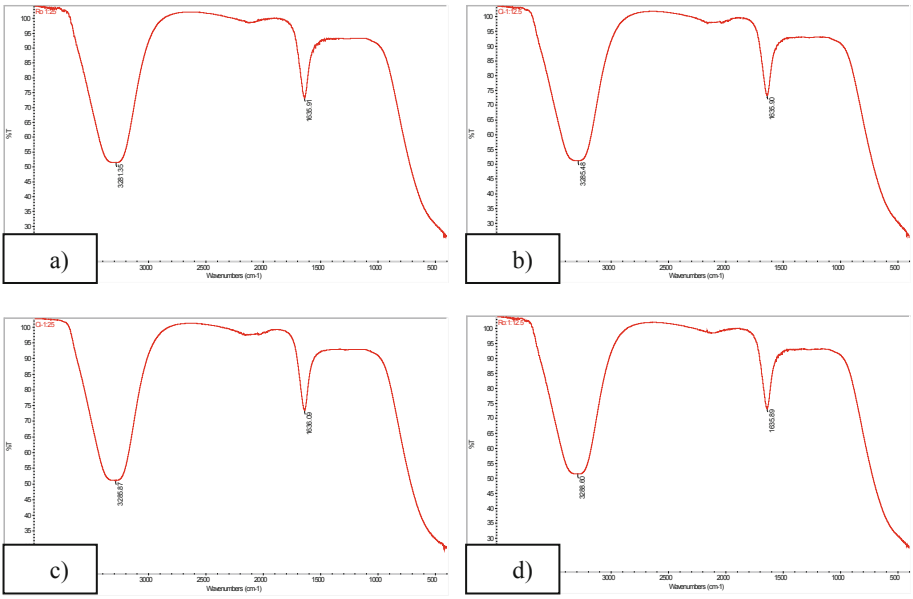


Fig. 7. FT-IR determination for a) Ci - Thyme (aqueous 1:25); b) Ci - Thyme (aqueous 1:12,5); c) Ro - Lemon balm (aqueous 1:25); d) Ro - Lemon balm (aqueous 1:12,5).

4 Conclusions

In order to fulfil the main purpose of this research there were used 8 galactogogue plants, extracted in water solvent, using 4 methods of extraction, as follows: maceration, infusion, decoction and microwave.

The present study demonstrates the high antioxidant activity, the phenolic and flavonoid content of lemon balm aqueous extracts that could be involved in sustaining the antioxidant status and protect against free radical damage. Other galactogogue plants, such as star anise or thyme, present strong antioxidant activity as well.

Artificial Neural network modelling showed that the antioxidant activity, the phenolic compounds and the flavonoid content can be predicted with high accuracy from several galactogogue plants extracts, using 4 extraction methods.

HCA technique separated the extraction methods into four clusters and identified a great similarity between cluster a, b and c.

Further studies on *in vitro* digestibility could be developed on several plant combinations and/or several concentrations of plants used as commercial ingredients in some foods.

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Oana-Viorela Nistor - the planning and interpretation of results.
Doina-Georgeta Andronoiu - writing the introduction.
Dănuț-Gabriel Mocanu - writing and explaining results.
Bogdan Ioan Ștefănescu - monitoring the experiments.
Sorin Ciortan - Artificial Neural Network simulation.
Elena Ioniță (Enachi) – Confocal laser scanning microscopy analysis.
Andreea Veronica (Botezatu) Dediu – FT-IR determinations.
Elisabeta Botez - the final reading of the manuscript.
All authors have read and agreed to the published version of the manuscript.

Conflict of Interests. The authors declare no conflict of interest.

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The Effect of Cow's Milk and Soy Beverage Ratio, Probiotic Culture and Fruit Concentrates on the Qualitative Aspects of Fermented Beverages

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Abstract. The study aims to determine the microbiological, sensory and nutritional properties of fermented beverages produced from probiotic culture (*L. acidophilus*), natural fruit aromas (strawberry, peach, pear, and apricot), and different mixtures of cow's milk and soy beverage. The ratios of cow's milk and soy beverage were 25:75%, 50:50%, and 75:25%, while 100% cow's milk and 100% soy beverage was considered as control samples. The parameters were analyzed at the end of fermentation and during 21 days of storage at + 4ºC. The highest viability of probiotic bacteria was recorded in the sample with an equal proportion of cow's milk and soy beverage (50:50%). The sensory properties of the samples were mainly affected by the ratio and type of milk used. Mixing cow's milk with soy beverages significantly improved the sensory properties of the product, especially its color, smell, and taste. The acceptability test showed good acceptance of fermented beverage samples by potential consumers other than a sample made from 100% soy beverage. Flavored drinks have a better sensory flavor score than non-flavored ones. Flavored drinks were also more desirable than non-flavored ones. Strawberry flavor was the most desirable one (100.00%), while peach and apricot flavors were less desirable (97.50%). The addition of fruit aromas to the fermented cow's milk beverage and soy beverage improved the sensory properties of the product, as well as its acceptability.

Keywords: Soy beverage · Cow's milk · Fermentation · Fruit aroma · Probiotics

1 Introduction

For many years, the nutritional value and good digestibility of fermented dairy products, especially yogurt as one of the main representatives of this group of products, makes

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these products interesting to study [1]. Soy-based products can provide additional health benefits to consumers due to their anticholesterol, antiatherogenic and hypolipid properties, as well as reduce allergenic effects [2]. However, consumption of soy beverage is limited due to the presence of an undesirable soy flavor [2–4]. Soy beverage contains various oligosaccharides, including stachyose and raffinose, which can lead to indigestion in consumers. Fermentation of soy beverage provides the ability to transform and improve flavor and texture [5–7]. Also, the fermentation of soy beverage is considered a good substrate for the development and production of functional foods. It is the fermentation of soy beverage by probiotic bacteria that reduces the level of oligosaccharides and raises the level of free isoflavones [2, 5, 8]. Since a certain number of people do not tolerate the aroma and taste of soy beverage, it can be processed into other products, including a fermented beverage. It is important to get information on how consumers accept such products. In such an assessment, a verbal hedonic scale according to Peryam with nine possible answers is most often used [1]. Thus, the research is conducted to assess and determine the effect of the combination of cow's milk and soy beverage and probiotic bacteria (*L. acidophilus*) on the physicochemical, sensory properties and acceptability of flavored soy-based beverages at the end of fermentation and the impact of juice or fruit concentrates as natural fragrances on sustainability and sensory properties during storage treatment [2].

2 Materials and Methods

For the preparation of flavored probiotic beverages, permanent UHT milk with 2.50% milkfat from the manufacturer Meggle (BiH) and soy beverage with 1.90% fat from the brand dmBio (GmbH + Co.KG, Germany) were used. Flavored beverages of cow's milk and soy beverage (50% cow's milk +50% soy beverage) were produced with thermophilic yogurt culture YF-L811 (Christian Hansen, Horsholm, Denmark) and probiotic culture *Lactobacillus acidophilus* La5 (Christian Hansen, Hansholm, Denmark) [9]. Samples were inoculated at +43 °C with 2.5% probiotic culture, 6% of commercial sucrose and 0.5% of peach, strawberry, apricot, and pear flavors were added to the flavored samples of fermented beverages. The fermentation of flavored fermented products is complete when a pH of 4.6 is reached. The produced samples of aromatized beverages were cooled and stored in a refrigerator at a temperature of +4 °C. The characteristics of the obtained flavored products were monitored during 21 days of storage at intervals of 1st, 7th, 14th, and 21st day. Changes in pH, titratable acidity, rheological and sensory properties were monitored in the storage sequence, and product acceptability was performed on the first day after storage. Acidity of milk and chemical composition (flavored beverages) were determined by standard analytical methods. The active acidity (pH) of probiotic beverages was determined by the pH meter pH3110 (Portable meter ProfiLine) and the titratable acidity by the Soxhlet Hönkel method and expressed as % of lactic acid) [5, 9]. A rotary rheometer (HAAKE VT500) was used to determine the rheological values of the properties of samples of flavored beverages at a temperature of 20 °C and in the range of shear rates from 0 to 500 s^{-1}. Sensory properties are rated by a weighted scoring method (ISO,1985) by a group of 5 trained sensory analysts [5]. Acceptability of fermented beverages was performed by testing 30 younger consumers using the verbal 9-point Hedonic Scale (Peryam) [9, 10]. The computer program XLSTAT - Pro 2014 was used for statistical analysis and data processing. Each experiment was repeated three times, and the results were presented as mean values.

3 Results and Discussion

Permanent UHT milk and soy beverages were used to produce flavored beverages. The results of analyzes of raw materials used for the production of aromatized beverages show that cow's milk differs from soy beverage in all analyzed parameters. The total dry matter of cow's milk was 11.33%, while the share of dry matter in soy beverage was 8.6% (Table 1).

Table 1. Chemical composition of milk and soy beverage (n = 3)

Chemical composition of milk (g / 100g)	Cow's milk	Soy beverage
Dry matter	11.33 ± 0.1	8.60 ± 0.12
Ash	0.87 ± 0.02	0.62 ± 0.03
Fat	2.50 ± 0.1	1.90 ± 0.10
Lactose / UH	4.58 ± 0.02	3.24 ± 0.02
Proteins	3.38 ± 0.02	2.84 ± 0.03
Water	88.67 ± 0.03	91.40 ± 0.30

Data represent the mean values of three replicates.

The soy beverage used for fermentation had on average a lower content of other analyzed components (fat, protein, carbohydrate content) compared to cow's milk. The lower content of fermentable carbohydrates in soy beverages can affect the longer fermentation of milk by probiotic lactic acid bacteria (BMK). However, the results of studies show that probiotic lactobacilli, especially bifidobacteria, ferment soy beverages well [11]. The duration of milk fermentation was approximately equal (360 min) for all flavored samples produced, as well as the acidity achieved at the end of fermentation (Table 2).

Table 2. Fermentation time and acidity of flavored yogurt samples produced

Parameters	Yogurt samples			
	50:50AK	50:50AM	50:50AB	50:50AJ
Fermentation time (minutes)	360	360	360	360
pH	4.68 ± 0.05^{a}	4.63 ± 0.03^{b}	4.64 ± 0.05^{b}	4.60 ± 0.02^{b}
% lactic acid	0.64 ± 0.001^{a}	0.65 ± 0.002^{a}	0.57 ± 0.003^{b}	0.60 ± 0.01^{b}

Legend: 50:50AB = 50% cow's milk + 50% soy beverage, peach aroma; 50:50AJ = 50% cow's milk + 50% soy beverage, strawberry aroma; 50:50AK = 50% cow's milk + 50% soy beverage, pear aroma; 50:50 AM = 50% cow's milk + 50% soy beverage, apricot aroma; Data represent mean values (± SD) of three repetitions. ^{abc} Duncan's Test confirmed the statistically significant difference between mean values (± SD).

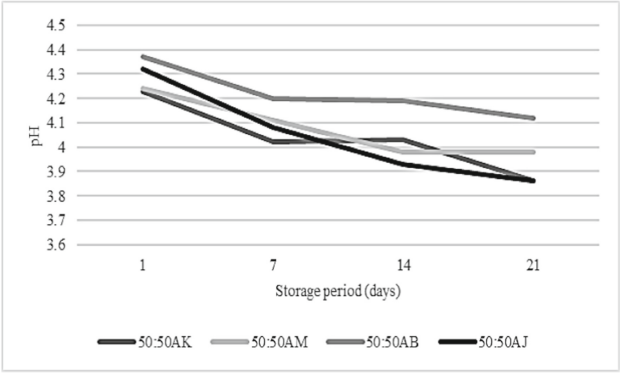
Table 3 shows the results of the acidity analysis of flavored samples during 21 days of storage at a temperature of + 4 ºC. The results obtained correspond to the usual range of acidity values for this analyzed type of beverages produced. Table 3 shows the changes in the pH drop over 21 days of storage of the samples in the refrigerator, and the dynamics of different decreases in pH in time were observed. The largest decrease in pH is evident in the sample with strawberry aroma, during the storage period, followed by apricots and pears. In contrast, the peach-flavored beverage did not have a significant drop in pH during storage days.

Table 3. Change in pH value and titratable acidity of flavored milk and soy beverages during storage at + 4 ° C

Samples	pH value				Titration acidity (°SH)			
	Storage days				Storage days			
	1	7	14	21	1	7	14	21
50:50AB	4.37 ± 0.01^{a}	4.19 ± 0.01^{a}	4.19 ± 0.02^{a}	4.12 ± 0.01^{a}	$25.66 \pm 0{,}5^{b}$	32.23 ± 1.70^{b}	31.70 ± 0.7^{c}	34.66 ± 2.08^{b}
50:50AJ	4.32 ± 0.02^{b}	4.08 ± 0.03^{b}	3.94 ± 0.12^{b}	$3.86 \pm 0{,}01^{c}$	$26.70 \pm 0{,}1^{b}$	36.50 ± 0.7^{a}	39.63 ± 0.7^{a}	39.40 ± 0.2^{a}
50:50AK	4.23 ± 0.02^{c}	4.02 ± 0.006^{c}	4.03 ± 0.16^{ab}	3.85 ± 0.006^{c}	28.23 ± 0.8^{a}	36.13 ± 2.1^{a}	38.63 ± 1.70^{a}	40.00 ± 1.6^{a}
50:50AM	4.24 ± 0.01^{c}	4.11 ± 0.01^{b}	3.98 ± 0.01^{b}	3.98 ± 0.07^{b}	28.73 ± 0.6^{a}	32.40 ± 1.2^{b}	36.50 ± 0.1^{b}	39.00 ± 2.0^{a}

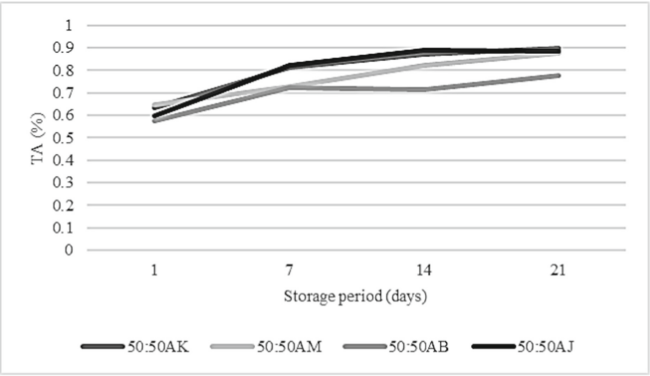
Legend: 50:50AB = 50% cow's milk + 50% soy beverage, peach aroma; 50:50AJ = 50% cow's milk + 50% soy beverage, strawberry aroma; 50:50AK = 50% cow's milk + 50% soy beverage, pear aroma; 50:50 AM = 50% cow's milk + 50% soy beverage, apricot aroma; Data represent mean values (± SD) of three repetitions. ^{abc} Duncan's Test confirmed the statistically significant difference between mean values (± SD).

These results were consistent with the research of Shahabbaspour et al. [12]. It was found that the titratable acidity (TA) increased proportionally with the decrease in pH (Figs. 1 and 2). Titratable acidity is expressed as a percentage of lactic acid.



Legend: 50:50AB = 50% cow's milk + 50% soy beverage, peach aroma; 50:50AJ = 50% cow's milk + 50% soy beverage, strawberry aroma; 50:50AK = 50% cow's milk + 50% soy beverage, pear aroma; 50:50 AM= 50% cow's milk + 50% soy beverage, apricot aroma; Data represent mean values (± SD) of three repetitions. abc Duncan's Test confirmed the statistically significant difference between mean values (± SD).

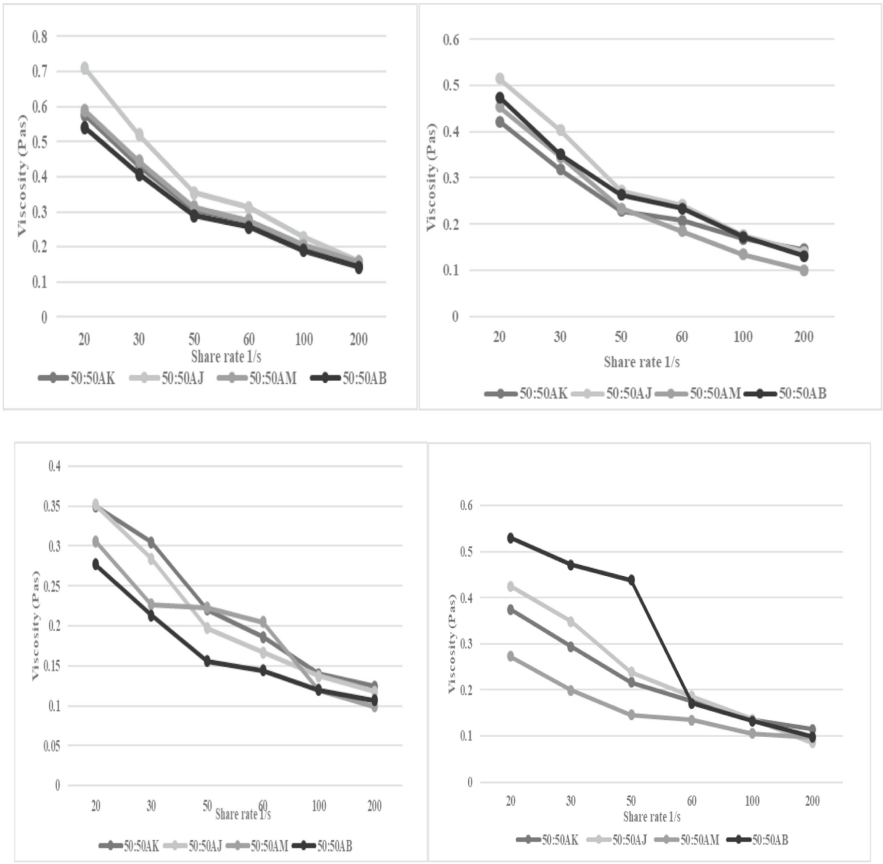
Fig. 1. Change of pH value during storage of flavored cow's milk beverage and soy beverage with La5



Legend: 50: 50AB = 50% cow's milk + 50% soy drink, peach aroma; 50: 50AJ = 50% cow's milk + 50% soy drink, strawberry aroma; 50: 50AK = 50% cow's milk + 50% soy drink, pear aroma; 50:50 AM= 50% cow's milk + 50% soy drink, apricot aroma; Data represent mean values (± SD) of three repetitions. abc Duncan's Test confirmed the statistically significant difference between mean values (± SD).

Fig. 2. Change of TA (%) during storage of flavored milk beverage and soy beverage with La5

Changes in viscosity of samples of flavored probiotic beverages during storage in the refrigerator at a temperature of + 4 ºC are shown in Fig. 3. The viscosity values of the samples are influenced by factors such as milk composition, standardization method, heat treatment, temperature, selected microbial culture, inoculum amount and duration of fermentation [9, 13]. The viscosity of all flavored beverage samples on the first day of storage varies in the range of 0.54 to 0.71 Pas, and then the viscosity begins to decline sharply. A significant decrease in viscosity was observed between the first and seventh day of storage in all samples, which is a characteristic of thixotropic systems that include yogurt. On the first day of storage, the highest viscosity at a shear rate of 20 s^{-1} was in the flavored strawberry beverage 50: 50AJ, while the lowest viscosity, at the same shear rate, had a sample with a peach aroma 50: 50AB. At other velocities, as on the first day, it is seen a sharp drop in viscosity (Fig. 3). At the 50:50 AM sample, there was a significant drop in viscosity at a shear rate of 20 s^{-1} from 0.59 Pa s to 0.27 Pa s. During the 14th and 21st days, there was no significant decrease in viscosity (Fig. 3) compared to the first and seventh days. For samples 50: 50AJ and 50: 50AB on the 21st day of storage, there was an increase in viscosity at a shear rate of 20 s^{-1}. In general, the viscosity of flavored beverages is higher than the viscosity of non-flavored samples, due to the addition of sucrose, ie an increase in dry matter [1].



Legend: 50:50AB = 50% cow's milk + 50% soy drink, peach aroma; 50:50AJ = 50% cow's milk + 50% soy drink, strawberry aroma; 50:50AK = 50% cow's milk + 50% soy drink, pear aroma; 50:50 AM= 50% cow's milk + 50% soy drink, apricot aroma; Data represent mean values (± SD) of three repetitions. abc Duncan's Test confirmed the statistically significant difference between mean values (± SD).

Fig. 3. Viscosity (Pa s) of samples of flavored beverages of a mixture of milk and soy beverage during 21 days of storage at + 4 °C

One of the main conditions and the biggest technological problems in terms of technology when it comes to fermentation with sensitive probiotic bacteria is to obtain a product with acceptable sensory properties [14]. The sensory quality of flavored milk and soy beverages was assessed using a scoring system using a unique scale of 20 weighted points. Table 4 shows the results of sensory characteristics of flavored beverages (strawberry, pear, apricot, and peach) produced with La5 and yogurt culture from a mixture of milk and soy beverage (50:50). When evaluating flavored beverages, the ratings for appearance, color, consistency, and aroma did not change significantly during the storage period. The odor ratings of flavored products increase for all samples throughout

the storage period. Taste ratings for all flavored samples also increase throughout the storage period.

Table 4. Sensory analysis of aromatized beverages during the 21st day of storage at + 4 ℃

Properties	Storage days	50:50AB	50:50AJ	50:50AK	50:50AM
Flavour (max 12)	1	9.50 ± 1.00^{a}	9.60 ± 1.14^{ab}	10.60 ± 0.65^{a}	10.00 ± 0.79^{ab}
	7	9.90 ± 0.74^{ab}	10.30 ± 0.78^{ab}	9.70 ± 0.83^{ab}	10.10 ± 0.74^{ab}
	14	10.60 ± 0.82^{a}	10.05 ± 1.2^{ab}	9.10 ± 0.74^{b}	10.30 ± 0.91^{ab}
	21	9.80 ± 0.91^{ab}	10.10 ± 0.65^{ab}	9.60 ± 1.67^{ab}	9.40 ± 1.34^{ab}
Odor (max 2)	1	1.47 ± 0.35^{a}	1.70 ± 0.20^{abc}	1.75 ± 0.35^{abc}	1.65 ± 0.48^{bc}
	7	1.79 ± 0.33^{abc}	1.84 ± 0.23^{ab}	1.82 ± 0.20^{ab}	1.91 ± 0.12^{ab}
	14	1.96 ± 0.08^{ab}	1.96 ± 0.09^{ab}	1.86 ± 0.21^{ab}	1.92 ± 0.11^{ab}
	21	2.00 ± 0.00^{a}	2.00 ± 0.00^{a}	1.95 ± 0.11^{ab}	2.00 ± 0.00^{a}
Appearance (max 1)	1	0.95 ± 0.11^{a}	0.95 ± 0.1^{a}	0.95 ± 0.11^{a}	0.95 ± 0.111^{a}
	7	0.83 ± 0.12^{a}	0.83 ± 0.11^{a}	0.83 ± 0.11^{a}	0.83 ± 0.11^{a}
	14	0.91 ± 0.12^{a}	0.91 ± 0.12^{a}	0.91 ± 0.12^{a}	0.91 ± 0.12^{a}
	21	0.86 ± 0.13^{a}	0.90 ± 0.13^{a}	0.91 ± 0.12^{a}	0.90 ± 0.14^{a}
Color (max 1)	1	0.85 ± 0.22^{a}	0.85 ± 0.22^{a}	0.85 ± 0.22^{a}	0.85 ± 0.22^{a}
	7	0.90 ± 0.13^{a}	0.90 ± 0.14^{a}	0.90 ± 0.14^{a}	0.90 ± 0.13^{a}
	14	0.86 ± 0.13^{a}	0.86 ± 0.12^{a}	0.86 ± 0.13^{a}	0.86 ± 0.13^{a}
	21	0.81 ± 0.11^{a}	0.81 ± 0.11^{a}	0.81 ± 0.11^{a}	0.81 ± 0.10^{a}
Consistency (max 4)	1	3.46 ± 0.55^{a}	3.40 ± 0.52^{a}	3.40 ± 0.52^{a}	3.42 ± 0.52^{a}
	7	3.59 ± 0.12^{a}	3.50 ± 0.00^{a}	3.55 ± 0.11^{a}	3.54 ± 0.09^{a}
	14	3.60 ± 0.13^{a}	3.55 ± 0.11^{a}	3.55 ± 0.11^{a}	3.55 ± 0.11^{a}
	21	3.40 ± 0.22^{a}	3.40 ± 0.22^{a}	3.38 ± 0.21^{a}	3.38 ± 0.22^{a}
Total (max 20)	1	16.23 ± 1.30^{aII}	16.51 ± 1.29^{abII}	**17.55 ± 1.26^{abI}**	16.99 ± 1.28^{abII}
	7	17.01 ± 0.73^{abII}	**17.42 ± 0.84^{abII}**	16.80 ± 0.83^{abII}	17.33 ± 0.86^{abII}
	14	**17.93 ± 1.01^{aI}**	17.33 ± 1.23^{abII}	16.30 ± 0.75^{abII}	**17.54 ± 0.98^{abII}**
	21	16.87 ± 0.76^{abII}	**17.21 ± 0.57^{abII}**	16.64 ± 1.76^{abII}	16.49 ± 1.27^{abII}

Legend: 50:50AB = 50% cow's milk + 50% soy beverage, peach aroma; 50:50AJ = 50% cow's milk + 50% soy beverage, strawberry aroma; 50:50AK = 50% cow's milk + 50% soy beverage, pear aroma; 50:50 AM = 50% cow's milk + 50% soy beverage, apricot aroma; Data represent mean values (± SD) of three repetitions. ^{abc} Duncan's Test confirmed the statistically significant difference between mean values (± SD).

According to Table 4, the samples 50:50 AJ (strawberry aroma) and 50:50 AM (apricot aroma) had the highest average for taste and smell while the 50:50 AK (pear aroma) sample received the lowest acceptability for taste and smell. The strawberry aroma treatment (50: 50AJ) was more satisfactory compared to other treatments. Of all samples on storage days, the 50: 50AJ sample showed the highest overall score,

while the 50:50 AK sample received the lowest overall score. In the overall assessment of sensory properties, the lowest values were recorded for the sample 50:50 AK (pear aroma), which decreases during the storage period and at the end of the 21st day of storage is 16.64. The highest values were recorded for the sample 50: 50AJ (strawberry aroma) where at the end of storage on the 21st day the value of the total sensory rating is 17.21. Higher amounts of sugar in flavored samples covered the unpleasant aroma of soy beverage and increased the viability of probiotic bacteria [15]. Figure 4 shows the overall sensory evaluations of samples of flavored probiotic beverages with La5 and yogurt culture during the storage period at + 4 ºC.

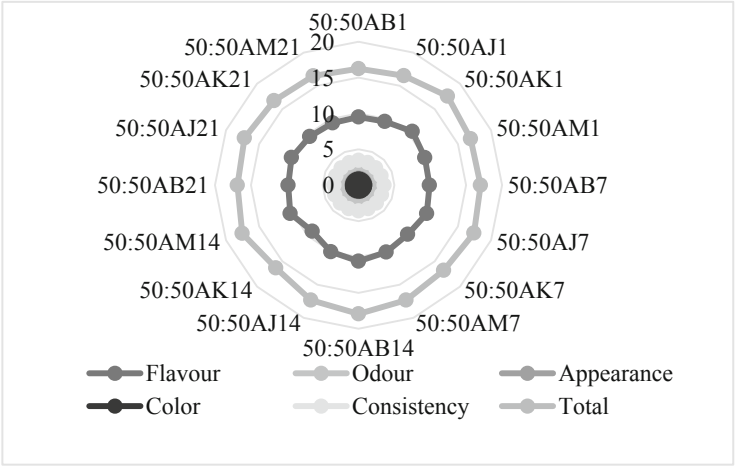


Fig. 4. Sensory characteristics of flavored milk and soy beverage with *L. acidophilus* and yogurt culture

The results of the acceptability test of flavored fruit beverages are shown in Table 5. Based on the data determined by the hedonic scale, the basic statistical parameters, as well as the desirability percentage, were calculated (Table 5). The results show the opinion of potential buyers (40) of new products. The sample of 50: 50AB (peach aroma), 50: 50AJ (strawberry aroma), and 50:50 AM (apricot aroma) was rated by the respondents according to the hedonic scale as "very satisfied" and "moderately satisfied", which shows that such flavored products would be well accepted by consumers. The 50: 50AK (pear aroma) sample for most potential consumers was rated as acceptable, ie according to the hedonic scale as "satisfied", which means that they would consume them. A similar study was conducted by Behrens et al. [16]. for fermented soy beverages with the addition of different types of fruits and nuts. According to their results, for most subjects, the fermented soy beverage with the addition of hazelnuts was unacceptable, while the addition of other fruits such as coconut, pineapple, kiwi, guava, and strawberry to the fermented soy beverage was acceptable by the respondents Behrens et al. [16].

Table 5. Scores obtained of flavored beverages after 1st day of storage at + 4 °C using a hedonic scale

Scores	Samples of flavored beverages			
	50:50AB	50:50AJ	50:50AK	50:50AM
9	8	8	6	4
8	13	10	5	3
7	11	16	14	14
6	6	4	5	10
5	1	2	0	8
4	0	0	5	1
3	1	0	3	0
2	0	0	2	0
1	0	0	0	0
Total	40	40	40	40
X	7.42^{a}	7.45^{a}	6.37^{bc}	6.55^{b}
S	1.27	1.08	2.03	1.25
Desirability (%)	**97.50^{a}**	**100.00^{a}**	75b	**97.50^{a}**
Cv	17.11	14.49	31.93	19.08

Data represent mean values (± SD) of three repetitions. abc Duncan's Test confirmed the statisti-cally significant difference between mean values (± SD). x = mean; s = standard deviation; Cv = variability coefficient..

4 Conclusions

Flavored dairy beverages of milk and soy beverage (50% cow's milk: 50% soy beverage) have been successfully produced with the addition of four different fruit flavors (strawberry, apricot, pear, and peach) and the probiotic bacterium *L. acidophilus*. In general, the viscosity of flavored beverages is higher than non-flavored, and this is caused by the addition of sucrose, ie an increase in dry matter content. The addition of fruit aromas to the fermented probiotic product of milk and soy beverage improved the sensory properties of the product. The results of the research showed that the type of probiotic culture, fruit aroma as well as the ratio of milk and soy beverage significantly ($P < 0.05$) influenced the acceptability and sensory properties of the samples. Different fruit aromas affected the general acceptability and sensory characteristics of the product during the cold storage treatment. The best results of product acceptability, as well as sensory characteristics with the probiotic culture of *L. acidophilus*, were recorded for the ratio of 50% milk and 50% soy beverage with strawberry aroma (50: 50AJ) and peach aroma (50: 50AB). In general, analysis of produced fermented beverages found that the ratio of 50% milk and 50% soy beverage is optimal for industrial production taking into account

overall aspects including probiotic sustainability, biochemical and nutritional properties of the product as well as soy protein function, acceptability, and sensory characteristics.

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Determination of Trace and Heavy Metals in Selected Samples of Oregano (*Origanum Vulgare* L.) from Bosnia and Herzegovina

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Abstract. Herbs are commonly used in traditional medicine and as spice around the world. They consist of a number minerals essential to human health and nutrition. Many of these microelements are crucial for various metabolic processes and needed for normal physiological functions. The concentration of some essential metals (Fe, Mn, Cu, Zn, and Cr) and heavy metals (Pb, Ni, and Cd) were analyzed in selected samples of oregano (*Origanum vulgare* L.) collected from different locations in Bosnia and Herzegovina. Preparation of samples was done using wet digestion with acids, in triplicate and determined by flame atomic absorption spectrometry. The mean concentration (mg kg^{-1}) of Fe, Mn, Cu, Zn, Cr, Pb, and Ni ranged from 68.79–152.6; 17.48–27.30; 4.63–6.96; 16.81–51.28; 0.023–0.036; 0.0020–0.0204; and 0.39–2.37, respectively. Cadmium was not detected in any analyzed samples of oregano. These results were in agreement with other published data except in the case of Pb which content was lower and within the permissible limit; and for Cd which do not detected during analysis. Thus, on the basis of experimental outcome, it can be concluded that oregano collected from various locations from BiH are safe and may not produce any harmful effect of metals toxicity during their applications as spices as well as in different pharmaceutical formulations.

Keyword: Trace metals · Heavy metals · Oregano · AAS

1 Introduction

Plants in the form of herbs and spices have been used in cuisine and different medicinal as well as pharmaceutical formulations since ancient times [14, 19]. They contain many various substances beneficial for human health but at the same time, there are harmful and toxic elements (e.g. heavy metals) in a wide range of concentrations. *Origanum vulgare* L. commonly known as oregano is often used as a spice in cuisines around the world to improve or increase aroma, taste and smell of food [11, 12]. Also, as a

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herb, oregano is used as a medicinal plant in different pharmaceutical formulations of traditional medicine as well as industrial preparations [2, 10].

A number of studies have demonstrated that *O. vulgare* L. has important health actions and medicinal properties such as antioxidant, antimicrobial, antifungal, antigenotoxic, anti-inflammatory effects, insecticidal and many others activities [3, 4]. Oregano contains highly chemically and biologically active substances including minerals and trace metals [16]. Metals have an important role in human health as essential components for a normal growth of cells, various functions of cells, they catalyze many biochemical reactions in the organism including the formation of red blood cells, hormones and vitamins, as well as in the processes of photosynthesis and respiration, oxidation and reduction in plants [2, 17].

The aim of this study was to determine the trace and heavy metals concentrations in selected samples of *Origanum vulgare* L. from different locations in Bosnia and Herzegovina.

2 Materials and Methods

Samples were collected from three different municipalities in Bosnia and Herzegovina: Bugojno, Travnik and Sarajevo. *Sample 1* as wild oregano were collected in Bugojno, distanced from roads and industrial pollution. Fresh leaves were cut into small pieces and air-dried in a dark place for two weeks, then stored in plastic bags until analysis. *Sample 2* as tea samples taken from a local Pharmacy in Travnik, declared as 100% oregano tea. *Sample 3* as a spice purchased at a local market in Sarajevo.

Sample Preparation and Analysis

Preparation of sample solutions for metal analysis from plant material was done by wet digestion using nitric and sulfuric acid by method of Lisjak et al. [15]. Mass of 0.5 g of dry plant matter was weighed into beakers of 100 ml, 5 ml of HNO_3 and 2 ml of H_2SO_4 was added and this solution was left at room temperature for 5–6 h. After that, the prepared solution was heated on a hot plate until the plant material has completely decomposed (about 30 min). After cooling, the solution was filtered through filter paper (blue band) into a 50 ml flask and diluted with redistilled water.

Metal content of collected samples of oregano was determined by FAAS (AAC-7000 Auto Atomizer Changer, Shimadzu, Japan) as recommended by the method of ISO 11047:1998. This instrument has a software program that directly reads the results based on the calibration curve equation obtained for each tested element.

Preparation of working solutions of standards for tested metals.

Working solutions of standards (series of standards) for the element analysis were prepared from original certified standard solutions (Merck, Darmstadt, Germany) of a concentration of 1000 ppm. The dilutions were made with redistilled water (Millipure Q water), and the concentrations in the series of standards for each element are selected by the expected average values of the content of the test element in the plant material.

All the chemicals and reagents were of analytical grade and purchased from Merck (Darmstadt, Germany). The sample and blank analyses were performed in triplicate, and values presented as a mean of analyzed metals mg kg^{-1}.

3 Results and Discussion

In this work, the content of trace and heavy metals concentration was determined in the plant species of *O.vulgare* L. as a medicinal and aromatic herb, collected from different locations in Bosnia and Herzegovina. The results of content of trace and heavy metals in three oregano samples from Bosnia and Herzegovina municipalities (Bugojno, Travnik, and Sarajevo) labeled as Sample 1, Sample 2, and Sample 3, were presented in Table 1.

Table 1. Trace and heavy metal content in different oregano samples from Bosnia and Herzegovina

Element	Sample 1	Sample 2	Sample 3
Cadmium, Cd mg kg−1	n.d.*	n.d.*	n.d.*
Chromium, Cr mg kg^{-1}	n.d.*	0.036	0.023
Copper, Cu mg kg^{-1}	6.96	4.63	4.87
Iron, Fe mg kg^{-1}	108.7	68.79	152.6
Lead, Pb mg kg^{-1}	n.d.*	0.0204	0.0020
Manganese, Mn mg kg^{-1}	26.28	17.48	27.30
Nickel, Ni mg kg^{-1}	0.39	1.46	2.37
Zinc, Zn mg kg^{-1}	51.28	34.48	16.81

Values presented as mean (mg kg^{-1}) of dry matter; *n.d.- not detected.

The concentrations of eight elements (Cd, Cr, Cu, Fe, Pb, Mn, Ni, and Zn) in the digested samples of oregano were determined by Flame Atomic Absorption Spectrometry (FAAS). Among the analyzed metals, lead (Pb) was at the detection limit of 0.002 mg/g and the concentration of the rest of metals are shown as mean of triplicate (mg kg^{-1}) (Table 1). The most abundant metal among the microelements was iron (Fe) while lead concentration was the lowest in tested samples of oregano. The content of trace and heavy metals in the selected three samples followed the trend: Fe > Zn > Mn > Cu > Ni > Cr > Pb, while cadmium (Cd) was not detected in any of the tested samples of oregano.

The concentration of iron was higher than other trace and microelements. According to the World Health Organization (WHO) concentration maximum or minimum for Fe in medicinal herbs has not been established. Our results as well as results from other authors show wide variations of iron in oregano species depending on various climatic and geographic conditions [3, 14, 18]. Results of this study are comparable to contents of iron found in work of Bukva [7] and colleagues which investigated the content of iron in oregano and different spices from BiH. Also, our findings for Fe are similar with the results of UAE [8]. Iron has a key function in the body including oxygen transport, energy production, and immunity. On the other hand, higher concentrations of iron are associated with toxicity and damage of various organs (e.g., liver damage) and impairments of many metabolic pathways and the cardiovascular system. Also, its

increase concentrations effects on plant growth which depended on soil properties and geographic region of growth of plant.

Zinc, after iron is the second trace element in higher concentrations that we detected. The range of Zn concentrations observed in this study was from 16.81 to 51.28 mg kg^{-1}. The content of zinc is the same or similar to the results of other studies [2, 3, 5, 6]. As one of the more important and essential microelements for normal growth and development of humans, Zn is responsible for protein and DNA synthesis and many other biochemical functions. Although our knowledge of zinc toxicity is scarce and not available yet it is well known that a high content of Zn of above permissible limits causes toxic effects on the human immune system, blood lipoprotein levels and copper levels.

Manganese is a necessary microelement for plant as well as animal growth. We found concentrations of Mn in the range of 17.48 to 27.30 mg kg^{-1} which are comparable with findings in studies that determined manganese and other trace elements in oregano plant. As detected concentrations of manganese are well below the max. limit and permissible levels it does not influence human and plant growth [12, 18].

The concentrations of copper varied from 4.63 to 6.96 mg kg^{-1} in Sample 1, Sample 2 and Sample 3, which is similar to the amount of copper observed in previous studies [10, 19]. In line with iron levels, the recommended limits by the WHO have not been established yet for the Cu in herbal medicine. Therefore, different countries have different referent and regulatory value limits for copper because this trace metal is an essential component of many enzymes and plays an important role in a number of physiological processes including free radical elimination, bone and specific tissues development and many others.

Nickel (Ni) concentrations in leaves are generally between 0.05 and 5 mg kg^{-1} in plants. Our results for concentration of this metal ranged from 0.39 to 2.37 mg kg^{-1} which is in line with the forementioned values [1, 18]. Nickel is an essential element for plants as well as humans. In the body it is a structural consistent of the enzyme urease, and necessary for the regulation of lipid content in tissues. Also, nickel in small quantities is involved in the formation of red blood cells. The WHO states that the permissible limit of nickel in medicinal plants is 1.5 mg kg^{-1} while daily requirement for humans is 1 mg kg^{-1}. However, Ni toxicity in humans is not a very common occurrence because its absorption by the body is very low [20]. Higher concentrations of Ni can cause severe diseases and various pathological effects and its toxicity might be attributed to nickel interference with the physiological processes of zinc and calcium [21].

Chromium concentrations detected in our study were below 5 mg kg^{-1}. This finding was in line with the findings of other investigations [23]. As chromium concentrations in plant depends of plant parts and it's decreased presence in the soil.

The content of lead in the analyzed Sample 3 and Sample 2 ranged from 0.0020 to 0.0204 mg kg^{-1}. In Sample 1 as a wild oregano plant Pb was not detected. The WHO maximum permissible limit of lead in medicinal herbs is 10 mg kg^{-1}. The obtained results showed that the concentrations of lead in Sample 2 and Sample 3 were below the permissible limit which is good having in mind that lead is a highly toxic heavy metal, especially from environmental pollution/pollutant sources [23]. Lead

can form complexes with crucial biomolecules and adverse effects or affects their functions. Its exposure may affect blood, immune, nervous, renal, muscular, reproductive and cardiovascular systems causing severe impairments and seriously defects [21].

Cadmium was not detected in any of the analyzed samples of oregano. In previous studies, the content of Cd was above the permissible limit set by WHO for medicinal herbs and plants (0.3 mg kg^{-1}). The higher level of cadmium has a serious toxicological effect on human health, especially for kidneys. Excretion of Cd is very slow and its accumulation in human kidneys during long periods of time causes irreversible and damaging effects on the liver, the vascular and immune system, and impairing of the renal tract [8].

4 Conclusion

Metal contents in oregano (*Origanum vulgare* L.) were determined by FAAS. In tested selected samples of this herb i.e. Sample 1, Sample 2, and Sample 3 had the lowest concentration of lead and cadmium was not detected in any analyzed samples. The results of this study indicated that selected oregano samples from different location in BiH are safe for use as tea, spice or medicinal herbs in various pharmaceutical formulations.

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Effects of Dry Fruit Supplement on Biscuit Quality

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Abstract. Biscuits belong to flour-based confectionery products where wheat white flour, fat and sugar are used as the basic raw materials. Dry fruits as a significant source of valuable macro- and micro-nutrients, as well as bioactive components, can improve the quality of biscuits, which was the subject of this research. Whole wheat flour biscuit samples were prepared with the addition of dried fruits: apricots, figs and prunes. The fruits in dry and rehydrated forms were added in a concentration of 30% calculated on flour weight. Control samples and the total of 6 samples were prepared in two replicates. The analysis of the biscuit samples included thickness and diameter increase, spread ratio, specific volume, hardness, moisture, pH, total titratable acidity and total phenolic content. Sensory evaluation was conducted using a 1–5 score scale on 5 selected properties: appearance, taste and melting, texture, smell and aroma, and overall acceptability. The results showed that the incorporation of dried and rehydrated fruits increased the total phenolic content of the biscuits compared to the control sample. It was found that the addition of dried fruits can improve the properties of the final product without drastically diminishing the sensory attributes. The dried fruits contributed better to the biscuit physical and chemical properties than the rehydrated fruits. The highest content of total phenol was found in the samples with prunes in their dry (7.17 mgGAE/gDM) and rehydrated (6.86 mgGAE/gDM) forms and it increased by 2.6 and 2.4 times respectively compared to the control samples. The best score according to the sensory evaluation had the sample with dried and rehydrated apricots.

Keywords: Biscuits · Dried fruits · Total phenolic components · Sensory evaluation

1 Introduction

Flour-based products are the most often consumed ones among confectionery products in general and their consumption has been constantly growing. Biscuits are classified as flour-based confectionery products and have a high caloric value due to the main ingredients such as wheat flour, mostly white, fat and sugar. However, they are low in micronutrients and are nutritionally poor.

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Biscuit production has transformed from a labour-intensive small craft production to the modern and mechanized industry based on sciences. Nowadays, biscuits are versatile snacks occupying a notable position due to their positive features like longer shelf life, varied attractive taste and fine texture. The wider consumption of biscuits among all bakery products has been observed and there is a number of ways of improving their nutritional quality to satisfy the huge consumer demand for biscuits with healthier ingredients and to make them more nutritious and tastier as well [1].

Flour-based confectionery products in general are considered good material for improvement by adding different ingredients which have important nutritive and non-nutritive valuable components. Fruits have received much attention recently as a source of biologically active substances and they have huge relevance for confectionary industry, especially for biscuits, cakes and other bakery products. The incorporation of fruit in different forms is a good way of improving not only the nutritive and non-nutritive values but also the physical and sensory quality of final products [2]. Dried fruits are widely used in biscuits and contribute to flavours and textures. The biscuits are usually small and thin and the fruits are supposed to be either small themselves or cut into small pieces for better fitting into the biscuit. For dry fruit incorporation into a biscuit formulation, the following is relevant: the final taste and aroma of the product, the size and appearance, the cleanliness in terms of dust, dirt, stones and other adventitious matter and the level of infestation or deterioration which has developed after harvesting and during storage [3].

Although a number of researches confirmed a possibility of developing different flour-based confectionery with improved functional characteristics using fruit products [4–7], and especially by-products from fruit processing as a source of considerable amounts of bioactive components [8–13], there are only a few researches that dealt with dry fruit incorporation into these products.

Dried fruits are a concentrated form of fresh fruits obtained by removing the moisture through sun-drying or using different modern drying techniques and they are nutritionally equivalent to fresh fruits in smaller serving sizes [14, 15]. The traditional drying technology leads to serious losses of bioactive compounds, but dried fruit can still be a valuable source not only of energy, dietary fibre and minerals, but also of antioxidant activity [16, 17].

Raisins, figs, dates, prunes and apricots are the most common dried fruits in the market and they are rich sources of essential nutrients and health-promoting bioactive compounds [15]. Prunes, dried apricots and dried figs accounted for 22% of the 2017/2018 dried fruit world total production in the amount of 242.666, 226.760 and 135.400 metric tons respectively [18]. These dry fruits are rich in carbohydrates and are a considerable source of sugars: 38.13% (prunes), 47.92% (dried figs) and 53.54% (dried apricots), among which the fructose and glucose are the most common ones. Despite high amount of sugar, dry fruit has a relatively low glycaemic index (GI), and the established GI values are 30 for dry apricots (low GI), 61 for dry figs (moderate GI) and 29 for prunes (low GI). The high presence of fibres, polyphenols and tannins are possibly responsible for this phenomenon [14].

The content of dietary fibres is rather high in prunes and it can be as high as 7.15%, in dried apricots 7.3% and in dried figs 9.8%, which meets the demands for human nutrition

and the latest dietary recommendations [14]. In general, dry fruits are excellent sources of minerals, especially copper, iron, magnesium and potassium. Dried figs are high in calcium (162mg/100g edible portion) and magnesium (68mg/100g edible portion), while dry apricots are rich in potassium (1162mg/100g edible portion) [14].

Dried plums are noted for their high content of dietary fibres, carotenes, iron and potassium [19, 20]. In addition, they have antioxidant polyphenolic phytochemicals and show antioxidant, anti-cancer, anti-hyperglycaemic, anti-hyperlipidaemic, anti-hypertensive, anti-osteoporosis, laxative and hepatoprotective characteristics [20, 21].

The incorporation of prunes into baking products has great benefits in terms of the production process: the high pectin content in dried plums provides added stability during baking, high sorbitol content helps to keep bakery products soft and moist over an extended shelf life and, with fructose and glucose, provide additional humectancy, dried plums contain about 2% of naturally occurring malic acid, which has proved to be an effective flavour enhancer, which helps to inhibit microbial spoilage and which can also serve as the natural acid component of chemical leavening systems [19].

According to research by Jesionkowska et al., among five different forms of product candy, fruit teas, cereals, bars, and cookies, consumers in some European countries (approx. 33% of respondents) considered cereals as the best product to which functional dried fruit could be added. The authors also concluded that dried fruits can be adopted as carriers of functional ingredients, especially when promoted as a source of antioxidants [16], and considerable opportunities exist in the dried fruit-based functional food products for expansion and innovation [15].

The objective of this study was to examine the physical, textural, chemical and sensory quality of whole wheat flour biscuits with the incorporation of dry apricots, dry figs and prunes in the amount of 30% on flour weight.

2 Materials and Methods

2.1 Procedure of Biscuit Making

All materials needed for biscuit making, namely whole wheat flour, sugar, vegetable fat and dry fruits, were purchased from the local market. For this research, dried apricots, figs and prunes were selected due to their frequent consumption, possibility of their cultivation in Bosnia and Herzegovina and their individual sizes.

Biscuit samples of whole-meal wheat flour were produced according to the AACC method 10-52 [22], by way of modification according to Džafić et al. [23]. Dry apricots, figs and prunes in dry (i) and rehydrated (ii) forms were added in the amount of 30% (w/w) calculated on flour weight.

Dry fruit was sliced manually by knife before being added to the dough. The preparation of rehydrated fruit included rehydration according to Akagić and Vranac [24], drained and sliced in the same way as dried fruit. Water corrections were made to ensure the unique consistency of the dough. The dough was sheeted to a thickness of 6 mm, cut using circular mould to 50 mm of diameter and placed on an aluminium tray.

The biscuit samples were baked at 205 °C for 11 minutes and then allowed to cool down to ambient temperature. Biscuits without dry fruit were produced as control samples. Two bakings for each biscuit sample (20 pieces) were performed and presented as replications.

2.2 Physical and Chemical Analysis of Biscuit Samples

The diameter and thickness of the samples were measured by a digital vernier calliper with the sensitivity of 0.01mm (MIB Messzeuge GmbH, Spangenberg, Germany) before and after the baking. The diameter and thickness of the samples were measured by taking an average value of 5 biscuits from each replication. The average of 10 individual pieces was recorded for each sample. Diameter (DI) and thickness increases (TI) were calculated as the ratio of the biscuit diameter and thickness before and after the baking and expressed in %. Spread ratio (SR) was calculated by dividing the values of diameter by the values of thickness. Specific volume (SV) was obtained as the ratio of volume (seed replacement method) to the weight of biscuit [25].

The hardness of the samples (N) was determined by the direct penetration method and by measuring the force required to pierce the analysed sample using TA.XT Texture Analyzer (Stable Micro System).

Moisture content (%), total titratable acidity (TTA) and pH value of the research samples were determined according to Kaluđerski and Filipović [26].

The total phenolic content (mgGAE/DM) (TPC) of biscuits was determined by spectrophotometric method (λ=765 nm) using Folin-Ciocalteu reagent [23].

2.3 Sensory Evaluation

Sensory evaluation was carried out by a panel of 11 trained members. They were selected and trained in accordance with the ISO 8586 guidelines [27]. Biscuit samples were evaluated by QDA using a 1-5 score scale on the following properties: appearance, which includes the assessment of the shape, colour, possible defects or fractures; taste and melting; biscuit structure; odour and aroma and overall acceptability. The sensory evaluations were obtained from 11 replicates where panellists were considered as replicates.

2.4 Statistical Analysis

Data were reported as a mean ± standard deviation (SD). A two-way analysis of variance (ANOVA) was used to evaluate whether significant differences existed between the biscuit samples depending on the type of dry fruit and on the form of the fruit added (dry and rehydrated). The determined differences were tested by the LSD test at a significance level of 0.05 using the SPSS 16 programme.

3 Results and Discussion

3.1 Physical and Chemical Properties of Biscuit Samples

The physical, textural and chemical properties of the biscuit samples are presented in Table 1. The thickness increase (%) (TI) of the biscuit samples with dry fruit added in

both forms, dry and rehydrated, differed significantly ($P < 0.05$) from the control samples and, as a consequence, they were lower than the control samples with no dry fruit added. Similar results were obtained by Mahloko et al. [28] in whose research the addition of 4% banana and 4% of prickly pear peel powder to wheat flour significantly decreased the biscuits height. Khouryieh and Aramouni [29] also noted that cookie height decreased as flaxseed incorporation increased in the cookies. Significant differences ($P < 0.05$) were not found between the biscuit samples with added dry and rehydrated fruit. The highest TI was recorded in the samples with figs in dry (48.43%) and rehydrated (53.20%) forms.

According to diameter increase (DI), the results showed significant differences between the control samples (3.09%) and the samples with added dry fruit where they ranged from 8.29% (samples with rehydrated prunes) to 12.64% (samples with prunes). Samples with dry fruit had a significant ($P < 0.05$), higher DI (%), possibly due to the higher water content in biscuits with rehydrated fruits available to gluten that allowed gluten to achieve a stronger matrix and due to the reduced spread of the samples.

The decrease in height with increasing levels of dry fruit is possibly a consequence of the inhibition of gluten development. The addition of non-gluten ingredients could reduce the total gluten content, affect the dough rising and the volume forming and simultaneously increase the width of baked products [30]. In addition, the high content of sugar in biscuits attracts water over the gluten proteins. High amounts of fat in biscuits also affect the development of the gluten matrix. Moreover, the addition of dry fruit makes the dough heavier and difficult to rise and retain its shape [2].

The biscuit spread ratio (SR) was significantly higher ($P < 0.05$) in the samples with dry fruit in both forms compared to the control samples (4.37) and it ranged from 5.98 (the samples with rehydrated figs) to 7.97 (the samples with dry apricot). Significant differences ($P < 0.05$) were not found between the samples with incorporated dry and rehydrated fruit. The SR is a measure of cookie quality and higher values are more desirable [1, 31]. However, the excessive SR is not quite eligible to consumers because it indicates that the product is extremely thin [2].

Replacing wheat flour with different types of non-wheat flour mostly affects their nutritional values and physical characteristics as well. Replacing wheat flour with the other flour such as barley, chestnut, defatted maize germ, potato flour and others decrease, while purple rice and sorghum increase the SR value [32]. The SR of the composite cookies had an increasing trend along with the increasing substitution level of amaranth flour [33]. The higher protein content of the flour due to a greater water binding ability reduces the SR while the SR increases when a non-wheat protein is present as the amount of lipids and fibres in the dough consequently rise [1].

The addition of dry and rehydrated figs into the biscuits significantly ($P < 0.05$) reduced the SV in the samples with dry and rehydrated figs compared to the control ones. The samples with dry apricots (1.51 cm3/g) had the highest SV and the samples with added prunes (0.84 cm3/g) the lowest. Differences in respect of the SV were not found between the samples with figs and prunes (Table 1).

The texture of the biscuit samples was significantly ($P < 0.05$) lower than the control ones (39.67N) as a consequence of the added dry fruit. In terms of the dry fruit form, the samples with added dry fruit showed significantly ($P < 0.05$) higher values than the samples with rehydrated forms. The texture of the samples with dry fruit ranged from

Table 1. Physical, textural and chemical properties of biscuit samples with dry and rehydrated fruits.

	Control	Dry				Rehydrated			
		Apricot	Fig	Prune		Apricot	Fig	Prune	
TI (%)	$65.74 \pm 10.11^{*}$	17.47 ± 4.02^{a}	48.43 ± 15.29^{b}	39.67 ± 18.07^{a}	*x*	37.07 ± 5.99^{a}	53.20 ± 11.52^{b}	28.10 ± 12.76^{b}	*x*
DI (%)	$3.09 \pm 1.89^{*}$	12.32 ± 3.36	12.50 ± 2.52	12.64 ± 2.21	*x*	11.59 ± 2.4	9.39 ± 2.39	8.29 ± 1.89	*y*
SR	$4.37 \pm 0.24^{*}$	7.97 ± 0.34^{c}	6.38 ± 0.76^{b}	6.81 ± 0.89^{a}	*x*	6.79 ± 0.28^{a}	5.98 ± 0.57^{b}	7.09 ± 0.59^{a}	*x*
SV (cm^3/g)	$1.29 \pm 0.22^{*}$	$1.51 \pm 0.14^{*a}$	0.97 ± 0.10^{b}	$0.84 \pm 0.25^{*b}$	*x*	$1.29 \pm 0.11^{*a}$	1.02 ± 0.01^{b}	$1.34 \pm 0.14^{*b}$	*x*
Hardness (N)	$39.67 \pm 0.43^{*}$	27.14 ± 6.25^{a}	20.22 ± 5.06^{b}	7.67 ± 0.29^{c}	*x*	6.32 ± 0.55^{a}	2.47 ± 0.28^{b}	2.60 ± 0.18^{c}	*y*
Moisture (%)	$3.24 \pm 0{,}12^{*}$	2.80 ± 0.36^{a}	6.65 ± 0.93^{b}	5.32 ± 0.60^{a}	*x*	16.60 ± 1.90^{a}	17.95 ± 0.24^{b}	16.46 ± 0.75^{a}	*y*
TTA	$1.65 \pm 0.75^{*}$	4.11 ± 0.13^{a}	$2.14 \pm 0.06^{*b}$	1.13 ± 0.98^{c}	*x*	1.31 ± 0.14^{a}	$1.24 \pm 0.07^{*b}$	1.09 ± 0.14^{c}	*y*
pH	$9.15 \pm 0.07^{*}$	7.65 ± 0.70^{a}	7.75 ± 0.70^{b}	7.70 ± 0.0^{c}	*x*	7.65 ± 0.07^{a}	8.50 ± 0.0^{b}	8.05 ± 0.70^{c}	*y*
TP (mgGAE/gDM)	$2.77 \pm 0.31^{*}$	5.29 ± 0.93^{a}	$3.22 \pm 0.04^{*b}$	7.17 ± 0.00^{c}	*x*	3.55 ± 0.54^{a}	$3.08 \pm 0.00^{*b}$	6.86 ± 0.06^{c}	*y*

Different letters in rows from *a* to *c* for each parameter indicate significantly different values among dry fruits at $P < 0.05$; different letters in rows from *x* and *y* for each parameter indicate significantly different values among forms of dry fruit (dry and rehydrated) at $P < 0.05$. Samples which are not different from the control at $P < 0.05$ are marked with *

TI – Thickness degree; DI – Diameter degree; SR – Spread ratio; SV – Specific volume; TTA – Total titrable acidity; TP – Total phenolic content; DM– dry mater.

2.47N (the samples with rehydrated figs) to 27.14N (samples with dry apricots) (Table 1). The rehydrated dry fruit had high moisture content that raised the moisture content in the samples and, consequently, these samples were more tender and softer.

The dry fruit increased the moisture content in the samples, with the exception of the sample with dry apricots that had lower moisture content (2.80%) than the control ones (3.24%). The samples with rehydrated fruit forms had significantly ($P < 0.05$) higher moisture content than the control ones and the samples with the added dry fruit (Table 1). This could be expected given the high water content in the rehydrated fruit. The moisture in the samples with rehydrated fruits was: 16.60 % (apricot); 17.95% (fig) and 16.46% (prune). These values are rather high for this kind of flour-based confectionery and present possible risk of spoiling and contamination.

Dry fruit also influenced the TTA (%) and pH values of the samples. The total acid content in dry fruit comes from the acid in fresh fruit. The total acid content in apricots is 0.6–1.1%; in plums 0.5–0.7 (%) [34] and in figs grown in subtropical region 0.4–1.2 (%) [35]. The samples with apricots and prunes in dry and rehydrated forms differed significantly ($P < 0.05$) from the control ones. The TTA was the lowest in the samples with rehydrated prunes (1.09) while the highest value was recorded in the samples with dry apricots (4.11), which is in accordance with literature data [34, 35]. The acids from dry fruit significantly ($P < 0.05$) decreased pH value in the samples with dry and rehydrated fruits. The lowest value was found in the samples with dry and rehydrated apricots (7.65) and the highest pH value was found in samples with rehydrated figs (8.5). The TP values significantly ($P < 0.05$) increased when apricots and prunes were added in dry and rehydrated forms in comparison to the control sample. The highest content of the TP was found in the samples with prunes in dry (7.17 mgGAE/gDM) and rehydrated forms (6.89 mgGAE/gDM) showing the increase of 2.6 and 2.4 times respectively compared to the control ones. An exceptionally high amount of TP is in accordance with the data reported by Alasalvar and Shahidi [14]. Nevertheless, they also reported higher amounts of TP in dry figs (960 mgGAE/100g) and in dry apricots (248 mgGAE/100g) [14], which does not correspond to the obtained results as they showed no significant increase of TP in the samples with added figs in both forms (Table 1). Vallejo et al. found also high concentrations of phenolic compounds which were present either in the skin (mainly anthocyanins) or in the pulp (mainly proanthocyanidins) of dry figs. However, they noted that harvest time and cultivar could have an influence on final phenolics concentration [36].

The content of total phenolics in the amount of 498.13 mgGAE/100g of dry apricots was determined by Čanadanović-Brunet et al [17] and the amount of 327 mgGAE/100g in dried apricots was also reported by Alasalvar and Shahidi [14]. The samples with dry forms of fruit had significantly ($P < 0.05$) higher content of TP than the samples with rehydrated fruit. A possible reason for this phenomenon is additional hydro-thermal treatment during the rehydration process which led to the decrease of the TP content in the samples with added rehydrated fruit.

3.2 Sensory Evaluation

According to the sensory evaluation significant differences ($P < 0.05$) were not found between the samples with added dry and rehydrated fruit, despite the fact that the biscuits

with rehydrated fruit were more tender and softer as they had significantly ($P < 0.05$) higher moisture and lower texture (Table 1).

The best scores had the samples with dry and rehydrated apricots in all tested sensory parameters (Table 1), especially regarding the external appearance (4.91 in dry and 4.82 in rehydrated form), and they significantly differed ($P < 0.05$) from the biscuit with dry figs and prunes. The colour of the biscuits with apricots was the lightest and that is one of the possible reasons for such a high score won.

A similar situation was found with regard to taste and melting. According to this sensory parameter, the samples with dry figs and prunes did not differ and they were also, evaluated as worse than the control sample ($P < 0.05$).

According to sample structure evaluation, the samples with all three types of dry fruits did not differ compared to the control ones, and the same was with odour and aroma. Differences between the samples with different dry fruit were not significant in terms of odour and aroma.

The panellists considered the samples with dry apricots the best in terms of overall acceptability (4.82 dry and 4.55 rehydrated form) while the samples with prunes in both forms were evaluated with the lowest score (3.73 dry and 3.64 rehydrated form) (Table 2). In spite of this, the samples with prunes were still acceptable without excessive and visible defects, and could be considered as moderately attractive.

Table 2. Sensory evaluation of the biscuit samples with dry and rehydrated fruits.

Sensory properties	Control	Dry			Rehydrated		
		Apricot	Fig	Prune	Apricot	Fig	Prune
Appearance	$4.09 \pm 032^{*}$	4.91 ± 0.30^{a}	$3.91 \pm 0.70^{*b}$	$3.82 \pm 0.98^{*b}$	4.82 ± 0.60^{a}	$3.91 \pm 0.54^{*b}$	$3.82 \pm 1.08^{*b}$
Taste and melting	$4.45 \pm 0.52^{*}$	$4.55 \pm 0.69^{*a}$	4.00 ± 0.77^{b}	3.45 ± 0.82^{b}	$4.27 \pm 0.65^{*a}$	3.64 ± 0.92^{b}	3.36 ± 0.81^{b}
Structure	$3.91 \pm 0.70^{*}$	$4.55 \pm 0.52^{*a}$	$3.91 \pm 0.54^{*ab}$	$3.73 \pm 1.23^{*b}$	$4.27 \pm 0.90^{*a}$	$4.09 \pm 0.83^{*ab}$	$3.64 \pm 1.03^{*b}$
Odour and aroma	$4.00 \pm 0.45^{*}$	$4.55 \pm 0.52^{*}$	$3.91 \pm 0.70^{*}$	$3.82 \pm 1.08^{*}$	$4.27 \pm 0.65^{*}$	$4.09 \pm 0.54^{*}$	$3.82 \pm 1.17^{*}$
Overall acceptability	$4.09 \pm 0.30^{*}$	4.82 ± 0.40^{a}	$3.91 \pm 0.54^{*b}$	$3.73 \pm 0.90^{*b}$	4.55 ± 0.69^{a}	$4.00 \pm 0.63^{*b}$	$3.64 \pm 0.81^{*b}$

Different letters in rows from a to c for each parameter indicate significantly different values among dry fruits at $P < 0.05$; Samples which are not different from the control at $P < 0.05$ are marked with *

4 Conclusion

The obtained results showed that dry fruit added into biscuit formulation affected its physical, chemical and sensory quality parameters. In general, the samples with dry fruit showed better quality than those with rehydrated fruit forms. In addition, the samples with rehydrated fruits had very high percentage of moisture which can affect the storage and shelf life of biscuits.

According to the presented results, an excellent way to improve wheat biscuits in terms of their total phenolic content is prunes, as they increase TP by 2.6 (dry form) and 2.4 (rehydrated form) times compared to the control sample. However, the sensory analysis showed that the biscuits with prunes are less attractive than the samples with dry and rehydrated apricots and figs.

Future researches should be carried out to analyse more types of dry fruit and find out the most convenient form of its incorporation into the flour-based confectionery to preserve bioactive components and ensure its acceptable and attractive sensory properties.

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Physical and Chemical Properties and Content of Heavy Metals in Honey Samples from the Area of High Herzegovina

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Abstract. Honey is a sweet, thick and viscous product with a characteristic taste and smell. It is produced by honeybees from nectar and honeydew. The quality of honey is influenced by the geographical area, the diversity of honey plants, the environment, the origin of bees and the treatment of beekeepers, and the way honey is kept and stored.

The goal of this research was to determine the physical and chemical properties and content of heavy metals in nectar honey samples and whether geographical location has an impact on these parameters. 20 samples of nectar honey collected from four municipalities from the territory of high Herzegovina were examined. Typical quality indicators such as water content, total acidity, electrical conductivity, determination of honey color and hydroxymethylfurfural (HMF) were determined. In addition to the above, the content of heavy metals and macroelements was determined, namely copper (Cu), lead (Pb), cadmium (Cd), zinc (Zn), iron (Fe) and potassium (K). The analysis showed that all samples have lower values from maximally allowed values set by on honey and other bee products [5] in the content of HMF, while certain samples do not meet the values of the Rulebook in the content of water, acidity and electrical conductivity. When it comes to heavy metals in the analyzed samples, the analysis found that all samples contain satisfactory concentrations of cadmium, zinc and potassium, while 9 out of 20 samples have higher values of copper, lead and iron than specified in the Rulebook on maximum allowed quantities of food contaminants [6]. According to the results of statistically processed data (one-factor ANOVA) between the examined samples there is no statistically significant difference in any parameter, which leads us to the conclusion that certain geographical factors of four selected municipalities in high Herzegovina have the same or similar impact on physico-chemical characteristics and honey quality from the same areas.

Keywords: Nectar honey · Physico-chemical analysis · Heavy metals · High Herzegovina

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1 Introduction

By definition, honey is a sweet, thick, viscous, liquid or crystallized product produced by honeybees from the floral nectar or from secretions of other insects (such as honeydew), which bees collect, add their own specific substances and deposited in honeycomb cells to mature [9].

Honey is not an industrial product, it's a completely natural source of carbohydrates. It contains sugars such as glucose, fructose, sucrose, maltose and other polysaccharides. Honey also contains other substances such as proteins, amino acids, enzymes, organic acids, minerals, pollen grains and other substances. Sometimes honey can also contain unwanted components such as heavy metals. These components are the cause of excessive environmental pollution. Bees are exposed to heavy metals through various sources: contaminated air, water, soil and food. Therefore, it is recommended to beekeeping in the most natural environment and as far away from roads and industrial zones as possible [3].

Significant factors that affect the quality of honey are the geographical area, the diversity of honey plant species, the environment, the treatment of bees (by beekeepers), and the way honey is stored. The chemical composition and physical characteristics of honey vary and will depend on the regional and climatic conditions in which the honey plant thrives, the botanical origin of the nectar and the length of storage (ripening) of honey in the hives.

Heavy metals are natural elements that have a high atomic weight and a density at least 5 times higher than water. Their multiple applications in industry, agriculture, medicine and technology have led to widespread use in the environment, but also to raising awareness due to their possible harmful effects on human health and the environment [9].

The most important heavy metals include arsenic (As), barium (Ba), bismuth (Bi), cadmium (Cd), antimony (Sb), chromium (Cr), cobalt (Co), copper (Cu), gold (Au), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni), platinum (Pt), silver (Ag), strontium (Sr), tin (Sn), vanadium (V), zinc (Zn). A large number of heavy metals have no ecological significance, because they are very rare or inaccessible, while some are bio elements and they are necessary for the normal functioning of living systems. Due to the high degree of toxicity to living organisms, as well as its wide distribution, elements that stand out are cadmium (Cd), lead (Pb), mercury (Hg), arsenic (As), chromium (Cr), nickel (Ni), as well as bio elements such as copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), cobalt (Co) and selenium (Se), which in low concentration are essential for various biochemical and physiological processes, but in higher concentration become harmful [4].

1.1 Material and Methods

For the purposes of this research, 20 samples of honey were collected, directly from producers from four municipalities in the territory of High Herzegovina: Prozor, Jablanica, Konjic and the area of Bijelo polje near Mostar. The samples were collected in the period from July to October 2020. All four municipalities had the same number of samples [5].

Sample analyzes were performed in the laboratories at the Faculty of Agriculture and Food, University of Sarajevo in the period October 2020 - January 2021.

Samples were taken in amounts of about 500 grams in glass containers.

1.2 Methods of Work

The following quality parameters were analyzed:

- water content in honey - refractometric method, [5–7],
- total acidity of honey - volumetric method [5–7],
- electrical conductivity of honey [5–7],
- content of hydroxymethylfurfural (HMF) in honey - method by White at two wavelengths [5-7],
- determining the color of honey using Lovibond comparator (NI 96785),
- determining the presence of heavy metals and macroelements (potassium, iron, zinc, lead, copper and cadmium) - using microwave incineration.

1.3 Sample Preparation for Analysis

Depending on the consistency of the honey, samples for analysis were prepared in different ways. If the honey was in a liquid state, it was gently mixed with a stick or shake before starting the analysis. If the honey was granulated, the closed sample vessel was heated in a water bath for 30 min at 60° C and, if necessary, at 65° C. During the heating process, the sample was stirred several times with a stick or shaken and then cooled to room temperature. When hydroxymethylfurfural was determined, honey was not heated.

1.4 Statistical Analysis

Based on the obtained results, statistical data processing was performed. The results of the research were processed using the statistical program IMB SPSS statistics 23. Descriptive statistics were performed first, followed by a test to determine whether there were differences in water content, acidity, electrical conductivity, HMF, and color between samples of specific localities, and whether the geographic area had an impact on the investigated parameters. The method that was used was one-factor ANOVA at a significance level of 95%.

1.5 Results and Discussion

The results of the analyzed parameters on honey samples are shown in Table 1.

According to the results, the water content in the analyzed honey samples ranged from 14.80 to 20.20%. From the attached table it can be seen that the group of samples from 1.M. to 5.M. has the lowest average value of water content. That can also be related to climate. Mostar has a moderate Mediterranean climate and fairly dry air, which also affects the free water content in honey. A study by Biber [1] states that the average value for water content ranged from 15,60 to 19,60%. Comparing the results obtained in the

Table 1. Average water content, acidity, electrical conductivity and hydroxymethylfurfural

Sample	Water (%)	Acidity (meqv./kg)	Electrical conductivity (mS/cm)	HMF (mg/kg)
1.K	15,60	21,00	**1,06***	2,35
2.K	**20,20***	**50,50***	0,32	4,35
3.K	17,40	22,50	0,39	3,20
4.K	17,40	30,50	**1,03***	0,70
5.K	15,80	20,00	0,70	2,71
1.J	18,00	46,00	**1,56***	0,71
2.J	17,80	35,50	0,74	3,14
3.J	15,40	45,50	**2,29***	3,61
4.J	16,80	44,50	**2,00***	2,67
5.J	17,20	**59,00***	**2,08***	1,69
1.P	16,20	49,50	**1,47***	1,93
2.P	16,00	42,00	**1,94***	1,37
3.P	17,80	18,00	0,20	1,84
4.P	16,20	26,50	0,38	1,78
5.P	18,20	39,00	**1,35***	2,50
1.M	15,80	46,50	0,78	3,13
2.M	15,20	**50,50***	**1,64***	4,05
3.M	14,80	35,50	**1,46***	1,74
4.M	17,20	32,00	0,66	3,52
5.M	17,20	33,00	0,72	2,91

* does not meet the values of the Rulebook (7)

paper with the results of other authors, it can be concluded that there are no significant differences in the water content in honey.

The mean value of acidity in the analyzed samples ranged from 18,00 to 59,00%. Too high acidity of honey generally means that the honey has fermented for some time resulting in the conversion of alcohol as a fermentation product into an organic acid [8]. Biber [1] states that the average acidity content for 30 samples of honey from the area of high Herzegovina ranged between 18,80 and 43,00 meqv./kg. By comparing the results obtained from this research with the results of other authors, it can be concluded that they partially fit into the variations cited by other authors.

The values of electrical conductivity of the analyzed samples ranged from 0,20 to 2,29%. Due to the high value of electrical conductivity in some samples of honey, it can be stated that such high values of conductivity indicate the presence of honeydew or the feeding of bees with sugar syrup. Some samples were dark and it is assumed that it is chestnut honey (samples from the territory of the municipality of Jablanica), which may

also be one of the reasons for the high electrical conductivity. Biber [1] states that the average conductivity for 30 samples of honey from the area of high Herzegovina ranged between 0,08 and 2,09 mS/cm. In the research from Biber [2], the analyzes showed that the electrical conductivity for 11 samples from the area of Konjic and Jablanica ranged from 0,85 to 1,22 mS/cm.

The HMF content in the analyzed honey samples ranged from 0,70 to 4,35 mg/kg. An elevated HMF content can be a good indicator of heating and improper storage of honey. Extremely high HMF values (above 100 mg/kg) can be an indicator of honey falsification. A study by Biber [1] states that the average value of HMF for 30 samples from the territory of high Herzegovina ranged between 1,95 and 72,16 mg/kg. Comparing the results obtained in this study with the results of other authors, it can be concluded that they partially fit into the variations cited by other authors.

The content of copper (Cu), lead (Pb), cadmium (Cd), zinc (Zn), iron (Fe) and potassium (K) is given in Table 2.

In 8 samples the concentration of cooper is higher than prescribed by the Rulebook.[1] According to the Rulebook on the maximum permitted amounts for certain contaminants in food, the maximum permitted amount of copper in all types of honey is 2,0 mg/kg. Copper plays a key role in the absorption of Fe and is therefore important for erythrocyte formation. Cu deficiency causes anemia.

Lead was detected in one honey sample. The cause of the increased content of lead in honey can be grazing of bees in areas located near industrial zones or highways. According to the Rulebook, the maximum permitted amount of lead in all types of honey is 0,10 mg/kg Cadmium was not detected in any sample. According to the Rulebook, the maximum allowed amount of cadmium in honey is not defined. Although, cadmium is sometimes present in honey, but these concentrations are very low. It is believed that cadmium very toxic heavy metal with mutagenic and carcinogenic effects.

In this study, the zinc content ranged from 2,90 to 19,34 mg/kg. According to the Ordinance, the maximum permitted amount of zinc for honey is not defined.

High iron content was detected in two honey samples. According to the Rulebook, the maximum permitted amount of iron in all types of honey is 20 mg/kg.

Based on the obtained results, the macroelement potassium (K) was the most present in all honey samples and its value ranged from 277,85 to 1967,12 mg/kg. Potassium is identified as an essential element in honey, and it is also necessary for the growth of the plants.

1.6 Determining the Color of Honey

The results of color measurements with the Lovibond comparator are shown in Table 3. Honey color intensity values expressed in millimeters of Pfund.

The average amount of values obtained for individual areas is shown in Fig. 1. The average color of honey from the area of Konjic is 44,60 mm on the Pfund scale, which places this honey in the category of extra light amber color (35–50 mm Pfund). For honey from the area of Jablanica the average color value is 97,90 mm of the Pfund scale, which

[1] Rulebook on maximum permitted quantities for certain contaminants in food (Official Gazette of BiH, No. 68/14)

Table 2. Content of macroelements and heavy metals in honey

Sample	Cu (Mg/Kg)	Pb (Mg/Kg)	Cd (Mg/Kg)	Zn (Mg/Kg)	Fe (Mg/Kg)	K (Mg/Kg)
1.K	1,36	ND	ND	3,33	19,03	1754,07
2.K	1,70	ND	ND	7,17	19,48	1055,87
3.K	1,66	ND	ND	4,50	10,15	950,15
4.K	1,90	ND	ND	9,21	10,98	1331,05
5.K	1,74	ND	ND	5,47	10,69	1035,61
1.J	1,96	**2,16***	ND	19,34	**21,36***	1473,13
2.J	1,36	ND	ND	6,86	11,50	843,36
3.J	**2,28***	ND	ND	6,32	13,73	1967,12
4.J	1,96	ND	ND	4,00	14,65	1749,77
5.J	**2,20***	ND	ND	4,69	12,58	1637,34
1.P	**2,09***	ND	ND	8,95	14,93	1211,50
2.P	**2,42***	ND	ND	5,63	15,42	1527,19
3.P	1,27	ND	ND	5,07	19,22	277,85
4.P	**2,05***	ND	ND	5,31	16,10	488,36
5.P	1,80	ND	ND	3,89	8,82	1250,95
1.M	1,92	ND	ND	4,35	12,39	784,19
2.M	**2,09***	ND	ND	10,27	13,88	1528,80
3.M	**2,53***	ND	ND	4,39	**20,71***	1534,57
4.M	**2,00***	ND	ND	4,56	16,42	704,09
5.M	1,72	ND	ND	2,90	7,72	654,32

ND – The content of a certain heavy metal was not detected
* does not meet the values of the Rulebook (7)

places this honey in the category of amber color (86–114 mm Pfund). The area of Prozor and Bijelo polje have approximate mean color values which are 69,40 for Prozor and 79,10 mm Pfund for Bijelo polje, and therefore this honey is classified in the category of light amber color (51–85 mm Pfund).

The analysis of variance determined the average color value of the analyzed honey samples depending on the individual groups of samples. The ANOVA statistical test determined that there is no statistically significant difference between individual groups of honey - the significance is 0.064. Although there is no statistically significant difference, a Post Hoc analysis was performed. Using the Tukey HSD test, it was determined that only the samples from the Konjic area differed statistically significantly from the samples from the Jablanica area.

Table 3. Honey color intensity according to the Pfund color scale

Sample	mm Pfund	Color of honey
1.K	43	Extra light amber
2.K	54,50	Light amber
3.K	26	White
4.K	69	Light amber
5.K	30,50	White
1.J	81	Light amber
2.J	82	Light amber
3.J	80	Light amber
4.J	108,50	Amber
5.J	138	Dark amber
1.P	81	Light amber
2.P	94,50	Amber
3.P	70	Light amber
4.P	17	Extra white
5.P	84,50	Light amber
1.M	107,50	Amber
2.M	78	Light amber
3.M	126	Dark amber
4.M	45,50	Extra light amber
5.M	38,50	Extra light amber

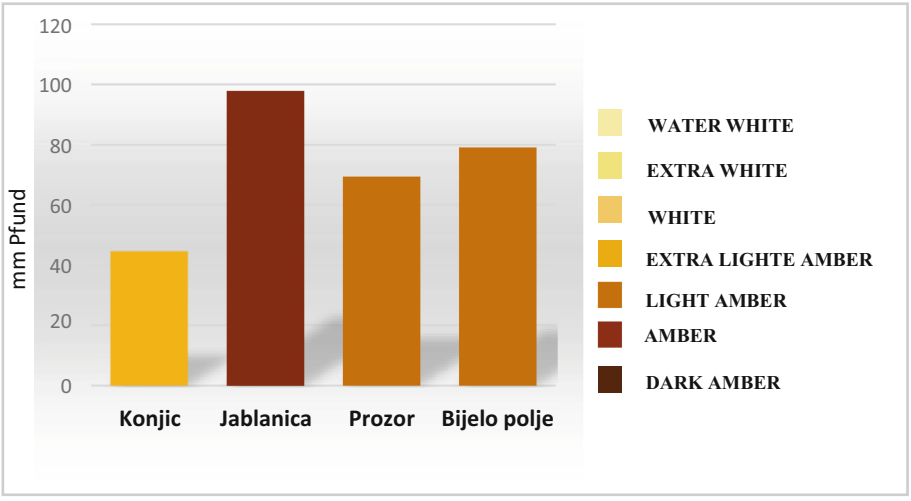

Fig. 1. Mean value of honey color for individual areas

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Artificial Neural Network Modeling of Marrow Slices (*Cucurbita Pepo Var. Giromontina*) by Convection and Combined Drying Methods

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Abstract. Drying is a complex thermal process that unfolds with simultaneous heat and mass transfer and involves a large number of variables. Thus, it is difficult to simulate the process and to predict the data related to raw materials or final products.

This paper is concerned with developing an artificial neural network (ANN) model for predicting the moisture content, moisture ratio and drying rate of convection and microwave combined drying of marrow chips.

To perform this study two drying methods were used: free convection (50, 60 and 70 °C) and a combined method consisting of first stage - hot air convection at 50, 60 and 70 °C, followed by hot air at 40 °C simultaneous with microwave at 210 W. The application of microwaves has shown a considerable reducing of drying time and drying rate and has improved the appearance of samples. Thus, the drying time of the combined drying method was reduced by 20–30% compared to the classical convection method.

Time, temperature and microwave power have been considered as the input variables to the topology of neural network. The output variables have been determined by moisture ratio and drying rate. A Multi-Layer Perceptron (MLP) network using BackPropagation (BP) learning algorithm with 5 respectively 6 neurons in hidden layer was used for modeling the data. The ANN architecture adopted by running 128 respectively 777 cycles allowed to establish important parameters in predicting the output data.

The program established insignificant errors (0.0098–0.0099), which indicates that the experimentally obtained data can be used successfully to simulate the drying process.

Keywords: Artificial Neural Networks · Mathematical modeling · Drying

1 Introduction

Drying is an old and important process in food industry involving heat applied to remove the water from vegetables to the level at which microbial spoilage reactions are greatly

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minimized [1, 2]. Drying is considered a simple and effective process of preservation, widely practiced [3].

Because the convection drying in hot-air is a process with high energy consumption, the increasing of the energy efficiency is indispensable. This could be reached by combining various drying techniques to solve the energy loss and to improve the quality of the products. One of the most common methods used to preserve the energy is microwave.

Microwave assisted drying is considered to be less harmful to the environment and humans. In present, microwave combined methods are used to improve the energetic efficiency of drying process by reducing the drying time and increasing the drying rate.

Drying is defined as a process of moisture removal due to simultaneous heat and mass transfer which depends on different factors such as: air temperature, power of microwave, air velocity, relative humidity of air, air flow rate, physical nature and initial moisture content of the drying material, exposed area and pressure [4, 5].

The knowledge of drying behavior is important in the design, simulation and optimization of drying process. The major objective of the researchers is to predict the final moisture content as a function of time and temperature. A way to predict the moisture content is to calculate it based on drying air parameters using physic models. Whereas drying process has a non-linear design, it is difficult to establish an accurate model for prolonged exposure, different temperatures or combined drying methods [6]. Similarly, it is difficult to define the moisture transfer in food products in mathematical terms [7, 8]. Empirical mathematical correlations usually give very accurate results for each specific experiment, but the equation is not valid for other conditions and there is no way to obtain a general equation for a range of drying parameters [5]. In this context, a great effort is being made within the drying area with the purpose of developing nonlinear models adapted to drying processes. A new set of methods has been developed recently, which applies Artificial Neural Networks to the tasks of modelling and prediction in drying systems. These works are supported by two of the most important capabilities of neural networks, namely the learning ability by optimizing an appropriate error function and the ability to approximate nonlinear functions [8].

Artificial Neural Networks (ANNs) have been the center of interest in various fields of science and technology. Artificial Neural Networks (ANNs) are an important tool in modeling, designing and controlling chemical or biochemical processes, because of its learning and generalization properties that confer it the full power of a self-organizing system.

Artificial Neural Networks (ANNs) are nonlinear statistical data models that replicate the role of biological NNs [9].

ANNs are able to learn both linear and highly nonlinear systems, and hence are capable of modeling very complex relationships as a dynamic response to external inputs. In addition, ANNs models can analyze multiple-input and multiple-output systems. Therefore, variability of multiple parameters in the development of an ANNs model is possible.

In drying technology, ANNs have been used for modeling, predicting, and optimization of heat and mass transfer, as well as quality indicators and physico-chemical properties of dried products [10, 11].

The present study aimed to perform, and model with ANNs the drying experiments (convection and combined method) used to obtain marrow chips. The drying kinetics in terms of moisture content and drying rate were observed.

2 Materials and Methods

2.1 Raw Material

A species of marrow *(Cucurbita pepo var. giromontina)* was used to carry out the research. Fresh marrows were purchased in July 2019 from a local supermarket in Galati, Romania and stored in a refrigerator at 6 ± 0.5 °C for 2 days. Prior to drying, vegetables were taken out of the refrigerator and prepared for experiments. A charge of 50.5 ± 0.2 g was used to be dried by once. So, the marrows were washed, manually peeled and cut into slices with 5 mm thickness and 18 mm diameter. The initial moisture content (M_0) of marrows was 84.65%. The equilibrium moisture content (M_e) which was reached after drying was 9.70%.

2.2 Drying Experiments

Two drying techniques have been studied. In order to identify the best drying method, convection drying method and combined microwave-convection drying method were studied. The drying studies were performed with a hybrid microwave oven (SHARP R-94ST Inventer Germany) in the laboratory of Unit Operations, Faculty of Food Science and Engineering Galati, Romania.

2.2.1 Convection Drying Method

For the study of the free convection drying process, the marrow chips were dried at 50, 60 and 70 °C. Marrow samples were dried from the initial moisture content (M_0) 84.65% to a final (equilibrium) moisture content of (M_e) 9.70%. Drying experiments were performed in triplicate for each drying temperature and the average of the values obtained was calculated. To determine the equilibrium moisture content, weightings were conducted at every 30 min. Abbreviations of dry samples are provided below: MS1 convection drying at 50 °C, MS2 - convection drying at 60 °C and MS3 convection drying at 70 °C

2.2.2 Combined Drying Method

A combined method consisting of first stage - hot air convection at 50, 60 and 70 °C, followed by hot air at 40 °C simultaneous with microwave at 210 W. For the combined microwave drying, the change in the samples' weight was recorded at intervals of 3 min.

Microwave combined drying method was carried out in a hybrid microwave oven with hot air convection and microwave functions (SHARP R-94ST Inventer Germany).

The encoding of dried samples for the combined methods is: MS I – convection drying at 50 °C/forced convection 40 °C + microwave at 210W, MS II - convection drying at 60 °C/forced convection 40 °C + microwave 210W and MS III – convection 70 °C/forced convection 40 °C + microwave 210W. A similar method was applied by Nistor et al. for drying of red beetroots [12].

2.3 Drying Kinetic

The moisture ratio (MR) and drying rate of marrow samples during drying experiments were calculated using the following equations [12]:

$$MR = \frac{M - M_e}{M_0 - M_e} \tag{1}$$

where: MR is the dimensionless moisture ratio; M, M_0 and M_e are the moisture content at any time, initial moisture content and equilibrium moisture content, respectively.

However, MR was simplified according to Akoy [1], Togrul [13], and Younis et al. [14] as

$$MR = \frac{M}{M_0} \tag{2}$$

The reduction of the moisture ratio with the drying time was used to analyze the experimental drying data. The moisture ratio (MR) represents the amount of moisture remaining in the marrow samples in relation to the initial moisture content.

$$DR = \frac{M_{t+dt} - M_t}{dt} \tag{3}$$

where, DR is drying rate, M_t, and M_{t+dt} are the moisture content at t and moisture content at t + dt (g water/g dry matter), respectively, t is drying time (min).

Several mathematical models have been proposed by researchers describing the moisture movement in various agriculture products like the exponential model, diffusion model, approximation of diffusion model and Lewis (Newton) equation model [15].

There are many methods to determine the drying parameters, of which the commonest is Fick's second law of diffusion (Eq. 4) [16, 17]. Fick's model of diffusion is often used in predicting the behavior of moisture removal.

$$\frac{\partial M}{\partial t} = -D_{eff}\frac{\partial^2 M}{\partial x^2} \tag{4}$$

Based on the assumptions of uniform initial moisture distribution, negligible external environment, negligible temperature gradients, insignificant shrinkage during drying and constant diffusion coefficient, the Eq. (4) can be solved using the analytical solution for cylindrical geometry, shown in Eq. (5):

$$\frac{(M - M_e)}{(M_0 - M_e)} = \frac{8}{\pi^2}\sum\nolimits_{n=0}^{\infty}\frac{1}{(2n+1)^2}exp\left(\frac{(2n+1)^2\pi^2 D_{eff}\,t}{L^2}\right) \tag{5}$$

where:

$8/\pi^2$ - the shape factor and depends on the geometry of the drying material ($4/\pi^2$ for a cylinder and $6/\pi^2$ for the sphere).

D_{eff} - the effective diffusivity, m^2s^{-1}.

L - half-thickness of slab, m

n - positive integer.

For long drying times, the Eq. (5) can be simplified as Eq. (6 and 7) by taking the first term of the series solution and expressed in a logarithmic form as follows [18]:

$$\frac{M - M_e}{M_0 - M_e} = \frac{8}{\pi^2} exp\left(-\frac{\pi^2 D_{eff}\, t}{L^2}\right) \tag{6}$$

$$\ln \mathrm{MR} = \ln \frac{8}{\pi^2} - \frac{\pi^2 D_{eff}\, t}{L^2} \tag{7}$$

Effective moisture diffusivity is actually calculated by using the slope of Eq. (6,7), namely, when the natural logarithm of MR versus time was plotted, straight line with a slope k and Eq. (8) was obtained:

$$\mathrm{k} = \frac{\pi^2 D_{eff}\, t}{L^2} \tag{8}$$

The effect of temperature on effective moisture diffusivity is expressed using an Arrhenius-type relationship, since temperature or microwave power have a significant effect over the drying process rather than the initial moisture content of the product. Using Arrhenius Equation, as a relationship between temperature and effective moisture diffusivity, the activation energy can be calculated using Eq. (9) for convection drying method and Eq. (10) for microwave combined method [17, 19]:

$$D_{eff} = D_0 exp\left(-\frac{E_a}{RT}\right) \tag{9}$$

$$D_u = D_0 exp\left(-\frac{E_a m}{P}\right) \tag{10}$$

in these equations, D_{eff} is effective moisture diffusivity ($m^2\ s^{-1}$), D_o is the pre-exponential factor of the Arrhenius equation ($m^2\ s^{-1}$), R is the universal gas constant (8.3143 J K^{-1}·mol^{-1}), E_a the activation energy (kJ/mol), T is the absolute air temperature in Kelvin [20], P is microwave output power (W) and m is the mass of raw sample (g) dried by combined method [16].

The activation energy expresses the effect of drying temperature on effective moisture diffusivity.

2.4 Modeling Drying Data

The drying curves were fitted to four well-known thin layer drying models that are widely used in most food and biological materials: namely, Newton (Lewis), Henderson and Pabis, and logarithmic models (Table 1). These models are generally derived by simplifying the general solution of Fick's second law. Henderson and Pabis model is the first term of a general series solution of Fick's second law [17].

The best model used to describe the drying characteristics of samples was chosen as the one with the highest coefficient of determination.

Table 1. Mathematical models used to describe for the approximation

Name	Mathematical model
Lewis	$MR = \exp(-kt)$
Henderson and Pabis	$MR = a \exp(-kt)$
Logarithmic	$MR = a \exp(-kt^n) + c$
Wang and Singh	$MR = 1 + at + bt2$

2.5 Rehydration Capacity

The rehydration capacity (RC) of dried marrow slices by convection and combined method were evaluated by immersing dried samples in a volume of 100 mL of distilled water at 24 °C for 180 min. The dynamics of the rehydration capacity was measured and calculated as the amount of water absorbed g per g of dry material at every 30 min. The rehydration capacity (RC) was calculated using Eq. (11), which is described as percentage water gain, and calculated from the samples weight difference before and after the rehydration [21].

$$RC = \frac{W_t - W_d}{W_d} * 100, \% \tag{11}$$

where RC is rehydration capacity, W_t is the weight of wet samples at any time and W_d is the initial weight of dry samples.

The experiments were performed in triplicates.

2.6 Statistical Analysis and Evaluation

A non-linear multiple regression analysis was performed using the drying mathematical models and the experimental data. The experimental data were interpreted by means of a non-linear regression and statistical analysis modeled with the CurveExpert Professional (CurveExpertPro 2.7.3., Hyams Development 2020, USA). It is a cross-platform solution for curve fitting and data analysis. Data were modelled using a toolbox of nonlinear regression models and smoothing methods. The drying models which are expressing the best experimental data were selected based on the sum of squares error (SSE), mean squared error (MSE), root mean squared error (RMSE), the coefficient of determination (R^2). Time series analyses are forecasted by including an automatic selection model procedure. Models include exponential smoothing, moving averages, random walks, linear and nonlinear trends. SSE is the sum of the squared differences between predicted data and actual empirical values of data. The mean squared error (MSE) represents the average of the squared difference between the original and predicted values in the data set. It measures the variance of the residuals. The root mean squared error is (RMSE) the standard deviation of the residuals (prediction errors). It measures the standard deviation of residuals.

2.7 ANN Modeling

The ANNs are a computational and intelligent model inspired by biological neurons system which is used to solve complicated problems in many applications such as optimization, prediction, modeling, clustering, simulation, and others (Fig. 1).

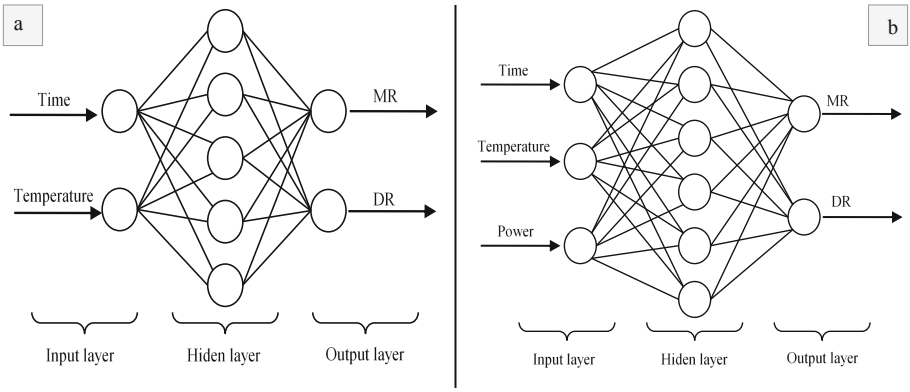

Fig. 1. ANN structure with one hidden layer a) convection drying method, b) combined drying method.

An Artificial Neural Network consists of interconnected identical simple processing units called neurons (perceptrons). A neuron is a basic processing unit of a neural network and performs two functions: the collecting of the inputs and producing of the outputs [22]. The ANN structure consists of three layers: the input layer, an output layer, and one or more hidden layers suitable to connect the input and output layer. This system forms a multilayer perceptron (MLP) structure. The multilayer perceptron has been considered as providing a nonlinear mapping between an input vector and a corresponding output vector. The neurons in the MLP are trained with the back propagation learning algorithm. MLPs are designed to approximate any continuous function and can solve problems which are not linearly separable. The major use cases of MLP are pattern classification, recognition, prediction and approximation [23, 24].

The main advantages of ANNs are non-linearity and complexity, short computing time and adaptive performance [25].

The Easy NN-plus 2017 program was used for modeling the drying data (moisture ratio and drying rate).

3 Results and Discussions

3.1 Drying Curves

Figure 2 shows the drying curves which represent the variation of moisture ratio with drying time for convection method (a) and combined method (b). Following experimental results show that the drying process takes place in two stages. A significant part (65.0 ± 5.0%) of the marrow moisture is lost during the first period (120, 150, 180 min,) of drying

process, which corresponds to a linear regression. In second period (240, 150, 90 min.) is needed much time to evaporate the remaining moisture to reach the equilibrium.

In both cases (convection drying and combined microwave drying methods), the moisture ratio (MR), decreases linearly with time, until the point of critical moisture content. The critical moisture content is the average material moisture content at which the drying rate begins to decline [26]. This is followed by a non-linear decrease of moisture ratio with t until, after a very long time, the solid reaches its equilibrium moisture content (M_e), and drying stops.

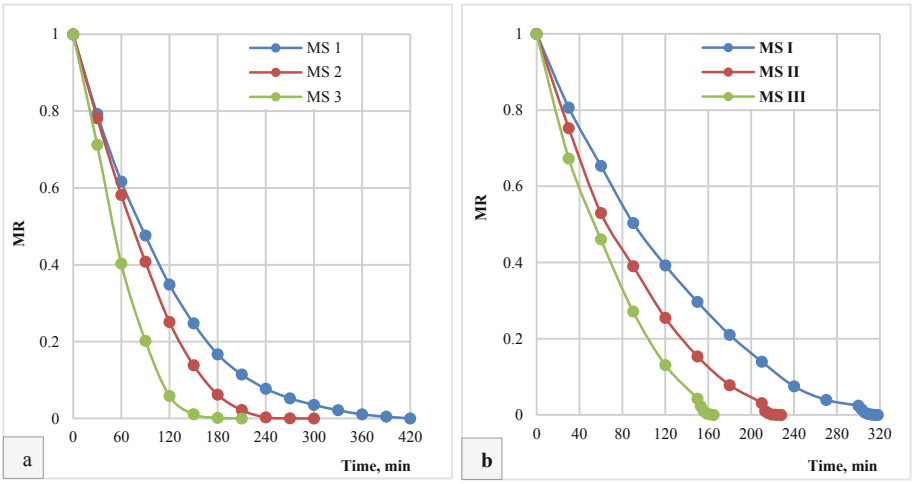

Fig. 2. Moisture content versus time: a) convection drying method, b) combined drying method.

By applying the microwave power of 210 W for reaching the point of critical moisture content (17.2 ± 3.5%), it was observed that the MR decreases much faster with 9.7 ± 2.1%, while the drying time is considerably reduced from 60–120 min to 15–18 min.

Removing water from the vegetable products subjected to the microwaves is based on the dipole character of the water molecule. The water molecules are oriented in the direction of the electric field that composes these waves [27]. The dipole orientation by changing the polarity of the field causes molecules friction between them, accompanied by release of heat, which is greater than the speed of the heat and mass transfer. Thus, the water molecules pass from the liquid state to the gaseous state, and the volume of the vapour formed does not pass through the semi-permeable membrane, forming an osmotic pressure, which leads to an increase in the volume of the product. At high pressure exceeding the mechanical strength of the tissues, they may be broken. So, drying through the microwave heating is effective in the field of the reduced values of relative humidity.

Similar data for dried mango fruits were reported by Akoy [1] and Mercer [28], Yoğurtçu [29] for zucchini and Zarein et al. [30] for apple slices.

The variation of drying rate with drying time are shown in Fig. 3. From drying rate curves, also can be seen that drying process takes place in two stages. In the first stage, drying rate reaches maximum levels (0.3428 – 0.4850 g water/g dry matter for convection method and 0.3755 – 0.4960 g water/g dry matter for combined method), because drying process happens at the surface of the marrow slices. After drying progresses, the second falling rate period starts inducing moisture diffusivity from parenchymal cells and their transportation to the surface.

The second stage drying process takes place with decreasing drying rate from 0.485–0.3428 g water/g dry matter to 0.0072–0.00217 g water/g dry matter. These results are in accordance with Darıcı and Şen for the kiwi drying process [31].

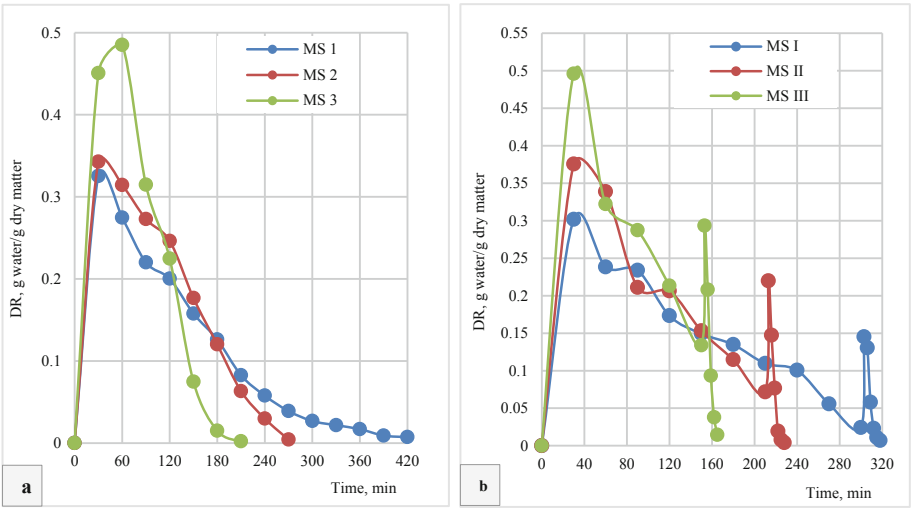

Fig. 3. Drying rate of marrow samples changes with drying time: (a) convection drying; (b) microwave combined method drying.

Drying rate decreases continuously with time and decreasing moisture content. A constant drying rate period was not detected in these drying experiments, and only one diffusional period was observed. The drying process of biological materials mostly occurs in the falling rate period: apple [13, 30], garlic [14], mango [1], celery leaves [16]. These findings could be explained by the rate of drying which is governed by the rate of internal movement of the moisture to the surface. During the drying falling rate period, mass transfer throughout the samples is considered to be controlled by vapor diffusion through the porous structure of marrow [32].

In the case of combined method, it could be observed two picks regarding the drying rate (first stage – 0.375–0.496 g water/g dry matter, second stage – 0.145–0.293 g water/g dry matter). The second stage is a consequence of the MW treatment which induces cellular walls disruption, thus intensifying the elimination of water from the product.

In combined drying method, it can be observed that in the second stage, when the microwaves are applied, the DR increases (up to 0.145–0.293 g water/g dry matter) followed by a linear decrease (until 0.014–0.004 g water/g dry matter).

Drying rate depends predominantly on the drying temperature or microwave power [33].

From the plots of moisture ratio (Fig. 2) and drying rate (Fig. 3) it was noticed that drying occurred predominately during the falling rate period for the marrow samples, commonly for most agricultural products: pumpkin [20], cocoyam slice [34] and soybeans [35].

3.2 Effective Moisture Diffusivity

The effective moisture diffusivity of marrow slices was described using the experimental drying data, by plotting experimental drying data in terms of *lnMR* versus drying time t in Eq. 7.

Depending on the drying temperature (50, 60, 70 °C), effective moisture diffusivities of marrow slices ranged from 3.3182×10^{-8} to 9.0429×10^{-8} ($m^2\ s^{-1}$) (Table 2).

Table 2. Effective moisture diffusivity and correlation coefficient at different temperatures for convection drying method.

Marrow samples	R^2	$D_{eff} \times 10^{-8}$ ($m^2\ s^{-1}$)
MS1	0.9743	3.3182
MS2	0.9188	4.9779
MS3	0.9193	9.0429

From Table 2, the results showed an increase in effective moisture diffusivity for marrow slices as drying temperature increased.

The moisture diffusivity depends on the intensity of thermal processing. An increased temperature leads to the decrease of water viscosity generated by water molecules activity. Therefore, the drying process is governed by thermal and humidity diffusion. This statement is also reported by Kutlu and Isci [18] for drying zucchini and Beigi [7] for convective drying of apple slices.

For combined drying method effective moisture diffusivities of marrow slices ranged from 3.0902×10^{-8} to 5.1167×10^{-8} $m^2\ s^{-1}$ in the first stage of drying process, and from 6.8847×10^{-7} to 7.8954×10^{-7} $m^2\ s^{-1}$ for the 210 W of microwave output power in the second stage (Table 3).

A similar behavior of moisture diffusivity, was observed in several studies performed on the drying of vegetable products: apple slices [30], dika nuts and kernels [36], Ginkgo Biloba Seeds [19].

Table 3. Effective moisture diffusivity and correlation coefficient at different temperatures for first and second falling rate periods for combined drying method.

Marrow samples	First stage		Second stage	
	R^2	$D_{eff1} \times 10^{-8}$ (m^2 s^{-1})	R^2	$D_{eff2} \times 10^{-7}$ (m^2 s^{-1})
MS I	0.9573	3.0902	0.9932	6.8847
MS II	0.9452	4.5341	0.9942	7.1727
MS III	0.9514	5.1167	0.9823	7.8954

The activation energy for convection dried samples was calculated at 46.37 kJ mol^{-1}. For microwave combined method, the activation energies were calculated as 27.41 W g^{-1} in first stage and 27.13 W g^{-1} s stage. Kutlu and Isci [18] for dried zucchini and Zarein et al. [30] for apple slices have reported comparable findings.

The activation energy of a drying process is closely related to its temperature and moisture diffusion. The higher value of the activation energy, the process will be slower. This is due to the fact that the activation energy is the one which overcomes before the proccess starts. Thus, activation energy can be reduced as the drying air temperature increases, or applying microwave power.

3.3 Modeling Drying Data

Different type of nonlinear regression equations was used to obtain each parameter value of every model (exponential, logarithmic, polynomial). The statistical results from models are summarized in Tables 4 and 5. In all cases, the statistical parameter estimations showed that R^2 and RMSE values ranged from 0.915 to 0.939, 0.066 to 0.093, respectively. Based on highest value of R^2, and lowest values of RMSE, it can be concluded that Logarithmic, Henderson and Pabis models gave better results than the other mathematical models.

Table 4. Non-linear regression analysis results for convection drying method

Mathematical model	Parameters value	SSE	MSE	RMSE	R2
Lewis $MR = \exp(-kt)$	a = −0.010	0.177	0.006	0.076	0.9351
Henderson and Pabis $MR = a \exp(-kt)$	a = 1.026 b = −0.011	0.175	0.006	0.076	0.9361
Logarithmic $MR = a \exp(-kt) + c$	a = 1.005 b = −0.012 c = 0.034	0.168	0.006	0.075	0.939
Wang and Singh $MR = 1 + at + bt^2$	a = −0.007 b = 0.001	0.257	0.009	0.093	0.9156

Table 5. Non-linear regression analysis results for microwave combined drying

Mathematical model	Parameters value	SSE	MSE	RMSE	R^2
Lewis $MR = \exp(-kt)$	a = −0.012	0.187	0.005	0.068	0.9372
Henderson and Pabis $MR = a \exp(-kt)$	a = 1.020 b = −0.010	0.184	0.005	0.069	0.9381
Logarithmic $MR = a \exp(-kt) + c$	a = 1.005 b = −0.012 c = 0.034	0.181	0.005	0.066	0.9390
Wang and Singh $MR = 1 + at + bt^2$	a = −0.005 b = 0.001	0.195	0.005	0.071	0.9365

The low values obtained for convection and microwave combined drying of SSE (0.181–0.195), MSE (0.005), and RMSE (0.066–0.071) involve high accuracy of a regression model. This means the model is closer to the experimental data. RMSE is positive, and a value of 0 would indicate a perfect fit to the data.

Residuals are a measure of how far from the regression line data points are; RMSE is a measure of how spread out these residuals are. The correlation coefficient registered values in the range of 0.936–0.939, which is sustained by the error values which are minimized to 0.009.

3.4 Rehydration Capacity

Rehydration of dried fruits and vegetables is a complex phenomenon affected by numerous factors such as: cellular structure, composition, drying technology and storage conditions. Thus, rehydration is an important property used for understanding the quality of dried material.

The rehydration capacity of marrow samples dried at different temperatures and combined method is shown in Fig. 4.

From the results presented in Fig. 4 can be observed that the rehydration capacity of marrow samples is decreased with increasing of the temperature (87.65% at 50 °C, 86.74% at 60 °C, 79.35% at 70 °C). Similar results have been obtained for microwave combined method – 84.98% for MS I, 85.22% for MS II, 78.46% for MS III). The lower rehydration values 79.35% and 78.46%, are evidenced at the highest drying temperature (70 °C) and in combination with microwave power (210 W) respectively. The explanation of this status is represented by the fact that the high temperature and MW treatment are breaking the cellular walls, which induces the prevention of water retention.

The results are in accordance with a few similar studies regarding convection drying and microwave assisted air drying of hawthorn fruit [37], zucchini [18] and sour cherries [21].

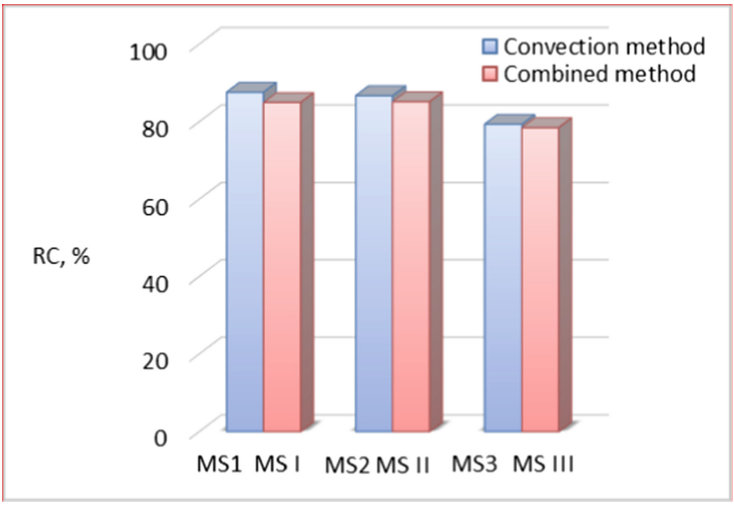

Fig. 4. Rehydration capacity of dried marrow chip by convection and combined method

ANN Modeling

The drying data were modeled using ANNs. The experimental and predicted drying parameters for the optimal ANN topology were shown in Fig. 5 and 6. For convection drying process, the network consists of two input layers (drying time and temperature), two output layers (MR and DR) and five hidden layers (it is the number which gives the best prediction results). At each node in a layer, the information is received, stored, processed, and communicated further to nodes in the next layer.

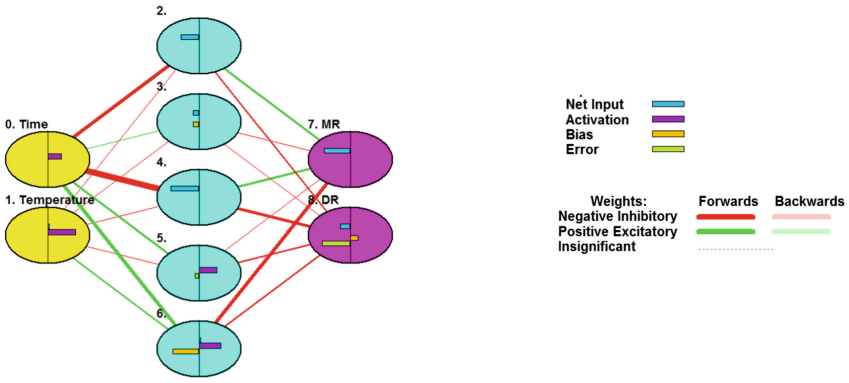

Fig. 5. Architecture of ANNs for convection drying method.

The network for microwave combined drying method consists of three input layers (drying time, microwave power and temperature), two output layers (MR and DR) and six hidden layers.

The learning procedure in this network was implemented by using the back-propagation algorithm. In back-propagation networks, the number of hidden neurons determines which is the best way to learn a problem. If too many hidden neurons are used, the network will tend to memorize the problem, and thus not generalize well later But, if there only a few neurons are used, will be reduced the convergence rate of the network. So, the right numbers of hidden neurons were determined by trial and error [38, 39]. Thus, after several attempts, the optimal number of five hidden nodes for convection method and six hidden nodes for combined method were set.

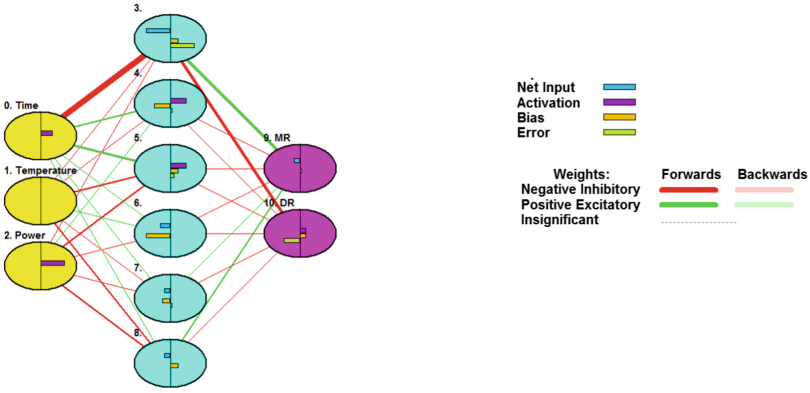

Fig. 6. Architecture of ANNs for combined drying method

The selection of a suitable number of hidden neurons, scale, activation function, learning rate, momentum coefficient and initial weights is very important to obtain a satisfactory prediction. Operation parameters values obtained for the network is given in Table 6 Predictive ability of the ANNs for drying parameters of marrow samples were found to be appropriate with the empirical results, as can be seen from the Fig. 7. Such results were also obtained by Zarein and Jaliliantabar [39], used ANN modeling of white mulberry drying by microwave and Özdemir et al. [40] for kiwi-fruit.

There was investigated the sensitivity of the drying process to the inputs and find the most dominant parameters of different drying methods. The most sensitive parameter is the time.

The drying time process is identified as the most important factor in the choosing of drying method, followed by air temperature and/or microwave power.

Training a neural network is the process of finding values for the weights and biases so that for a given set of input values, the computed output values closely match the known, correct, target values. To predict the outputs, a number of 128 learning cycles, respectively 777 were applied for convection and combined drying methods. Neural network momentum is a simple technique that often improves both training speed and accuracy. The momentum factor value could influence the final quality of the prediction. The momentum factor in practice is chosen between 0.1 and 0.8. The value of the momentum (0.806) indicates the optimum outputs values, which is attached to a proper learning rate. An optimum momentum also helps in smoothing out the variations, if the gradient keeps changing direction. A right value of momentum can be either learned by hit and trial.

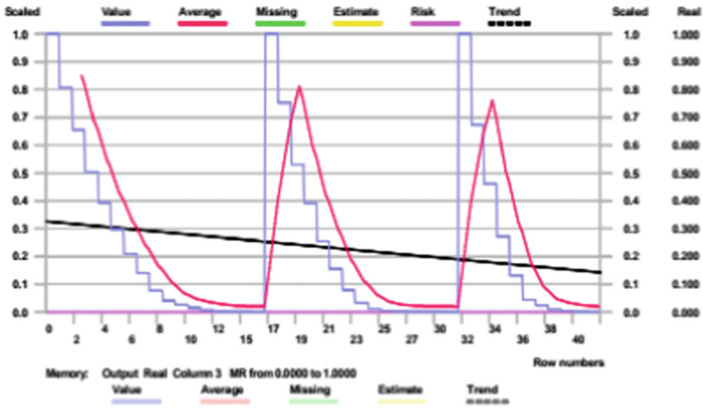

Fig. 7. ANN modeling of MR for combined drying of marrow samples.

Table 6. Configurations of Artificial Neural Network model

ANN parameters	Convection drying method	Combined drying method
Learning cycles	128	777
Learning rate	0.65012513	0.65012513
Weights	10	18
Momentum	0.80635853	0.80635853
Accelerator	1.00000000	1.00000000
Max Training error	0.09440771	0.10546582
Ave. Training error	0.00983808	0.00999401
Min. Training error	0.0000002	0.00000249
Target error	0.01000000	0.01000000
Training examples	34	43

4 Conclusions

Nowadays consumers demand of high-quality processed food led to the need to optimize the report between quality assurance and costs. In conclusion, several technical solutions and operation procedures should be adapted to the novel necessities to increase simultaneously the products' quality and to reduce the energy consumption. Vegetables drying seems to be an extremely important process, that could influence the consumers acceptability and commercial success.

The present findings determined that the drying temperature, time and respectively the microwave power, all had an effect on the moisture content and the drying rate. The analysis of moisture ratio, drying rate and shrinkage of the marrow chips showed that the most promising method is combined drying, which ensures high quality at the lowest possible energy consumption.

ANNs modeling was applied to predict the moisture ration and drying rate values. The results showed that the ANNs are appropriate to model the convection and microwave combined drying experiments. ANNs topology had the capability to predict the network outputs with an insignificant error.

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Determination of Chloride Content in Baby Food

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Abstract. Infant formula based on milk is the best alternative to breast milk. The development and improvement of infant formula aims to achieve the quantitative and qualitative characteristics of breast milk. However, the fact is that breast milk is irreplaceable, and it is therefore recommended that infant formula be used exclusively if there is no possibility for breast-feeding. One of the essential ingredients that is a necessary component of the infant formula is the chloride ion. Insufficient amounts of chloride in baby's diet can cause poor muscle control, delayed speech and slow growth. Today there are several different types of infant formula with different chloride content. If baby is fed by baby food, there is practically no other ways to have enough chlorides in a diet. Since baby food contains all ingredients that are essential to life, it is very complex, and determination of chemical parameters including chloride content is rather hard. Due to the problem of determining the content of chloride in baby food, the aim of this study was to examine the possibility of determining chloride in baby food samples by means of automatic potentiometric titration at several temperatures, using an ion selective electrode (ISE) and classical Mohr's titration. Apart from measuring the chloride content, the moisture content (by two methods) and water activity were also determined. The results were statistically evaluated by means of statistical techniques, one-way ANOVAs and Tukey tests. The accuracy and precision were calculated. The most precise method was ISE. The study found that the automatic titration method (room temperature) showed the most accurate results.

Keywords: Baby food · Chloride · Automatic titration · Potentiometric · ISE · Mohr

1 Introduction

Chlorine is an essential element that the body needs for many critical functions. It is present in the blood as a negatively charged ion and is the dominant anion in blood accounting for 70% of the total anion content. It is the main extracellular anion, therefore, it is found mainly in the fluid around the cells, and its main role is to regulate the fluid movement in the blood vessels and to maintain normal kidney function [1]. In addition, chlorides help reduce excess acid in the body. When the pH balance is disturbed, which is the case when too many foods that increase acidity are taken into the body, the body tries to neutralize this situation as quickly as possible. Chlorides act as neutralizers helping

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regulate the pH balance in the body [2]. In addition to regulating the pH balance, chlorides help transport CO_2, a waste product of respiration from the body, and transport electrical impulses throughout the body [3]. In the stomach, chlorides react with hydrogen to form hydrochloric acid, which is a necessary component in the process of digestion. It helps break down food so that nutrients are absorbed in the small intestine.

The chloride ion is a very important nutrient for the growth and development of infants. Children consuming baby food in the early stages of development have no other significant source of chloride. Therefore, the chloride ion is one of the very essential ingredients of baby food. The safe and adequate daily intake of chlorides for babies is 0.3 g/day [4]. Infants with low chloride content in the body are found to suffer from failure to thrive, constipation, food refusal, muscular weakness, and delayed psychomotor development associated with metabolic alkalosis [5]. Chlorides that are most often included in infant formulas based on milk are: sodium chloride, magnesium chloride, potassium chloride and calcium chloride.

Some compounds that contain chlorine, but are not salts, are also called chlorides. Such a compound is choline chloride with a chemical formula $C_5H_{14}ClNO$. Choline chloride is a loose powder that contains 60% of the active substance, and generally qualifies as a member of vitamin B group, more precisely, as vitamin B4 [6].

Baby food is very complex in Its composition [7] which is why it is rather hard to determine the amounts of ingredients in a correct and precise manner, especially if utilizing low cost equipment and chemicals. Several methods can be used to measure the contents of chlorides in food samples. First, and well known, are classical [8] and automated methods of titration [9] using precipitation and potentiometric end point determination. Aside from titrations, an ion selective chloride electrode can also be used [10]. In addition to these rather inexpensive methods, there are several methods that use expensive instrumentation such as ion chromatography [11], or spectrophotometry [12].

It is the need to have the content of chloride in baby food analysed precisely, accurately and with low cost that gave rise to the aim of this study: to find the best method for chloride determination in baby food samples, not requiring the use of expensive equipment and chemicals.

2 Materials and Methods

The material used for the purposes of this paper was infant formula based on milk from three different manufacturers. It was, for the sake of the experiment, named: Baby food 1, 2 and 3 (Table 1). The baby food used in the research is classified as water soluble baby formula. The baby food samples used were for infants 6–12 months of age. After purchase, the formulas were kept sealed at room temperature until the start of laboratory analysis.

The baby food samples basic composition is shown in Table 1.

2.1 Determination of Water Content

It is important to have the water content measured in order to obtain the results closer to the real value and to present the amount of chlorides in baby food formula based on

Table 1. Basic components of baby food samples per 100 mL of prepared feed.

Sample	Fat	Carbohydrate	Protein	Salt	Chloride
Baby food 1	3.4 g	7.0 g	1.5 g	0.07 g	47 mg
Baby food 2	3.3 g	8.6 g	1.5 g	0.05 g	54 mg
Baby food 3	3.6 g	7.7 g	1.7 g	0.05 g	45 mg

dry matter. For the purposes of water content determination, we used a classical heating oven and an IR heater.

Determination of Water Content by Classical Oven Method. Determination of water content was performed using the Memmert UN110 oven. The weighting glass dish was dried for 30 min. in the oven set at 105 °C, then cooled in a desiccator to room temperature. Then, 2 g to 4 g of the test sample was transferred into the prepared vessel weighed with the contents to the nearest 0.001 g. The samples were heated for 2 h in the oven set at 105 °C. The vessel and its contents were cooled for 15–20 min, and then weighed. The heating, cooling and measurement operations were repeated until the results of two consecutive measurements separated by 1-h heating time differed by less than 0.1% by weight of a portion of the test sample.

Determination of Water Content with an IR Dryer. In order to compare the results, the moisture was also measured using a HC103 Mettler Toledo moisture meter. This determination is based on the principle of infra-red drying of the sample and measurement of mass loss. No preparation of either the container or the samples is required for this moisture measuring method. The sample was placed on an aluminum plate located in the instrument, after which the instrument was closed. The drying temperature was 101ºC $\pm$ 3. The result, expressed as a percentage, was read on the instrument display after a few minutes, and recorded.

2.2 Determination of Water Activity

Since water activity (aw) in foods is influenced by water content and NaCl content (chloride) [13] this parameter was also measured. The aw value of the baby food was measured using a Rotronic Hygrolab 3 aw-meter. When measuring the aw value, the standard Ca $(NO_3)_2 \times 2H_2O$ with aw value at 0.560 was used. As the samples were in powder form, no additional sample preparation was required. The samples were placed in plastic chambers which were then placed in the recess of the aw-meter. After the sound signal, we recorded the measured aw values shown on the aw-meter display. We performed three independent measurements for each sample, on the basis of which a mean value was calculated.

2.3 Determination of Chloride Content

Three different methods were applied for this purpose: Mohr's method, automatic titration and ion selective electrode (ISE):

Classical Titration (Mohr's Method). 2 g of the sample was weighed into a beaker and then 100 ml of boiling distilled water was added. The solution was heated for about 10 min and then cooled, after which 2 ml of 5% K_2CrO_4 was added and titrated with 0.1 M $AgNO_3$ until a stable red-orange colour appeared.

Automatic Titration. For determination of chlorides in baby food by means of automatic titration [14], a G20 Mettler Toledo instrument was used. Before starting the titration, the desired titrant and its concentration were adjusted, in this case 0.01 mol/L of $AgNO_3$. In addition, the mixing speed (stirrer) was adjusted to 30%. The forced termination of the titration was defined (if the endpoint of titration is difficult to achieve) and this is usually at a 30 mL titrant consumption. Prior to the analysis, the sample was prepared as follows: the amount of baby food contained in 6 spoons was weighed and transferred into a beaker which was then filled with 180 mL of distilled water. This was stirred for 5 min at 700 rpm. After adjusting the instrument, we pipetted 15 mL of HNO_3 1:49 and 15 mL of prepared food into the sample holder. The concentrations of chloride ions were determined by automatic titration in samples at different temperatures: 24 °C–26 °C (room temperature), 30 °C, 50 °C and 70 °C.

Ion Selective Chloride Electrode (ISE). For determining the chloride content with ISE, an Mettler Toledo chloride electrode was used. (EPA, 1996). Four standards, 5, 10, 20 and 40 ppm, were prepared. From a stock solution of 1000 ppm Cl, these amounts were taken in a 50 ml volumetric vessel and then 3 ml of 5 mol/dm^3 $NaNO_3$ was added and supplemented with 0.05 mol/dm^3 HNO_3. Before determining the chloride in the standard solutions, the blank value was first measured which was later subtracted from the standard and the sample values. 5 g ($\pm$0.0001 g) of baby food was weighed on the analytical balance, followed by addition of 100 mL of HNO_3 (0.1 mol/L) and 6 ml of ISA solution ($NaNO_3$ 5 mol/L). The solution was placed onto magnetic stirrer at 300 rpm until read.

2.4 Statistical Evaluation

Due to this fact, it is assumed SPSS statistical program, version 20.0 (SPSS Inc., Chicago, IL, USA), by way of the following analyses:

Descriptive statistics (arithmetic mean or average, standard deviation).

One-way ANOVA: When the p-value was less than 0.05, the difference between the compared groups was considered statistically significant. Otherwise, the differences between the compared groups were not considered statistically significant.

Tukey test: The differences between the mean values are separated by the Tukey test. When the p-value was less than 0.05, the difference between the compared groups was considered statistically significant.

Accuracy, precision and trueness of the applied methods: Accuracy is defined as closeness of agreement between a quantity value obtained by measurement and the true value (JCGM, 2004). Precision is the closeness of agreement between independent test results obtained under stipulated conditions (ISO, 1994). The closeness of agreement between the average value obtained from a large series of test results and an accepted reference is defined as trueness [18].

3 Results and Discussion

3.1 Determination of Basic Quality Parameters

The results of measurements of moisture and water activity content are shown in Table 2. All results are presented as value ± standard deviation.

Table 2. Results of moisture and water activity measurement

		a_w	Moisture, % (drying oven)	Moisture, % (IR dryer)
Baby food 1	1.	0.135 ± 0.009	2.53 ± 0.006	2.06 ± 0.163
	2.	0.124 ± 0.009	2.53 ± 0.006	2.17 ± 0.163
	3.	0.118 ± 0.009	2.54 ± 0.006	2.38 ± 0.163
Mean		**0.126 ± 0.009^{b*}**	**2.53 ± 0.006^{b}**	**2.20 ± 0.163^{b}**
Baby food 2	1.	0.135 ± 0.003	2.44 ± 0.124	2.05 ± 0.111
	2.	0.135 ± 0.003	2.22 ± 0.124	1.83 ± 0.111
	3.	0.140 ± 0.003	2.43 ± 0.124	1.97 ± 0.111
Mean		**0.137 ± 0.003^{b}**	**2.36 ± 0.124^{b}**	**1.95 ± 0.111^{b}**
Baby food 3	1.	0.298 ± 0.002	4.62 ± 0.021	3.42 ± 0.021
	2.	0.299 ± 0.002	4.63 ± 0.021	3.39 ± 0.021
	3.	0.302 ± 0.002	4.59 ± 0.021	± 0.021
Mean		**0.300 ± 0.002^{a}**	**4.61 ± 0.021^{a}**	**3.41 ± 0.021^{a}**

*Significant differences between results are presented with different small letters in superscript.

Moisture content was determined by two methods**:** a classical oven and an IR dryer. The average moisture content obtained by the classical drying method for the three types of baby food was 3.17%. Results obtained by the IR for baby food 3 had the highest moisture content, where the average moisture content was 4.61%. The average moisture content in baby food 2 was 2.36%, and in baby food 1 2.54%. The obtained results indicate that the percentage of moisture in baby food 3 is significantly higher than in the other two types of food. Analysis of variance showed that there were statistically significant differences ($p < 0.05$) in the percentage of moisture measured by the classical oven method. The Tukey test showed that the moisture percentage in baby food 3 was statistically significantly ($p < 0.05$) different from the moisture percentage in baby food 1 and 2, and that the moisture percentage in baby food 1 and baby food 2 did not differ significantly ($p > 0.05$). The average moisture content obtained by the method of drying in the oven for the three types of baby food was 3.17%, while the average moisture content obtained by an IR dryer measurement for the three types of food was 2.54%. According to Regulation 80/04 (2004), the moisture content of dehydrated baby food must not exceed 7%. The product declarations do not state the moisture content. The results of moisture content obtained by an IR dryer are in every case lower than those obtained by a classical oven measurement. The reason for this is that the measuring of

moisture on an IR dryer took only a few minutes, as opposed to a drying to constant mass method which lasted about 10 h. Due to this fact it is assumed that not all the moisture was able to come out of the samples when measured on the hygrometer. Nevertheless, moisture measurement with a hygrometer proved to be quite good, especially due to the measurement speed. The average aw value of the three types of milk-based baby food was 0.19. The highest aw value was measured in baby food 3 samples with a mean value of 0.30. The other two samples had significantly lower aw values: baby food 2–0.14 and baby food 1–0.13. The measurement of the aw value by the aw-meter was very accurate, since the deviations were very small during three consecutive measurements.

Determination of Chloride Content

The amounts of chlorides reported by means of several different methods are shown in Table 3. The results are presented as average values from three replicates ± STD.

Table 3. Results of chloride content measurements by different methods of analysis

	Sample/probe	Classical titration mg/100 mL	Automatic titration, mg/100 mL				ISE mg/100 mL
			24 °C	30 °C	50 °C	70 °C	
Baby food 1	1.	62.20 ± 4.32	48.06 ± 1.89	49.73 ± 0.51	50.97 ± 0.41	42.35 ± 1.07	55.36 ± 2.15
	2.	56.43 ± 4.32	45.96 ± 1.89	48.81 ± 0.51	50.26 ± 0.41	42.07 ± 1.07	57.99 ± 2.15
	3.	53.48 ± 4.32	49.64 ± 1.89	48.83 ± 0.51	50.25 ± 0.41	44.10 ± 1.07	59.74 ± 2.15
Mean		**57.37 ± 4.32^{b}**	**47.89 ± 1.89^{b}**	**49.12 ± 0.51^{b}**	**50.49 ± 0.41^{b}**	**42.84 ± 1.07^{b}**	**57.70 ± 2.15^{b}**
Baby food 2	1.	63.69 ± 1.74	57.83 ± 2.63	56.59 ± 0.25	58.24 ± 1.68	47.44 ± 2.19	67.08 ± 0.91
	2.	65.92 ± 1.74	53.44 ± 2.63	56.37 ± 0.25	58.06 ± 1.68	46.24 ± 2.19	67.45 ± 0.91
	3.	62.40 ± 1.74	52.94 ± 2.63	56.88 ± 0.25	61.12 ± 1.68	50.58 ± 2.19	68.85 ± 0.91
Mean		**64.00 ± 1.74^{b}**	**54.74 ± 2.63^{a}**	**56.61 ± 0.25^{a}**	**59.14 ± 1.68^{a}**	**48.09 ± 2.19^{a}**	**67.79 ± 0.91^{a}**
Baby food 3	1.	46.03 ± 2.90	39.53 ± 1.48	37.18 ± 1.41	38.30 ± 1.20	34.88 ± 1.90	51.92 ± 1.17
	2.	51.27 ± 2.90	39.12 ± 1.48	40.13 ± 1.41	39.01 ± 1.20	33.78 ± 1.90	51.31 ± 1.17
	3.	51.32 ± 2.90	36.67 ± 1.48	38.56 ± 1.41	40.75 ± 1.20	37.65 ± 1.90	53.67 ± 1.17
Mean		**49.54 ± 2.90^{a}**	**38.44 ± 1.48^{c}**	**38.62 ± 1.41^{c}**	**39.35 ± 1.20^{c}**	**35.44 ± 1.90^{c}**	**52.30 ± 1.17^{c}**

Significant differences between the results are presented with different small letters in superscript.

The results for average chloride content determined by means of classical titration are graphically shown in Fig. 1.

As we can see from Fig. 1, the highest concentration of chloride ions was found in baby food 2 samples, with an average value of 62.49 mg/100 m. They are followed by samples of baby food 1 containing 55.91 mg of chloride per 100 ml of baby food. Baby food 3 samples had the lowest chloride concentration, 47.26 mg/100 ml. The average concentration of chloride ions obtained by the classical titration method for the three types of food was 55.22 mg/100 ml. When we compare these values with the values presented in the product declaration, we can notice that the values obtained by classical titration are much higher than the declared values. The high difference between the values determined by way of classical titration and declared values is caused by high titration error, since the indicator color change is difficult to be seen. Analysis of variance

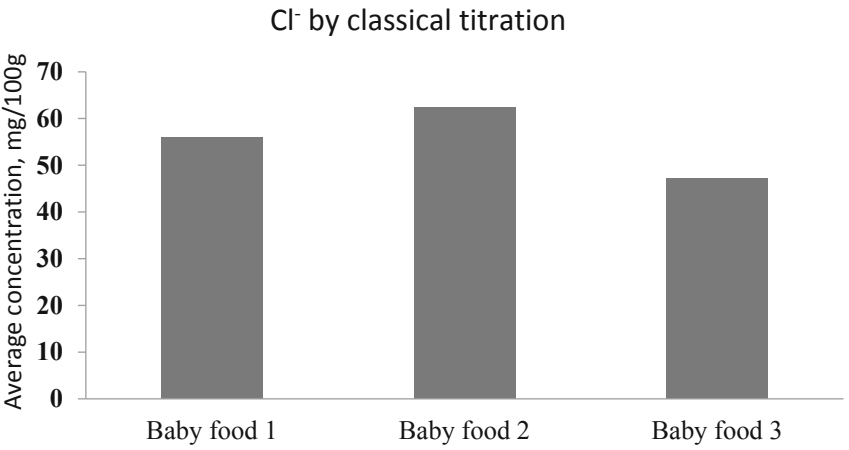

Fig. 1. Chlorides determined by classical titration

showed that there were statistically significant differences ($p < 0.05$) in the concentration of chloride ions of the tested samples. Tukey test results showed that there are statistically significant differences ($p < 0.05$) in the concentration of chloride ions between baby food 1 and baby food 3, as well as between baby food 2 and baby food 3 samples. There were no statistically significant differences ($p > 0.05$) in chloride concentration between baby food 1 and 2. The reason for differences between baby food 3 and other two samples could be in the fact that baby food 3 showed different characteristics than other baby food samples: baby food 3 had much higher moisture content, much higher water activity and much lower chloride content. The baby food 3 is an organic baby food. It is also likely that baby food 3 has some other characteristics different from the other samples.

Determination of Chloride Content by Means of Automatic Titration

Results of an average chloride content measured by means of automatic titration with a chloride electrode and at several different temperatures are shown on Fig. 2.

Figure 2. shows the average concentrations of chloride ions at temperatures of 24 °C, 30 °C, 50 °C and 70 °C for the three types of food. The highest concentration was found in the samples of baby food 2 at a temperature of 50 °C (57.75 mg/100 ml), and the lowest in the samples of baby food 3 at 70 °C (33.80 mg/100 ml). When looking at Fig. 2, we can notice that the concentration of chloride ions in all three types of food increased as the temperature increased. We can further notice that the highest concentration of chloride ions in all three types of food was at a temperature of 50 °C, after which the chloride concentration decreased significantly (at 70 °C). At the highest temperature (70 °C), the lowest concentrations of chloride were recorded in all three types of food. Therefore, we can say that high temperatures interfered with the contents of chloride determined by automatic titration method. Analysis of variance showed that there is a statistically significant ($p < 0.05$) influence of food type and temperature on the concentration of chloride both individually and jointly. The Tukey test showed that there were statistically significant differences ($p < 0.05$) between chloride ion concentrations at 24 °C and 50 °C,

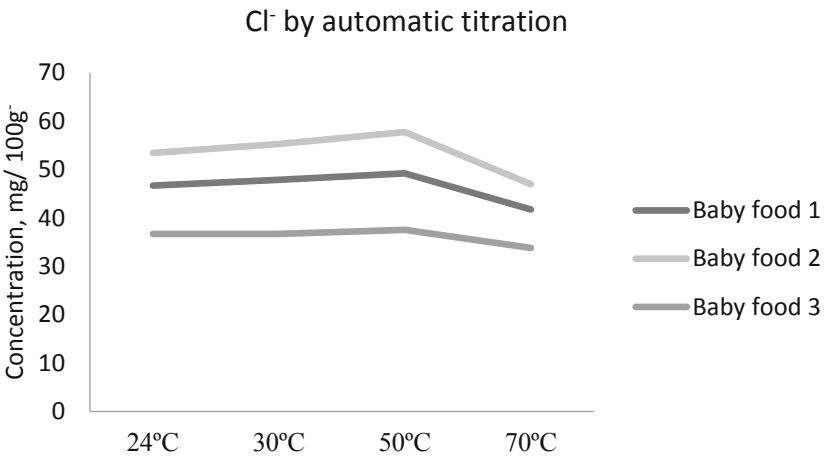

Fig. 2. Chlorides determined by automatic titration

while the difference between Cl^- concentrations at 24 °C and 30° was not statistically significant ($p > 0.05$). The Tukey test also showed that the difference between chloride ion concentrations at 30 °C and 50 °C was not statistically significant, but the difference in Cl^- concentrations at 30 °C and 70 °C was statistically significant ($p < 0.05$) as well as the difference in Cl^- concentrations at 50 °C and 70 °C. As we can see, the concentrations of Cl^- at 70 °C were statistically significantly different from concentrations at all other temperatures. The Tukey test further showed that there were statistically significant differences ($p < 0.05$) in the concentration of chloride ions between all three types of food.

Determination of Chloride Content by Means of ISE

Figure 3 below shows the results of average concentration of chloride determined by means of ion selective chloride electrode.

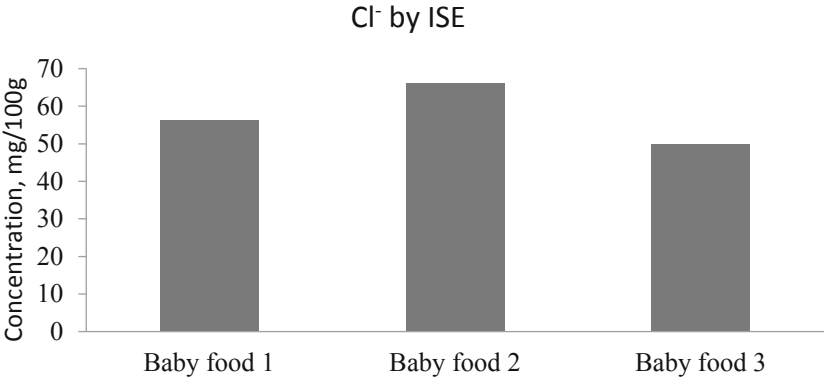

Fig. 3. Amount of chlorides determined by ion selective chloride electrode

Figure 3 presents the concentrations of chloride ions in the samples of the three types of food. Chloride ion concentrations were obtained using an ion selective chloride electrode. From the Fig. 3 we can see that this method resulted in the highest concentration of chloride ions in baby food 2 samples (66.20 mg/100 ml), then baby food 1 samples (56.23 mg/100 ml), while the lowest concentration of Cl^- was found in the samples of baby food 3. Analysis of variance showed that there were statistically significant differences ($p < 0.05$) in the concentration of chloride ions in the tested samples. The results of the Tukey test show that there are statistically significant differences ($p < 0.05$) in the concentration of chloride ions between all three types of the tested samples.

3.2 Accuracy of Methods - Comparison with Declared (True) Value

The concentration of chloride ions in baby food was determined using three methods in order to compare the results and see which of these methods shows the closest results to those declared on the product label. Table 4 below shows both the declared values and the results of chloride content per 100 mL of prepared feed obtained by all three of the methods, presented as mean values.

Table 4. Chloride content label values and average values of chloride in mg/100 mL of prepared feed obtained by three different methods

	Label values	Classical titration	Automatic titration				ISE
			24 °C	30 °C	50 °C	70 °C	
Baby food 1	47	55.91	46.67	47.88	49.21	41.75	56.23
Baby food 2	54	62.49	53.45	55.28	57.75	46.95	66.20
Baby food 3	45	47.26	36.67	36.84	37.54	33.80	49.89

The closest value to the one on the product declaration was observed in baby food 1, which was 46.67 mg/100 ml, while the declared value was 47, the difference being only 0.33 mg. Baby food 2 samples had an average of 53.45 mg/100 ml of chloride ions, which is 0.55 mg less than the concentration displayed on the label (54 mg/100 ml). Thus, determination of chloride in the samples of baby food 1 and 2 by automatic titration at 24 °C resulted in very small deviations of Cl^- concentration from the values listed in the product label. When it comes to baby food 3 samples, as seen from the Table 4, the chloride concentrations obtained by automatic titration are significantly lower (for about 10 mg) than the values on the label, although all of the food types tested have similar values declared on the product label. Baby food 3 shows lower chloride content than the other samples in all of the applied methods. The reason for the low value of chloride content in baby food 3 could be the lower solubility of certain compounds that contain chlorides, or the fact that baby food 3 is organic in origin, and chlorides may be hard to be extracted in a reasonable amount of time.

When it comes to the results obtained by the ion selective chloride electrode, we can see from Table 4 that these values are the highest in all three types of food as compared

to the ones obtained by the other two methods. The reason for this is probably a very large number of ions from the solution, especially monovalent anions (NO_3^- from ISA solution), which interfered with the adequate reading of the chloride concentration. The concentrations obtained by this method were higher than the concentrations displayed on the declaration, namely: by 9.23 mg in baby food 1, by 12.2 mg in baby food 2 and by 4.89 mg in baby food 3 samples.

The calculated accuracy of methods applied is shown in Table 5.

Table 5. Accuracy of the methods applied.

		Classical titration	Automatic titration				ISE
			24 °C	30 °C	50 °C	70 °C	
Baby food 1	% Error	18.95	**−0.70**[a]	**1.87**	**4.70**	11.17	19.63
Baby food 2		15.72	**−1.02**	**2.31**	**6.94**	13.05	22.59
Baby food 3		**5.02**	−18.51	−18.18	−16.57	−24.89	10.86

[a]The errors below 10% are bolded

The lower the error, the more accurate the method is. It is hard to say what percentage of error is too high for certain method, but the most often found rule is 10%.

The most accurate method for determination of chloride content in infant formula, as seen from Table 5, is automatic titration at temperatures from 24–50 °C, with almost zero error found in the case of automatic titration at room temperature. The highest error was seen with ion selective electrode method. This very high error is explained by the fact that complex matrix of baby foods interferes with the electrode.

3.3 Precision of the Methods Applied

Standard deviations with all three of the methods within each type of food were quite high (Table 3). The highest standard deviation was observed with baby food 3 samples. Based on the standard deviation, we can say that the most precise method in case of baby food 1 is automatic titration at all applied temperatures, while in case of baby food 2, at 30 °C. The highest deviations from mean value, as much as 4.32, was observed with the method of classical titration, making this method the least precise in this respect. The reason for this is probably inadequate recognition of the end point of the titration i.e., the first colour change of the system. Transition from orange to red colour is hard to discern close to the end point of titration, so titration error is always higher than it should be. Determination of end point is therefore much better to be made with potential change.

4 Conclusions

In most cases, automatic titration proved to be the most precise method because it showed the smallest deviations from the mean value (in case of baby food 1 at all applied temperatures). Furthermore, the automatic titration method showed the most

accurate results, i.e., the concentrations obtained by this method were almost identical to the concentrations of chloride stated on the product declaration of baby food 1 and 2. The calculated error for determination of chloride in baby food was the smallest (-0.70) at room temperature. For this reason, we recommend automatic titration to be applied when determining chloride content in milk-based baby food. All the food types that were subject to this research had a chloride content that is in accordance with proscribed values (EFSA, 2014).

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Effects of Wet Gluten Adjustment on Physico-Chemical and Rheological Characteristics of Three Types of Wheat Flour

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Abstract. The aim of this study was to examine the effects of increasing and decreasing gluten content on the physical, chemical and rheological properties of dough obtained from different types of wheat flour. For the research, samples of three types of flour were used: T-500, T-710 and T-850, obtained by milling the wheat variety *Falado*. Corrections were made to the gluten content based on the content of wet gluten in the wheat flour samples, as follows: by increasing it to 33% by adding vital gluten and by decreasing it to 22% by adding wheat starch. After adjusting the gluten content, the total of nine flour samples were obtained. Physico-chemical analyses included the determination of moisture, ash, protein, wet and dry gluten content, titrable acidity, sedimentation value and quality of gluten by visual evaluation. Rheological analyses included those performed on a farinograph: flour water absorption, dough development, stability, degree of softening, quality number and quality group, and those performed on an extensograph: area under extensograph curve, dough resistance, extensibility, R/E ratio. The statistical analysis showed a significant influence of flour type and gluten content on all physical, chemical and rheological parameters, except on the dough development. The results showed that the vital gluten addition increased and that the wheat starch addition decreased protein, ash and wet gluten content, acidity and sedimentation value, flour water absorption, degree of softening, area under extensograph curve and extensibility of dough in the samples of all types of flours with certain exceptions (samples T 500/22, T 710/33 and T 850/33).

Keywords: Wet gluten content · Type of flour · Physical and chemical parameters · Rheological parameters

1 Introduction

Gluten proteins are storage proteins in wheat. They are located in the kernel endosperm and are responsible for the unique viscoelastic properties and water absorption capacity

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of dough [1–4]. The total protein and wet gluten (WG) content has the most important influence on the processing suitability of flour [5] and plays critical roles in determining the volume of baking products as one of their crucial quality characteristics [6].

The amount of WG in wheat flour for making bread is usually between 20–35%. The high gluten content ensures favourable rheological characteristics and the high volume of bread. The evaluation of the rheological properties of wheat flour dough is the most important for the successful manufacturing of all bakery products, because they determine its behaviour during mechanical handling and consequently, affect the quality of finished products [7–10].

The vital wheat gluten (VG) is a protein concentrate (approximately 80% protein) obtained from wheat flour during the wet milling of the wheat flour for starch production and treated carefully to retain its baking properties [3, 11, 12].

It was established that the sources of gluten have a strong effect on the dough rheology and the baking performance [13].

The adding of the VG for the purpose of improving weak flours is a common practice and it serves as a compensation for the lack of protein quality and quantity. For making wheat bread from frozen dough, the VG was added in defined amounts of 2%, 4%, 5%, and 6% of flour weight and the results showed that the most stable samples were those with 4% and 6% of the VG (for white flour) and those with 4% and 5% of the VG (for whole-wheat flour) [14]. The VG in the amount of 2.5%, in combination with other plant-origin materials (defatted Syrian cephalaria flour, rosehip and malt flour) showed significant effects on the specific volume, acidity, colour, and textural properties of whole wheat bread [15]. Pasta flour, bread and biscuit products enriched with the VG also showed appreciable results [16].

In addition, the gluten quantity and quality are important for confectionary products based on wheat flour such as biscuits, cookies, crackers, wafers, cakes, waffles and others. The flours suitable for these products are soft wheat flours with low to medium protein content 7–10% (on a 14% moisture basis), low water absorption, low damaged-starch content, fine flour granulation and satisfactory dough consistency [10, 17], and also with low WG content and low sedimentation value [18]. The quality of flour for production of these products is not always satisfactory or uniform and various corrections are often required particularly in the WG content as the gluten controls shape, dimensions and weight of finished products. Product dimensions are strictly defined and any minor deviation can lead to manufacturing problems, especially during packaging [19]. The major problem in the control process is the maintenance of the product size and shape because the gluten in the dough is more or less elastic and also because dough pieces shrink after cutting and during the early stages of baking. The amount of this shrinkage depends on the flour quality [20] and modifications to the gluten quantity and quality are often necessary. A possible reduction in gluten content can be achieved by adding wheat starch (WS), which can also contribute to the optimisation of plastic properties of the dough [21].

The objectives of this study were to evaluate physical, chemical and rheological parameters of different flour types with the adjustment of the WG content in flour samples according to recommendations for the bread and flour-based confectionary making

process and this by increasing the WG content by adding VG to 33% and by decreasing the WG content by adding WS to 22%.

2 Materials and Methods

2.1 Materials

Flour samples obtained by industrial milling of wheat (*Falado* variety) from the wheat-growing region of Derventa, Bosnia and Herzegovina (crop year 2019), were used for the conducted research. The physico-chemical properties (test weight: 77.8 kg/hl and total proteins: 12,0%) of wheat were in accordance with the values for this variety declared by the seed producer. The wheat sample was characterised by low amylolytic activity (maximum viscosity of suspension: 770 BU and FN: 410), which indicated that corrections in the amylolytic activity were required for its further use in the production of bakery products.

Table 1. Physico-chemical properties of the sample of wheat, *Falado* variety, used in the research

Parameter	Value
Moisture (%)	12.38
Ash (% d.m.)	1.68
TA	1.90
Proteins N × 5.7 (%)	12.20
Sedimentation value	44.0
Test weight (kg/hL)	77.85

TA – titrable acidity

2.2 Milling of Wheat

The wheat milling process was performed in industrial conditions in the mill *Ljubače*, Bosnia and Herzegovina. After milling, the samples of three types of wheat flour were obtained: T-500 (white), T-710 (semi-white) and T-850 (brown) and used for further research.

2.3 Corrections of the Gluten Content of Wheat Flours

Based on the content of WG in the wheat flour samples, corrections were made in the gluten content, as follows: by increasing it to 33% by adding VG and by decreasing it to 22% by adding WS. The VG and the WS were manufactured by *Fidelinka - Skrob d.o.o.* /Ltd/. The VG corresponded to the current standard [22]. The required quantities (%) of the VG and the WS for corrections were obtained by calculations using the Paerson

square method [21]. The gluten adjustment was done by preparing wheat flour mixtures with VG/WS in the quantity of about 10 kg of mass which were homogenised by a spiral Diosna mixer operating at slow speed for 15 min. After the gluten adjustment/preparation of the mixtures, a total of 9 wheat flour samples were obtained. The samples were marked on the basis of the type of wheat flour and the content of the WG, as shown in Table 2.

Table 2. Wheat flour samples obtained after gluten adjustment

Type of wheat flour	Content of WG (%)	Sample mark
500	22.00 ± 0.2	T 500/22
	25.88 (native)	T 500/N
	33.00 ± 0.2	T 500/33
710	22.00 ± 0.2	T 710/22
	28.36 (native)	T 710/N
	33.00 ± 0.2	T 710/33
850	22.00 ± 0.2	T 850/22
	26.98 (native)	T 850/N
	33.00 ± 0.2	T 850/33

2.4 Physical and Chemical Analysis of Wheat Flour Samples

Physical analyses included the determination of gluten quality and a sedimentation test. The gluten quality was determined by the sedimentation test according to Zeleny and by an elasticity and extensibility test [23].

Moisture, ash and total protein (Kjeldahl; N × 5.7) contents were determined using the standard methods of analysis [24]. The titrable acidity (TA) was determined by the Shulerd method using an extraction of acidic substances with 67% ethanol [23]. The content of wet and dry gluten was determined by means of the device Glutomatic 2200.

2.5 Rheological Analysis of Wheat Flour Samples

Flour water absorption (WA; %), dough development time (DDT; min), dough stability (DS; min), softening degree (SD; BU), quality number (QN) and quality group (QG) were determined on Brabender farinograph (model SEW), while the area under extensograph curve (AUEC; cm^2), dough resistance (DR; BU), extensibility (mm) and R/E ratio were determined on Brabender extensograph (type DM90/40) [23].

2.6 Statistical Analysis

All results are expressed as mean ± standard deviations (SD). A two-way analysis of variance with interactions (ANOVA) was used to evaluate whether significant differences existed between the wheat flour samples depending on flour type and WG content. The established differences were tested by the Tukay test for $P < 0.05$.

Table 3. Physico-chemical properties of wheat flour samples

Flour type	T-500				T-710				T-850						
WG content	22%	N	33%	T	22%	N	33%	T	22%	N	33%	T	T	G	TxG
Moisture (%)	12.75 ± 0.05c	13.63 ± 0.10a	13.16 ± 0.07b	x	12.73 ± 0.06b	13.31 ± 0.15a	12.74 ± 0.04b	x	12.51 ± 0.06b	13.25 ± 0.11a	12.65 ± 0.06b	x	*	*	*
Ash (% d.m.)	0.50 ± 0.01c	0.52 ± 0.01b	0.55 ± 0.01a	z	0.68 ± 0.01c	0.72 ± 0.01b	0.74 ± 0.01a	y	0.80 ± 0.01c	0.85 ± 0.02b	0.90 ± 0.01a	x	*	*	*
TA	1.27 ± 0.06a	1.27 ± 0.12a	1.33 ± 0.15a	z	1.53 ± 0.06a	1.60 ± 0.10a	1.77 ± 0.06a	y	1.80 ± 0.10a	1.83 ± 0.21a	2.03 ± 0.06a	x	*	*	ns
Proteins N × 5.7 (%)	9.84 ± 0.03c	11.97 ± 0.32b	14.07 ± 0.02a	x	9.86 ± 0.01c	13.00 ± 0.10b	14.10 ± 0.02a	x	9.83 ± 0.01c	13.07 ± 0.21b	14.12 ± 0.01a	x	*	*	*
Dry gluten (%)	7.49 ± 0.11c	8.72 ± 0.11b	11.91 ± 0.13a	x	7.51 ± 0.12c	9.68 ± 0.18b	11.93 ± 0.06a	x	7.42 ± 0.10c	9.60 ± 0.31b	11.74 ± 0.17a	x	*	*	*
Sedimentation value	25.33 ± 0.58b	34.33 ± 2.08a	38.00 ± 1.00a	x	18.33 ± 1.53b	28.67 ± 1.53a	27.00 ± 3.00a	y	13.00 ± 0.00b	21.33 ± 1.15a	24.67 ± 3.21a	y	*	*	ns
Visual evaluation of gluten quality	Very stretchable	Stretchable	Elastic		Very stretchable	Stretchable	Elastic		Very stretchable	Stretchable	Elastic				

Different letters in rows from a to c for each parameter indicate significantly different values among WG contents at $P < 0.05$; Different letters in rows from x to z for each parameter indicate significantly different values among flour types at $P < 0.05$.

T – flour type; G – WG content; T × G – interaction between flour type and WG content; TA – titrable acidity. ns – not significant; * – significant differences at P-value below 0.05.

3 Results and Discussion

3.1 Physical and Chemical Properties of Wheat Flour Samples

All evaluated physico-chemical properties were significantly influenced by the flour type and WG content. The wheat flour samples of the same type of flour differed significantly in terms of moisture, ash, protein and dry gluten content and sedimentation value, which was an expected consequence of gluten adjustment and changes in the chemical composition of the samples.

The chemical analysis showed that moisture and ash contents of wheat flour samples were in accordance with the current legislation [26]. Sample T 500/N had moisture content, and samples T 500/22, T 500/N and T 500/33 had ash content similar to those obtained by Keran et al. [27] for the same type of flour. The content of dry gluten in all wheat flour samples was in accordance with the results reported by Hajek [28].

The sedimentation value provides information about the quantity and quality of wheat flour gluten. It was established that there is a positive correlation between the sedimentation value and the gluten strength, as well as with the bread volume [21]. According to the ICC Standard 116/1 [25], the sedimentation value of the T 500/N and T 500/33 samples in the range of 30–40 meant good baking properties for the production of bakery products, while the sedimentation value of the samples T 710/22 and T 850/22, which was below 20, meant that these flours are suitable for the biscuit production. Other samples had sedimentation values ranging from 20 to 30, which indicates that they have average baking properties for the baking production.

The visual evaluation of the gluten behaviour [23] showed that the gluten consistency changed from “stretchable” to “very stretchable” with the addition of WS while with the addition of VG, it changed from “stretchable” to “elastic” in all types of flour.

As expected in all types of flour, the addition of VG caused an increase, while the addition of WS caused a decrease in the content of ash, protein and dry gluten, TA and sedimentation value in relation to their values in standard wheat flour samples with native gluten content. Exceptions were observed in the cases of the sample T 500/22 where, with the addition of WS, there was no change in the TA, and in the sample T 710/33 the sedimentation value decreased slightly with the addition of VG. These exceptions were not significant. The mentioned increases are in accordance with the research of the authors Codina et al. [29], who pointed out that the addition of the VG increases the technological potential of wheat flour up to a certain dose. It was confirmed that VG increases the protein content and thus the nutritional value of the final product [30–32]. The increase in ash and protein content and sedimentation value by increasing the WG content in flour samples of type 500 was in accordance with the results obtained by Oručević et al. [18].

3.2 Rheological Properties of Wheat Flour Samples

The statistical analysis showed significant influence of flour type and WG content on all examined farinograph properties with the exception of DD.

The farinograph properties of all wheat flour samples were within the range of values obtained by Hajek [28].

WA ranged from 52.13 (sample T 500/22) to 59.77% (sample T 850/33) and was in accordance with the results reported by Rakita [33]. According to Oručević Žuljević [21], WA ranges from 50 to 60% depending on the quality of the flour. All examined wheat flour samples had WA in accordance with this literature data, while the WA of wheat flour samples of types 500 and 850 was lower than those reported by Keran et al. [27] for the same types of flour. The higher WA indicates the ability of flour to absorb a larger amount of water to achieve a standard consistency and, consequently, the dough from such flour will have stronger properties [34] while flours with a lower value of the WA are generally used for biscuit production [21]. WA ranging from 50 to 54% is a generally used criterion for flours used for biscuit production [35]. Only samples T 500/22 and T 710/22 had the values suitable for biscuit production. According to Oručević Žuljević [21], in practice, flours cannot be compared on the basis of WA if they are flours of different types, while in flours of the same type WA depends on the content and quality of gluten. The higher gluten content and its better quality increase the WA. According to Đaković [6], these two parameters are in positive correlation. Therefore, it was expected that in samples of the same type of flour this parameter will increase with the addition of VG (increase in the gluten content) or decrease with the addition of WS (decrease in the gluten content), as shown in Table 4.

The analysed wheat flour samples had fairly uniform values of DDT ranging from 2.00 to 2.50 min, and were in line with the values for this parameter which are considered suitable for bakery products [21].

It is considered that weak flours have DS less than 3 min, moderately strong flours 5.5–7.0 min, while strong flours are characterised by DS ranging from 7.5 to 9.0 [33]. Based on the obtained DS values, the analysed wheat flour samples are classified as weak flours and are suitable for production of hard biscuits [35], except for the samples T 850/22 and T 850/33, which are classified as moderately strong flours. The DS of these two samples were in accordance with the results reported by Minarik [36].

The SD is a farinograph indicator which is most often taken into consideration during flour quality assessment. According to literature data [21], the SD in the range between 70 and 120 BU is considered suitable for bakery products. Experience from the production in *Mlin i Pekara d.d. Ljubače* /Mill and Bakery JSC Ljubače/ showed that the optimal values of this parameter are from 50 to 80 BU. The wheat flour samples of type 850, samples with native and WG content of 22% of types 500 and 710 had values of the SD corresponding to the stated experimental parameters. In all types of flour, the addition of VG caused a decrease, and the addition of WS caused an increase of the SD compared to its values in standard flour samples with native gluten content. An exception is the sample T 850/33 whose SD increased with the addition of VG, but this was not significant (Table 4). These decreases in the SD with the increasing WG content are in accordance with the literature data [6] and with the results obtained by Oručević [18].

The samples with native gluten content belonged to quality group B1 and with the addition of VG they moved into quality group A2. On the other hand, the addition of WS did not cause any changes in the quality group of the analysed wheat flour samples. It is possible to assume that the favourable quality of *Falado* wheat gluten is the reason for this dough behaviour.

Table 4. Farinograph properties of wheat flour samples

Flour type	T-500				T-710				T-850						
WG content	22%	N	33%	T	22%	N	33%	T	22%	N	33%	T	T	G	TxG
WA (%)	52.13 ± 0.12c	56.17 ± 0.15b	58.22 ± 0.10a	x	53.20 ± 0.10c	58.67 ± 0.49b	59.20 ± 0.10a	x	54.03 ± 0.06b	59.37 ± 0.10a	59.77 ± 0.10a	x	*	*	*
DDT (min)	2.00 ± 0.00a	2.17 ± 0.29a	2.17 ± 0.29a	x	2.00 ± 0.00a	2.00 ± 0.00a	2.00 ± 0.00a	x	2.00 ± 0.00a	2.00 ± 0.00a	2.50 ± 0.50a	x	ns	ns	ns
DS (min)	0.50 ± 0.00a	0.50 ± 0.00a	0.67 ± 0.29a	y	1.67 ± 0.58a	0.67 ± 0.29b	0.50 ± 0.00b	y	6.00 ± 0.00a	0.50 ± 0.00b	5.67 ± 0.58a	x	*	*	*
SD (BU)	76.67 ± 5.77a	56.67 ± 5.77b	40.00 ± 0.00c	x	63.33 ± 5.77a	46.67 ± 5.77b	33.33 ± 5.77c	x	70.00 ± 0.00a	53.33 ± 5.77b	56.67 ± 5.77b	x	*	*	*
QN	63.03 ± 0.23b	64.80 ± 0.17b	71.40 ± 0.17a	x	64.07 ± 0.51c	69.07 ± 2.19b	71.80 ± 1.14a	x	69.30 ± 0.00a	65.13 ± 0.12b	70.37 ± 0.29a	x	*	*	*
QG	B1	B1	A2		B1	B1	A2		B1	B1	A2				

Different letters in rows from a to c for each parameter indicate significantly different values among WG contents at $P < 0,05$; Different letters in rows from x to y for each parameter indicate significantly different values among flour types at $P < 0.05$.

T – flour type; G – WG content; T × G – interaction between flour type and WG content; WA – water absorption; DDT – dough development time; DS – dough stability; SD – softening degree; QN – quality number; QG – quality group. ns – not significant; * – significant differences at P-value below 0.05.

Table 5. Extensograph properties of wheat flour samples

Flour type	T-500				T-710				T-850						
WG content	22%	N	33%	T	22%	N	33%	T	22%	N	33%	T	T	G	TxG
AUEC (cm^2)	70.67 ± 1.15c	93.67 ± 4.16b	129.33 ± 3.06a	x	90.00 ± 4.58b	117.33 ± 10.07a	133.00 ± 8.19a	x	74.33 ± 9.29b	85.00 ± 2.00b	118.00 ± 5.00a	x	*	*	*
DR (BU)	306.67 ± 11.55b	313.33 ± 11.55b	356.67 ± 11.55a	y	346.67 ± 11.55b	396.67 ± 5.77a	383.33 ± 28.87a	x	413.33 ± 11.55a	323.33 ± 5.77c	370 ± 10.00b	x	*	*	*
Extensibility (mm)	140.00 ± 3.00b	162.33 ± 2.52a	165.33 ± 1.53a	x	130.67 ± 2.08b	156.33 ± 7.23a	161.67 ± 1.53a	x	108.67 ± 8.14b	146.00 ± 3.46a	156.00 ± 1.73a	x	*	*	*
R/E ratio	2.19 ± 0.13a	1.92 ± 0.05a	2.15 ± 0.08a	y	2.65 ± 0.11a	2.54 ± 0.14a	2.37 ± 0.20a	x	3.81 ± 0.26a	2.21 ± 0.05b	2.37 ± 0.05b	x	*	*	*

Different letters in rows from a to c for each parameter indicate significantly different values among WG contents at $P < 0,05$; Different letters in rows from x to y for each parameter indicate significantly different values among flour types at $P < 0.05$.

T – flour type; G – WG content; T × G – interaction between flour type and WG content; AUEC – area under extensograph curve; DR – dough resistance. ns – not significant; * – significant differences at P-value below 0.05.

All extensograph properties were affected significantly with the flour type, as well as with WG content and, consequently, the wheat flour samples of the same type of flour differed significantly in the values of the AUEC, DR and extensibility, while in the case of R/E ratio, significant differences were observed only in wheat flour samples of type 850.

The addition of VG led to an increase in the AUEC and extensibility, while the addition of WS led to a decrease of these properties in the wheat flour samples. These changes are in accordance with the literature data [6, 21]. Also, the AUEC and extensibility were within the range of values obtained by Hajek [28]. Based on AUEC values (below 80cm^2), the samples T 500/22 and T 850/22 belonged to weak flours [21] and were similar to soft wheat flour quality [38] and, according to available literature data [6, 37], they are suitable for use in biscuit production. On the other hand, samples T 500/33 and T 710/33 belonged to strong flours according to AUEC values (120–200 cm^2) and could classified as top-quality bread flour [38]. According to this parameter (range from 80 to 120 cm^2), all the other samples belonged to medium strong flours [21] (Table 5).

The ratio of DR in respect of stretch and extensibility is a ratio between elastic and plastic properties of the dough. It is considered that doughs with the R/E ratio value in the range of 1.5–3.0 have optimum ratio of strength and extensibility of the dough, which is necessary for the entire process of bread production [21]. All analysed wheat flour samples had optimal values of this parameter, except for the sample T 850/22, which had a slightly higher value.

4 Conclusions

The results presented here show that the addition of the VG improves the nutritional value due to the increase in the total protein content, and also improves the farinograph and extensograph properties of the flour. Flour like this is in demand on the market for the production of noodles and pies. There is a growing demand for this flour and, in general, the interest for flour with a defined gluten content is growing. On the other hand, although the addition of WS leads to a decrease in the value of certain rheological properties, such flours are suitable for the flour-based confectionery industry, which is very important because the market is currently looking for special flour with strictly specified gluten quantity and quality. However, it is necessary to emphasise that interventions on flour imply high initial quality and it could be seen in this research that the wheat variety *Falado* was characterised by desirable quality parameters and that it provides different types of flour that can be corrected and adjusted to the needs for different kinds of bakery and confectionery products.

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Efficiency of Dairy Value Chain in Bosnia and Herzegovina

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Abstract. Dairy farming production is a strategic part of agriculture and certainly the most important branch of livestock production in Bosnia and Herzegovina. Dairy farming is the basis for the development of this sector, given that a large number of farms that produce milk for the market, which generates household subsistence income from this production. The main problem in this sector is the low level of efficiency of the value chain. Therefore, it is very important to research and to know the structure of the value chain as well as the opinions of its participants. Precisely because of this, the general goal of the research in this paper was to analyze the efficiency of the value chain of dairy products, based on a survey conducted with value chain participants (farmers, dairies and distributors). Collaboration among all chain members is very important in order to reach higher levels of value chain efficiency. The performance of the value chain is shaped by the efficiency of the chain, which is shaped by the way and intensity of information exchange, but also by the level of alignment of the business goals of the chain actors. The level of information exchange and alignment of business goals define the level and intensity of cooperation within the chain, which is reflected in the business success of each individual actor in the chain, which finally defines the level of chain efficiency, or its ability to create added value for all actors with availability of resources and achieving synergetic effects.

Keywords: Value chain · Information sharing · Goal alignment

1 Introduction

Milk production, processing, distribution and sale is an extremely important strategic sector in the agricultural economy of Bosnia and Herzegovina. The importance of the sector is also emphasized by the fact that milk and dairy products belong to the group of basic foods irreplaceable in human consumption. They are basic food products, which, in addition to energy-valuable substances, also provide the body with certain protective ingredients (Havranek and Rupić 2003).

To remain competitive in a business environment that is increasingly driven by consumer behavior, only companies that have the ability to offer consumers products exactly

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the way they want, to understand them better than themselves, succeed. To achieve this, a business concept called the value chain was developed. Competition increasingly involves entire value chains and the importance of close cooperation of all members of the chain is gaining in importance, in order to achieve higher levels of value chain performance. Emerging markets, such as the market in Bosnia and Herzegovina, usually suffer from inefficiencies resulting in mismatches between supply and demand in the value chain. This is why the importance of researching the performance of the value chain of dairy products is emphasized.

Insight into the scientific literature related to value chain analysis as well as theory, the conceptual elements that affect the value chain performance have been explored. The main precondition for an efficient value chain is the relations between the participants in the value chain as well as the exchange of information between them, and for this reason the analysis of the existing elements was performed. Also, the alignment of business goals of the participants in the value chain was considered, which is also the main prerequisite for close cooperation between the partners of the value chain. Lack of cooperation among participants in agri-food value chains has been observed very often (Naspetti et al. 2011; Pauwelyn 2014). Also, similar conclusions were reached by Kotilla et al. (2010), who found that European chains are insufficiently integrated and that is why they do not function well. More precisely, the cooperation existed in dyadic relations, but not at the level of the entire agri-food chain. In general, the importance of strong collaborative relationships as a mechanism for improving overall value chain performance has been widely demonstrated (Velker et al. 2008).

According to Naspetti et al. (2011), one way to achieve growth in the dairy sector, is certainly aimed at improving cooperation and trust among the strategic partners in the chain who add the most value. Within these strategic partnerships, the level of information exchange and joint decision-making needs to be significantly improved, and each value chain actor needs to establish clear courses of action in achieving common chain goals and levels of effectiveness. A shared vision and values and trust among the actors that ensure the qualitative exchange of information are crucial for the creation of such strategic partnerships. Every member of the chain needs to show commitment, loyalty, so that profit is guaranteed, fair distribution of income and long-term business relationships (Stevenson 2009).

However, in general, for most European agri-food chains (Naspetti et al. 2011; Pauwelyn 2014), it can be said that the actual levels of cooperation, information exchange and alignment of business objectives are too low to allow for such stable strategic partnerships. Also, Kvam and Bjorkhaug (2014) came to the conclusion based on a review of European agri-food markets that it would be necessary to select chain partners who share the same values in order to develop mechanisms for building trust, transparency and joint decision-making. A close relationship of chain actors is crucial for small-scale value chains, and building such relationships ensures fair profit distribution and social sustainability (Stevenson et al. 2011). However, building such a strong chain integration with strategic partners in the chain is extremely difficult and requires significant resources (Naspetti et al. 2011; Pauwelyn 2014).

2 Material and Methods

The survey was based on a sample of 34 value chain participants, and their structure is shown in Table 1. For this research convenience sample was selected, because only such a sample could be realized because of the organizational and technical reasons.

Table 1. Structure of value chain participants

	Frequency	*%*
Producers/farmers	23	67,65
Processors/dairies	5	14,71
Distributors (wholesalers, retailers, supermarkets)	6	17,65
Total	34	100,00

The research was conducted in the direction of perceiving the efficiency of the value chain through a survey directed to farmers, dairies and distributors. The aim of the survey was to obtain answers and gain insight into the relationships and exchange of information among value chain participants and the alignment of business objectives of value chain participants. In this way, have been identified areas that reduce the efficiency of the value chain, i.e. areas in which there is a need for improvement, in order for each actor to strengthen its ability to strengthen its competitive advantages.

Two hypotheses are set in this research, which referred to the efficiency of the value chain:

H1 *- The value chain is not efficient enough because the exchange of information between the participants in the value chain is not adequate.*

H2 *- The value chain is not efficient enough because the business goals of the participants in the value chain are not aligned.*

The questionnaire for participants in the value chain of the dairy sector, farmers, dairies and distributors was partly taken from the work of Bram Pauwelyn (2014), and the other part was created by the author of this paper. Data was collected through a survey questionnaire, applied to participants of the dairy value chain (from May to July 2017). The survey questionnaire was designed in order to analyze the efficiency of the value chain in terms of:

– exchange information between participants.
– alignment of business goals of the participants in the value chain.

In this paper, the focus is on the three participants in the value chain, farmers, dairies and distributors. In this way, simplicity in research is enabled and the most relevant actors of this agri-food chain are focused. Each question concerning one specific relationship was asked in two directions, i.e. to both actors participating in the relationship. In our case, farmers were asked about their relationship with dairies, while dairies made the same statements regarding their relationship with farmers.

MANUFACTURERS/FARMERS ↔ PROCESSORS/DAIRIES ↔ DISTRIBUTORS.

Monitored relationships in the value chain of milk and dairy products:

Manufacturer/farmer - processor/dairy processor/dairy - distributor.

Respondents in this study were tasked with evaluating five conceptual statements on a 5-level Likert scale, according to their agreement with a particular statement.

3 Results and Discussion

The performance of the value chain is shaped by the efficiency of the chain, which is shaped by the way and intensity of information exchange, but also by the level of compliance of the business goals of the chain actors. The level of information exchange and alignment of business goals define the level and intensity of cooperation within the chain, which is reflected in the business success of each individual actor in the chain, which finally defines the level of chain efficiency, i.e. its ability to create added value for all actors, with available resources and achieving synergetic effects. The more efficient the chain, the more successful the actors are, and small and medium farmers find it easier to operate because they operate in a stable market, which ultimately strengthens the entire agricultural sector and its development. That is why in the following text, the first level and intensity of the exchange will be considered, but also the type of information they exchange, and then the alignment of business goals of all actors.

The exchange of information is the basis for the functioning and survival of the value chain (Renko and Popović 2013). Also, sharing information in the value chain is very important, and is a key factor for improvement and connection between the actors involved in the chain. Transparency through the value chain is necessary and it leads to the creation of trust and loyalty, and the establishment of strong alliances that affect the development of the entire sector.

The results of the survey indicate that all distributors and dairies share some information they receive with their chain partners. A higher percentage of farmers, 61% of them, pointed out that they share information with their partners in the chain, while 39% pointed out that they do not. This suggests that farmers are still isolated and that there is not enough horizontal communication and cooperation among actors who have even the lowest level of power within this chain.

When it comes to the information they share with their suppliers, the answers were given by distributors and dairies, since the survey did not include suppliers from farmers, and therefore, in this case, farmers were not taken into consideration. The largest percentage of distributors, 50% of them pointed out that the information they exchange with their suppliers, refers to the wishes of customers, 33% of them stated that this information is related to price changes, while 17% of distributors pointed out that it is some other information, mainly those related to quality and preferably lower prices. Dairies pointed out in a large percentage of 40% that price changes are the most common information they exchange with their suppliers. Also, a high percentage of 40% of dairies state that they exchange information with their suppliers regarding the tidyness of the service, and 20% of them point out that the information they exchange with their suppliers most often refers to the wishes of customers.

The survey made it possible to find out what information the participants in the value chain share with their customers. The answers to this question were given by dairies and farmers, so the largest percentage of dairies of 60% pointed out that this information is related to price changes, while 20% of them pointed out that the information most often exchanged with their customers are mainly concerned with the wishes of suppliers and some other information that is a trade secret, so that dairies have not disclosed what kind of information it is. The highest percentage of farmers of 33%, states that the information they exchange with their customers (which are classified under the category "other"), related to unpaid debts, the state of new technologies, quality information, information related to milk samples and quality of delivery. Also, a significant percentage of 27% of farmers point out that the most common information they exchange with their customers is related to supplier wishes and price changes, while 13% stated that the production volume forecast is the information most often exchanged with customers. Therefore, the information that is exchanged is of an internal character, i.e. it refers to the details of cooperation within the chain, e.g. prices and services. Actors are focused on themselves and not on consumers or market conditions, which is in line with previous research by Nikolić et al. (2011).

When it comes to the exchange of information and statements evaluated by all participants in the value chain, the following chart shows the average values of responses of participants in the dairy value chain for each of the offered statements related to the exchange of information between them (Fig. 1).

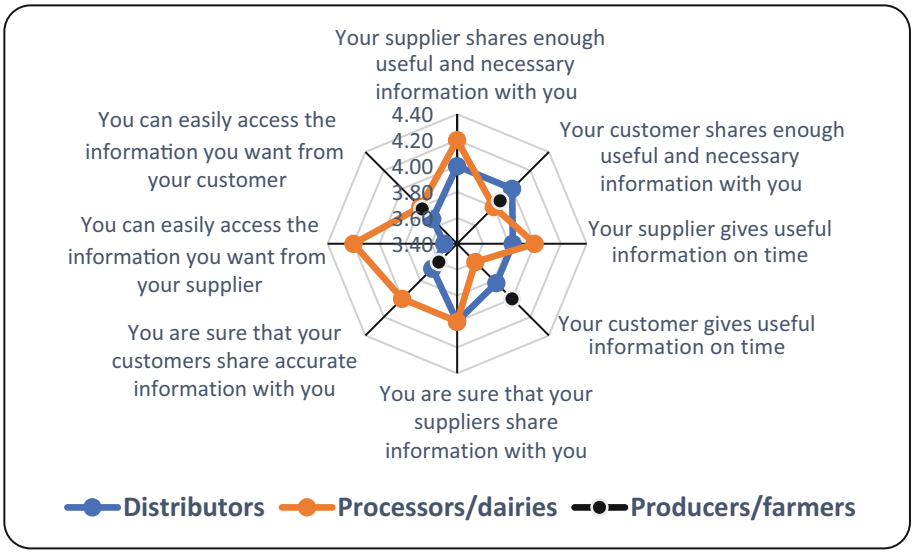

Fig. 1. Exchange of information of dairy value chain participants (1 strongly disagree, 5 strongly agree)

Note: The questions concerning the relationship of value chain participants with suppliers were not answered by farmers, given that four actors were included in this research, and for this reason, farmers' answers are presented by black dots on the chart.

There is an exchange of information between the actors and the level of satisfaction of the chain's actors is different, where farmers are the least satisfied, but on average the actors rated all communication characteristics with a score over 3.4, which indicates a positive attitude of all. The results indicate that the stronger in the chain are more satisfied, which is to be expected, considering that they are the ones who dictate the conditions and achieve the largest share in the newly created added value. It is interesting to note that dairies provide information to farmers on time, and vice versa, while the attitude of distributors towards dairies is significantly different. In addition, it can be seen that the level of trust between farmers and dairies is slightly lower than that between dairies and distributors. This is a very important aspect of the value chain. The low level of trust of farmers towards other actors in the chain is a real obstacle to the improvement of cooperation that should lead to the improvement of milk quality and productivity on farms. This is certainly an obstacle to faster growth of production volume on farms, and it certainly does not motivate the exchange of ideas and innovation.

Table 2 and 3 represent the relationship between farmers and dairies on the one hand and dairies and distributors on the other. Each question concerning one specific relationship is asked to both actors whose relationship is being monitored, thus enabling reciprocal information to be obtained. Namely, farmers were asked about their relationship with dairies, and dairies were asked the same question about their relationship with farmers (Table 2). The relationship between dairies and distributors was investigated in the same way (Table 3). Based on the data from Table 2 and 3, it is seen that the scores expressing satisfaction with the exchange of information are quite high (minimum 3.50), which suggests that all participants in the value chain are satisfied with the way they receive information from their partners in the value chain. The student's t-test did not prove statistically significant differences (at a demanding significance level of 0.05) between different participants in the dairy supply chain, in terms of their different attitudes regarding the exchange of information, which is presented in Tables 2 and 3. However, it is important to emphasize that the ratings of the "stronger" actor in the chain are higher and this indicates that the communication is still "one-sided", i.e. only the interest of the "stronger" actor is being talked about. When it comes to the first research group, the overall lower result was reported by farmers, compared to dairies. The only item in which both agree is that it relates to exchanging useful information with each other on time (4.00). When it comes to the second group, it can be noticed that dairies show a slightly lower overall result compared to distributors. The only item in which dairies show a higher degree of agreement with distributors is that they can easily access the desired information (3.80) compared to distributors (3.50).

The relationship between the actors of the value chain is, among other things, defined by the exchange of information. Value chain actors talk only about price and price reduction, there is no exchange of information on the scope of business, ways to strengthen cooperation, ways to improve technology and business efficiency, greening, use of "by products", strengthening energy efficiency, waste management, new technologies, introduction "Smart" communications (digitization of the same, etc.). There is no exchange

Table 2. Mean values of statements of value chain participants, concerning the exchange of information between producers/farmers and processors/dairies (1 strongly disagree, 5 strongly agree)

Sharing information	*Role in the chain*					
	Producers/farmers (n = 23)		*Processors/dairies (n = 5)*			
	Mean	*SD*	*Mean*	*SD*	*tp*	
Sharing enough useful and necessary information	3,87	0,63	4,20	0,84	– 1,011	0,321
Useful information on time	4,00	0,30	4,00	0,71	0,000	1,000
Sharing information that is accurate	3,60	0,66	4,00	0,71	– 1,194	0,243
Easy access to desired information	3,78	0,67	4,20	0,45	– 1,318	0,199

Mean-arithmetic mean, SD-standard deviation, t-realized value of Student t test, p-probability of rejecting the null hypothesis with a risk of 5%

Table 3. Mean values of statements of value chain participants, concerning the exchange of information between processors/dairies and distributors (1 strongly disagree, 5 strongly agree)

Sharing information	*Role in the chain*					
	Processors/dairies (n = 5)		*Distributors (n = 6)*			
	Mean	*SD*	*Mean*	*SD*	*tp*	
Sharing enough useful and necessary information	3,80	0,84	4,00	0,63	−0,452	0,662
Useful information on time	3,60	0,89	3,83	0,98	−0,408	0,693
Sharing information that is accurate	4,00	0,71	4,00	0,89	0,000	1,000
Easy access to desired information	3,80	1,10	3,50	1,22	0,424	0,682

Mean-arithmetic mean, SD-standard deviation, t-realized value of Student t test, p-probability of rejecting the null hypothesis with a risk of 5%

of information about this, and a good relationship and efficient communication is the point due to which a synergistic effect occurs, that is in other words strengthening the performance of the whole chain and each individual. This means that it is a very old-fashioned value chain, which is formed by "inertia", ie. narrowed market opportunities and low innovation of actors. What is positive is that there is communication, that a significant level of trust has been expressed, which is the basis for strengthening the chain.

When it comes to the importance of business goals of actors in the chain, it should be emphasized, first of all, that goals must be defined and clear to all participants in the value chain so that each of them can contribute to achieving these goals, which is only possible if all partners in the chain working together (Molnar 2010). The alignment of the goals of all actors in the value chain is in fact the extent to which the participants in the value chain share their knowledge and contribute to the achievement of common goals. It is important to emphasize that all goals of chain actors should be directed in the same direction so that products can be delivered to end consumers as quickly and efficiently as possible (Narayanan and Raman 2004). Thus, the goal of each participant in the chain should be to "meet consumer demand as efficiently as possible", which should be translated into specific goals. The only acceptable way to achieve the above is to improve the relationships between the partners in the chain (Aertsens 2011).

In order to properly explore the importance of the business goals of the value chain actors, a comparison of responses was made between farmers and dairies on the one hand, and dairies and distributors on the other.

Based on the data from Table 4, which monitors the relationship between farmers and dairy, it can be clearly seen that the student's t-test proved statistically significant differences (at a demanding significance level of 0.05), for business purposes: growth (increase in market share, sales), response (to meet customer needs as soon as possible), efficiency (maximizing production at minimum cost) and product quality (food safety, attractiveness).

For farmers, the most important thing is to achieve fair prices and product quality, while the least important thing is to meet customer needs and achieve efficiency. So, product quality is an extremely important business goal for farmers because that is exactly what dairies require of them. The most important thing for dairies is to achieve growth, ensure efficiency (cost reduction) and quality, while ecological production is the least important to them. For dairies, efficiency as a business goal is extremely important because it essentially refers to maximizing production with minimal costs, which ultimately results in higher profits, so it seems logical to set efficiency as an extremely important business goal for them. Also, for dairies, the important business goal is growth (sales) as well as product quality, which is to be expected because the requirements addressed to them are certainly, among other things, in the direction of food safety. A major problem is the fact that farmers do not care about increasing market share and efficiency. This mismatch also suggests a low ability to create a synergistic effect and reflects the different levels of power of these two groups of actors in this value chain. This is confirmed by the findings related to the exchange of information.

Table 4. Mean values of the statements of the participants in the value chain, concerning the importance of the stated business objectives (1 extremely unimportant, 5 very important)

Business goals	*Role in the chain*					
	Producers/farmers (n = 23)		*Processors/dairies (n = 5)*			
	Mean	*SD*	*Mean*	*SD*	*tp*	
Growth (increase in market share, sales…)	4,13	0,626	5,00	0,000	−3,06	**0,005**
Response (to meet customer needs as soon as possible)	4,00	0,426	4,60	0,548	−2,72	**0,012**
Efficiency (maximizing production at minimum cost)	4,04	0,475	5,00	0,000	−4,44	**0,000**
Product quality (food safety, attractiveness)	4,30	0,470	5,00	0,000	−3,26	**0,003**
Organic production	4,22	0,518	4,40	0,548	−,71	0,486
Sustainable cooperation with my partners in the chain	4,26	0,449	4,60	0,894	−1,27	0,216
Fair prices	4,35	0,487	4,80	0,447	−1,90	0,068

Mean-arithmetic mean, SD-standard deviation, t-realized value of Student t test, p-probability of rejecting the null hypothesis with a risk of 5%

The comparison of dairies and distributors is shown in Table 5, which clearly shows that the student's t-test did not prove statistically significant differences (at a demanding significance level of 0.05) between the mentioned participants in the value chain of dairy products, when it comes to the importance of business goals for their business. Nevertheless, it can be noticed that dairies attach more importance to all the stated business goals (growth, response, efficiency, product quality, organic production and fair wages, fair prices) compared to distributors, except in the case of sustainable cooperation with chain partners, as a business goal to which distributors attach more importance (4.83) compared to dairies (4.60).

The results shown in Table 5 clearly indicate that product quality comes first for both dairies and distributors, which speaks to their developed awareness of the importance of marketing a quality and safe product that is at the same time attractive to the end consumer. It is interesting that for them, in addition to quality, good relationships with partners in the chain are the most important. They are not focused on organic production or efficiency (distributors), which can make it difficult to green the chain, but they can be drivers of innovation and technical-technological improvement because quality and

Table 5. Mean values of statements of value chain participants, concerning the importance of the stated business objectives (1 extremely unimportant, 5 very important)

Business goals	*Role in the chain*					
	Processors/dairies (n = 5)		*Distributors (n = 6)*			
	Mean	*SD*	*Mean*	*SD*	*tp*	
Growth (increase in market share, sales…)	5,00	0,00	4,50	0,55	2,023	0,074
Response (to meet customer needs as soon as possible)	4,60	0,55	4,50	0,55	0,302	0,770
Efficiency (maximizing production at minimum cost)	5,00	0,00	4,00	1,26	1,752	0,114
Product quality (food safety, attractiveness)	5,00	0,00	4,83	0,41	0,905	0,389
Organic production	4,40	0,55	4,17	0,41	0,811	0,438
Sustainable cooperation with my partners in the chain	4,60	0,89	4,83	0,41	−0,576	0,579
Fair prices	4,80	0,45	4,67	0,52	0,452	0,662

Mean-arithmetic mean, SD-standard deviation, t-realized value of Student t test, p-probability of rejecting the null hypothesis with a risk of 5%

relationship with actors are important, but since there is no communication with farmers, their focus is only on the upper part of the chain.

As can be clearly seen in Fig. 2, the business objectives of the dairy value chain actors are not aligned and complementary. The only thing common to all actors is the lesser importance of organic production for all actors. This indicates that the willingness to innovate (organic production is innovative production) is at a lower level and that they follow a traditional business policy that promotes efficiency as a top goal, and added value for stakeholders is not the focus. What is positive is that for all actors, quality (especially safety) is very important. Of course, this is a reflection of the nature of the products, i.e. their perishability and daily consumption. It should be emphasized, however, that safety is a prerequisite for marketing the product. A product that does not meet the requirements of the legislator regarding health safety cannot be placed on the market at all. Nevertheless, this may be the basis for further strengthening the value chain. But what is worrying is the difference in the importance of business goals related to efficiency. This is essentially a reflection of the level of knowledge of farmers and distributors and their ability to apply business improvement measures, especially various measures that

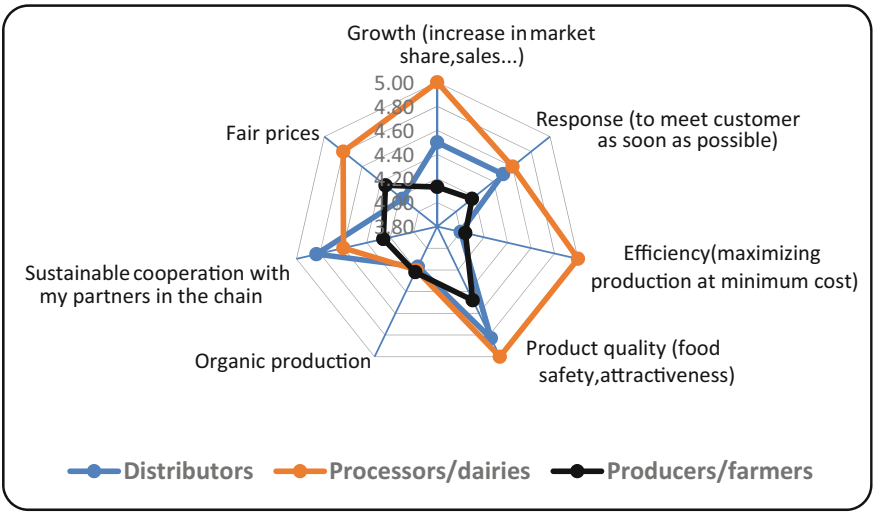

Fig. 2. Business goals of participants in the dairy value chain (1 extremely unimportant, 5 very important)

increase technical and technological efficiency, and thus all processes related to reducing negative environmental impact (especially carbon footprint and energy efficiency).

Coordination of business goals among actors in the value chain was also assessed on the Likert scale of 5 levels, so based on the answers obtained, it can be concluded that dairies believe that business goals among actors in the value chain are coordinated to the greatest extent (3.60), in relation to distributors (3.17) and farmers (3.17), who in relation to them are of the opinion that business goals among the actors are less coordinated.

One of the business objectives of value chain actors is certainly to achieve a fair share of total profits in the chain, and for this reason a question was asked to all actors of the value chain as to whether they believe they have a fair share of total profits in the chain. Distributors are of the opinion that they have a fair share of total profit, while dairies are divided on this issue, so 60% of them think that they have a fair share of total profit in the chain, while 40% do not think they have a fair share of total profit in the chain. Also, a divided opinion on this issue is shared by farmers, so 56.52% of farmers believe that they have a fair share of total profit in the chain, and 43.48% of farmers do not think that they have a fair share of total profit in the chain. This is an important weakness of the chain and essentially confirms the lower level of trust of farmers towards other actors in the chain. Distrust and dissatisfaction expressed in this way do not motivate farmers to expand production or improve productivity.

Dairies who feel that they do not have a fair share of the total profit in the chain, stated the following when it comes to profit:

"Most of the profits go to large shopping centers, which are constantly putting pressure on additional rebates."

"Most of the profits go to large retail chains that set high product margins."

It is important to emphasize that mostly smaller dairies were included in this research. The largest of them has a market share of 8.21%.

Farmers who think that they do not have a fair share of the total profit in the chain, stated some of the following statements:

"Dairies take most of the money".

"The state takes the most money from farmers."

"A huge amount of money goes to VAT, and incentives from the state are minimal."

"The biggest profits are made by retail chains, and we farmers get only a small part."

Several farmers, when asked where most of the profits go, said they did not know.

The results of the survey clearly show that the actors of the value chain do not agree with each other and that there is no alignment of business goals. Namely, differences in the understanding of the importance of business goals and in the approach to business show that there is no communication and that relations are "superficial", as well as that they do not focus on the development aspects of the value chain. All of the above is a big problem and it makes sense that the actors of the value chain are not customer-oriented.

4 Conclusion

Based on all the above, we can conclude that the value chain of dairy products is the result of spontaneous market developments and that there is little or no activity aimed at coordinating and organizing chain actors to improve business, strengthen the ability to create added value and follow market trends. The chain is "old-fashioned" and the actors do not understand that cooperation will bring them more. The actors of the chain are still self-centered, so farmers are focused on fair prices, and dairies are still promoting a culture of "business secrets", i.e. they are not ready for dialogue and joint coordination of business activities. Low efficiency, low level and frequency of information exchange and focus on the exchange of information that is not aimed at the transfer of good practices, and inconsistency of business goals prevent the setting of modern business practices that focus on social responsibility and could contribute to all actors in the chain clearly distancing on the market and to build specifics that will ensure the further development of all actors, and thus the agri-food sector. Based on this, we can say that the hypotheses set out in this study: "(I)" The value chain is not efficient enough because the exchange of information between participants value chain is not adequate ", and (II)" The value chain is not efficient enough because the business goals of the participants in the value chain are not harmonized", they accept.

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Food Forensics - A Case of Honey

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Abstract. As the population increases, the need for food increases too. Therefore food producers strive to produce as much food as possible with small investments. Because of that, there is a lot of food in market with non-nutritive components, as a cheap substitute for original, healthier food products. The term food forensics involves the possibility of using innovative scientific methods to verify the authenticity and traceability of foodstuffs. It's necessary to differentiate between authentication and traceability. Authentication is a procedure which is used to verify certain properties declared on a product and makes it possible to ascertain if a product is counterfeit. Traceability is a procedure used to check the connection between food and raw materials used in it's production. Also, traceability allows tracking a product through certain stages of production, processing and distribution. Food products must meet certain conditions before they can be placed on the market, and in order to check whether certain conditions are met, controls are carried out using appropriate methods. As consumer awareness grows, their demand for food, which is characteristic for specific areas, is growing. Because of that, products contain a designation of geographical origin, which is also a proof of quality that product. The most used analytical methods in food forensics are: biochemical, molecular genetic, spectroscopic, spectrometric, and separation methods. To make it easier for forensics to detect counterfeit products, they need to know the products that are usually counterfeited and the methods that can be used for that purpose. Honey is one of the most commonly counterfeited products. Adding sugar directly into honey is the most common way to counterfeit honey, which affects the authenticity of this product. In this project, the presence of sugar in honey will be determined experimentally using a modified iodometric method according to Luff Schoorl.

Keywords: Authentication · Traceability · Food forensics · Counterfeited · Analytical methods · Honey

1 Introduction

Modern food production is constantly evolving and offering a variety of food products to consumers. However, as the food market generates large revenues, food has become a suitable target for counterfeiting. Food fraud has increased in the last few years because of supply chain complexity, and consumer demand for high-quality food [1]. Consumers demand reliable information about the food they consume, while food producers need

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to protect themselves from competition [2]. The geographical origin of food can be marked with two types of protected designations: The Protected Designation of Origin (PDO) and the Protected Geographical Indication (PGI). Products that have a protected geographical indication for consumers create the belief that these are products with special properties, quality and authenticity. Because of that information consumers will always pay more. In that way a mark of origin for consumers becomes synonymous for products with quality assurance [3]. Food forensics is a scientific discipline that allows you to check whether a food is authentic or not. As the notion of traceability is usually associated with authentication, it is necessary to differentiate between these two terms, because there is a certain difference between authentication and traceability. Authentication is a procedure which is used to verify a certain property declared on a product, and allows to determinate whether the product is counterfeit, while traceability is a procedure which is used to verify the relationship between food and the raw materials used in the production. Traceability makes it possible to track a product through certain stages of production, processing, and distribution [4]. Forensic sciences are often used to investigate and solve a crime, which is the first association when you hear term forensic. This scientific discipline investigates "crimes" related to food counterfeiting where cheap substitutes for original, healthier food products are used [5].

The term food forensics has been used to solve the problem of unauthorized behaviour and counterfeiting of food, also to solve the problem of mislabeling or fraud, and other issues related to the safety and authenticity of food products and ingredients [6]. Geographical origin, counterfeiting, and contamination of food products can be tested using modern analytical methods. The main goal of „food forensics" is to check the correctness of the properties of food indicated on the product, and to detect the possibility of counterfeiting food products [7]. In the European Union, before food is placed on the market, it is checked according to the prescribed food safety controls. Therefore, food prepared for the market must perform certain standards, and in order to check certain standards, controls are carried out using appropriate analytical methods.

Analytical methods used in food forensics are divided into four groups:

- biochemical,
- molecular genetic,
- spectroscopic and spectrometric, and
- separation methods.

The appropriate analytical method is selected based on several factors, such as: precision, accuracy, repeatability, boundary detection and quantification, selectivity and working environment [8].

In food forensics, biochemical methods are often used to detect immunoenzymatic reactions during the examination of a certain substance. Biochemical methods include: enzyme-linked immunosorbent assay, immunochromatographic assay, and Western blot analysis or protein immunoassay. The use of enzyme-linked immunosorbent assay is very widespread in food forensics, where it is used to determine food allergens such as peanuts, hazelnuts, eggs, milk, and to determine contamination by microorganisms and antibiotics. Immunochromatographic tests are used to determine allergens in food, as

well as to determine pathogens and mycotoxins in food. Western bolt analytical analysis, also called protein immunoassay, is used to detect a specific protein in a sample [9].

DNA-based analytical methods have developed rapidly in recent years, and they are very important in food forensics. Most DNA-based methods rely on the polymerase chain reaction technique because of their sensitivity, simplicity, and specificity, which allows the identification of certain components even in complex and processed food [10].

Molecular genetic methods are based on the analysis of the deoxyribonucleic acid molecule, so polymerase chain reaction - PCR is mainly used for DNA analysis. In addition to PCR, numerous modifications are used as well as electrophoresis in a crosslinked polyacrylamide gel polymer. Using modified PCR methods, the proportion of selected genes in foods can be determined from a variety of animal and plant species, so competitive PCR and qPCR in real time are used for such quantitative measurements [9].

Methods based on the physical properties of the DNA sequence are used to determine the genotype of the species present in the product, i.e. to determine the authenticity of the food. Three approaches are most commonly used, namely: determination of a single nucleotide polymorphism (SNP), determination of a variable number of consecutive repeating sequences, and the use of the restrictive fragment length polymorphism method. The SNP analysis determined the traceability and authenticity of olive oil, as well as the origin of beef and pork, while the RFLP method determined the authenticity of fish and seafood meat, as well as the proportion of fruit in jams. Molecular genetic methods include the electrophoretic method, and they are used to analyze microbial communities present in food.

Spectroscopic methods used in food forensics include: mass spectrometry, nuclear magnetic resonance, ultrasound spectroscopy, infrared spectroscopy, fluorescence and vibrational spectroscopy. By mass spectrometry it is possible to separate and analyze many complex compounds in a short period of time, and data on the compounds present in the food can also be obtained. Mass spectrometry is often used in combination with liquid and gas chromatography. The combination of mass spectrometry with liquid and gas chromatography is often used and is a very powerful tool in food forensics. Mass spectrometry to determine the proportion of isotopes - IRMS is used to determine the geographical origin of foods such as honey, potatoes, dairy products, as well as to verify the authenticity of fruits and vegetables, wine, whiskey, honey and fruit juices. Spectroscopic methods include the method of nuclear magnetic resonance and in food forensics it is used to determine the geographical origin, as well as the sensory's properties of certain grape and wine varieties, the quality of olive oil, dairy and meat products. Infrared spectroscopy can determine the functional groups of molecules in a particular food. If all the functional groups of a molecule in a particular food are taken into account, this method can provide a "stamp" of the target molecule or macromolecule, and this stamp is used in food forensics to determine the identity of molecules present in food. Fluorescence spectroscopy is a useful method that can be performed quickly, with very short sample preparation and is often used to determine the botanical and geographical origin of honey, then used to analyze and determine the forgery of olive oil, as well as to

determine the proportion of muscle, fat and connective tissue in meat products. Vibrational spectroscopy is also widely used as a fast and inexpensive method for assessing the quality and authenticity of food [9].

Separation methods include: gas chromatography, liquid chromatography and capillary electrophoresis. Chromatographic methods are one of the most important methods used in food analysis. These methods are based on the adsorption or distribution of analytes between the mobile and stationary phases, and they are classified depending on the form of the stationary phase, stationary and mobile phase. Gas chromatographic methods have a gaseous mobile phase, while liquid chromatographic methods have a solid stationary phase applied in a column and a mobile phase pumped through the column. Gas chromatography is a separation method used to analyze and quantify volatile compounds. Gas chromatography is often used in combination with mass spectrometry. The advantages of gas chromatography are high capacity, repeatability, sensitivity, as well as the speed of performing this method. Some of the newer applications of gas chromatography in food authentication are the analysis of triglycerols in dairy products, determination of fatty acid markers and the like. Liquid chromatography is a separation method used to separate substances based on the distribution between the mobile and stationary phases. High performance liquid chromatography is often used to determine individual food ingredients. Polar and non-polar compounds can be analyzed by HPLC, so HPLC is often used to determine the authenticity of food products [7].

Capillary electrophoresis is a separation method and is often used in food forensics. It is used to determine the geographical origin of wine by determining carbohydrates, organic acids, inorganic anions and cations, then to determine the botanical origin of honey by determining phenolic compounds, to determine the geographical origin of honey by determining sugar, then to determine olive variety by determining protein. Also, in food forensics, capillary electrophoresis is used to determine metal cations such as calcium, potassium, magnesium in milk and dairy products, as well as to determine the presence of melamine in milk and dairy products, as well as in ice cream and eggs. All these methods have certain advantages and disadvantages, but if they are used in combination with other methods, e.g. mass spectrometry in combination with gas or liquid spectrometry can provide accurate and precise data on each food product. Thanks to the described methods, forensic scientists can determine whether the food produced complies with the stated declaration and whether it complies with legal regulations [9]. In order to facilitate the identification of the non-nutritive and illicit components present in the product, it is necessary to know the products which are most often counterfeited as well as the analytical methods which can be used for that purpose. Among the products that are most often counterfeited is honey. Counterfeiting honey with the addition of sugar syrups of various origins is the most common type of fraud, but also the biggest problem that affects the authenticity of honey. Honey can be counterfeited with cheaper sweeteners such as corn syrup (CS), invert syrup (IS), and glucose-fructose syrup (HFC). Honey counterfeiting with sugar syrup is used to increase volume and can be direct or indirect. Direct counterfeiting involves the addition of foreign components to honey, most commonly sugar syrups, while indirect counterfeiting involves feeding bees to improve yields and is very difficult to detect. The authenticity of honey with regard to geographical

origin is important to determine, so that the consumer has reliable information about the origin and quality of honey [9].

Various chromatographic methods are used to determine the identity of honey of different floral origin, as well as to detect the presence of added sugars. High performance liquid chromatography-HPLC and gas chromatography are used to quantify glucose, sucrose and fructose. Biomolecular methods are increasingly used to verify the authenticity of honey. Gas and liquid chromatography in combination with mass spectrometry are used to separate and identify volatile and semi-volatile components in honey [11]. In addition to the methods above that can be used to determine the authenticity of honey, as well as to verify whether honey is counterfeit, it is also possible to use other methods that are not so sophisticated and do not require modern laboratories and equipment. In this paper, a modified Luff Schoorl iodometric method was used to examine the possibility of honey forgery.

2 Materials and Methods

2.1 Materials

For this research 5 samples of honey were collected from the territory of Bosnia and Herzegovina. The samples were submitted to the Faculty of Agriculture and Food, University of Sarajevo, where the experimental part of the work was carried out. All chemicals were obtained from commercial sources and used without further purification.

2.2 Method

Determination of sugar content was performed by a modified luff schoorl iodometric method. The principle of this method is that the reducing sugar (natural invert) converts Cu^{2+} ions into Cu^{+} ions. Unused amount of Cu^{2+} is re-titrated with a standardised thiosulfate solution. The difference between reagent volumes used for the blank and the sample coresponds to a certain sugar amount given in the table. The non-reducing disaccharide (sucrose) must first be inverted, ie hydrolyzed to reducing monosaccharides by acid, and then determined by copper sulphate solution. In this way, data on the total amount of sugar in the tested sample are obtained. The difference between the obtained total invert and the natural invert gives the amount of reducing sugars formed by the inversion of sucrose.

2.3 Determination of Directly Reducing Sugars

About 2 g of honey was dissolved with 20 mL of warm deionised water. The pH was adjusted to 7 with a 15% Na_2CO_3 solution. The solution was boiled for 5 min to destroy the enzymes. About 1 g of celite was added to remove the proteins. The percipitate was washed with deionised water and filtered. The filtrate was made up to 250 mL.

Determination of directly reducing sugars is done immediately. 7 cm^3 of the filtrate was pipetted off into a 250 or 300 cm^3 Erlenmayer flask, then 10 cm^3 of Feling I and Feling II solution was added, and deionized water so that the volume is 50 cm^3. The

Erlenmayer flask with the solution is heated over an asbestos mesh, with such a suitable burner until the precipitatation of the red Cu_2O ceases (an electric stove was used instead of a burner). The solution was then cooled rapidly to 20 °C, 25 cm^3 of 20% KJ, 10 cm^3 of 25% H_2SO_4 was added and, with constant stirring, it was immediately titrated with 0.1 M $Na_2S_2O_3$ until a very slight yellow color. Towards the end of the titration, a few drops of starch solution are added and carefully titrated until the color changes, ie. the disappearance of the blue hue. A blank test is performed in parallel (all solutions except the solution in which the sugars are determined).

2.4 Total Invert Content Determination

50 cm^3 of the basic filtrate was pipetted into a 100 cm^3 volumetric vessel, a little deionized water and 0.5 cm^3 of concentrated HCl were added and heated for 30 min to boiling. After inversion, the sample was cooled and neutralized by adding NaOH dropwise and made up to 100 cm^3 with deionized water. It is best to have a pH of 6.00–6.50. When the contents have been neutralized, the samples are left overnight. The further procedure is the same as for the determination of direct reducing sugar [12].

3 Results and Discussion

For this research, 5 samples of honey from the territory of Bosnia and Herzegovina were collected. Analyzes were performed on the collected honey samples to determine the content of reducing sugars (glucose and fructose), and the content of sucrose. 2 replicates were performed for each sample, so that based on the obtained data for the content of reducing sugars and sucrose we could conclude whether there is a possibility of counterfeited honey, or whether the product meets certain quality requirements according to the Ordinance on honey and other bee products. The results obtained are shown in the Table 1.

Table 1. Content of reducing sugars and content of sucrose in samples

Samples	Content of reducing sugars (g/100g)	Content of sucrose (g/100g)
2	74,75	**11,02**
3	76,98	1,53
4	66,68	**16,41**
5	67,64	4,27
6	75,98	1,95

The content of reducing sugars ranged from 66.68–76.98 g/100 g. The lowest content of reducing sugars was in sample 3 and amounted to 66.68 g/100 g, while the highest content of reducing sugars had sample 2, and was 76.98 g/100 g. According to the

Ordinance on honey and other bee products, the content of glucose and fructose in meadow honey must not be less than 60 g/100 g [13]. Based on the obtained results, it can be concluded that the content of reducing sugars in all samples is in accordance with the Ordinance on honey and other bee products.

According to the Ordinance on honey and other bee products, the sucrose content in meadow honey must not exceed 5 g/100 g [14].

Based on the obtained results, it can be concluded that the allowed sucrose content was exceeded in two samples (sample 1 and sample 3). The sucrose content in sample 1 is 11.02 g/100 g, while in sample 3 the sucrose content is 16.41 g/100 g, which does not correspond to the prescribed values. Increased sucrose content in honey can be the result of counterfeiting honey with invert sugar (increasing the sucrose content) or counterfeiting with table sugar sucrose (direct addition to honey or feeding bees). [14]. Sample 2 has the lowest sucrose content of 1.53 g/100 g, followed by sample 5 containing 1.95 g/100 g of sucrose, and sample 4 containing 4.27 g/100 g of sucrose, so these samples are in in accordance with the Ordinance on honey and other bee products.

Comparing the samples 1 and 3 whose sucrose contents exceed the maximum content allowed by the Ordinance, it can be concluded that sample 3 shows a higher degree of counterfeiting given that it also has the lowest content of reducing sugars.

4 Conclusion

In this study, a simpler, modified Luff Schoorl iodometric method was used to test for the presence of sugar in honey samples. The content of reducing sugars in honey according to the Ordinance on honey and other bee products for meadow honey mustn't be less than 60 g/100 g. The content of reducing sugars in all tested samples met the quality requirements according to the Ordinance, and ranged from 66.68–76.98 g/100 g.

The sucrose content according to the Ordinance on honey and other bee products must not exceed 5 g/100 g. In the examined samples, the allowed sucrose content was exceeded in two samples. Sample 1 and sample 3 showed a higher sucrose content than allowed by the Ordinance, where the sucrose content of sample 1 was 11.02 g/100 g, while the sucrose content of sample 3 was 16.41 g/100 g. Other samples are in accordance with the Ordinance on honey and other bee products and meet quality requirements.

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Heat Treatment Influence on the Content of K, Mg, Fe, Mn, P, Zn in Chicken Meat

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Summary. Meat is a major source of minerals in the human diet. The mineral content in meat is variable and depends on various factors such as animal breed, nutrition and processing. The amount of mineral substances ranges from 0.8% to 1.0%. Sufficient quantities of minerals present in chicken meat are: phosphorus, iron, zinc, potassium, magnesium and manganese.

The aim of this paper is to determine the influence of heat treatment regime on the content of essential minerals in chicken meat products.

The paper analyzes four experimental groups of boiled chicken meat samples. During the experiment, the following parameters were varied: temperature 55–75 °C, boiling time 80–107 min and relative humidity 74–86%. Samples were prepared by microwave oven digestion. Analysis of mineral content was performed using atomic absorption spectrophotometry (ICP OES technique). The content of the following elements was determined: K, Mg, Fe, Mn, P and Zn.

The mineral content in the finished products had the following values: K 460.27–486.27 mg/kg, Mg 47.12–51.53 mg/kg, Fe 0.67–0.74 mg/kg, Mn 2.83–3.11 mg/kg, P 410.15–430.77 mg/kg and Zn 1.21–1.26 mg/kg.

Change in the heat treatment parameters affected the content of the tested elements. In all finished product samples, the content of mineral substances was higher in relation to the raw meat samples. Statistical analysis of the results showed no significant differences in Fe, Mn and Zn content in the final products, while the content of K, Mg and P differed significantly depending on treatment.

Keywords: Heat treatment · Chicken meat · Mineral substances

1 Introduction

Meat is known as an excellent source of essential trace elements such as iron (Fe), zinc (Zn), selenium (Se), vitamins A, B12, and folic acid [1–3]. They are important for the human body as the body cannot create them on its own. Many functions of the human organism depend on these nutrients [4]. Meat is an important source of several micronutrients due to the fact that some of them are exclusively present in meat or their bioavailability is much higher than in plant sources [5, 6]. Many of the minerals contained in meat are vital to the human body and participate in its various significant functions

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[7]. Poultry meat is very popular in consummation. The reasons for the increase in the production and consumption of chicken meat are the strong influence of the knowledge about its nutritional value, nutritional properties and lower cost price. Chicken meat is considered a healthy dietary choice as it has a lower fat content as well as a higher polyunsaturated fatty acid content compared to other types of meat [8]. It is characterized by a high content of proteins and vitamins [9]. It is a biologically valuable food with a favorable amino acid composition, low fat content and high digestibility. Chicken meat is a good source of minerals and vitamins. Compared to red meat (except pork) it contains more calcium, magnesium, phosphorus and sodium. The iron content is almost the same as in pork [10].

A large number of studies have been conducted on the content of mineral substances in food and meeting the nutritional needs of the population in certain regions in the country and the world [11–13]. The amount of mineral substances ranges from 0.8% to 1.0%. Minerals present in chicken meat in sufficient quantities are: phosphorus, iron and zinc, potassium, magnesium and manganese.

Heat treatment can be defined as a physical method of food preservation that uses high temperature to destroy microorganisms, inactivate tissue enzymes and make the raw material edible, digestible and microbiologically safe [14, 15]. Heat treatment plays an important role in ensuring the digestibility, sensory properties and safety of meat and meat products [16]. The heat treatment results in an increase in the temperature of the raw material, as well as chemical and biochemical changes that lead to structural changes [15].

Heat treatment of the raw material (product) results in appropriate sensory properties (taste and texture) in food [13].

Heat treatment processes affect the concentration (increase or decrease) of some minerals in the samples as shown in the research of the following authors [15, 16]. In some cases, boiled beef meat showed lower concentration of K, and higher concentrations of Ca, Cu, Fe, Mn and Zn, compared to fresh samples [15]. Higher temperatures increase the content of these nutrients, probably due to the evaporated water what causes the minerals to concentrate.

This research is focused on the influence of the modified heat treatment method (as a part of industrial production) on the content of mineral substances (K, Mg, Fe, Mn, P and Zn) in chicken meat.

During heating, heat penetrates the raw material/product, after which the product becomes digestible and microbiologically acceptable [17, 18]. During heat treatment, the raw material (product) changes, which results in appropriate sensory properties (taste and texture) in food [19].

This research is focused on the influence of the modified method of heat treatment (as a part of industrial production) on the content of mineral substances (K, Mg, Fe, Mn, P and Zn) in chicken meat.

1.1 Materials and methods

Sample Peparation

During the research in this study, 48 chickens fattened for 42 days were used, after which the slaughter process was approached. After slaughter, the meat samples (breast meat)

were cooled for 24 h at a temperature of about 1 °C. After cooling (temperature < 4 °C in the sample center), the pieces of breast meat were brined. The brine mixture consisted of sodium chloride (2.5%) and nitrite salt (0.3%). Pepper and garlic were used as spices. The brining process was carried out under controlled conditions: temperature 2–4 °C, duration 10 days. From the chicken meat samples, 48 pieces of meat were divided into 4 experimental groups (12 samples each). For each experimental group, the process parameters of heat treatment were defined and determined separately, as stated in Table 1 (Universal Thermodynamic Chamber manufactured by Doleschal, Austria).

After the heat treatment, the products were vacuum packed, using plastic bags impermeable to air and water vapor. The products were stored at 1–2 °C. Before the analysis, four random meat samples were taken from each experimental group for further analysis.

Table 1. Heat treatment parameters used during thermal treatments process of chicken meat

Thermal treatments		*Temperature (°C)*	*Time (hours, minutes)*	*Air flow (m^3/min)*	*Relative humidity (%)*
Experimental group I (EG I)	I drying	65	20 min	7.1	74–76
	I smoking	70	5 min	7.1	74–76
	Boiling	72	30 min	7.1	74–76
	Roasting	75	10 min	7.1	74–76
	II smoking	70	15 min	7.1	74–76
Experimental group II (EG II)	I drying	65	20 min	9.7	78–82
	I smoking	65	5 min	9.7	78–82
	Boiling	72	35 min	9.7	78–82
	Roasting	75	10 min	9.7	78–82
	II smoking	65	20 min	9.7	78–82
Experimental group III (EG III)	I drying	65	20 min	9.7	78–82
	I smoking	60	5 min	9.7	78–82
	Boiling	72	40 min	9.7	78–82
	Roasting	75	10 min	9.7	78–82
	II smoking	60	25 min	9.7	78–82
Experimental group IV (EG IV)	I drying	65	20 min	12.7	82–86
	I smoking	55	5 min	12.7	82–86
	Boiling	72	42 min	12.7	82–86
	Roasting	75	10 min	12.7	82–86
	II smoking	55	30 min	12.7	82–86

Heat treatment default combination of heat treatment by drying, smoking, boiling, frying, and smoking of the samples in the four experimental groups lasted for some time at the appropriate temperature as indicated in Table 1.

Mineral Content Analysis

The mineral content was carried out in the Laboratory for Chemical Safety of Products, Faculty of Technology, Banja Luka. Samples of fresh meat and heat-treated meat were thawed (temperature 4 °C), minced and homogenized in a homogenizer with rotating stainless steel knives (Tecator 1094; Foss, Hillerod, Denmark). All meat samples were microwave digested in duplicate, and ICP OES analysis was performed three times in each digest (duplicate). The sample digestion procedure was performed in accordance with NF EN 13805 standard»Food-Determination of trace elements-Digestion under pressure. The obtained solutions were used to determine the concentration of the analyzed elements (K, Mg, Fe, Mn, P and Zn). The Optima 8000 Optical Emission Spectrophotometer (ICP OES) instrument, Perkin Elmer, USA, was used to determine the mineral content in samples of fresh meat and chicken meat products.

Statistical Analysis of Results

The difference between treatments was examined using a one-factor analysis of variance of different groups (ANOVA), which compared the average values of these groups for one continuous variable. For all measurements, a mean value and standard deviation (SD) were calculated by descriptive statistical analysis. All statistical analysis was performed in the statistical program IBM SPSS, Statistics 26.

2 Results and discussion

Table 2 shows the results obtained by determining the content of mineral substances in four experimental groups of heat-treated chicken in industrial conditions, when changing different parameters (temperature, time, relative humidity). During the digestion process, the content of individual elements changed depending on the process and the experimental group. Measured average values of K, Mg, Fe, Mn, P, Zn concentrations were the highest in experimental group III (smoking/boiling, temperature 60–75 °C, time 100 min and relative humidity 78–82%) and the lowest in EG I (smoking temperature 65–75 °C, time 80 min and relative humidity 74–76%).

In fresh samples of all experimental groups the mineral content was similar, while in the digestion process the values for all elements were higher.

The results show the highest average potassium content (K) measured in EG in fresh meat samples, and the lowest in EG III. The results also show the highest average magnesium content (Mg) measured in EG IV in fresh meat samples, and the lowest in EG II. The highest average value of iron (Fe) content was measured in EG II, while the lowest was measured in EG III. The highest average value of manganese content (Mn) was measured in EG I, and the lowest in EG II. The measurement results show that the highest average value of phosphorus (P) content in EG I, and the lowest P content in EG IV. The measurement results show that in fresh meat samples the highest average value of zinc (Zn) content was measured in EG III, while the lowest Zn content was measured in EG I.

In heat treated meat samples, the highest average value of K content was measured in EG III, and the lowest in EG I. The highest average value of Mg was measured in EG III, and the lowest in EG I. The highest average value of Fe content was measured in EG III, while the lowest average value was measured in EG I. The highest average value of Mn was measured in EG III, and the lowest in EG I. The highest average value of P was measured in EG III, and the lowest in EG I. The highest average value of Zn was measured in EG III, while the lowest values of Zn were measured in EG IV.

Table 2. Descriptive indicators of mineral substances in fresh and heat treated chicken meat

Eksperimental group	*Meat samples*	*K (mg/g)*	*Mg (mg/g)*	*Fe (mg/g)*	*Mn (mg/g)*	*P (mg/g)*	*Zn (mg/g)*
Eksperimental group I	Raw	272.79 ±6.00	28.01 ±1.75	0.38 ±0.02	1.67 ±0.07	252.18 ±12.55	0.01 ±.002
	Heat treated	460.27 ±10.83	47.12 ±1.45	0.67 ±0.09	2.83 ±0.15	410.15 ±2.04	1.24 ±0.06
Eksperimental group II	Raw	275.43 ±4.81	26.82 ±2.05	0.38 ±0.03	1.62 ±0.02	241.84 ±13.11	0.02 ±0.003
	Heat treated	478.60 ±6.65*	50.09 ±2.23*	0.71 ±0.05	3.06 ±0.21	417.65 ±4.72*	1.26 ±0.06
Eksperimental group III	Raw	270.30 ±9.37	28.02 ±1.62	0.34 ±0.06	1.65 ±0.05	246.51 ±5.77	0.021 ±0.002
	Heat treated	486.27 ±11.11*	51.53 ±3.27*	0.74 ±0.02	3.11 ±0.26	430.77 ±9.7*	1.31 ±1.62
Eksperimental group IV	Raw	273.36 ±3.39	28.99 ±1.03	0.36 ±0.04	1.66 ±0.06	240.47 ±5.98	0.02 ±0.002
	Heat treated	462.41 ±9.52	48.70 ±1.78	0.68 ±0.07	2.84 ±0.15	411.50 ±6.32	1.21 ±0.11

Legend:$\bar{x}$-Mean, *statistically separated group variance.

The influence of treatment within the experimental groups on the content of K, Mg, Fe, Mn, P, Zn in the sample of boiled chicken meat was investigated by one-factor analysis of variance. It was shown that there is no statistically significant difference ($p > 0.05$) between the mean values of Fe, Mn and Zn in samples of boiled meat between experimental groups, while for other elements a statistically significant difference was found, and the experimental group was separated on the basis of maximum value. As previously mentioned, during the boiling process, the maximum value for all tested elements in chicken meat was in EG III (boiling temperature 60–75 °C, time 100 min). The lowest average values of the elements K, Mg, Fe, Mn, P were recorded in EG I (boiling temperature 65–75 °C, time 80 min), while for Zn the lowest average value was determined in EG IV.

In fresh samples all experimental groups show similar mineral content, while when the digestion processes the values for all elements were higher in relation to the mineral content in fresh samples.

Several studies have addressed the impact of heat treatment processes on the mineral content of meat. As the authors: Rekanović et al. [19] Gerber et al. [20], Tomović et al. [21] and Campos et al. [22] stated in their research, the temperature and boiling time significantly affect the mineral content, where the values for all elements in the cooking process were higher than the mineral content in fresh meat. The obtained average values of minerals in fresh samples were lower compared to the values obtained by Mendes et al. [23] in the mineral composition of fresh and boiled chicken meat, followed by the values in Balouchi lamb examined by Norouzian and Ghiasi [24].

In some studies, the values of certain elements such as Ca and Mg were analyzed. The values for trace elements Fe, Zn, Cu, Mn, Se during digestion obtained in this paper were similar to the values obtained by Lucarini et al. [25] in Italian ham but less in terms of values than reported by Jimémez-et al. [26] for Iberian prosciutto. Purchas et al. [27] compared the mineral content in fresh and cooked lean beef, the results show a decrease in Na and K content and an increase in Ca, Cu, Fe, Mn and Zn in cooked compared to the values in raw meat.

These results show that divalent minerals are better retained during cooking than Na and K. This may be because their greater binding to proteins prevents a decrease in the content of divalent minerals during cooking.

Also, the results of increasing and/or decreasing mineral content in fish samples were found [28, 29]. Baking process increases the content of K, Ca, Zn and Cu in the fish. Although the authors did not explain the differences in mineral content in the muscle tissue of fish cooked by different methods, mineral gain may result from water loss that occurred during cooking, or possible migration of such elements from the chambers used to cook fish. Heat treatment increased the values of these elements, especially by the boiling process, probably due to evaporated water causing the concentration of water-soluble minerals [30].

3 Conclusions

From a nutritional point of view, different meat processing had affected the content of significant mineral nutrients. Heat treatment affected the concentration of all elements whose content was higher than their content in fresh samples.

Technological processes of digestion affect the content of K, Mg, Fe, Mn, P, Zn in chicken meat. By increasing the temperature and prolonging the time during the digestion process, the value of these minerals increases. In conditions of higher boiling and smoking temperatures there is an increase in the content of mineral substances in the samples of finished products, although the values obtained in the mentioned treatment of Fe, Mn and Zn did not have statistically significant values ($p > 0.05$) in relation to K, Mg, P, whose values stood out significantly.

Treatment (EG III) with elevated temperatures (60–75 °C) and prolonged boiling time (100 min) based on the obtained results for the tested minerals proved to be optimal and favorable for the technological process of heat-treated chicken meat. These results

can be a recommendation for producers to choose the most efficient way of cooking meat to maintain or improve its nutritional quality.

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Do School Menus in Zagreb Municipality Offer Enough Fruits and Vegetables?

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Abstract. Adequate consumption of fruits and vegetables (FV) can have a positive effect on health and World Health Organization (WHO) recommends to eat at least 400 g of FV to achieve this. In Croatian schools, children from 1st to 4th grade can stay up to 8 h in school, therefore, school menus should offer an adequate amount of FV to help children reach the recommended daily intake. The aims of this study were to evaluate whether school menus follow the recommendation for the daily amount of FV, to estimate which meals contain the most FV, and to determine which FV are most commonly offered in school menus. Quantity and frequency of FV (fresh, frozen and canned FV except potatoes, dried legumes and nuts) was estimated in annual schools' menus from 14 primary schools in the Zagreb municipality. The WHO recommendation was proportionally adjusted according to number and meal type towards Croatian national guidelines. School menus offer on average 62% of the recommended amount of FV daily. However, there was a great variability between schools (from 33% to 149%). The FV was served within 97% of lunches, 54% of breakfast and 27% of snacks. Annually school menus offered 97% fresh/frozen fruits and 82% fresh/frozen vegetables. The most frequently-served category of vegetables was bulbous (40%) and of fruits was pome (47%). School menus do not meet the recommendation for daily serving quantities of FV. It is necessary to increase the availability and diversity of FV in school menus.

Keywords: Frequency · Fruits · School menus · Vegetables

1 Introduction

It is well known that diet has a significant impact on health. Particular focus is placed on adequate intake of fruits and vegetables (FV), as they provide fiber, micronutrients, and phytochemicals [1]. Moreover, adequate intake of FV is associated with a healthy lifestyle [2]. Accordingly, the recommendation of the World Health Organization (WHO) is to eat at least 400 g FV per day to maintain health and reduce the risk of non-communicable diseases [1].

Nowadays, a dietary shift is observed in countries around the world, with an increase in the consumption of processed foods high in salt, sugar and fat, and a decrease in the

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consumption of unprocessed foods such as FV [3]. In line with this observation, results from WHO European Childhood Obesity Surveillance Initiative show that only 42.5% of children consume fruits and 22.6% of children consume vegetables daily [4]. Such inadequate eating behaviors in childhood may continue into adulthood [5] and increase the risk of developing obesity in adulthood [6]. Therefore, children must be provided with an environment in which they develop healthy eating behaviors [7].

Apart from the family environment, schools provide an important environmental setting which can support activities that promote healthy behavior, e.g. healthy nutrition and physical activity [7, 8]. To promote healthy dietary behavior, different stakeholders should be included in the process of implementation of school curriculum activities and organization of school food service. Also, according to the school food system, nutritional policies should be applied to best support healthy behaviors that are maintained for the proper growth and development of school-age children [8].

In Croatia, the Act on Education in Primary School and Secondary Schools [9] and the National Pedagogical Standard for Elementary Education [10] form the legal basis for the organization and functioning of the school food system. In accordance with these documents, primary schools are obliged to organize school meals for children according to the standards prescribed by the Ministry of Health. A working group of the Ministry of Health has developed *National guidelines for school meals for children in primary schools* in order to improve the quality of nutrition in schools [11]. Therefore, the national guidelines include information on the organization and management of school catering, the definition of public procurement contracts, nutrition recommendations, preferred food preparation methods, practical guidance on nutrition planning and menu design. In national guidelines, nutrition recommendations include reference values for daily energy, macronutrient, micronutrient, and water intake, as well as information on the number, type, timing, and recommended energy value of meals. School nutrition is organized at the community level, and at Zagreb City the school procurement process involves the schools (principals), the Office of Education, Culture and Sport, and the Office of Public Procurement. Procurement of school meals must be done in accordance with the Law on Public Procurement [12], and the criterion of lowest prices is used. Funds for food procurement are provided from the state budget, the budget of local and regional self-government and from parents. Children from socially disadvantaged families, families without income and children of war veterans have the right to free or subsidized meals. The price of meals is fixed in all public schools in Zagreb City and the budget for breakfast is €0.67 (HRK 5.00), lunch is €1.20 (HRK 9.00) and snack is €0.34 (HRK 2.50). The schools in Zagreb City must provide at least one meal, but for children with day stay (from 1st to 4th grade) schools are obliged to organize the possibility for children to have three meals a day (breakfast, lunch and snack). All students from 1st to 8th grade can consume school meals for which they are registered by their parents one month in advance.

In order to increase the intake of fruits, vegetables and dairy products, schools in Croatia have the opportunity to participate in a School Scheme program in addition to the common meal provision. Participation in the School Scheme is voluntary and financially supported by the European Commission. Participating schools receive a free meal of fresh fruits and vegetables (100–150 g per student) once a week throughout the

school year and dairy products (150–250 ml per student) once a week for 12 weeks in the school year. Participating schools can choose suppliers of fruit (apples, pears, citrus fruits, berry fruits, sweet and sour cherries, plums, peaches, apricots and nectarines), vegetables (carrots, beetroot, celery, tomatoes, radishes and other root vegetables) and dairy products (milk, lactose-free milk, yogurt and other fermented dairy products with no added sugar, fruits, flavorings, walnuts and cocoa) from registered local suppliers [13, 14].

Taking everything mentioned above into account, schools have all the requirements to provide children with the recommended amounts of fruits and vegetables, however a nutritionist is not involved in meal planning, while other school staff are (e.g. the chef, the principal, the secretary or the biology teacher). This means that school nutrition is not under professional supervision, which can lead to poor implementation of national guidelines and consequently inappropriate menu planning [15]. Prolonged inadequate nutrition can lead to impaired growth and development of children, especially those who eat up to three meals at school [1, 2, 8]. Therefore, the objectives of this study were: (1) to assess whether school menus follow the recommendation for the daily amount of FV, (2) to determine which meals contain the most FV, and (3) to determine which FV are most commonly offered in school menus.

2 Methods

2.1 Study Design and Settings

This was an observational study within the pilot project named "Pilot Project: School meals and fruit and vegetable intake in schools with and without a garden", part of the Horizon 2020 project "Strengthening European Food Chain Sustainability by Quality and Procurement" (Strength2Food, H2020-SFS-2015–2, contract no. 678024). The study was performed according to the Declaration of Helsinki and approved by the Ethics Committee of the Institute for Medical Research and Occupational Health (100–21/16–8). The Croatian Ministry of Science and Education and the Education and Teacher Training Agency approved engagement of primary state schools from Zagreb city in the pilot project (602–01/16–01/00388).

The selection of schools was done as follows. Firstly, we approached schools that serve at least one meal per day. Secondly, among these schools were selected those which participated in the School Scheme program to reduce the possibility of bias that may occur due to differences in the ability of fruit and vegetable procurement. Finally, headmasters at 87 public state schools were approached to participate in the project, and 14 (13% of public state schools) headmasters consented to implementation of the pilot project in schools. These schools were located in the city center and suburbs, in more and less affluent areas according to the official poverty rate in the locality of the school.

2.2 Assessment of Quantity and Frequency of Offered FV in School Menus

Quantity and frequency of FV was estimated in the annual menus of all 14 participating schools. A total of 2469 daily menus were collected from the secretariats and/or

finance offices of the schools, while the recipes and standard quantity of ingredients were obtained by directly interviewing the cooks of the schools. The quantity of ingredients per child was calculated from the received recipes and entered into the software "Nutrition" (Infosistem d.d.). Excel spreadsheets were then extracted from the software with annual school menus, from which the quantities and frequencies of fruits and vegetables were filtered for further analysis.

The estimate of FV offered included all fresh, frozen, and canned FV except potatoes, dried legumes, and nuts. The quantity of FV offered was compared with the WHO recommendation of 400 g FV per day per child [1]. The WHO recommendation was proportionally adjusted by meal number offered and meal type (Table 1) to the recommendations from the Croatian national guidelines for school meals for children in primary schools [11]. For frequency analysis, first FV was divided into fresh/frozen and canned FV. Then fresh/frozen FV was divided into categories according to the national guidelines [11].

Table 1. Adjusted quantity of WHO recommended fruit and vegetables toward Croatian national guidelines for school meals for children in primary schools [1, 11]

School food service model	Offered meals	Recommendation for daily offer of fruits and vegetables
Model A	breakfast+lunch+snack	240 g (60% of recommendation)
Model B	breakfast+lunch	200 g (50% of recommendation)
Model C	lunch	140 g (35% of recommendation)
Model D	breakfast	60 g (15% of recommendation)

2.3 Statistical Analysis

Data were analyzed descriptively using SPSS for Windows 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Continuous data were reported as mean and standard deviation and categorical data as percentages.

3 Results

Among all 14 school enrolled in project, 12 of them had the food service model with three meals per day (breakfast, lunch and snack), 1 of them with two meals per day (breakfast and lunch) and 1 of them with two meals per day whereby children consume only one meal (lunch or breakfast) depending on the class shift. In total 2469 daily menus from 14 annual school's menus were collected, within which were 2379 breakfasts, 2376 lunches and 1223 snacks.

3.1 Quantity of Offered FV in School Menus

School menus offered on average 143.2 ± 102.2 g of FV daily, which is 62.0 ± 44.3% of the recommended amount. However, there was a great variability in quantity of offered FV between schools (Table 2). Four schools (29% of enrolled schools) offered on average less than 100 g of FV daily, nine schools (57% of enrolled schools) offered on average between 100 and 200 g of FV daily, while two schools (14% of enrolled schools) offered on average more than 200 g of FV daily. Only one school (ID75) offered an average amount of FV that met the adjusted recommendation for FV (Fig. 1). The other 13 schools offered FV below the recommendation, with an average daily FV offering between 33% and 89% of the recommendation.

Table 2. Daily amount of offered fruit and vegetable in one school year in 14 primary schools

School ID	School food service model	Minimum offer (g)	Maximum offer (g)	Average offer (g)[a]
ID 8	Model A	0.0	447.4	121.6 ± 75.1
ID 10	Model B	0.0	342.2	127.7 ± 79.3
ID 11	Model A	93.8	288.5	178.7 ± 44.1
ID 15	Model A	0.0	262.9	87.4 ± 52.5
ID 18	Model A	0.0	325.0	101.7 ± 72.8
ID 30	Model A	0.0	532.1	121.3 ± 90.3
ID 41	Model A	26.0	460.4	170.8 ± 85.0
ID 50	Model C and D[b]	0.0	203.8	32.4 ± 44.3
ID 55	Model A	7.1	492.0	205.6 ± 81.4
ID 63	Model A	0.0	254.8	98.7 ± 56.3
ID75	Model A	87.3	688.2	359.6 ± 73.6
ID 88	Model A	0.0	329.7	127.4 ± 68.9
ID 95	Model A	0.0	234.0	81.0 ± 50.8
ID 102	Model A	0.0	423.1	185.9 ± 76.6

[a]mean ± standard deviation,
[b]depending on the class shift children consumed lunch (model C) or breakfast (model D)

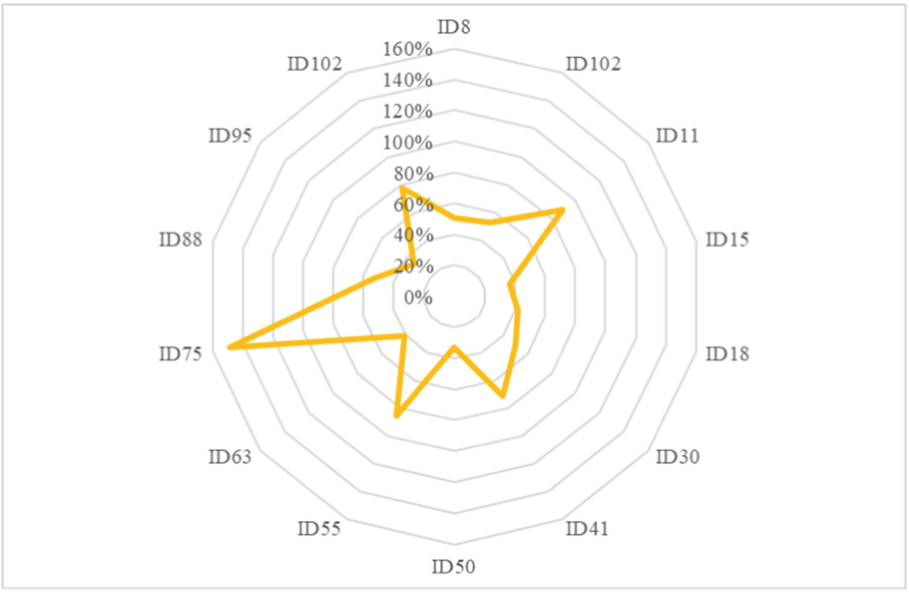

Fig. 1. Average daily percentage meeting the recommendation for fruits and vegetables in annual menus of 14 primary schools

3.2 Frequency of Offered FV in School Menus

According to the different types of school meal (Fig. 2), FV was served within 97% of lunches, 54% of breakfasts and 27% of snacks. School food services didn't offer any kind of FV within the majority of snacks (73%) and 46% of breakfasts. Fruits were most often offered at breakfasts (48%) and snacks (27%), while vegetables at lunches (94%).

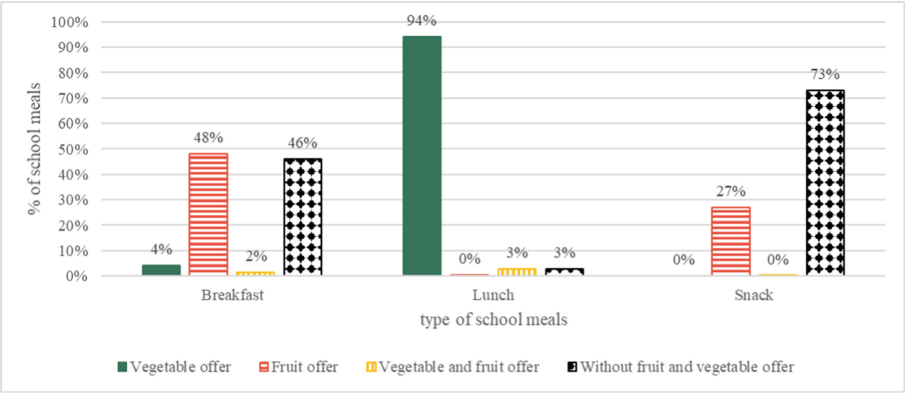

Fig. 2. Proportion of meals in which were offered fruits and/or vegetables in annual school menus of 14 primary schools

3.3 Diversity of Offered FV in School Menus

Annually, schools procured 97% fresh/frozen fruits and 82% fresh/frozen vegetables. According to the National guidelines, offered fresh and frozen fruits were divided into 5 categories. The most frequently-offered categories of fruits were pome (47%) fruits followed by tropical (23%), citrus (20%) and stone (7%) fruits, and berries (3%) (Fig. 3). Vegetables were divided into 6 categories in line with National guidelines. School food services most frequently-offered bulbous (40%) vegetables and then root (25%), cabbages (13%) and leafy and stalky (10%) vegetables, fresh legumes (7%), and fruit (5%) vegetables (Fig. 4).

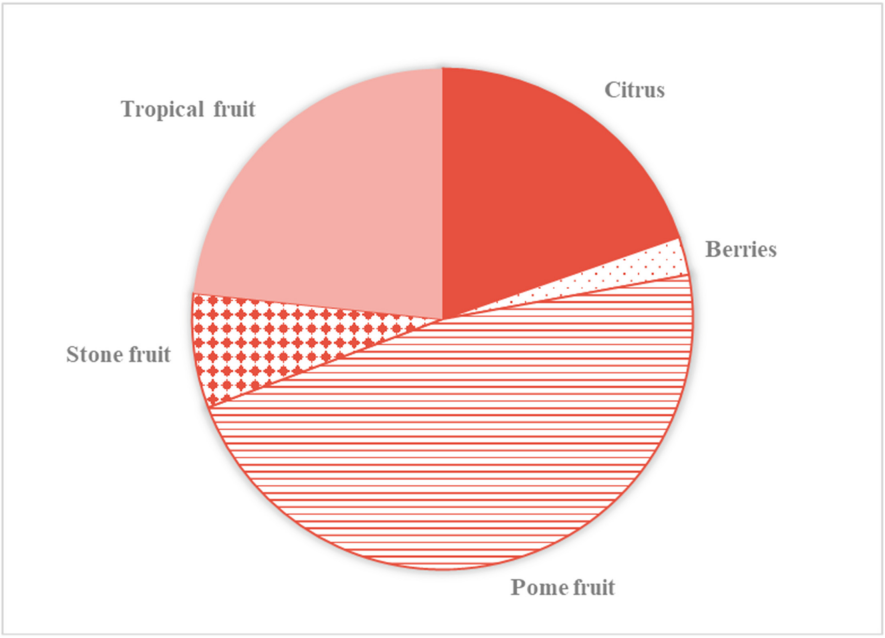

Fig. 3. Distribution of procured types of fruit categories in annual school menus of 14 primary schools

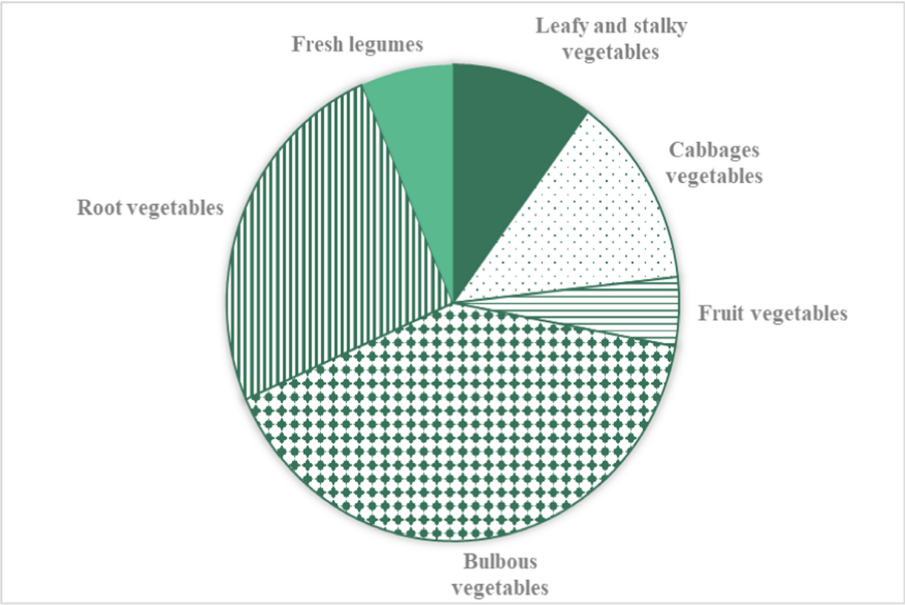

Fig. 4. Distribution of procured types of vegetable categories in annual school menus of 14 primary schools

4 Discussion

According to the available literature, this is the first study that evaluates the availability of FV in school meals in Croatia. This study shows that school menus contain inadequate amounts of FV as well as a low variety of FV offerings.

In Croatia, schools serve up to three meals with only one meal option. As children do not have the right to choose between the individual components of the meal (e.g. an option between different vegetable side dishes), schools are responsible for the quality of the whole meal they offer during mealtime. The quality of meals has a direct impact on children's health, development and growth and also on good eating habits through balanced, high quality and tasty meals. School meals provide opportunities for children to learn to enjoy food, practice healthy eating behaviors, try new foods, and socialize with their peers [7, 8, 16]. It should also be noted that school meals reduce inequalities in food access among children [17–19].

The results of the analysis of school menus in Zagreb City show that schools with three meals (breakfast, lunch and snack) offered between 81.0 ± 50.8 g and 359.6 ± 73.6 g FV, schools with two meals (breakfast and lunch) offered 127.7 ± 79.3 g FV and school with one meal (breakfast or lunch) offered 32.44 ± 44.3 g FV. Considering that the national guidelines apply to all schools [11], it was expected that there would be no difference in the amount of FV in the meals. However, schools are responsible for menu planning and it is usually the head teacher and cooks who decides on the amounts of ingredients used in meals. Accordingly, it is possible that differences in the amount of fruit and vegetables offered could exist due to knowledge of national

guidelines and nutrition, as well as the skills of kitchen staff. Many other factors could also have an influence, such as the number of kitchen staff, the equipment in the kitchen, the daily number of school meals served, the location of food preparation (on-site or a combination of on-site and central kitchen), the reduction of food waste by not adding dishes that children throw away in large quantities to the menu, the length of the lunch period, school finance, etc. These factors were not examined in this study, but would be of interest in the following.

According to the recommendation of WHO, it is necessary to eat at least 400 g FV per day to support proper growth and development and reduce the risk of developing non-communicable diseases [1]. Given that children spend a part of the day at school, it is necessary to provide an adequate amount FV through school meals. According to national guidelines, school breakfast should cover 15% of the daily recommendation, school lunch 35% and school snack 10%. Considering that children spend up to 8 h in primary school they may eat more than one meal at school. Therefore, children who eat breakfast and lunch should meet 50% of the recommendation through school meals, and children who eat breakfast, lunch, and snack should meet 60% of the recommendation [8, 11]. Intake of FV should be distributed throughout the day, so while taking into consideration previously mentioned recommendations [1, 11] to support adequate intake of fruits and vegetables, schools could serve 240 g FV to children who eat 3 meals, 200 g FV to children who eat breakfast and lunch, 140 g FV to children who eat only lunch, and 60 g to children who eat only breakfast. The results of this study show that the offer of FV in school meals was below the recommendations. Only one school that offered three meals offered more than 100% of the recommended amount of FV while 11 schools offered between 34% and 86% of the recommended amount of FV. One school that offered breakfast and lunch met 89% of the FV recommended amount, and one school that offered lunch or breakfast met 33% of the FV recommended amount. There are also concerns about meal quality in Canada [20] and Australia [21], where studies indicate that school day lunches contribute to lower intakes of orange and dark green vegetables and also lower intakes of whole fruits. In these two countries, there are no national lunch programs; instead, school meals are funded by provincial, community, and nongovernmental organizations, parents, and donors [15, 21]. At the same time, school meals in the United States are offered at a low price or free of charge through the National School Lunch Program (NSLP) and contain the required amount of FV [22]. Although children may compensate for a lower FV intake of school meals with the other meals throughout the day, the availability of healthy foods in school meals promotes healthy eating behaviors in elementary school children [16]. Also, according to the European Union Action Plan 2014–2020, it is necessary to promote healthier school environments to reduce childhood obesity. The plan includes providing access to healthier meals and limiting access to snacks and less healthy meals [23].

Fruit was most often offered during breakfast because it can be eaten both in the dining hall and later in class when children cannot eat a large amount during breakfast (e.g., sandwich and apple). The problem is that almost 50% of breakfasts do not offer fruits or vegetables, though they can be integrated into meals (e.g. dried fruits in porridges, tomatoes or lettuce in sandwiches, fresh fruits in homemade fruit yogurts, etc.). A school lunch in Croatia typically consists of a main dish (meat/fish with side dish, stew,

pasta or risotto), salad, optional bread, dessert and fresh water. Accordingly, vegetables are most often served at lunch. Schools can increase their FV offerings over lunch by substituting fruit for sweet desserts, especially on days when vegetables are served in smaller quantities over entrees. In the United States, 9 out of 10 lunches contain fruit, and 50% of these contain fresh fruit, while others contain canned fruit or 100% fruit juice. In addition, about 96% of lunches have one or more vegetable options, not counting salads in the salad bar [24].

The results of this study suggest that schools have a low variety of FV offered. Bulbous (40%) and root vegetables (25%) were the most commonly procured vegetables. This trend continues as onions, garlic, carrots, parsley and celery, for example, are used to prepare most dishes, especially stews and soups. Although these vegetables are most commonly found in school meals, they are present in the smallest quantities, unlike cabbage or leafy greens. Of the cabbage vegetables, cabbage was most frequently offered in salads and kale in stews, while cauliflower and broccoli were offered in stew form with less frequency. From the categories of leafy and stem vegetables, lettuce was most frequently offered in salads and spinach and chard in combination with potatoes as a side dish. Fruity vegetables are mainly served as salads in spring (tomatoes and cucumbers). Among the fruit categories, the most frequently offered category was pome fruit, as apples are often served. The second most frequently offered fruit category was tropical fruits (bananas), and the third was citrus fruits due to the frequent offering of bananas and mandarins, respectively. Berries and stone fruits were offered less than 10 times in a school year. The most frequently offered fruits were strawberries and grapes from the berry category and peaches from the stone fruit category. Similar to this study, school menus across America are less variable in terms of fruit offered, and it is suggested that variability should be increased. This recommendation specifically refers to increasing the availability of melons and berries [24]. Various fruits and vegetables in the diet provide different vitamins, minerals, fibers, and phytochemicals that have health-promoting properties [1, 25]. Therefore, it is recommended to increase the offer of fruits and vegetables in the school menu, which are not so much in the menu so far. One of these is legumes, which are a good source of plant protein and fiber, which has been linked to a lower risk of obesity and cardiovascular disease [26–29]. Also, schools could increase the supply of berries, as they are a good source of folic acid, vitamin C, vitamin E, polyphenols and fiber [25, 30]. Expect, for the already known positive effect of consumption, eating berries can improve cognitive performance in children [1, 25, 30, 31]. The nutritional quality of school meals depends on the variety and quantity of foods. In institutional food service settings, such as schools, food selection is influenced by public procurement policies, food policies, and national school nutrition guidelines [16, 32]. Food policy and school feeding guidelines provide the basis for food procurement in line with procurement policy [8, 11]. However, there are several factors that may influence the inconsistency between school nutrition and procurement policies, such as the number of kitchen staff, the size and equipment of the kitchen, the financial resources of the school, limited knowledge about nutrition, etc. [15].

Interest in the quality of school meals has increased worldwide. As a result, public food procurement policies focus on healthy and high quality foods that can potentially increase the overall demand for healthier and more environmentally sustainable foods

and make them available to the general public [33, 34]. Programs are being developed in the United States of America and European countries to support the sourcing of local and organic foods to maintain the sustainability of the food system. This implies increased availability of plant-based foods, especially FV, which consequently leads to increased quality of school menus. In addition to supporting the sourcing of local and organic foods, these programs also include other activities such as nutrition and cooking education, school gardens, salad bars, student field trips to farms, etc. [33, 35–39]. The European Commission and the European Parliament have launched the EU School Fruit Scheme, EU Milk Scheme and New School Scheme projects, which offer healthy options and increase daily consumption of fresh fruits and vegetables, healthy food and water intake in schools [23]. Schools in the city of Zagreb participating in this project can choose 100–150 g per student from FV once a week throughout the school year [13, 14]. Schools choose fruits more often than vegetables, and although schools have a wider range of fruits (apples, pears, citrus fruits, berry fruits, sweet and sour cherries, plums, peaches, apricots and nectarines) and vegetables (carrots, beetroot, celery, tomatoes, radishes and other root vegetables), they most often choose apples, mandarins and tomatoes.

The present study did not examine the impact of school location and sociodemographic areas. Although children living in suburbs with a garden around the house may have a higher intake of fruits and vegetables because they are more familiar with fruits and vegetables and have a greater availability of them [40, 41]. In addition, "healthier" behaviors (higher intake of fruits and vegetables) were significantly associated with a higher socioeconomic status [42]. Accordingly, it was hypothesized that school staff may include meals on the menu that are less likely to be wasted and more likely to be accepted by children attending the school, which could affect the amount and frequency of fruits and vegetables served. Therefore, the inclusion of schools from different locations and different socio-demographic areas ensures the representativeness of the sample. The influence of school location and sociodemographic on the availability of fruits and vegetables in school menus may be of interest for further study as one of the environmental factors. This study only observed the amount and frequency of FV served as part of the school menu, but not the actual amount of fruits and vegetables eaten by children during school meals. Therefore, future research should estimate the intake of FV through school meals among school-age children. To assess the impact of school meals on children's overall diet, further research should also estimate the availability of other foods through school meals and their proportion of total daily intake.

5 Conclusions

School menus do not meet the recommendation for daily portion sizes of FV. There is a need to increase the availability and variety of FV in school menus. This indicates that the national guidelines are poorly implemented in the school food system. Through this study it was shown that there is a need for better quality control of school menus and the employment of professional staff, nutritionists, in the organization of the school food system in Croatia.

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Improving the Quality of Wheat Bread by Using Chia (*Salvia hispanica* L.) Seeds and Psyllium (*Plantago ovata*) Husk

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Abstract. The important direction in field of functional food is development of functional bakery products. Bread is suitable for enrichment with ingredients that have pronounced functional properties. Chia (*Salvia hispanica* L.) seeds, psyllium (*Plantago ovata*) husk and extra virgin olive oil are interesting ingredients for bread enrichment due to their high nutritional properties. The aim of the paper is to determine impact of these ingredients on physical, chemical and sensory characteristics of wheat semi-white bread and proportion of ingredients that provides the best bread quality. Two groups of bread samples were produced: without oil addition (i) and samples with addition of 3% of extra virgin olive oil (ii). Control and five samples with addition of chia seeds and psyllium husk were produced in each group. The addition of chia seeds and psyllium husk was the same for all samples (10%), but their ratio varied and amounted: 100/0; 75/25; 50/50; 25/75 and 0/100. The results show that psyllium husk significantly degrade physical and sensory bread quality in contrast to chia seeds, especially volume, porosity, texture and appearance. Olive oil addition significantly improves physical and sensory bread quality, but causes a lower total phenolic content. Results show that chia seeds and psyllium husk addition in total amount of 10% could improve general bread quality and significantly increase total phenolic content. The 75/25 ratio of chia seeds and psyllium husk with olive oil provides the best bread quality.

Keywords: Bread quality · Chia seeds · Psyllium husk · Olive oil

1 Introduction

Balanced diet has a very important function in prevention of modern lifestyle diseases such as cardiovascular diseases, diabetes, cancer, digestive system diseases and obesity. Bioactive compounds in food have a positive effect in prevention and treatment of these diseases. Therefore, there is an increase in the importance and use of functional food that contain some of bioactive compounds. The development of functional bakery products is a relatively new trend compared to other functional products.

The quality of bread is determined by its physical properties, sensory properties, chemical composition and nutritional value. Recently, the nutritional value of bread has

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become increasingly important as a determinant of bread quality. Bread is generally characterized as a source of energy, especially white wheat bread which is poor in nutritional components. Improving the nutritional value of bread mainly refers to the introduction of new ingredients [1]. Bread is suitable for enrichment with functional components because it is a basic food that is consumed almost every day [2]. Various additives are used to enrich bread and other bakery products such as whole grains and pseudocereals, various seeds, plants, spices, fruits and vegetables, and bioactive components such as fiber, vitamins, minerals, essential amino acids and fatty acids and antioxidants [3]. Enrichment of bread affects the change of chemical composition and improvement of nutritional value, but should not negatively affect other quality parameters and sensory acceptability of bread, nor significantly complicate the production process, which is a challenge for the bakery industry [1].

Chia (*Salvia hispanica* L.) is an annual herbaceous plant belonging to the Lamiaceae family. This plant is native to Mexico and Guatemala and it is one of the oldest plants cultivated by the Aztecs. Due to their very good chemical composition, chia seeds have become a popular all over the world [4]. Chia seeds (CS) have an excellent fatty acid composition that is suitable for human health. Most of the fatty acids in CS oil are polyunsaturated fatty acids, primarily ω-3 fatty acids [5]. The protein content in the seeds is higher than in most cereals, they do not contain gluten, as well as antinutrients that can interfere with the metabolism and absorption of proteins. CS contain a large amount of dietary fiber that swells in the presence of water and forms a gelatinous layer around the seeds. Insoluble dietary fibers predominate in the composition of CS [6]. These seeds are also very rich in micronutrients. Chia is a significant source of antioxidants (phenolic compounds), minerals and vitamins (B vitamins) [7]. CS are mainly used to improve the nutritional value of food and contribute to the functionality of the product mostly due to the content of ω-3 fatty acids, dietary fiber and polyphenolic compounds. Therefore, CS are associated with a reduced risk of cardiovascular disease, cancer, allergies, inflammation, constipation, diabetes, hormonal dysfunction and obesity [8].

Psyllium (*Plantago ovata* Forskal) has been used in the world since ancient times, primarily for medical purposes. *Plantago ovata* is native to India and Pakistan [9]. The word "Psyllium" is used for plant, seed and husk. Psyllium or Ispaghula is a common name for several species within the genus Plantago from whose seeds mucous substances are obtained. Of all the Plantago species, *P. ovata* and *P. psyllium* are most commonly grown. The seeds of the species *Plantago ovata* are known as white or Indian psyllium [10]. Psyllium husk (PH) is the main product of the Psyllium plant and it consists of a seed coat and disturbed outer layers of dried and mature seeds of *Plantago ovata* Forsk [9]. Due its hydrophilic nature the PH absorbs water and a pure colorless mucous is formed increasing the volume by ten times or more. The most important ingredients of PH are carbohydrates (over 80%) and minerals [11]. Almost the total amount of carbohydrates in PH consists of dietary fiber (polysaccharides), and in their composition are predominantly present soluble fiber. PH has a very low energy value. Dietary fiber of psyllium is the reason for almost all the benefits of consuming this food [12]. Psyllium is used for digestive problems such as constipation or diarrhea, irritable bowel syndrome, intestinal inflammation, colon cancer, diabetes and hypercholesterolemia [10].

Olive oil is a highly valued and quality vegetable oil. Extra virgin olive oil (VOO) is the highest quality olive oil that is consumed due to its high content of bioactive components [13]. All the ingredients of olive oil can be divided into two fractions. The first fraction is the saponifiable fraction, which makes up about 98–99% of all ingredients and includes triacylglycerols, free fatty acids and phosphatides. The second fraction is an unsaponifiable fraction made up of a large number of compounds (over two hundred compounds) [14]. Although they make up a very small proportion of the oil, the unsaponifiable ingredients are very important for the sensory and nutritional quality and stability of olive oil [15]. This fraction consists of microcomponents: sterols, vitamins, waxes, aliphatic alcohols, antioxidants, aromatic compounds [14]. The content of unsaturated fatty acids and antioxidants is the main reason for the health benefits of consuming olive oil. Significant antioxidants in VOO are: tocopherols, β-carotene, phytosterols, pigments, terpenic acids and phenolic compounds [16].

The aim of the paper is to determine impact of chia seeds, psyllium husk and extra virgin olive oil on physical, chemical and sensory characteristics of wheat semi-white bread and proportion of ingredients that provides the best bread quality.

2 Materials and Methods

2.1 Materials

The following raw materials were used to produce the bread samples: semi-white wheat flour-type 710 (Klas, Bosnia and Herzegovina), extra virgin olive oil (Constanza, Italy), ground psyllium husk (dm-drogerie markt, Germany), chia seeds (EuroCompany, Bosnia and Herzegovina), dry yeast (SAF-NEVA, Russia), sugar (Agrana, Bosnia and Herzegovina) and salt (Solana Tuzla, Bosnia and Herzegovina). All materials were purchased at local stores. Ascorbic acid (Sigma-Aldrich, Germany) was also used as a flour improver.

2.2 Methods

The first part of the experiment was the production of bread in laboratory conditions. The second part was the analysis of the quality of the produced bread samples, which included the analysis of the baking, physical, chemical and sensory characteristics of the bread. Two groups of bread samples were produced: without oil addition (i) and samples with addition of 3% of extra VOO (ii). Control and five samples with addition of CS and PH were produced in each group. Total amount of the added ingredients (CS and PH) was the same for all samples (10%), but their ratio varied and amounted: 100/0; 75/25; 50/50; 25/75 and 0/100.

Bread Samples Preparation. Bread samples were produced by direct process such as hearth bread. The basic recipe and samples producing method were according to Oručević Žuljević [17]. The recipe for bread samples is shown in Table 1. The preparation of raw materials included: sifting the flour, grinding the CS, homogenization of dry ingredients, yeast activation and tempering the water to mixing temperature of 30 °C. The CS were ground in an electric mill to achieve approximately the same granulation as the ground PH. Yeast activation meant dissolving the same amount of sugar and

yeast in 40 ml of water at a temperature of 30 °C. The activation process took 10 min. Determining the required amount of water for kneading was done empirically according to the consistency of the dough. All ingredients were mixed using stand mixer at minimum speed for 72 s. Thereafter, mixing was continued at maximum speed for 33 s. After mixing, manual kneading and formation the dough for 2.5–3 min followed.

The dough was fermented in bulk at temperature of 30 °C and relative humidity of 75% for 30 min. After the first fermentation, the dough was kneaded by hand for 2 min and divided into dough pieces weighing 100 ± 1 g. The round shaped dough pieces were covered with a cloth and finally fermented at room temperature for 50 min. A pot with boiling water was placed in the oven, heated to 200 °C, in order to provide a sufficient amount of water vapor in the first phase of baking. The bread was baked at a temperature of 200 °C for 6–7 min, and then at a temperature of 180 °C for 6–9 min. The total baking time was adjusted to the type of bread sample and lasted from 12 to 15 min. The baked bread was covered with a cloth and left to cool for 1–2 h.

Table 1. Recipe for bread samples

Ingredients (g/100 g)	Control		P100/C0		P75/C25		P50/C50		P25/C75		P0/C100	
	–	+	–	+	–	+	–	+	–	+	–	+
Wheat flour	100	100	100	100	100	100	100	100	100	100	100	100
Water	57.5	56.5	74.5	74.5	78.5	76.5	77.5	76	75	74.5	70	66
Salt	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Dry yeast	1	1	1	1	1	1	1	1	1	1	1	1
Sugar	1	1	1	1	1	1	1	1	1	1	1	1
Ascorbic acid	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
Olive oil	0	3	0	3	0	3	0	3	0	3	0	3
Psyllium husk	0	0	10	10	7.5	7.5	5	5	2.5	2.5	0	0
Chia seeds	0	0	0	0	2.5	2.5	5	5	7.5	7.5	10	10

Control - without the addition of CS and PH; P100/C0 - contains only PH; P75/C25 - PH to CS ratio is 75:25;; P50/C50 - PH to CS ratio is 50:50;; P25/C75 - PH to CS ratio is 25:75; P0/C100 – contains only CS; (–) denotes a group without VOO; (+) denotes a group with VOO.

Baking Characteristics of Bread. The method of determining the baking chracteristics is according to Oručević Žuljević [17]. The following baking chracteristics of bread samples were determined: bread yield (Eq. 1) and weight loss during baking and cooling (1h) of bread (Eq.2).

$$\text{Bread yield} = \frac{\text{mass}(bread) * \text{dough } yield}{\text{mass}(dough)} * 100(\%) \quad (1)$$

$$\text{Weight loss} = \frac{\text{mass}(dough) - \text{mass}\,(bread)}{\text{mass}(dough)} * 100(\%) \quad (2)$$

Physical and Chemical Characteristics of Bread. The volume of bread samples was determined by the method of seed replacement using millet seed according to Keser [18]. According to this method, the volume of bread is equal to the volume of the seed squeezed out of the calibrated container. The specific volume was obtained from the ratio of volume to mass of bread [19].

The method for determining the shape of bread is according to Oručević Žuljević [19]. The shape of the bread represents the ratio of the height and width of the bread measured at the largest part of the cross section. Hearth bread has an appropriate shape if the values are from 0.7 to 0.9. The surface of cross-section of bread was determined by measuring this surface area using a planimeter [20]. To determine the crumbliness of the bread, the bread was cut with an electric knife in the middle and the crumbs collected. The collected crumbs were weighed and their mass represented the crumbliness of the bread [17]. The thickness of the crust was determined by measuring the thickness of the upper and lower crust in the cross section of the bread by digital vernier caliper, sensitivity 0.01 mm (MIB Messzeuge GmbH, Spangenberg, Germany) [20]. The method of determining the porosity of bread is according to Kaluđerski and Filipović [20]. The porosity of bread was determined by comparing the cross-sectional appearance with Dallman scale of porosity. Grades for porosity were marked with numbers from 1 to 8 according to Dallman scale.

The firmness of the crust and the crumb of the bread was determined by the method of penetration the bread sample and measuring the force required for penetration. The method is according to Jarni [21]. The TA. XT Texture Analyzer (Stable Micro System) was used to measure the firmness and the results were processed and read in Exponent Connect. A 25 mm thick slice of bread was used to determine the texture of the crumb. A slice of the same thickness from the end of the bread was used to determine the firmness of the crust, with the crust facing the probe. The firmness was expressed in Newton (N).

The color of the upper crust and the crumb of the bread was determined using a Konica Minolta colorimeter. The method of color determination was based on the CIE L * a * b* system. Color Data Software SpectraMagic NX was used to read the values of L *, a * and b *.

The moisture content was determined by a two-step drying process according to Kaluđerski and Filipović [20]. In the first stage, the samples were dried at 50 °C (±2 °C) for 12 h and the content of separated moisture (MC_1) in this drying stage was calculated. The sample was then dried at 130 °C (±1 °C) to constant weight. The moisture content (MC_2) was also calculated in the second drying phase. The total moisture content was calculated according to Eq. 3.

$$\text{Total moisture content} = \text{MC1} + \text{MC2} - \frac{\text{MC1} * \text{MC2}}{100}(\%) \tag{3}$$

The content of total phenols was determined by Folin-Ciocalteu spectrophotometric method at a wavelength $\lambda = 765$ nm. The content of total phenols in the samples was calculated using the prepared calibration curve [22]. The method of sample preparation was adjusted according to Romankiewicz et al. [23]. The chopped sample (crust and crumb) in amount of 1 ± 0.05 g was mixed with 10 ml of solvent. The solvent was a mixture of methanol and water (1: 1). The pH of solvent was adjusted to pH = 1 with the addition of concentrated HCl. Extraction was performed by shaking using a laboratory

shaker for 30 min, with a shaking speed of 120 rpm. The produced extract was then centrifuged for 10 min at 13000 rpm. The supernatant was used for analysis.

Sensory Evaluation. Sensory evaluation of bread samples was performed by a five-member trained sensory panel. The assessment was performed using a scoring test with grades from 1 to 5. The scale was ranged from very good (grade 5) to unsatisfactory (grade 1). The following quality parameters were evaluated: shape and appearance, appearance of crust and outer surface, appearance and porosity of crumb, elasticity, structure of crumb, odor and taste. Samples were evaluated two hours after baking.

Statistical Analysis. Methods of descriptive statistics, two-factor analysis of variance (ANOVA) and Tukey's test within post hoc analysis were used for data processing. The statistical significance was 95%.

3 Results and Discussion

3.1 Baking Characteristics

The results of baking characteristics of bread samples are shown in Table 2. According to the bread yield results, all samples had significantly different yields compared to the control. The addition of CS and PH to bread significantly increased the yield of bread. Within the group without VOO, the highest yield was recorded in the sample with 75% PH and 25% CS (170.51%), and in the group with the addition of VOO, the highest yield had the sample with 10% PH (171.70%). The results show that PH increased bread yield more than CS. This effect is explained by the chemical composition of CS and PH, because they are both very rich in fibers that have a great ability to bind and retain water. According to the research of Romankiewicz et al. [23], the addition of chia in the amount of 2–8% causes a decrease in bread yield, which is in contrast to the obtained results and characteristics of CS. A study by Man et al. [25] has shown that the addition of PH (5%, 10% and 15%) increased the ability to bind water in the dough, weight and yield of bread, which is in accordance with the presented results. The addition of VOO also had a statistically significant effect on bread yields. Almost all samples (except for the sample with 75% PH and 25% CS) had a higher yield of bread in group with the addition of VOO.

Results of weight loss from Table 2. do not show statistically significant differences in weight loss between the control sample (Control), the sample with 10% PH (P100/C0) and the sample with 10% CS (P0/C100). Significant and higher data were observed in samples with a combination of PH and CS. The largest weight loss within the group without VOO was observed in the sample with 50% PH and 50% CS (11.17%), and within the group with VOO the largest value was in the sample with 75% PH and 25% CS (12.03%). Higher weight loss values may be related with a with higher water content in the dough in these samples. The research of Romankiewicz et al. [23] shows that the addition of CS in larger quantities (6–8%) has the effect of reducing losses, while the obtained results show that the addition of only CS (10%) does not significantly affect this parameter. Statistical analysis of the data found a significant effect of VOO on weight loss, but the effect varied depending on the type of sample.

Table 2. Baking characteristics of bread samples

	VOO	Control	P100/C0	P75/C25	P50/C50	P25/C75	P0/C100
Bread yield *(%)*	$-^{x}$	145.48 ± 0.81^{b}	169.68 ± 0.53^{a}	170.51 ± 0.42^{a}	169.44 ± 0.44^{a}	168.39 ± 0.41^{a}	162.62 ± 1.02^{c}
	$+^{y}$	147.65 ± 0.90	171.70 ± 1.32	169.56 ± 1.10	170.93 ± 1.18	169.56 ± 1.11	163.49 ± 1.17
Weight loss *(%)*	$-^{x}$	9.50 ± 0.51^{c}	9.62 ± 0.27^{ac}	11.08 ± 0.22^{b}	11.17 ± 0.23^{ab}	10.55 ± 0.22^{ab}	9.78 ± 0.57^{ac}
	$+^{y}$	9.27 ± 0.55	10.02 ± 0.69	12.03 ± 0.57	11.09 ± 0.62	11.11 ± 0.58	10.29 ± 0.64

Control - without the addition of CS and PH; P100/C0 - contains only PH; P75/C25 - PH to CS ratio is 75:25;; P50/C50 - PH to CS ratio is 50:50;; P25/C75 – PH to CS ratio is 25:75; P0/C100 – contains only CS; (–) denotes a group without VOO; (+) denotes a group with VOO; The displayed values are given as mean ± standard deviation; Values in the same order marked with different letters differ statistically significantly according to the Tukey test (significance level 95%); Groups without VOO and with VOO marked with different letters indicate a statistically significant influence of VOO on a certain parameter (significance level 95%).

3.2 Physical and Chemical Characteristics

The results of the physical and chemical characteristics of bread are shown in Table 3. It was found that the weight of the control sample (Control), the sample with 10% PH (P100/C0) and the sample with 10% CS (P0/C100) did not differ significantly. Samples with a combination of PH and CS (P75/C25, P50/C50, P25/C75) had significantly lower weight. The measured weight of the samples is directly related to the weight losses (higher losses caused lower weight of bread), which is also shown by the statistics. The addition of VOO affected the weight of the bread in the same way as in the case of weight loss. VOO had a statistically significant effect on the height and width of the bread. According to the results from Table 3, it is noticeable that VOO increased the height as well as the width of the bread with the exception of the sample with 10% CS. It was expected, because the oil makes the dough softer and more elastic, which allows the dough to rise better. The sample with 10% CS (P0/C100) had the lowest height while the other samples did not differ statistically significantly in height from the control sample. Samples with a higher share of PH (P100/C0 and P75/C25) had a smaller width than the others and differed significantly from the control sample in width.

The shape of all the bread samples was at the lower limit of the optimal values and the values were not close to the spherical shape of the bread. The best shape was observed in samples with a higher share of PH and the values for the shape of these samples were 0.71 (P100/C0) and 0.70 (P75/C25), but although the ratio of height and width was favourable, these samples had a small height and width. The less desirable shape of the bread samples can be explained by the presence of a larger amount of water which makes the dough heavier and by the presence of the CS and PH added that hinder the rising process. No significant effect of VOO on the shape of bread has been determined. Haros [26] in his study obtained similar values for the shape of bread with the addition of CS, but with a smaller amount of CS (5%). In the same paper, no statistically significant difference in the shape of bread was found between the control sample and the sample with the addition of CS, which is in accordance with the obtained results. Beikzadeh

et al. [27] stated in their paper that the addition of PH over 7.5% in the biscuit affects the symmetry and shape of the biscuit.

Table 3. Physical and chemical characteristics of bread samples

	VOO	Control	P100/C0	P75/C25	P50/C50	P25/C75	P0/C100
Weight *(g)*	$-^{x}$	90.94 ± 0.25^{c}	90.84 ± 0.48^{c}	89.11 ± 0.40^{a}	89.21 ± 0.34^{ab}	89.79 ± 0.28^{abc}	90.88 ± 0.62^{bc}
	$+^{y}$	90.94 ± 0.72	90.43 ± 0.50	88.43 ± 0.47	89.20 ± 0.51	89.26 ± 0.60	89.92 ± 0.83
Height *(mm)*	$-^{x}$	51.68 ± 0.32^{a}	49.05 ± 1.05^{ab}	50.71 ± 2.31^{ab}	53.20 ± 0.74^{a}	52.15 ± 0.53^{a}	48.26 ± 1.05^{b}
	$+^{y}$	54.36 ± 1.41	54.70 ± 1.85	50.47 ± 0.49	51.97 ± 0.66	52.97 ± 0.85	51.05 ± 1.01
Width *(mm)*	$-^{x}$	83.95 ± 1.48^{a}	69.94 ± 7.09^{b}	73.08 ± 1.25^{bc}	81.05 ± 2.21^{a}	77.20 ± 0.43^{ac}	83.61 ± 0.99^{a}
	$+^{y}$	84.71 ± 0.50	77.66 ± 2.56	77.17 ± 3.92	82.11 ± 1.74	83.37 ± 0.16	81.62 ± 0.72
Shape *(/)*	$-^{x}$	0.62 ± 0.01^{ac}	0.71 ± 0.08^{b}	0.70 ± 0.04^{bc}	0.66 ± 0.01^{abc}	0.68 ± 0.01^{abc}	0.58 ± 0.02^{a}
	$+^{x}$	0.64 ± 0.02	0.71 ± 0.01	0.66 ± 0.03	0.63 ± 0.01	0.64 ± 0.01	0.63 ± 0.02
Specific volume *(cm^3/g)*	$-^{x}$	3.70 ± 0.15^{a}	2.10 ± 0.23^{b}	3.11 ± 0.24^{c}	3.18 ± 0.12^{c}	3.68 ± 0.09^{a}	2.34 ± 0.17^{b}
	$+^{y}$	4.27 ± 0.13	2.58 ± 0.34	3.56 ± 0.15	3.26 ± 0.14	4.07 ± 0.05	3.04 ± 0.06
Crust thickness *(mm)*	$-^{x}$	2.21 ± 0.08^{ab}	2.56 ± 0.06^{a}	2.61 ± 0.09^{a}	1.93 ± 0.06^{bc}	1.87 ± 0.11^{c}	1.79 ± 0.16^{bc}
	$+^{y}$	1.96 ± 0.05	2.03 ± 0.23	1.79 ± 0.05	1.85 ± 0.11	1.68 ± 0.07	2.10 ± 0.12
Surface of cross-section *(cm^2)*	$-^{x}$	40.05 ± 0.58^{a}	28.33 ± 0.94^{b}	31.95 ± 0.85^{c}	34.58 ± 1.32^{cd}	35.25 ± 0.33^{d}	35.55 ± 0.42^{d}
	$+^{y}$	41.98 ± 0.62	31.30 ± 2.67	34.93 ± 1.40	36.58 ± 0.38	38.73 ± 0.56	37.43 ± 0.47
Crumbliness *(g)*	$-^{x}$	0.0715 ± 0.018^{a}	0.0572 ± 0.016^{ab}	0.0249 ± 0.002^{b}	0.0174 ± 0.003^{b}	0.0219 ± 0.002^{b}	0.0237 ± 0.001^{b}
	$+^{x}$	0.0652 ± 0.017	0.0220 ± 0.005	0.0157 ± 0.002	0.0162 ± 0.001	0.0292 ± 0.000	0.0319 ± 0.004
Porosity *(/)*	$-^{x}$	3.50 ± 0.58^{a}	8.00 ± 0.00^{b}	7.25 ± 0.50^{bd}	5.75 ± 0.50^{ac}	4.25 ± 0.50^{a}	6.25 ± 0.96^{cd}
	$+^{y}$	4.50 ± 0.58	6.75 ± 0.50	6.75 ± 0.50	4.75 ± 0.50	4.00 ± 0.82	5.25 ± 0.50
Crust firmness *(N)*	$-^{x}$	6.588 ± 0.601^{a}	12.501 ± 2.935^{b}	7.944 ± 1.224^{ab}	7.509 ± 2.108^{ab}	6.057 ± 1.187^{a}	6.089 ± 1.425^{ab}
	$+^{y}$	5.073 ± 1.596	7.316 ± 1.323	5.879 ± 0.461	6.389 ± 1.087	5.253 ± 0.878	6.558 ± 2.797
Crumb firmness *(N)*	$-^{x}$	2.896 ± 0.279^{a}	5.731 ± 1.083^{b}	4.162 ± 0.242^{a}	3.459 ± 0.243^{a}	3.585 ± 0.483^{a}	3.452 ± 0.147^{a}
	$+^{y}$	2.604 ± 0.046	3.670 ± 0.693	2.703 ± 0.498	2.467 ± 0.484	1.856 ± 0.342	2.202 ± 0.261
Moisture content *(%)*	$-^{x}$	36.40 ± 0.43^{b}	40.22 ± 0.94^{ac}	42.09 ± 1.93^{a}	40.16 ± 1.45^{ac}	39.60 ± 0.28^{abc}	37.91 ± 0.39^{bc}
	$+^{x}$	34.64 ± 0.66	40.38 ± 1.19	42.68 ± 1.08	39.31 ± 0.54	39.01 ± 0.49	36.10 ± 2.06
Total phenol content *(mgGAE/100gDM)*	$-^{x}$	238.99 ± 0.00^{a}	269.15 ± 138.15^{ab}	457.95 ± 119.79^{ab}	990.53 ± 80.02^{ab}	1103.7 ± 767.74^{ab}	1182.45 ± 65.03^{b}
	$+^{y}$	141.88 ± 83.18	182.27 ± 170.79	308.45 ± 192.79	544.46 ± 276.37	693.88 ± 387.53	731.47 ± 10.18

Control - without the addition of CS and PH; P100/C0 - contains only PH; P75/C25 - PH to CS ratio is 75:25;; P50/C50 - PH to CS ratio is 50:50;; P25/C75 - PH to CS ratio is 25:75; P0/C100 – contains only CS; (–) denotes a group without VOO; (+) denotes a group with VOO.

The sample with 25% PH and 75% CS (P25/C75) did not differ statistically significantly from the control in specific volume and these two samples had the largest volume. This indicates that this ratio of PH and CS do not significantly worsen the volume of bread. All other samples had significantly lower values of specific volume compared to the control. The lowest volume was noticed in the sample with 10% PH (2.10 cm^3/g) and in the sample with 10% CS (2.34 cm^3/g), which indicates that the CS and PH in large quantities decrease the volume of bread. Romankiewicz et al. [23] in their study confirmed a significant decrease in bread volume with the addition and increase of CS, and such an impact was explained by the difficult development of the gluten network and low spread in the presence of CS. According to a study by Man et al. [25], there was a decrease in the volume of bread with an increase in the proportion of PH. Beikzadeh et al. [27] in their paper also noted a decrease in the volume of the biscuit containing PH. VOO had a statistically significant effect on the volume of bread and surface of cross-section. In the group of samples with VOO, a significantly higher specific volume

and larger surface od cross-section of bread were found. Higher volume is associated with the influence of VOO on increasing the height and width of bread. Osuna et al. [28] stated that replacing the fat in bread with VOO causes an increase in specific volume. The results for volume and surface of cross-section of bread are strongly correlated. All samples had a statistically significantly smaller surface of cross-section than the control sample. Such results can be related to the reduction of the relative gluten content in the samples with additives, because PH and CS do not contain gluten [17]. The smallest cross-sectional surface was found in the sample with 10% PH (28.33 cm^2 - without VOO and 31.30 cm^2 - with VOO), while higher cross-sectional surfaces were recorded with increasing CS content, as in the case with volume.

The sample with the thinnest crust and which statistically significantly differed from the control sample was bread with 25% PH and 75% CS (P25/C75), whose crust thickness was 1.87 mm (without VOO) and 1.68 mm (with VOO). A thicker crust was observed in samples with a higher share of PH (2.56–2.61 mm) compared to samples with more CS (1.79 – 1.87 mm). The significant effect of VOO on the thickness of crust was confirmed. The crust of the bread is thinner with the addition of VOO, except for the sample with 10% CS. No statistically significant effect of VOO on bread crumbliness was recorded. All samples, except the sample with 10% PH (P100/C0), had statistically significantly lower crumbliness compared to the control sample. PH addition caused higher bread crumbliness than CS addition.

Olive oil had a statistically significant effect on the porosity of bread. In most cases, VOO contributed to a better porosity and appearance of the bread crumb. No statistically significant difference in porosity was found between the control sample, the sample with 50% PH and 50% CS (P50/C50) and the sample with 25% PH and 75% CS (P25/C75). Samples with a higher PH content had poorer porosity, and the structure of the crumb was much more compact. According to research by Romankiewicz et al. [23] and Haros [26], samples with CS addition did not differ significantly from the control sample in porosity. These studies confirm that CS does not negatively affect the porosity of bread. According to the research of Man et al. [25], the addition of PH affects the reduction of bread porosity, which is in line with the obtained results.

Based on the texture results shown in Table 3, the only sample that was statistically significantly different from the control in crust and crumb firmness is the sample with 10% PH (P100/C0). It is possible to conclude that CS does not significantly affect the firmness of the crust and the crumb, but PH with a higher share contributes to the harder crust and the crumb of the bread. According to Haros [26], the addition of CS did not affect the texture of the crumb of the bread, which is in line with the obtained results. According to Romankiewicz et al. [23], the addition of CS caused a decrease in the firmness of the bread crumb, which is explained by the presence of gums, mucus and fat. Beikzadeh et al. [27] stated in their work that PH in the amount of 10–15% caused an increase in the firmness of the biscuit, which is also shown in the case of the results for bread. The addition of VOO had a statistically significant effect on the texture of the crust and the crumb of the bread. VOO reduced the firmness of the bread and made the crust and crumb of the bread softer and more elastic.

According to the obtained results, the sample with 25% PH and 75% CS (P25/C75) and the sample with the addition of CS only (P0/C100) did not differ statistically significantly from the control sample in moisture content. Other bread samples had a significantly higher moisture content compared to the control sample. According to Romankiewicz et al. [23] and Haros [26], the addition of CS did not affect the moisture content in bread, which confirms the obtained results. Man et al. [25] with their research confirmed that the addition of PH increases the moisture content in bread. Olive oil did not significantly affect the moisture content of the bread.

The sample with 10% CS (P0/C100) had a statistically significantly higher content of total phenols compared to the control sample. This sample had a statistically the highest phenol content which was 1182.45 mg GAE/100 g DM without the addition of VOO, and with the addition of VOO 731.47 mg GAE/100g DM. Other samples did not differ statistically significantly from the control sample or from the sample with 10% CS in phenol content. It was found that CS significantly increase the phenol content in bread while it was not confirmed for PH addition, and the content of total phenols increases with increasing CS content. Romankiewicz et al. [23] also found a positive effect of CS on phenol content in bread. In the paper of Melini et al. [29] it is stated that the addition of CS in the amount of 5–10% in pasta has a significant effect on increasing the content of total phenols.

The results show a statistically significant influence of VOO on the content of total phenols. The addition of VOO to the bread resulted in a reduction in the content of phenolic compounds, which is unexpected because extra VOO also contains a certain amount of phenols. Marinopoulou et al. [30] found that the addition of black and green olive pulp increases the phenol content and antioxidant activity of bread. Lower phenol content in samples with VOO could be caused by different chain oxidation reactions, interactions with other components in the bread composition, but also by difficult phenol extraction during the analysis due to the presence of oil.

The results for the color of the crust and the crumb of the bread are presented in Table 4. It was determined that VOO did not have a statistically significant effect on the color of the crumb of the bread. On the other hand, VOO had a statistically significant effect on the color of the crust, with significant differences in the a* and b* values in the samples with the addition of VOO. In most samples, the addition of VOO caused higher a* values (more red tones) and higher b* values (more yellow tones) for the crust of the bread. Osuna et al. [28] stated that by replacing the fat with olive oil in the bread, the color of the bread crust changed, but significant changes were recorded in the L* and b* values.

PH affected the brightness of the crust more than CS. The crust of bread with a higher PH content is darker. The addition of PH and CS did not significantly affect changes in a* values for the crust compared to the control sample, however, samples with PH had higher a* values compared to samples with CS. These additives caused significant changes in b* values for the crust of bread. Both PH and CS caused lower b* values, or less yellow tones on the crust. PH and CS had a significant effect on the brightness of the crumb. The darkest crumb was observed in the sample with 10% PH and the sample with 10% CS. All samples had higher a* values for the crumb than the control sample. PH contributed more to the appearance of higher a* values for the crumb than CS. All

Table 4. Color of the crust and the crumb of bread samples

	VOO	Control	P100/C0	P75/C25	P50/C50	P25/C75	P0/C100
CRUST							
L* value	$-^{x}$	66.18 ± 1.28^{a}	63.77 ± 1.32^{ab}	61.08 ± 1.76^{b}	61.05 ± 2.68^{ab}	64.22 ± 0.36^{ab}	65.08 ± 2.02^{ab}
	$+^{x}$	65.95 ± 2.96	64.48 ± 1.00	59.82 ± 3.86	62.73 ± 1.48	59.80 ± 1.41	62.40 ± 2.17
a* value	$-^{x}$	6.71 ± 1.93^{ab}	6.49 ± 1.17^{ab}	9.08 ± 1.06^{a}	8.96 ± 1.16^{a}	6.31 ± 0.95^{ab}	3.80 ± 1.30^{b}
	$+^{y}$	9.16 ± 1.91	6.83 ± 1.39	9.92 ± 1.38	8.80 ± 0.14	9.27 ± 0.59	6.96 ± 1.48
b* value	$-^{x}$	35.61 ± 2.63^{a}	29.38 ± 0.74^{b}	31.59 ± 0.26^{b}	32.86 ± 0.14^{ab}	29.97 ± 1.90^{b}	27.21 ± 1.33^{b}
	$+^{y}$	36.10 ± 0.42	29.57 ± 2.14	32.09 ± 1.61	31.47 ± 0.40	32.14 ± 0.77	32.27 ± 1.36
CRUMB							
L* value	$-^{x}$	68.68 ± 1.84^{a}	57.24 ± 1.35^{b}	61.56 ± 2.86^{ab}	61.99 ± 2.97^{ab}	63.83 ± 1.87^{ab}	60.68 ± 2.56^{b}
	$+^{x}$	66.07 ± 3.52	60.08 ± 0.39	60.65 ± 2.79	63.44 ± 0.42	64.60 ± 3.19	59.28 ± 2.41
a* value	$-^{x}$	-0.15 ± 0.18^{a}	2.54 ± 0.32^{b}	1.70 ± 0.18^{c}	1.13 ± 0.18^{cd}	0.52 ± 0.31^{d}	1.14 ± 0.42^{cd}
	$+^{x}$	-0.40 ± 0.19	2.24 ± 0.25	1.53 ± 0.38	1.38 ± 0.15	0.55 ± 0.33	1.07 ± 0.32
b* value	$-^{x}$	21.17 ± 0.50^{a}	20.78 ± 0.29^{a}	18.84 ± 0.85^{c}	18.23 ± 1.00^{c}	17.09 ± 0.85^{b}	18.89 ± 0.22^{c}
	$+^{x}$	20.58 ± 0.76	20.64 ± 0.62	18.54 ± 0.13	19.77 ± 0.87	16.64 ± 0.25	17.91 ± 0.46

Control - without the addition of CS and PH; P100/C0 - contains only PH; P75/C25 - PH to CS ratio is 75:25; P50/C50 - PH to CS ratio is 50:50; P25/C75 - PH to CS ratio is 25:75; P0/C100 – contains only CS; (–) denotes a group without VOO; (+) denotes a group with VOO.

samples had significantly lower b* values for the crumb of the bread than the control sample, except for bread with 10% PH. CS had more of an effect on the appearance of less yellow tones in the crumb of the bread. In general, PH and CS affected the color of the crumb much more than the color of the bread crust.

3.3 Sensory Evaluation

In Table 5 are presented the results of sensory evaluation of produced bread samples. Based on the obtained results, VOO had a statistically significant impact on all analyzed sensory quality parameters. Samples with the addition of VOO were significantly better evaluated and more acceptable from the sensory aspect. Better elasticity, appearance, porosity and structure of the crumb of bread with VOO are correlated with results for texture indicating a softer crumb, and results for porosity indicating better porosity and structure of crumb with the addition of VOO. Better grades for the appearance of bread, crust and outer surface are consistent with texture results, colorimetry results and results for crust thickness. Sensory evaluation determined that darker color of the crust with VOO was more acceptable and that the crust in these samples was smoother and shinier. Also, higher scores for the shape and appearance of bread with VOO are consistent with a significantly higher volume in the group of samples with the addition of VOO.

Based on the shape and appearance of the bread, no statistically significant differences were found between the control sample and the samples with additives, except in the case of the sample with 10% PH. This sample was rated as the sample with the worst shape and appearance with a score of 3.3 (without VOO) and 3.6 (with VOO). A higher PH content in bread negatively affected this quality parameter. The highest score (5.0) for the shape and appearance of the bread was given to the control sample with VOO.

Table 5. Sensory characteristics of bread samples

	VOO	Control	P100/C0	P75/C25	P50/C50	P25/C75	P0/C100
Shape and appearance	$-^{x}$	4.80 ± 0.27^{a}	3.30 ± 0.84^{b}	3.90 ± 0.55^{ab}	4.40 ± 0.42^{a}	3.80 ± 0.45^{ab}	4.00 ± 0.00^{ab}
	$+^{y}$	5.00 ± 0.00	3.60 ± 0.89	4.20 ± 0.76	4.70 ± 0.45	4.70 ± 0.45	$4{,}9 \pm 0.22$
Appearance of crust and outer surface	$-^{x}$	4.10 ± 0.74^{a}	3.20 ± 0.45^{b}	3.60 ± 0.55^{ab}	4.20 ± 0.27^{a}	4.10 ± 0.42^{a}	$4.00 \pm 0{,}00^{a}$
	$+^{y}$	4.90 ± 0.22	3.30 ± 0.45	4.10 ± 0.22	4.70 ± 0.45	4.90 ± 0.22	$5.00 \pm 0{,}00$
Appearance and porosity of crumb	$-^{x}$	4.20 ± 0.27^{a}	2.20 ± 1.10^{b}	3.10 ± 0.74^{ab}	3.60 ± 0.55^{ab}	3.60 ± 0.55^{a}	3.60 ± 0.42^{a}
	$+^{y}$	4.70 ± 0.45	2.90 ± 0.74	3.90 ± 0.74	4.00 ± 0.71	4.60 ± 0.42	4.20 ± 0.57
Elasticity	$-^{x}$	4.40 ± 0.55^{a}	3.20 ± 1.10^{a}	3.50 ± 0.50^{a}	4.20 ± 0.45^{a}	4.20 ± 0.27^{a}	3.40 ± 0.55^{a}
	$+^{y}$	4.90 ± 0.22	4.30 ± 0.67	4.50 ± 0.71	5.00 ± 0.00	4.90 ± 0.22	4.70 ± 0.67
Structure of crumb	$-^{x}$	4.30 ± 0.45^{a}	2.80 ± 0.84^{b}	3.40 ± 0.55^{ab}	3.60 ± 0.55^{ab}	3.50 ± 0.50^{ab}	3.60 ± 0.89^{ab}
	$+^{y}$	4.40 ± 0.42	3.40 ± 0.55	3.70 ± 0.45	4.00 ± 0.71	4.10 ± 0.22	4.30 ± 0.67
Odor and taste	$-^{x}$	4.20 ± 0.45^{ab}	3.50 ± 1.12^{a}	3.30 ± 0.67^{a}	4.10 ± 0.42^{ab}	5.00 ± 0.00^{b}	4.60 ± 0.55^{ab}
	$+^{y}$	4.74 ± 0.37	3.90 ± 1.03	4.30 ± 0.57	4.80 ± 0.45	5.00 ± 0.00	4.90 ± 0.22

Control - without the addition of CS and PH; P100/C0 - contains only PH; P75/C25 - PH to CS ratio is 75:25;; P50/C50 - PH to CS ratio is 50:50;; P25/C75 - PH to CS ratio is 25:75; P0/C100 – contains only CS; (–) denotes a group without VOO; (+) denotes a group with VOO.

The sample with 10% PH got the worst scores (3.2) for the appearance of the crust and outer surface and differed statistically significantly from the control. Other samples did not have a statistically significantly different appearance of the crust and outer surface compared to the control sample. PH affected the appearance of pale, uneven and rough crust. The appearance, porosity and the structure of the crumb are related and rated very similarly. According to these parameters, none of the samples, except for the sample with 10% PH, differed statistically significantly from the control. The sample with 10% PH won significantly the worst scores for the appearance and porosity of the crumb (2.2) and the structure of the crumb (2.8). In general, PH had a more negative effect on the appearance and structure of the crumb than CS. Based on odor and taste, no sample differed statistically significantly from the control, and all samples were acceptable as control sample. The highest scores for taste and odor (5.0) were given to the sample with 25% PH and 75% CS. Samples with a higher CS content were evaluated as samples with better taste and odor, compared to samples with a higher amount of PH.

4 Conclusions

The results show that olive oil had a positive effect on the sensory properties and almost all physical parameters of bread quality, but caused a decrease in the content of total phenols. The results showed that psyllium husk significantly degrades physical and sensory bread quality in contrast to chia seeds, especially regarding specific volume, porosity, texture and appearance. The PH to CS ratio that ensured the best bread quality was 25/75. Only this ratio of all analyzed ensured a good specific volume of bread. Bread with 25/75 ratio of PH and CS was sensory acceptable as well as control bread, and the addition of PH and CS in this amount did not negatively affect almost any of the analyzed parameters of bread quality. Although bread with 10% CS had the highest

phenol content, there was no significant difference compared to bread with 2.5% PH and 7.5% CS. Based on this research, it can be concluded that it is possible to produce bread with the addition of PH and CS, which is high sensory acceptable and has appropriate quality and increased content of total phenols.

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Level of Pollution of the Miljacka River

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Abstract. Most pollution, according to the ecologists, comes from sewage waters from populated zones, industry, and illegal dumps. Environmentalists warn that any, even the smallest changes in the physical and chemical quality of water affects the organisms that live in it. Water resources, which define life on Earth and biodiversity, oblige us to be rational, and to take care of them. The dynamic development of society and the increasing pressure on the natural environment, and thus on water, are becoming one of the key issues of sustainable development, because water pollution in the ground and on the surface further affects the reduction of the water supply. The level of impact on water quality becomes higher as land uses intensify through the spectrum of agriculture, timber harvesting, housing, industry, and roads.

This research paper provides an overview of the analysis of water samples from the Miljacka river from ten different places in the city of Sarajevo from Bentbaša to the estuary of the river Miljacka. All analyzes were performed in the Laboratory for Chemistry of the Faculty of Agriculture and Food in Sarajevo and the laboratory EURO INSPEKT ltd. - "Real INSPECT" Sarajevo. Physicochemical parameters examined in this research work were: odor, taste, color, total hardness, turbidity (measured 9.61–39.81 NTU), pH value (6.6–7.15), consumption of KMnO4 (0.977–4.23 mg O_2/l), ammonia (1.76–2.83 mg N/I), nitrates (0.98–2.44 mg/l), nitrites, lead (3.60–6.24 μg/l) and mercury (0.10–0.60 μg/l). Based on the conducted research, we can conclude that the river Miljacka has a high content of nitrates, ammonia, mercury and inadequate turbidity. This may be due to anthropogenic activities such as municipal wastewater discharge, lack of infrastructure and environmental awareness of citizens.

Keywords: Water pollution · Miljacka river · Physicochemical analysis

1 Introduction

Water is a necessary raw material in industrial production, energy, food industry, for communal needs and more. Water scarcity is a global problem. The health of billions of people is endangered due to the lack of hygienically correct water. According to the WHO (2011), about 1.1 billion people do not have access to drinking water, and about 2.5 billion people do not have basic sanitation. Today, waters are usually under

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the influence of a higher load from various sources of pollution, which resulted in the need for constant control of water quality and timely application of appropriate methods in order to protect them. Human activities commonly affect the distribution, quantity, and chemical quality of water resources. The range in human activities that affect the interaction of ground water and surface water is broad. Most rivers in the Federation of Bosnia and Herzegovina are polluted downstream from larger cities and settlements, as wastewater is discharged into watercourses mostly without any treatment. Riverbeds are very often a place for solid waste disposal, which additionally affects pollution (Černek 2018). Water pollution is generally a fairly broad term, and it usually means a reduction in water quality due to subsequently received impurities. The effects of water pollution are far-reaching and affect not only the environment but also human beings and animals (Weiner 2000).

Watercourses are mostly polluted by wastewater from settlements (sewers) and the industry. It is important to emphasize the growing importance of water pollution with chemical substances (nitrates, nitrites, ammonia, heavy metals, detergents, pesticides, etc.). A large number of harmful bacteria comes from human and animal excreta (canal water), while chemical pollution comes mainly from the industry (Heberer et al. 2020). The only way to protect rivers from microbiological and chemical pollution is to treat the wastewater of settlements and industry before discharging it into watercourses. Therefore, some activities significantly pollute the water. If the process water is not treated before discharge (Bunčić 2018). Water quality monitoring is important for environmental protection, management of waterways and their tributaries, and identification of pollution.

A special property of surface waters is self-cleaning, the so-called auto purification. In the event that surface waters become polluted with organic matter, large amounts of bacteria and other types of microorganisms will develop in the water, which will carry out mineralization with or without the presence of air (Novaković et al. 2018). Mineralization in this case represents the decomposition of organic pollutants. If the process takes place in the presence of oxygen, it will be performed by aerobic microorganisms, mineralization will take place quickly and without an unpleasant odor (Štrkalj 2014). According to all problems with river stream and pollution problems point of this research work was to get realistically results about pollution water in the river of Miljacka.

2 Material and Methods

The experimental part of the work was divided into three stages. The first stage involved sampling, the second stage the preparation of samples for analysis, while the third stage was the analysis of samples and reading the obtained values. Samples were taken from ten places along the Miljacka River from Bentbasha to the estuary of the river. Physico-chemical analyzes were performed in the Laboratory for Chemistry of the Faculty of Agriculture and Food Sciences University of Sarajevo and the laboratory EURO INSPEKT ltd. - "Real INSPECT" Sarajevo (Fig. 1).

Atomic spectroscopy with a graphite furnace (EN ISO 15586: 2003, IDT; ISO 15586: 2003, IDT) was used to determine trace elements. The AA Duo instrument manufactured by Agilent is equipped with a graphite furnace, graphite cuvettes, equipped with a background correction system and the necessary hollow cathode lamps.

Fig. 1. Miljacka river basin with hydrological stations (Source: http://www.centar.ba/upload/documents/BFC/1._Strateski_dokumenti/2.%20Sektorski%20dokumenti/Miljacka-I%20faza.pdf)

For pre-treatment of samples and solutions for preparation, chemicals and solutions of the highest possible purity were used, as follows: water of the first class as specified in the standard ISO 3696: 1987 ($\leq$0.01 mS/m), nitric acid concentrated (HNO_3) = 14.4 mol/l, or $\approx$ 1.4 kg/l (65%), standard solution, c = 100 µg/l for lead, blank dilution solution, palladium nitrate / magnesium nitrate modifier and inert gas (argon).

Determination of mercury was performed by AAS atomic absorption spectrometry method with and without enrichment BAS EN ISO 12846 - modified method.

A non-enrichment hydride technique was used which can perform determinations of 0.05 µg/l. The equipment manufacturer's recommendation is that the limit is 0.20 µg/l. Since this is a new instrument, the quantification limit was not practically determined, but the request of the equipment manufacturer was respected.

Table 1. Standardized methods for determining physicochemical analyzes used in research

Turbidity/NTU	EN ISO 7027–1:2017
Electroconductivity of water /µS/cm	BAS EN 27888:2002
pH	BAS EN ISO 8467:2002
Suspended matter/mg/l	BAS ISO 11923:1997
Nitrates, NO_3- /mg N/l	APHA 4500-NO3-B
Amonium, NH_3/mg N/l	BAS ISO 7150–1:2002

3 Results

Water turbidity was expressed in NTU (Nephelometric Turbidity Units). Based on the obtained results, we can say that the lowest water turbidity is in the profile PT1 (9.61) and PT6 (5.99), and the highest in PT8 (36.14). This was reasonably so because this tributary came from a mountain massif at a time of melting snow and intensified activity.

Table 2. Results of physical and chemical analysis of water from the river Miljacka

Faktor	PT1	PT2	PT3	PT4	PT5	PT6	PT7	PT8	PT9	PT10
Turbidity/NTU	9.61	15.77	39.81	22.83	13.73	5.99	24.1	36.14	25.02	23.39
Electroconductivity /μS/cm	4.28	/	265	358	401	377	396	352	343	476
pH*	6.6	/	7.12	7.15	6.85	7	6.84	6.71	7	6.89
Suspended matter/mg/l*	12	15	37	48	8		74	26	870	14
Nitrates. NO_3-mg N/l**	1.8	1.7	1.32	2.44	0.98	1.58	2.82	3.36	2.04	2.9
Amonium. NH_3 /mg N/l**	1.76	2.76	0.25	2.83	2.12	0.05	1.23	4.08	1.89	3.16
Consumption of KMnO4/gO_2/L*	4.23	/	/	/	0.97	/	7.49	/	/	/

Suspended particles most often occur in surface waters into which they reach by leaching from the soil or by the erosive action of water in watercourses. As we can see, the results tell us that the largest presence of suspended matter is in the PT4 (48 mg/l) and PT9 (870 mg/l) profile.

Based on the obtained results, we can say that the concentration of nitrate is the highest in the PT8 (3.36 mg NO_3-/N/l) profile and the lowest in PT5 (0.98 mg NO_3-/N/l). Sources of nitrates in surface and groundwater are natural and artificial fertilizers, municipal wastewater, overflow septic tanks, dead animals, fish excrement and the like.

The presence of ammonia in the upper stream higher than allowed is an important indicator of fecal contamination. As we can see the most polluted are PT2 (2.76 mg NH_3 /mgN/l) and PT4 (2.83 mg NH_3 /mgN/l, and PT8 (4.08 mg NH_3 /mgN/l) and PT10 (3.16 mg NH_3 /mgN/l). The ammonia content in water depends on the temperature and pH of the water. The higher the temperature and pH, the higher the toxicity of ammonia. Nitrates and nitrites are formed by the process of nitrification, which is a process of microbiological oxidation of ammonium. Therefore, they are often associated with older fecal contamination or older water contact with organic matter. Thus, nitrates indicate the occurrence of organic contaminants in the last stage of oxidation or the presence of mineral fertilizer residues (Rajković 2003).

Potassium permanganate consumption is the oldest method on the basis of which the load of water with organic matter can be estimated. Data from table shows the results of determining the content of $KMnO_4$ consumption. Sample 1 has a much higher value of $KMnO_4$ consumption (4.23), compared to sample 5 where the content decreased (0.977). According to the Decree on classification and categorization of surface and groundwater Official Gazette the permitted content of $KMnO_4$ is 4.0–7.0 mg l–1. The condition of surface waters is especially bad in the dry summer period when the water level of rivers is extremely low, which leads to the concentration of nutrients and organic components and the creation of unpleasant odors near watercourses.

Contaminants may be present in water or in air as a result of natural processes or through mechanisms of displacement and dispersal related to human activities. Contaminants from point sources discharge either into ground water or surface water through an area that is small relative to the area or volume of the receiving water body (Winter et al. 2016).

Trace elements

Considering that these are surface water bodies, the comparison of the determined values with the limit ones was made on the basis of the Decision on surface and groundwater characterization, reference conditions and parameters for water status assessment and water monitoring (Official Gazette of FB and H, No. 1/14).

Sample 4 (3.60 μg/l) has the lowest concentration of lead in water, while sample number 6 (6.24 μg/l) has the highest content. The lead content in surface waters varies widely depending on the source of pollution, the lead content in the sediment and the properties of the environment (pH, temperature, etc.).

Table 3. Lead and mercury content in used samples

Sample	Lead (Pb)	Mercury (Hg)	Unit of meassure	Reference value, μg/l
1	4.26	0.50	μg/l	
2	4.12	0.55	μg/l	Pb = 7.20
3	4.22	0.50	μg/l	Hg = 0.07
4	3.60	0.60	μg/l	
5	4.12	0.60	μg/l	
6	6.24	0.10	μg/l	
7	5.20	0.15	μg/l	Pb = 7.20
8	6.20	0.10	μg/l	Hg = 0.07
9	5.60	0.10	μg/l	
10	5.08	0.10	μg/l	

Based on the Decision on the characterization of surface and groundwater, reference conditions and parameters for water status assessment and water monitoring ("Official Gazette of FB and H", No. 1/14), the reference value of mercury is 0.07 μg/l. According to the obtained results of the mercury content test of the tested sites of the Miljacka river, it has been observed that water samples from site number 4 and 5 (0.60 μg/l) have the highest concentration, and samples 8, 9 and 10 (0.10 μg/l) have the lowest content values of mercury in the water.

The main sources of mercury pollution include anthropogenic activities such as agriculture, municipal wastewater discharge, mining, incineration and industrial wastewater discharge (Milanov 2014).

4 Discussion

Nitrates indicate the presence of organic pollutants in the last stage of oxidation or the presence of mineral fertilizer residues. The amount of dissolved substances depends on the origin of the water. Organic substances in water are most often the result of microbiological decomposition of plant and animal organisms.

The effects of human activities on the quantity and quality of water resources are felt over a wide range of space and time scales (SSWMS 2013). In areas where agricultural activity is more pronounced, the nitrate content in the water may be increased. Nitrogen fertilizers and organic fertilizers also contribute to the increase of nitrate concentration in water. Apart from them, the sources of pollution are emissions from combustion engines and improper waste disposal. Ammonia is a colorless, gaseous compound of hydrogen and nitrogen, highly soluble in water. It is most often found in water as a product of the decomposition of organic matter, and as a product of wastewater containing ammonia and fertilizers.

Based on the examined water quality, which was conducted in the area of the river Miljacka and its tributaries, it can be seen that they did not fully comply with the regulations.

From the physico-chemical analysis of the water from the river Miljacka from the mentioned profiles, it can be concluded that the values of the concentration of ammonia, nitrates, suspended solids and other parameters increase to the PT8 profile, after which they decrease. The increased content of ammonia and nitrate leads to an unpleasant smell and taste of water if it is for drinking. The development of bacteria increases, and the transparency of water decreases. Water is polluted aesthetically, ecologically and hazardous for health (Omanović-Mikličanin et al. 2017). With the development of the human community, with the increase in the number of inhabitants, the amount of organic matter has increased significantly (Dural et al. 2007). Their concentrated discharge into rivers prevents the process of self-purification and natural biological purification.

Also, the water from the PT5 profile shows reduced concentrations of suspended solids, ammonia, as well as the consumption of permanganate, which may indicate possible self-purification on that profile by the influx of larger amounts of water, which means that water in that area has self-cleaning properties. Only water in which biological balance has been achieved can have the ability to self-cleanse (Veljković 2008).

5 Conclusions

According to the results of water control from river Miljacka we can conclude that water from different sites of sampling was polluted with different pollution material that could cause problem for the flora and fauna in the river stream. Despite the legal obligations to preserve watercourses, no one is still complying. In accordance with the obtained results of the analysis, it is necessary to take one big step in the use of these obligations and to protect the entire watercourse of the river Miljacka from all types of pollution. Special attention should be paid to organic pollution, which is an indicator of fecal pollution in the very center of the city of Sarajevo. At the end it can be said the measured values do not meet the values according to the Regulation on classification and categorization of surface and groundwater.

Conflict of Interest. The authors declare that there are no conflicts of interest regarding the publication of this paper.

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The Effects of Pretreatments on the Physicochemical and Sensory Properties of Frozen Carrots

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Abstract. The aim of this research was to investigate the effects of thermal pretreatments on the physicochemical and sensory properties of frozen carrots. The experiment includes three parts, of which the first focuses on defining the optimal texture of carrot samples depending on applied temperature and power. The second part shows the production of frozen samples after pretreatment, such as blanching in water at 85 °C and in water vapor for 30, 60, 90 and 120 s, as well as microwave heating at medium power of 400–500 W for 30, 60, 90 and 120 s. The physicochemical (texture, total dry matter, L-ascorbic acid and total phenols) and sensory analyses of the produced samples, both before freezing and after defrosting, are presented in the third part. The results show a statistically significant effect of pretreatments on the analyzed physicochemical and sensory properties before freezing and on physicochemical properties of defrosted samples. In terms of preserving all analyzed physicochemical and sensory properties, blanching in water vapor has shown to be the most optimal pretreatment for carrots set for freezing.

Keywords: Microwave heating · Blanching · Texture · Total phenols · L-ascorbic acid

1 Introduction

The cultivation of vegetables is a seasonal job and in order to consume the produce throughout the year a significant part is preserved via different methods. Freezing is considered to be the optimal method. It includes lowering the temperature, separating water or ice crystals, which results in stopping all chemical and microbiological processes. However, to preserve the quality of the raw material during these procedures, it is necessary to carry out pretreatments. The most commonly used pretreatment is blanching, which is a thermal process of short-term food processing. The main goals of blanching are inactivating enzymes that catalyze the degradation reactions in raw or frozen products and the prevention of microbial activity in the product [1]. In blanching, temperatures usually range from 70 °C to 100 °C and the duration of the treatment is from

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1 to 15 min [2]. Blanching can improve the quality of vegetables in terms of stability and preservation of color, texture and reduction of the number of microorganisms [3]. The texture also changes during the blanching process. The most commonly used blanching methods are blanching in water, vapor and microwave heating [4]. A vegetable that is highly valued by consumers due to its sensory and nutritional components is the carrot (lat. *Daucus carota L.* ssp. *sativus* Hoffm). It contains significant amounts of dietary fibers, minerals, antioxidants – particularly carotenoids, followed by phenols and vitamins [5–8]. In the food industry, it is used in fresh, dehydrated, frozen or marinated form [8]. Due to the growing consumer demand for products with slightly modified properties in terms of raw material, the need for frozen carrots is growing. However, besides the loss of nutritional components, especially those soluble in water, freezing also causes irreversible changes to the texture of food. Therefore, in order to preserve the physicochemical and sensory properties of frozen carrots, it is necessary to perform certain pretreatments. Therewith, this study aims to examine the effects of pretreatments - blanching in water and vapor and microwave heating - on the physicochemical and sensory properties of frozen carrots.

2 Materials and Methods

2.1 Materials

The main material used in this study includes carrots bought in the supermarket. In addition to fresh carrots, used to define the optimal texture, a commercial sample of frozen carrots purchased in the same market was analyzed.

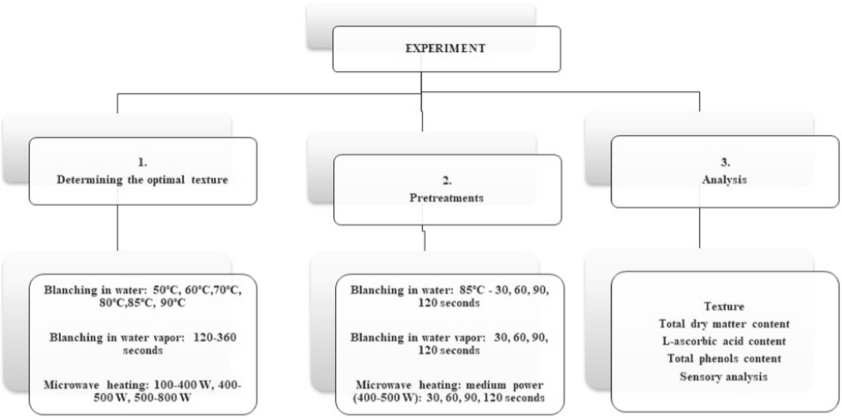

Fig. 1. Experimental part of the research

2.2 Methods

Determining the Optimal Texture

In order to determine the optimal texture carrot samples were cut into 5 mm thick slices. The samples were pretreated by blanching in water at 50 °C, 60 °C, 70 °C, 80 °C, 85 °C and 90 °C, by vapor for 120, 180, 240, 300 and 360 s, and heated in a microwave oven at moderate low power, moderate power, moderate strong power (Table 1). Blanching in water was carried out in a water bath, water vapor blanching in a vapor cooker (Philips AVENT) and a microwave oven (Končar M20BM) was used for microwave heating. After the pretreatments, the texture, i.e., the hardness, of the samples was determined by the method of direct piercing and the samples were frozen in liquid nitrogen. Twenty-four hours later, the samples were defrosted and the hardness was measured again. At the same time, the hardness of the commercial sample of frozen carrots, used for defining the optimal texture of the produced samples, was determined. A texture in the range of 5.32–10.25 N was used in selecting the optimal treatment time for each pretreatment performed (Table 1). In relation to the findings, the following pretreatments were selected: blanching in water at 85 °C for 30, 60, 90 and 120 s, blanching in water vapor for 30, 60, 90 and 120 s and microwave heating at moderate power of 400–500 W for 30, 60, 90 and 120 s (Fig. 1).

Table 1. Selection of optimal treatment time for carrot samples

Pretreatment	Texture before freezing (N)	Texture after defrosting (N)
Blanching in water		
50 °C – 5 min	20.82	0.88
60 °C – 4 min	15.67	1.15
70 °C – 3 min	14.49	1.31
80 °C – 2 min	12.71	1.41
85 °C – 1 min	4.89	3.17
90 °C – 0,5 min	8.25	0.87
Blanching in vapor		
2 min	20.98	5.91
3 min	13.18	5.26
4 min	6.54	2.40
5 min	8.10	1.94
6 min	2.80	2.56
Microwave heating		
Moderate low power (100–400 W)	7.88	1.86
Moderate power (400–500 W)	7.11	3.00
Moderate strong power (500–800 W)	5.89	3.15
Commercial sample	–	3.17

The Production Process of Frozen Carrots

The production of pretreated frozen carrots was carried out in the second part of the study. The process included the following stages: inspection and washing of the carrots, peeling with a ceramic knife and cutting into 5 mm thick slices, pretreating by blanching in water (W), water vapor (V) and microwave heating (M) and freezing samples in liquid nitrogen and storing them at −20 °C. Also, the control sample (C) was prepared in the same way but without pretreatments.

Determining the Optimal Texture

The inspection of the texture (hardness) was carried out before freezing and after defrosting the carrot samples. Hardness is a physical property of products and it was determined with the help of a texture analyzer (TA.Xt plus - Stable Micro System, Surrey, UK) by the direct piercing method, with the value of the force used expressed in N.

Determining Total Dry Matter Content

The content of total dry matter was determined by the standard AOAC [9] method, by drying the samples at 105 °C until constant weight. For the analysis, 3.5 g of previously prepared sample was taken.

Determining L-Ascorbic Acid Content

The content of L-ascorbic acid was determined by the standard AOAC [9] method, using 2,6-dichlorophenolindophenol.

Determining Total Phenols Content

Total phenols content was determined using the Folin-Ciocalteu spectrophotometric method [10]. Methanol mixed with 1% butylated hydroxytoluene (BHT), to prevent oxidation, and 3% formic acid was used as the extraction agent, according to Escarpa and Gonzales [11]. Ten milliliters of the extracting agent were added to 10 g of crushed carrot sample. The prepared mixture was placed in an ultrasonic bath for 30 min for extraction, after which the sample was centrifuged at 10,000 rpm for 7 min to separate the liquid from the solid matter. For the analysis, 0.2 ml of aliquot was taken and 1.8 ml of distilled water was added to the test tube. Folin-Ciocalteu reagent (1:10 dilution) was added to the solution and left to react for 30 s. Then, 8 ml of 7.5% Na_2CO_3 was aliquoted into the test tubes. These chemicals were mixed in a test tube and left for 2 h. After that, the absorbances measured 765 nm on the spectrophotometer (Shimadzu, UV-1700, Japan). Gallic acid was used as the standard to produce the calibration curve and the total phenols content was expressed in g GAE/100 g of fresh weight.

Sensory Analysis

Sensory analysis was conducted before freezing and after defrosting the carrot samples. An average sample was made from the three repetitions of all produced samples. The samples were presented using the Latin Square method, with four slices of carrot on a clear plate with a three-digit code number. In this analysis, a scoring method and a unified grading scale were applied. The evaluation was carried out by 10 trained evaluators that

passed a screening test based on recommendations given in ISO 8586. They assessed the following sensory attributes: appearance, taste, firmness, and overall acceptability. Each attribute was rated on a five-point scale, where 1 is dissatisfactory and 5 is excellent [12].

Statistical Data Analysis
The analysis of the statistical data was performed in the SPSS program (Version 23) and it included a two-factor analysis of variance to analyze the impact of pretreatment and treatment time, before freezing and after defrosting, on the L-ascorbic acid content, total phenols, total dry matter, texture and sensory attributes. The determined statistically significant differences were tested by the post hoc Tukey test. The significance level was $p \leq 0.05$. Principal component analysis (PCA) of the samples was performed in the Past 3.22 program.

3 Results

In this section are presented results for texture, total dry matter content, L-ascorbic acid content, total phenols content, sensory analysis and PCA analysis.

3.1 Texture

Figures 2 and 3 show the texture of carrot samples before freezing and after defrosting. The statistically significant effect of pretreatment, as well as the interaction of influencing factors, was found. However, the treatment time did not show any effect on the texture of carrot samples. It is evident that the texture, or hardness, was lost by extending the duration of the pretreatment. Generally, blanching in vapor preserved the best texture of defrosted samples (Figs. 2 and 3). Paciulli [13] examined carrots previously blanched in water at a temperature of 100 °C for 2.5 min and in a microwave oven at moderately low power of 450 W for 10 min. The effect of the pretreatment on the microstructure and texture of industrial frozen carrots was analyzed. After blanching in water, the carrot samples had dehydrated and the cells were separated with the cell wall swelling. Blanching caused cellular dehydration and tissue separation with the same intensity in both fresh and treated carrots. Microwave heating caused tissue dehydration and rapid removal of water from the peripheral zone, which probably increased the mechanical strength of the tissue, thus increasing the crystallinity of cellulose and hemicellulose in the cell wall. Kidmose [14] also investigated the influence of different blanching media on carrot texture. Microwave heating resulted in a disturbed, dehydrated texture. Xu et al. [15] pointed out that certain processing processes directly affect the physical characteristics of vegetables. The study found that blanching significantly reduces the hardness of soybeans by 17.28% in a sample blanched at 100 °C for 2.5 min. The reduction in hardness was more intense with the extension of the blanching duration. When talking about changes in the texture of defrosted samples, Zaritzky [16] reported that the main reasons for this phenomenon are water migration and ice recrystallization. The texture of the carrot samples was changed so that in defrosted samples it was significantly lower compared to the soybean whose hardness increased. In order to

preserve the texture, it is suggested to minimize temperature fluctuations or introduce internal barriers between the product and the packaging. In the current study, the texture of the samples blanched in water ranged between 7.8 N to 15.0 N with slightly visible changes during storage. These values are slightly lower than the ones given by Zaritzky where only the sample blanched for 120 s belongs to the specified range for texture. Reyes de Corcuera et al. [4] proposed blanching as a pretreatment that will even allow the formation of ice crystals during the production of frozen vegetables. Neri et al. [17] monitored the effect of thermal treatments (55–90 °C) on carrot texture. The research showed that by extending the treatment time and increasing the temperature the texture significantly decreases, by a temperature of 90 °C. Samples blanched at 75 °C showed an increase in hardness. According to the authors G´omez Galindo et al. [18], this phenomenon is characteristic for treatments carried out at relatively low temperatures, up to 75 °C, and this can be attributed to the activation of polyphenol esterase (PE) at temperatures between 55 °C and 70 °C. PE, in fact, hydrolyzes the methyl ester in the pectin molecule before thermal inactivation, releasing methanol and free galacturonic groups. Free carboxyl groups can create bonds between pectin polymers by forming a salt bridge with divalent cations, especially Ca^{2+}, that are naturally present in fruit tissue or in blanching water. On the contrary, during treatment at high temperatures, PE is rapidly inactivated and certain reactions affect the solubilization, degradation and gelling of the cell wall which leads to softening of the plant tissue. Xu et al. [19] investigated the effect of water blanching pretreatment, at 60 °C and 90 °C for 4 min, on carrot texture. The study found that the properties of texture, elasticity and strength were higher at lower temperatures, while the sample blanched at 90 °C had poorer structure and elasticity, but increased viscosity. The main reasons for this phenomenon may be changes in the cell membrane, loss of turgidity and dissociation of the cell wall matrix.

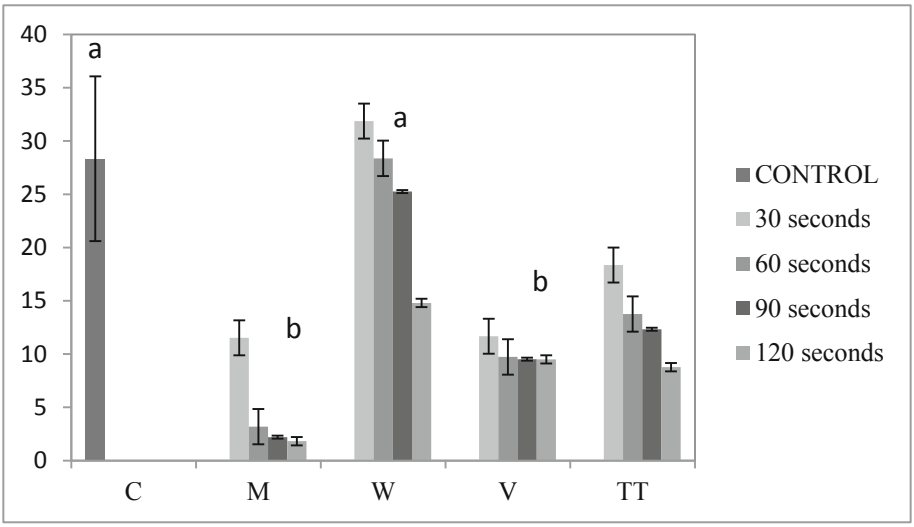

Fig. 2. Texture of samples before freezing (N) (Letters a-b represent statistically significant differences between pretreatments at probability level $p \leq 0.05$). Explanation of symbols: C-Control; M-Microwave heating; W-Blanching in water; V-Blanching in water vapor; TT-Treatment time;

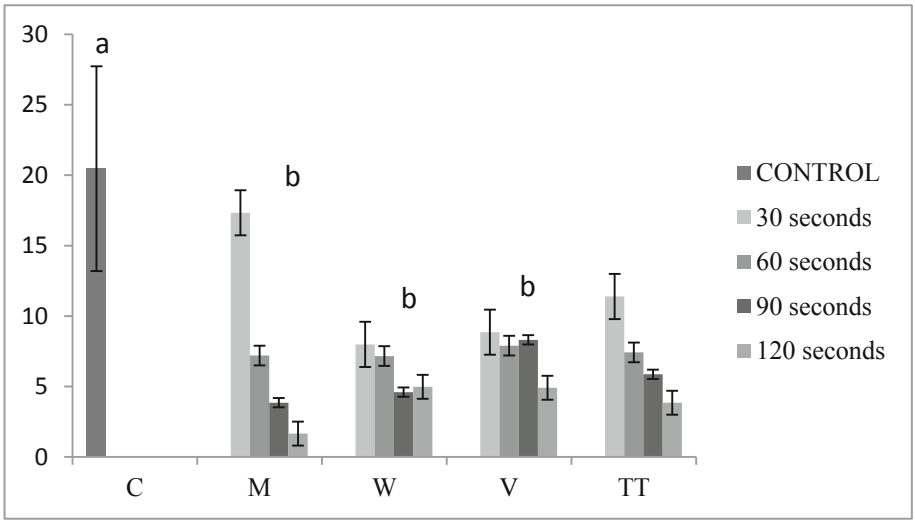

Fig. 3. Texture of samples after defrosting (N) (Letters a-b represent statistically significant differences between pretreatments at probability level $p \leq 0.05$). Explanation of symbols: C-Control; M-Microwave heating; W-Blanching in water; V-Blanching in water vapor; TT-Treatment time;

3.2 Total Dry Matter Content

The two-factor analysis of variance revealed a significant influence of pretreatment as well as the treatment time on the content of total dry matter in samples before freezing and after defrosting. Significant interaction between these two factors was also found (Figs. 4 and 5). In samples before freezing, an increase in total dry matter was observed in microwave heating (from 18.1–24.43%) and blanching in vapor (12.64–18.16%) by extending the pretreatment, while blanching in water decreased the content (14.38–11.93%). Defrosted carrots without pretreatment had a higher content of total dry matter after defrosting compared to that before freezing. The reason for this occurrence may be the concentration of chemical components due to water loss. A possible explanation for why samples that underwent blanching in water had the lowest values for total dry matter is the loss of components soluble in water. By reducing the soluble dry matter, the total dry matter is also reduced. Kidmose [14] investigated the influence of three different blanching media (water, water vapor, and microwave) on the content of total dry matter, and found that the content is higher after microwave treatment compared to the other two media. Benko et al. [20] analyzed the chemical parameters of seven carrot cultivars. The total dry matter content of the investigated varieties of fresh carrots ranged from 9.83 to 15.15% and is in line with the obtained results (11.58%). The relatively high values of dry matter content in the studied carrot varieties prove the high nutritional value, the same as in this study.

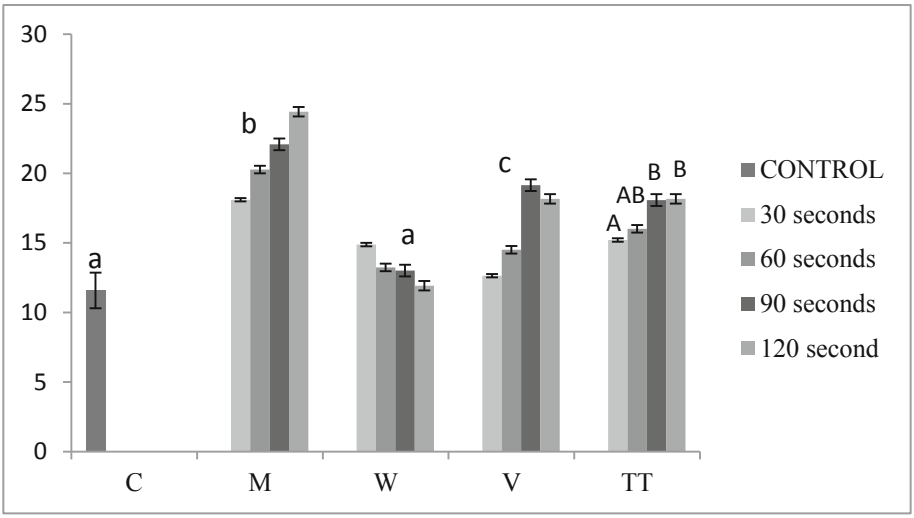

Fig. 4. Content of total dry matter before freezing (%) (Letters a-c represent statistically significant differences between pretreatments and letters A-B between treatment times at probability level $p \leq 0.05$). Explanation of symbols: C-Control; M-Microwave heating; W-Blanching in water; V-Blanching in water vapor; TT-Treatment time;

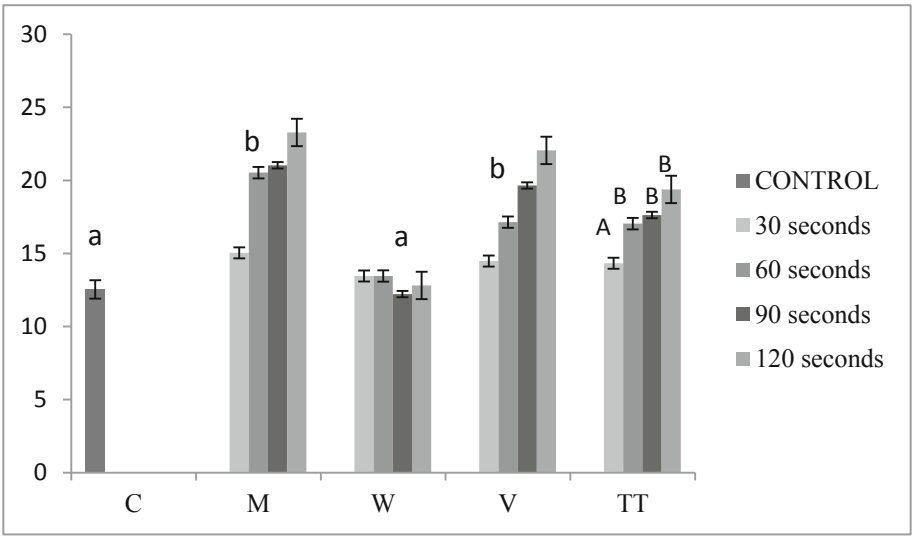

Fig. 5. Content of total dry matter after defrosting (%) (Letters a-b represent statistically significant differences between pretreatments and letters A-B between treatment times at probability level $p \leq 0.05$). Explanation of symbols: C-Control; M-Microwave heating; W-Blanching in water; V-Blanching in water vapor; TT-Treatment time;

3.3 L-Ascorbic Acid Content

A significant effect of pretreatment and its duration, as well as its interaction, on the content of L-ascorbic acid was evident for both carrot samples (Figs. 6 and 7). In the case of pre-freeze samples, the highest content of L-ascorbic acid was determined by microwave heating for 30 s (0.0034 g/100 g of sample) and showed the best preservation of L-ascorbic acid. After defrosting, the carrot samples blanched in vapor for 30 s had the highest content of L-ascorbic acid (0.0022 g/100 g sample). There is an evident oscillation in the content of L-ascorbic acid and a slight decrease related to the extension of the pretreatment duration. The reason for this may be the extreme sensitivity of L-ascorbic acid to various factors. One of them is the sensitivity to microwave heating and longer exposure to the treatment. Also, preparing the samples affects the L-ascorbic acid content due to the tendency for oxidative processes. Muftugil [21] examined the effect of blanching on L-ascorbic acid content in beans and found a higher content in samples blanched in water compared to water vapor. On the other side, the content was highest in samples heated in the microwave. The content of folic acid and L-ascorbic acid in previously treated green beets was analyzed by Osinboyejo et al. [22], who found that folic acid is more stable considerate to pH, temperature, and oxygen factors than L-ascorbic acid. The results of the same study showed that the content of L-ascorbic acid decreases significantly with the extension of the pretreatment at the same temperature. The reason for the drop in L-ascorbic acid content is the water-soluble character and sensitivity to high temperatures. According to Sikora et al. [23], the content of L-ascorbic acid in fresh carrots ranged from 0.0040–0.0049 g/100 g. In this research, the content of L-ascorbic acid in fresh carrots was 0.0028 g/100 g and the reason for the deviation may be the cultivar, agrotechnical properties, and the method used for determining L-ascorbic acid.

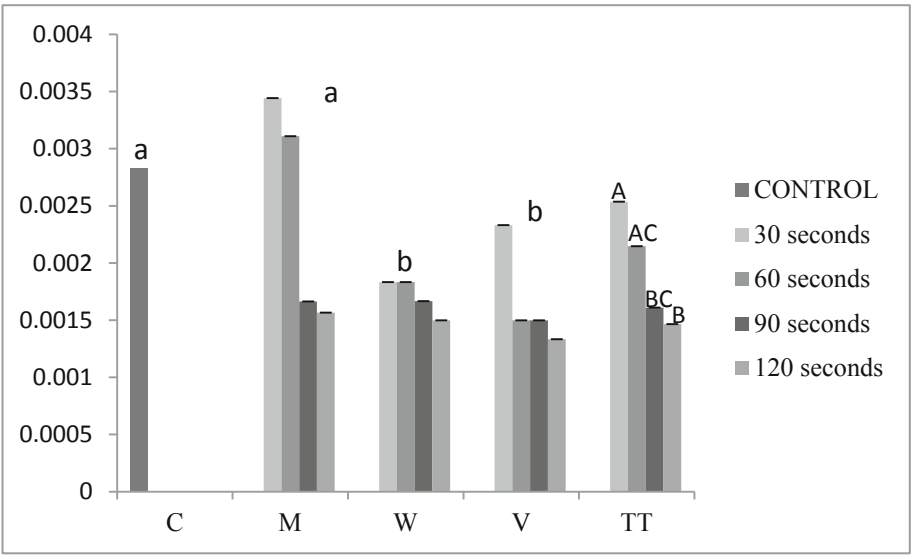

Fig. 6. Content of L-ascorbic acid before freezing (g/100g) (Letters a-b represent statistically significant differences between pretreatments and letters A-C between treatment times at probability level $p \leq 0.05$). Explanation of symbols: C-Control; M-Microwave heating; W-Blanching in water; V-Blanching in water vapor; TT-Treatment time;

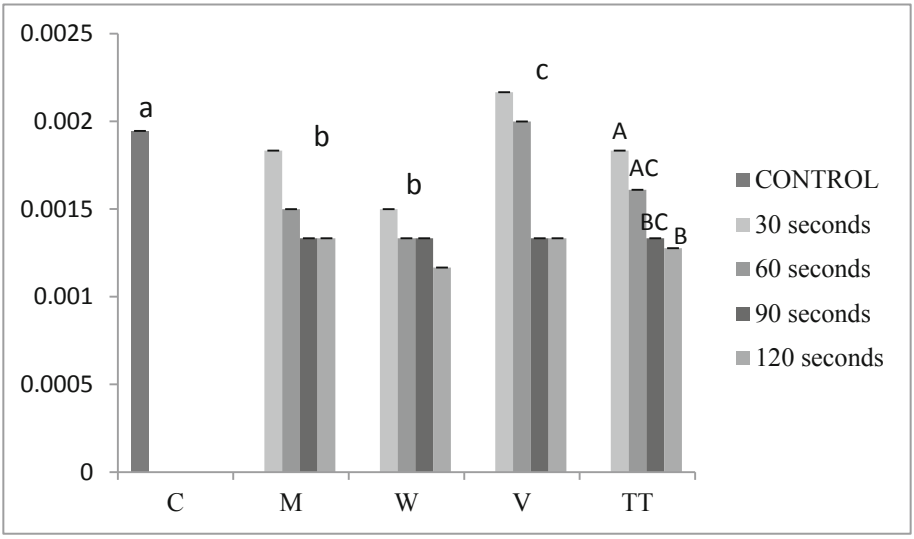

Fig. 7. Content of L-ascorbic acid after defrosting (g/100g) (Letters a-c represent statistically significant differences between pretreatments and letters A-C between treatment times at probability level $p \leq 0.05$). Explanation of symbols: C-Control; M-Microwave heating; W-Blanching in water; V-Blanching in water vapor; TT-Treatment time;

3.4 Total Phenols Content

The investigated pretreatments and their duration, as well as their interaction, showed a statistically significant effect on the total phenols content in both samples - before freezing and after defrosting. There was a considerable increase in the content of total phenols in the samples that were microwaved, as well as a decrease in the samples blanched both before and after defrosting. The reason for the increase in content in relation to extending the microwave pretreatment is the concentration of phenolic components due to the evaporation of water during microwave heating and the formation of colored compounds because of the caramelization of sugars present in carrots. Compounds formed during microwave heating were able to react with the Folin-Ciocalteu reagent and show a higher content of total phenols. Ma et al. [24] investigated the influence of blanching, cooking and baking on the content of total phenols in fresh carrot juice and concluded that heating in a microwave oven can increase the content. Ismail et al. [25] emphasize that phenolic compounds are very sensitive to heat treatment, even in a very short period of time. By analyzing polyphenolic components in carrots, Koidis et al. [26] noted that peeling affected the polyphenols content in the finished product. It has been determined that cleaned carrots have a higher amount of polyacetylene (phenolic compound), but by washing them after cutting the content is significantly reduced due to removal with water. Possible reasons for the lower content of total phenols in the treated carrot samples are operations such as peeling, cutting and washing. Rawson et al. [27] and Kramer et al. [28] claim that blanching and rapid freezing increase the retention rate of certain polyphenolic components in carrots during storage in the frozen state. Kidmose et al. [29] found an increase in the content of falcarinol, a polyphenolic compound, in frozen

carrots blanched before freezing. The content of total phenols in orange varieties of carrots was 0.0348 g GAE/100 g [30], while similar results, showing 0.0293 g GAE/100 g to 0.0396 g GAE/100 g for fresh carrots [31]. These results are much lower than in this experiment (0.087 g GAE/100 g), which may be due to differences in the variety, environmental conditions, soil type, harvesting time, storage, and so on (Figs. 8 and 9).

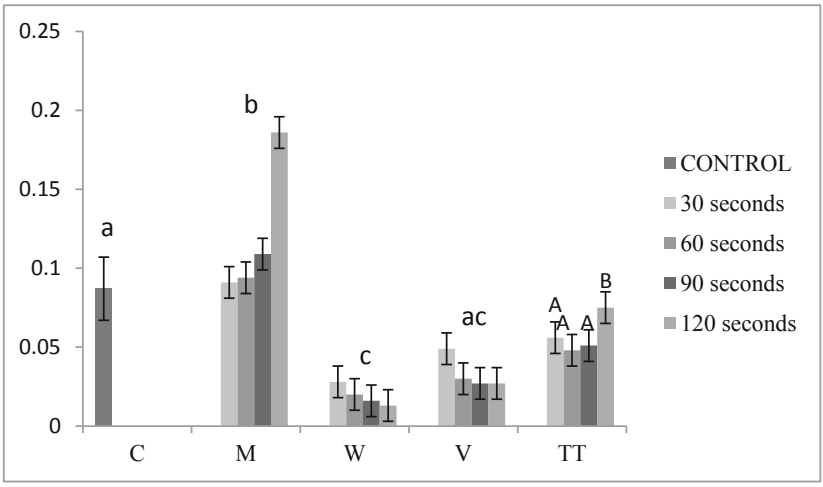

Fig. 8. Content of total phenols before freezing (g GAE/100 g of fresh matter) (Letters a-c represent statistically significant differences between pretreatments and letters A-B between treatment times at probability level $p \leq 0.05$). Explanation of symbols: C-Control; M-Microwave heating; W-Blanching in water; V-Blanching in water vapor; TT-Treatment time;

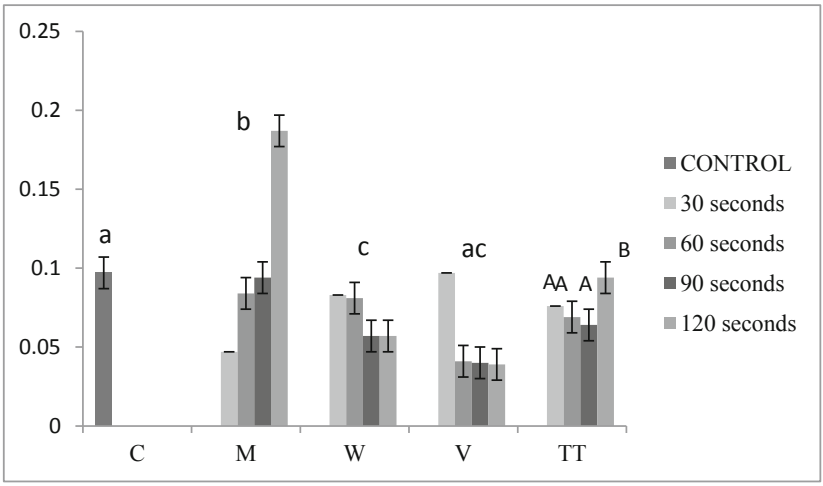

Fig. 9. Content of total phenols after defrosting (g GAE/100 g of fresh matter) (Letters a-c represent statistically significant differences between pretreatments and letters A-B between treatment times at probability level $p \leq 0.05$). Explanation of symbols: C-Control; M-Microwave heating; W-Blanching in water; V-Blanching in water vapor; TT-Treatment time;

3.5 Sensory Analysis

Rating for appearance, taste, firmness and general acceptability given during the sensory analysis are shown in Tables 2 and 3. The two-factor analysis of variance revealed a significant effect of pretreatment on the analyzed sensory properties before freezing and an effect of the treatment time on taste. There is no effect of these factors on the analyzed properties after defrosting. The interaction between the factors is significant in terms of appearance and general acceptability in all analyzed samples. On average, samples blanched in water received the highest rating, while samples that underwent microwave heating received the lowest numbers. A decrease in the acceptability of the samples' firmness related to the extension of the treatment is evident, which corresponds to the low values of the texture values of samples treated in the microwave. Samples blanched in water vapor after defrosting received the highest scores for the analyzed sensory properties, while samples blanched in water received the lowest scores (Table 3). Shamaila et al. [32] analyzed the influence of different blanching treatment time in water (30, 60, 90, 120, 180, 240 and 300 s) on the sensory properties of carrots. The rating for the analyzed sensory properties decreased with the extension of the blanching duration, except for the carrot flavor which received higher scores. However, the evaluators "appreciated" the aroma of "cooked carrots" more. Ratings for the flavor of the samples were lowered by prolonging the blanching treatment. This is in agreement with the results of the conducted work in which the evaluators gave the taste of blanched samples the lowest scores, especially for defrosted samples. However, carrot samples had the sweetness that Albran et Mabrouk [33] attributed to sugars, primarily sucrose and fructose, as well as free amino acids such as glutamic acid, and also pointed out that the taste and texture were influenced by the initial processing of the carrot and the process itself because the concentration of ingredients that contribute to the taste is reduced and the original structure of the fruit is disturbed. To preserve optimal sensory properties, the authors suggest limiting blanching time, rapid cooling, and the use of alternative blanching methods.

Table 2. Ratings of analyzed sensory properties before freezing (1–5)

	Sensory property			
Sample	Appearance	Taste	Firmness	General acceptability
Control	4.3 ± 0.27a	4.4 ± 0.15a	4.5 ± 0.27a	4.3 ± 0.27a
M 30 s	4.6 ± 0.22	4.4 ± 0.22	4.5 ± 0.24	4.6 ± 0.22
M 60 s	3.8 ± 0.25	4.0 ± 0.37	3.8 ± 0.37	3.8 ± 0.25
M 90 s	3.5 ± 0.27	3.7 ± 0.21	3.1 ± 0.38	3.5 ± 0.27
M 120 s	3.0 ± 0.15	3.1 ± 0.23	2.6 ± 0.27	3.0 ± 0.15
Total average $\overline{M}$	3.7 ± 0.22b	3.8 ± 0.26b	3.5 ± 0.31b	3.7 ± 0.22b
W 30 s	4.5 ± 0.31	4.5 ± 0.31	4.2 ± 0.18	4.5 ± 0.31
W 60 s	4.0 ± 0.21	4.1 ± 0.31	4.2 ± 0.20	4.0 ± 0.21
W 90 s	3.8 ± 0.29	3.8 ± 0.25	3.2 ± 0.37	3.8 ± 0.29

(*continued*)

Table 2. (*continued*)

	Sensory property			
Sample	Appearance	Taste	Firmness	General acceptability
W 120 s	3.9 ± 0.28	3.8 ± 0.33	3.8 ± 0.38	3.9 ± 0.28
Total average $\overline{W}$	4.1 ± 0.27ab	4.1 ± 0.30ab	3.9 ± 0.28a	4.1 ± 0.27ab
V 30 s	3.7 ± 0.26	3.7 ± 0.21	3.5 ± 0.31	3.7 ± 0.26
V 60 s	3.8 ± 0.25	3.8 ± 0.25	3.7 ± 0.37	3.8 ± 0.25
V 90 s	4.1 ± 0.21	4.2 ± 0.25	3.9 ± 0.31	4.1 ± 0.21
V 120 s	3.7 ± 0.21	3.4 ± 0.27	3.7 ± 0.30	3.7 ± 0.21
Total average $\overline{V}$	3.8 ± 0.23ab	3.8 ± 0.25b	3.7 ± 0.32b	3.8 ± 0.23ab

Total average – Average of all scores for different treatment times (microwave heating -M, blanching in water -W and vapor-V)
Letters a-b represent statistically significant differences between pretreatments at probability level $p \leq 0.05$

Table 3. Ratings of analyzed sensory properties after defrosting (1–5)

	Sensory properties			
Sample	Appearance	Taste	Firmness	General acceptability
Control	3.4 ± 0.23	3.8 ± 0.24	3.3 ± 0.23	3.4 ± 0.23
M 30 s	3.6 ± 0.22	3.6 ± 0.16	3.6 ± 0.13	3.5 ± 0.22
M 60 s	3.6 ± 0.16	3.5 ± 0.17	3.2 ± 020	3.6 ± 0.16
M 90 s	3.7 ± 0.25	3.8 ± 0.20	3.7 ± 0.30	3.8 ± 0.25
M 120 s	2.9 ± 0.25	3.1 ± 0.23	2.9 ± 0.18	2.9 ± 0.25
Total average $\overline{M}$	3.5 ± 0.22	3.5 ± 0.19	3.4 ± 0.20	3.5 ± 0.22
W 30 s	3.9 ± 0.23	3.7 ± 0.34	3.7 ± 0.30	3.9 ± 0.23
W 60 s	3.6 ± 0.22	3.7 ± 0.30	3.7 ± 0.30	3.6 ± 0.22
W 90 s	3.2 ± 0.22	3.1 ± 0.31	3.2 ± 0.33	3.2 ± 0.22
W 120 s	2.9 ± 0.25	3.2 ± 0.31	3.1 ± 0.31	2.9 ± 0.25
Total average $\overline{W}$	3.4 ± 0.23	3.4 ± 0.32	3.4 ± 0.31	3.4 ± 0.23
V 30 s	3.5 ± 0.17	3.6 ± 0.16	3.5 ± 0.22	3.5 ± 0.17
V 60 s	3.8 ± 0.20	3.7 ± 0.21	3.8 ± 0.20	3.8 ± 0.20
V 90 s	3.7 ± 0.15	3.3 ± 0.21	3.4 ± 0.16	3.7 ± 0.15

(*continued*)

Table 3. (*continued*)

	Sensory properties			
Sample	Appearance	Taste	Firmness	General acceptability
V 120 s	3.9 ± 0.18	3.8 ± 0.25	3.8 ± 0.20	3.9 ± 0.18
Total average $\overline{V}$	3.7 ± 0.18	3.6 ± 0.21	3.6 ± 0.20	3.7 ± 0.18

Total average – Average of all scores for different treatment times (microwave heating-M, blanching in water-W and vapor-V)

3.6 PCA Analysis

As shown in Fig. 10, samples distinctly differed according to the analyzed physicochemical and sensory parameters. Carrot samples treated in the microwave for 30 s (before freezing) are notably distinguished among other samples by the content of L-ascorbic acid. A significant correlation between total dry matter and total phenols content is present and samples with the highest contents are carrots treated in the microwave oven for 120 s, both before freezing and after defrosting. Carrot samples blanched in water, regardless of the treatment duration, demonstrated the highest hardness and are clearly separated from other samples by component number 2. Sensory attributes show a high mutual correlation, which can be seen in fresh carrot samples before freezing. Samples bleached in water vapor show greater homogeneity and similarity in all analyzed parameters, thus this treatment provides the optimal preservation of the desirable nutritional, bioactive and sensory carrot properties.

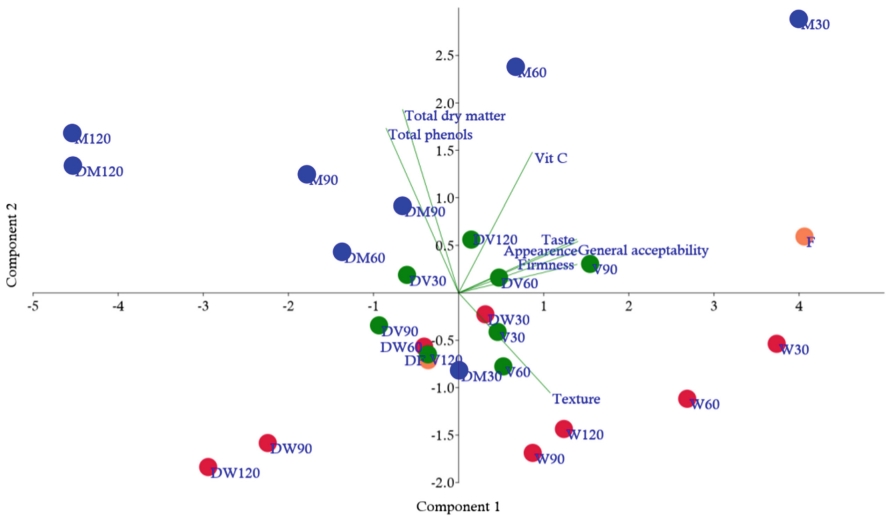

Fig. 10. PCA analysis of carrot samples

Explanation of symbols:
W30, W60, W90, W120 - Blanching in water (30–120 s, before freezing).
DW30, DW60, DW90, DW120 – Blanching in water (30–120 s, after defrosting).
V30, V60, V90, V120 - Blanching in water vapor (30–120 s, before freezing).
DV30, DV60, DV90, DV120 - Blanching in water vapor (30–120 s, after defrosting).
M30, M60, 90, M120 – Microwave heating (30–120 s, before freezing).
DM30, DM60, DM90, DM120 – Microwave heating (30–120 s, after defrosting).

4 Conclusion

The best pretreatment for preserving the physicochemical and sensory properties of carrot samples before freezing and after defrosting is vapor blanching, followed by microwave heating and water blanching. Shorter treatment times, specifically for 30 and 60 s, have shown to be more effective in preserving all desirable properties compared to other analyzed modes. Blanching pretreatments have a significant positive effect on the preservation of certain chemical components and on the sensory acceptability of frozen carrots. Future research should focus on testing other, alternative blanching methods to get a more detailed picture of the optimal method or pretreatment.

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Antibiotic Resistance of Wild Enterococci Isolated from Travnički/Vlašićki Cheese, B&H

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Abstract. Antibiotic resistance poses safety risk to public health, involving the spreading of antimicrobial resistance between animals, humans and the environment, also consequently along the food chain. Limited studies have considered the spread of antibiotic resistance due to wild enterococci isolated from traditional cheese. *Enterococcus faecalis* and *Enterococcus faecium* are the most common isolated enterococci from food, they can be found in different food sources (cheeses, meat, olives and vegetables). Variety of traditional cheeses made from raw and pasteurized milk contained enterococci as an essential part of natural microflora. The purpose of this work is preliminary examination of antibiotic resistance in enterococci isolated from traditional *Travnički/Vlašićki* cheese. As a result of 16S rRNA sequence analysis, 14 of the 21 enterococci strains were identified as *E. faecalis*, 6 as *E. faecium* and 1 strain as *E.durans*. The 21 *Enterococcus* strains were tested for susceptibility to 9 different antimicrobial agents by agar dilution method. A total of 19 *Enterococcus* strains displayed resistance to low concentrations of aminoglycosides, streptomycin (STR), gentamycin (GEN) and kanamycin (KAN) were analyzed with MIC > 64 $\mu g\ mL^{-1}$, MIC > 64 $\mu g\ mL^{-1}$ and MIC > 32 $\mu g\ mL^{-1}$, retrospectively. Only 2 *Enterococcus* strains were sensitive to above mentioned MIC of aminoglycosides. On the susceptibility/sensitivity of *Enterococcus* species to β-lactams, all *Enterococcus* strains were sensitive to ampicillin (AMP) (MIC > 16 $\mu g\ mL^{-1}$). However, 3 of the 14 *E.faecalis* strains as well as 3 of the 6 *E.faecium strains* and a *E.durans* strain showed susceptibility to MIC > 16 $\mu g\ mL^{-1}$ of penicillin G (PEN G). A total of 12 *Enterococcus* strains (57%) were sensitive to MIC > 16 $\mu g\ mL^{-1}$ of tetracycline (TET). All cheese enterococci strains displayed sensitivity to very high MIC > 8 $\mu g\ mL^{-1}$ of erythromycin (ERY) as well as chloramphenicol (CHL) (MIC > 4 $\mu g\ mL^{-1}$), those breakpoints were commonly used for clinical isolates. 50% of *Enterococcus* strains showed resistance to MIC > 32 $\mu g\ mL^{-1}$ of vancomycin (VAN), even if vancomycin-resistant enterococci (VRE) are important opportunistic pathogens with limited therapeutic options.

Keywords: Antibiotic resistance · Wild enterococci · Traditional *Travnički/Vlašićki* cheese

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1 Introduction

Traditional food plays an important role in regional and national cultural identity. Traditional cheeses are one of the food product that have become the image of different countries or region of origin, they differ from each other by their making process, time and way of ripening, type of milk used, texture, color, flavor, coagulation type (enzymatic and/or acid, etc.) [1]. Among these traditional cheeses, white-brined or white-pickled cheese varieties like *Feta*, *Domiati*, and related cheeses (e.g., *Brinza, Beli Sir, Telemes, Kareish, Beyaz Peiniri*) evolved in the eastern Mediterranean and Balkan regions [2, 3, 4, 5, 6]. On the Balkan Peninsula the most popular white pickled cheese varieties are *Bieno Sirenje* or *Beaten* cheese (Macedonia), *Beli Sir u Kriskama* or *Srpski*, *Sremski, Sjenicki, Homoljski, Zlatarski, Svrljski* (Serbia), *Pljevaljski, Polimsko-Vasojevaski, Ulcinjski* (Montenegro*), Travnicki/Vlasicki* [4, 7]. The central region of Bosnia and Herzegovina (*Vlašić* mountain) has a long tradition of sheep production, most of them *Travnička (Vlašička* or *Dubska) Pramenka* breed. This autochthonous breed has been selected for its rusticity, adaptability to the environment and lower milk yield compared to other breeds [8]. Practically all sheep's milk is used to make cheeses. Most of the brined cheeses are produced in family enterprises or in small artisanal units [9]. The increased demand for cheeses matured in brine has created the need for standardized techniques for their manufacture [10]. In this context, the continuity of traditional pastoral systems and milk production is the key to the protection of this breed, the preservation of its production systems as well as for the denomination of origin "*Travnicki/Vlašićki* cheese" [7, 9, 11]. Total quantity of traditional *Travnički/Vlašićki* cheese are still made from raw sheep's milk, without starter culture addition, the autochthonous microflora of the milk contributes to ripening process [7, 9, 11]. Enterococci are dominant microbiota of many artisanal cheeses [12]. Enterococci may be present to large numbers in dairy products (up to 10^8 CFU g−1) [13], in many artisanal cheeses enterococci are part of the non-starter lactic acid bacteria (NSLAB) [12, 13]. They are among the most common lactic acid bacteria in raw milk [16], which they access from dairy environment, animals, and humans [13, 14, 15, 16]. The presence of these bacteria in dairy products is usually associated with inadequate hygiene practices as a consequence of fecal contamination [17, 18]. Despite their relation to the intestinal microbiota of humans and dairy animals, fecal contamination does not seem to play an important role upon entrance of enterococci into the dairy production chain [19]. The commercial application of enterococci in probiotic preparations and foods for human consumption is limited because of insufficient information on their safety-related properties and health benefits as well as unfavorable regulatory environment [19, 20].

The most frequent species belonging to the *Enterococcus* genus found in dairy products are *Enterococcus faecium* and *Enterococcus faecalis* [19] as well as *Enterococcus durans* and *Enterococcus gallinarum* [19, 21]. *E. faecium* and *E.faecalis* might represent a public health issue for their resistance to antibiotics [22]. Antimicrobial resistance and virulence genes can be carried on mobile genetic elements [23, 24]. Therefore, enterococci in milk can contribute to the spread of potentially pathogenic, antimicrobial-resistant strains to humans through food.

The Commission Regulation (EC) No 1441/2007 of 5th December 2007 allows derogation from Regulation (EC) No 2073/2005 of 15th November 2005 'on microbiological

criteria for foodstuffs' declaring that enterococci in food are not always due to fecal contamination and sets no limit for their presence in foods [25]. However, The European Food Safety Authority (EFSA) requires as part of its Qualified Presumption of Safety approach to the safety assessment of bacteria deliberately introduced in the food chain, that acquired resistance determinants to antimicrobials of clinical importance are absent [26–28]. Because presence of virulence factors in enterococci is strain-dependent, a genus- or species-wide decision on their safety status has been hampered. The Scientific Committee of the European Food Safety Agency has accordingly decided not to grant them Qualified Presumption of Safety (QPS) [19, 29], and neither has the US Food and Drug Administration granted them Generally Regarded as Safe (GRAS) status [19, 29, 30]. The lack of QPS/GRAS status significantly hinders application of enterococci as food cultures and conveys one more justification to why research on enterococcal safety-related traits is of utmost importance [19].

The purpose of the present study was to analyze antimicrobial resistance in cheese enterococci isolates and to prevent the spread of pathogenic, antimicrobial-resistant enterococci in dairy products. In the current study, the MRS agar medium supplemented with antibiotics was compared with the closely defined M17 agar medium for antimicrobial susceptibility testing of cheese enterococci [31].

2 Material and Methods

Cheese Sampling

Traditional white-pickled *Travnički/Vlašićki* cheese samples were taken directly from manufacturers on the mountain *Vlašić* (central Bosnia and Herzegovina). The samples were placed into sterile plastic bags and transported in a cooling box to the Laboratory of Microbiology at the Faculty of Agriculture and Food Sciences, University of Sarajevo. Samples were stored at a temperature of 4 °C until their examination within 24 h.

Isolation and Identification of Enterococci

Isolation of cultivable enterococci from cheese samples was performed as follows: ten grams of each cheese sample was homogenized and transferred to 90 mL sterile 2% (w/v) tri-sodium citrate solution (Himedia, India). Decimal dilutions to 10^{-8} of the homogenates were prepared with sterile 0.9% (w/v) sodium chloride and were plated on KF Streprococcus agar/ pH 7,2 (Himedia, India) with aseptically added 10 ml of 1% 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) (FD057) after cooling to 45–50 °C, then on de Man, Rogosa and Sharpe (MRS) agar/pH 5,7 (Himedia, India) and on M17 agar/pH 7,2 (Himedia, India) [32]. Incubation of inoculated media was performed at 37 °C for KF Streprococcus agar in aerobic conditions, at 30 °C for M17 agar as well and at 30 °C in anaerobic conditions with Anaerocult A (Merck) for MRS agar media.

After 24 h of incubation at 37 °C, typical colonies of presumptive enterococci on KF Streprococcus agar (which appear surrounded by a black halo) were observed [32, 33]. Five presumptive LAB colonies were randomly picked from one of the highest dilution plates of each of the KF/37 °C, MRS/30 °C and M17/30 °C agar media. Colonies were cultured in MRS or M17 broth for 24 h, this procedure was repeated twice (from agar

medium to broth) in order to obtain pure cultures. Single colonies were transferred to 10 mL of MRS or M17 broth and thus were considered as pure strains. After 24 h of cultivation, bacterial pure cultures were centrifuged, rinsed twice with 0.9% NaCl sterile solution and stored at −20 °C in sterile MRS or M17 broth (Himedia) supplemented with 20% (v/v) glycerol. Identification analysis was performed to the genus or species level by phenotypic identification based on physiological and biochemical methods, also Rapid ID32 Strep system (Biomerieux, France) was used for identification of presumptive coccus-shaped of LAB, according to the manufacturer instruction.

All isolates were tested for Gram stain, morphology and catalase production. Coccus-shaped isolates were analyzed for ability to grow at temperature (15 °C, 37 °C, 45 °C), in 2,4 and 6.5% NaCl broth and at pH 9,6. Reduced number of isolates were tested for production of CO_2 from glucose in reconstituted MRS broth with inverted Durham tubes, production of acetoin from glucose (Voges-Proskauer test), time required for the formation of curd in reconstituted skim milk and exopolysaccharides (EPS) production as described previously [34, 35]. Identification analysis was performed to the genus or species level by phenotypic identification based on physiological and biochemical methods, also Rapid ID32 Strep system (Biomerieux, France) was used for identification of presumptive coccus-shaped of LAB, according to the manufacturer instruction.

16S rRNA Sequence Analysis

A modified version of the Marmur procedure for bacterial DNA extraction was used [36]. Quality of isolated DNA were visualized through 1% (w/v) agarose gel (Sigma Chemical Co., Poole, United Kingdom) electrophoresis and stained via ethidium bromide. Polymerase chain reaction (PCR) was done on genomic DNA to amplify genes for the 16S rRNA. The universal primers 5′-GAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′ (*Escherichia coli* positions 9–27 and 1510–1492 respectively) were used for amplification of an approximately 1450 bp DNA fragment of the 16S rRNA gene [23]. The primers were synthetized by Invitrogen Ltd. (Paisley, Scotland). PCR reactions were carried out in a 50 μL reaction mixture containing 1 μL of each 20 pmol primer, 5 μL 10 × PCR buffer, 1 μL 10 mM dNTP, 1 μL DNA template and 0.25 μL 5U μL^{-1} Taq Polymerase (Finnzymes Oy, Espoo, Finland) and 40,75 μL dd H_2O. The PCR reaction was carried out in a DNA-Thermal Cycler (PTC-200, Waltham, MA, USA) using the following program: initial denaturation at 94 °C for 10 min; 30 cycles consisting of denaturation at 94 C for 45 s, primer annealing at 56 °C for 45 s, elongation at 72 °C for 90 s; a final extension step at 72 °C for 10 min.

The PCR products were purified using QIA-quick PCR purification Kit (QIAGEN, Germany), according to the procedure recommended by the supplier. Spectrophotometric analysis (NanoDrop Spectrophotometer, NanoDrop Technologies, Thermo Fisher Scientific) was used for preliminary evaluation of nucleic acid quality, via the assessment of the absorbance ratios $A_{260/230}$ and $A_{260/280}$.

Sequencing was done using a BigDye v3.1 terminator cycle sequencing kit, the primers 5′- CAGCAGCCGCGGTAATAC-3′ and 5′-ACGGGCGGTGTGTAC-3′ (*E. coli* positions 519–536 and 1406–1392, respectively) and the sequencing device ABI Prism 377 DNA (Applied Biosystems) [37]. The PCR reactions were carried out using the following program: initial denaturation at 94 °C for 10 min; 30 cycles consisting of denaturation at 94 °C for 45 s, primer annealing at 56 °C for 45 s, polymerization and

ddNTPs incorporation at 72 °C for 90 s. Sequences were edited using BioEdit software (Abbott, CA, USA) and analyzed using BLAST (basic local alignment search tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.1 Antimicrobial Susceptibility

The 21 *Enterococcus* strains were tested for their antimicrobial susceptibility by agar dilution method, according to modified the Clinical and Laboratory Standards Institute guidelines [38]. The inocula were prepared by suspending colonies from fresh overnight grown cultures in 5 mL of physiological solution (0.85% NaCl, w/v) until a density of 0.5 McFarland standard was reached. The cell suspensions were swabbed for confluent growth onto MRS and M17 agar media in duplicate (Merck, Darmstadt, Germany).

Media Preparation
To determine antibiotic resistance in enterococci, the agar dilution method on MRS/pH 5,7 and on M17/pH 7,2 agar media (Merck, Darmstadt, Germany) prepared according to the procedure recommended by the supplier. After the medium had cooled to 45 °C, a sterile solution of antibiotic was added to give a final concentration. A modified version of an internationally accepted procedure, such as those published by the Clinical and Laboratory Standards Institute [38] resistance of lactic acid bacteria to aminoglycosides were performed according to [40], in which Meuller-Hinton medium was replaced by MRS agar and M17 agar [31]. The agar dilution method is performed by incorporation of different concentrations of the antimicrobial agent into a molten agar medium, followed by the inoculation of a standardized microbial inoculum to the surface of the agar plate. The agar plates are evaluated by visually comparing varying strains in the series [39].

Nine antimicrobial compounds commonly used for the treatment of human and animal infections were tested. The antimicrobial belonged to different families: penicillins-penicillin (PEN-MIC > 16 μg/ml) and ampicillin (AMP-MIC > 16 μg/ml), glycopeptides-vancomycin (VAN-MIC > 32 μg/ml), macrolides-erythromycin (ERY-MIC > 8 μg/ml), tetracyclines-tetracycline (TET-MIC > 16 μg/ml), phenicols-chloramphenicol (CHL-MIC > 4 μg/ml) and aminoglycosides–low level gentamicin (GEN-MIC > 64 μg/ml), low level streptomycin (STR-MIC > 64 μg/ml) and low level kanamycin (KAN-MIC > 64 μg/ml).

Susceptibility Testing: Classification of the enterococci isolates into sensitive, intermediate and resistant groups was based on specific ability of each strain to grow on the whole surface area of Petri plates (without measuring the size of the inhibition zone). Cut-off values to differentiate among resistant and susceptible groups were defined on the basis of the growth distribution of the population after incubation at 37 °C for 18 h, the inhibition were determined and the strains classified as resistant (R), intermediate resistant (IR), or susceptible (S) according to the CLSI [38]. The minimum inhibitory concentration (MIC) was determined for each IR or R strain on a given antimicrobial.

3 Results and Discussion

Studies on prevalence and safety issue of enterococci from foods are limited in Bosnia and Herzegovina. This study was performed to evaluate the duel role of enterococci in food technology, although enterococci are dominant microflora of autochthonous cheeses produced in Southern Europe. In autochthonous white pickled *Travnički/Vlašićki* cheese produced in mountain-dairies, the counts of enterococci on KF Streptococcus agar media ranged between 2,3 log CFU/gr and 6,69 log CFU/gr [40]. Preliminary research on autochthonous LAB isolated from artisan dairy products of the Western Balkan region has indicated that about one third of all isolated LAB are *Enterococcus* species [41–47].

All enterococci strains used in this study were isolated from white-pickled *Travnički/Vlašićki* cheese samples [18] manufactured from raw sheep's milk and collected from nomadic pastoralists on the mountain *Vlašić* plateau in a period from 2006 to 2009. A total of 90 presumptive *Enterococcus* colonies were picked from KF Streptococcus agar media surface, also 180 colonies were collected from M17 and MRS plates. After Gram- staining, morphological examination under microscope and catalase tests, a total of 53, 30 and 31 isolates from KF Streptococcus, M17 and MRS plates, respectively, were selected. Most of primary isolated bacterial strains were catalase positive. Once the isolates were purified, phenotypic and technological tests were carried out. The isolates were stored as frozen stocks at −20 °C in MRS broth containing 20% (v/v) glycerol.

Initial LAB Characterization

To group of gram positive and catalase negative coccus-shaped isolates [82] were analyzed as presumptive enterococci, 42 isolates were bacilli.12 isolates show weak growth at 45 °C and 2 isolated showed weak growth in 6.5% NaCl, 50% of isolates produced acetoin and all isolates showed low acidification capacity during growth in milk for 48 h. Determination of technological and functional potential of enterococci is the ultimate goal in exploring their diversity. Phenotypic characterization of the enterococci strains with regard to production of CO_2 from glucose, acetoin production (VP^+), milk acidification and EPS production was performed for all isolated, the procedure was described for seven isolates primary isolated on KF Streptococcus media [41].

According to the phenotypic sugar fermentation profile characterizations using Rapid ID32 Strep system (Biomerieux, France), the coccus-shaped isolates from *Travnički/Vlašićki* cheese belonged to the genera *Enterococcus* [7], *Lactococcus* [15] and *Leuconostoc* [6], also 8 isolates could not be identified. The microbiologically and physiologically positive strains not identified by Rapid ID32 Strep system were excluded from further evaluation. Statistically significant difference in sugar fermentation profiles (patterns) of coccus-shaped isolates was observed, also the occurrence of lactococci and enterococci was different and correlated with phase of cheese ripening. These differences could be ecologically significant. All strains isolated on KF Streptococcus agar were assigned to the genus *Enterococcus.* Due to the presence of selective inhibitory components, such as 2,3,5-Triphenyl Tetrazolium Chloride (TTC), KF Streptococcus

agar has been shown to be selective for the isolation of cheese enterococci. Majority of isolates from MRS and M17 agar media also belonged to the genus *Enterococcus.*

Diversity of the Enterococci in Cheese

A total of 21 enterococcal strains isolated from *Travnički/Vlašićki* cheese samples were identified to species level by 16S rRNA gene sequencing analysis. The BLAST search produced percentages of identity with sequences available in NCBI database of at least 97%, which is the minimum level of similarity required between 16S rRNA genes from two strains to be considered as belonging to the same species [48]. 21 strains were confirmed to belong to 3 species: *Enterococcus faecalis* (14/21), *Enterococcus faecium* (6/21) and *Enterococcus durans* (1/21).One strain could not be classified as any of species because it shared only 96% identity with *E.faecalis.* The most common species were *E. faecalis* (66%) and *E. faecium* (30%), that is similar like in traditional Slovak *Bryndza* cheese [49]. Only one strain of *E.durans* was isolated in this study, also in different types of dairy products in the Western Balkan region (mainly cheeses) out of 636 natural dairy enterococci isolates, only five strains belonging to *E. durans* [50].

Antibiotic Resistance Is Common Among Cheese Enterococci in Bosnia and Herzegovina

Out of the 21 enterococci isolates tested for antibiotic sensitivity, more than 50% of isolates were resistant to one or more antibiotics. Resistance includes both full resistance and intermediate resistance, unless otherwise stated. The *E.durans* strain, three *E.faecium* strains and three *E.faecalis* strains were intermediate resistant to MIC > 16 μg/ml penicillin and all enterococci were sensitive to the same MIC > 16 μg/ml ampicillin. Small growth rate of intermediate penicillin-resistant enterococci comparing with the growth on standard MRS and M17 agar media associated on intermediate resistance. The resistance of enterococci to β-lactams is a characteristic feature that appears to be related to the low affinity of their penicillin-binding proteins (pbp's). Although rare, the resistance to β-lactams in *E. faecalis* may also be associated with β-lactamases [51, 52]. Penicillin or ampicillin resistance among enterococci due to β-lactamase production has been reported very rarely [38].

Low-level resistance to MIC > 64 μg/ml of amynoglycosides (gentamycin and streptomycin) was documented for all tested enterococci. Low level gentamicin resistance can also be associated with decreased permeability to the antibiotic [53]. All strains showed identical growth on M17 and MRS agar plates supplemented with MIC > 64 μg/ml gentamycin and streptomycin. Kanamycin also belonged to aminoglycosides-all *Enterococcus* strains were resistant or intermediate resistant to MIC > 32 μg/ml of kanamycin. Some studies have confirmed the existence of resistant strains of enterococci to MIC > 2,048 μg /ml of kanamycin [53]. According to the data given in the FEEDAP Panel, enterococci are the most resistant to kanamycin, streptomycin and neomycin, with a sensitivity threshold MIC > 1,024 μg/ml [40]. The tested concentration of kanamycin was about 32 times lower than microbiological breakpoint for enterococci (EC, 2005). Despite the growth rate on the surface of M17 and MRS media containing the antibiotic, enterococcal strains could not be considered as kanamycin resistant. The same growth rate was detected on M17 and MRS agar media with kanamycin.

Table 1. The antibiotic resistance of enterococci

Antimicrobial class	Antibiotic	Conc μg/ml	*E.durans n = 1*			*E.faecalis n = 14*			*E.faecium n = 6*			Total no of enterococci		
			S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Beta-lactams	PEN	>16	0	1	0	11	3	0	3	3	0	14	7	0
	AMP	>16	1	0	0	14	0	0	6	0	0	21	0	0
Glycopeptides	VAN	>32	1	0	0	5	1	8	4	1	1	10	2	9
Macrolides	ERY	>8	1	0	0	12	2	0	5	1	0	18	3	0
Tetracyclines	TET	>16	0	1	0	8	1	5	4	1	1	12	3	6
Phenicols	CHL	>4	1	0	0	12	2	0	5	1	0	18	3	0
Amynoglycosides	KAN	>32	0	0	1	0	2	12	0	1	5	0	3	18
	STP	>64	0	0	1	0	1	13	0	1	5	0	2	19
	GEN	>64	0	0	1	0	0	14	0	0	6	0	0	21

One *E.faecium* strain and five *E.faecalis* strains were resistant to MIC > 16 μg/ml of tetracycline. On both agar media tetracycline-resistant enterococci showed equal growth. Although the growth of other enterococcal strains were excellent on MRS agar media supplemented with tetracycline but the M17 agar media supplemented with tetracycline did not support the growth. Three species were intermediate resistant and it was decided that 12 strains those did not grow on duplicate M17 agar media were sensitive to MIC > 16 μg/ml tetracycline. According to [31], the choice of culture medium will significantly affect the classification of LAB strains into susceptible, intermediate and resistant categories. There are as yet no guidelines available on the interpretation of susceptibility tests for food bacteria. For this purpose, the current study compared the performance of the two complex and nutrient rich agar media those are not commonly used in antibiotic susceptibility [31].

All analyzed isolates of enterococci were sensitive to MIC > 8 μg/ml erythromycin on both supplemented agar media. The presence of a wide range of erythromycin resistance genes in *Enterococcus* species has been reported elsewhere [54].

Three *E.faecalis* strains were intermediate resistant to MIC > 4 μg/ml. The use of chloramphenicol in food and for the treatment of animals is banned by the European Union, due to the possibility of development irreversible and fatal aplastic anemias in humans, who consume meat or milk contaminated residues of this antibiotic [55].Vancomycin resistance was the most prevalent (49%), eight *E.faecalis* and one *E.faecium* strains showed high vancomycin resistance (MIC > 32 μg/mL). Transferable resistance to vancomycin is plasmid - encoded and is associated with species of the genus *Enterococcus* [56]. In 2002, Giraffa and collaborators [13] highlighted the role played by food reservoirs of VRE in the dissemination of antibiotic resistance traits in the environment. VRE and tetracycline-resistant enterococci have been found among food animals in several countries in Europe [57].

4 Conclusion

Enterococci resistant to one or more antibiotics including erythromycin, tetracycline and vancomycin were isolated from cheese samples. They are frequently resistant to commonly used antibiotics in veterinary and human medicine. Antibiotic resistance in enterococci can be intrinsic and acquired, therefore they may be considered as opportunistic pathogens. This work points out a need for detailed characterization of enterococci isolated from dairy products, since they could be reservoirs of antibiotic resistance and virulence genes.

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Microbiological Safety of Dairy Products of Individual Producers That Are Not Under the Supervision of Veterinary and Sanitary Inspection in FB&H

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Abstract. Milk products have been utilized by humans for many thousands of years. Food safety begins on the farm and continues through processing and transportation processes until the milk or milk product is consumed. Several studies suggest that bacteria in milk stem not only from external colonization and an endogenous route of bacterial transmission has been proposed. Food poisoning can be caused by different types of pathogenic microorganisms. The presence of food borne pathogens in milk products is due to direct contact with contaminated sources in the dairy farm environment, to excretion from the udder of an infected animal or by contamination in the production process.

The purpose of this study is to examine the microbiological safety of dairy products of small producers that are not under the supervision of veterinary-sanitary inspection. The aim is to point out the importance of monitoring the microbiological safety of dairy products of all producers and to investigate the relationship of applied technological procedures in the production of dairy products, as well as the category of dairy products, and microorganisms causing food-borne diseases. A total of 62 samples of dairy products were collected and available for sale in the producers' households or at points of sale that are not under the control of the veterinary-sanitary inspection, in 10 FB&H municipalities. Out of 62 analyzed samples, 35 (56.45%) samples corresponded and 27 (43.55%) samples did not comply with the applicable legislation on the microbiological safety of food. Of the analyzed samples (n = 62), 50 (80.65%) were from cow's milk and 12 (19.35%) from sheep's milk. Among the samples of dairy products that were not microbiologically correct (n = 27), the presence of pathogenic microorganisms of the genus *Salmonella* was detected in 1 (3.7%) sample, and the presence of pathogenic bacteria of the species *Listeria* sp. in 6 samples (22.22%).

Keywords: Dairy products · Food safety · Veterinary-sanitary inspection · Food poisoning

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1 Introduction

Farm animals represent a major source of pathogens that can be transferred to milk. Raw milk provides a potential growth medium for the development of bacteria that can be controlled or destroyed through the pasteurization process [1]. *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Campylobacter* are the most frequent potential pathogens associated with milk or dairy products in industrialized countries [2] and are the main microbiological hazards linked to raw milk [3, 4] and raw cheese [5].

Milk is the nutritious fluid secreted by all the mammalian species to fulfill the nutritional requirements of their neonates, and today it has become a major part of the human diet. Milk being diverse in its composition, high water activity, and neutral pH has made it highly prone to different microbiological contaminations which ultimately spoil the raw milk as well as the milk products [6]. Therefore, proper care has to be taken starting from the feed material of the animals to milking equipment, health conditions, and other environments [7]. Microorganisms as well as the released toxins are a major threat for food industries as they significantly affect the productivity and present risk regarding the food safety and well-being of humans and animals [8]. The raw milk is contaminated either from the feed material or improper handling of equipment and the animals during and after milking. The main factors responsible for the contamination in milk and dairy products are climatic variations of the region [9].

The production of indigenous dairy products in Bosnia and Herzegovina has been preserved for centuries, despite numerous wars, displacement, and frequent migrations of the population to cities. These products are characterized by great diversity [10].

Cheese is made from milk by coagulating the casein present in milk by using the enzyme rennet. The important phase during cheese production is the ripening process in which bacteria and fungi develop on the cheese surface and are responsible for the organoleptic and textural characteristics of cheese. The ripening is achieved due to the proteolytic and lipolytic activities of these microorganisms leading to the production of ammonia and sulfur compound [11]. Fresh (young) cheese is probably the oldest and most popular type of cheese produced in households. It is characterized by a high water content, low milk fat content, and high acidity. It has a characteristic taste, smell, color, and consistency. Traditional fresh cheese is usually produced from raw milk left to naturally become acidified at room temperature (22 °C). The acidification process, also called curdling, naturally lasts 1 to 2, but no more than 3 days [12]. Vlasic (Travnik) cheese is originally produced from unpasteurized, fresh sheep milk immediately after milking. The cheese originates from the mountain of Vlasic, just above the town of Travnik in central Bosnia and Herzegovina. It is produced on the mountain in cheese huts (katuni) and is usually kept for 2 to 3 months on the mountain to mature. Vlasicki cheese belongs to a group of white soft cheeses ripened in souse. Traditionally, it is made from sheep milk [13]. Cheese from bellows (cheese presses, cheese in skin sack) is characteristic for the entire region of Herzegovina. With minor differences, the same technology is used to produce cheese from cow, sheep, and goat milk, or their mixtures. Full-fat and skim milk is also used. Maturation takes place in sheep or goat bellows. The pronounced and piquant taste, flavor, and aroma of these cheeses originate from intensive lipolysis and proteolysis as a result of specific anaerobic conditions inside the skin sack,

autochthonous microorganisms from raw milk and skin, as well as their manufacturing procedure [10, 14]. Skimmed cream (skorup, kajmak) is a specific product, characterized by high content of fat, proteins, and the process of maturation. In the classification, it is located between cheese and butter and is closest to the group of products that are based on milk fat. The traditional method of making skimmed cream is to boil the milk slowly, then simmer it for two hours over very low heat. After the heat source is shut off, the cream is skimmed and left to chill (and mildly ferment) for several hours or days [10]. However, the number of people consuming unpasteurized products continues to increase all over the world due to a growing demand for natural and unprocessed foods [15].

Numerous types of dairy products appear on the markets in Bosnia and Herzegovina, produced from unpasteurized or pasteurized milk, with or without starter cultures of bacteria, yeasts or molds, etc. Each type of production in dairy-making brings specific challenges, even certain microbiological risks, and are especially recognized risks of traditional non-industrial production and uncontrolled sales of smaller producers in conditions that are not under veterinary and sanitary supervision. This paper aims to determine the microbiological safety of soft, semi-hard cheeses, butter, cream, skimming cream from pasteurized and unpasteurized milk and dairy products most often consumed in our society, which are not part of regular veterinary and sanitary control and are sold in unregistered places, in uncontrolled conditions.

2 Materials and Methods

2.1 Sample

For the research, samples of dairy products (n = 62) of individual, small producers that are not under veterinary and sanitary supervision, in non-original packaging, in places that are not registered for sale and distribution were collected. The total sample (n = 62) was composed of 16 samples of cream, 1 sample of butter, 21 samples of soft fresh cheese, 22 samples of semi-hard cheese, and two samples of cream (Table 1). Dairy products were collected on the territory of the Federation of Bosnia and Herzegovina, in 10 different municipalities, in February - March 2021. Samples were delivered in portable refrigerators at a temperature of 4 to 8 °C to the microbiological laboratory of the Health Ecology Service of the Public Health Institute Federation of Bosnia and Herzegovina in Sarajevo. All samples collected during the early spring season were taken from an outdoor market and selling spots, where conditions were not controlled (open market without refrigerated cases). During the sampling of dairy products, data were collected on the owner of the sample, the type of dairy product and the basis of the technological process of production, the location of the producer, and the date of sampling.

Table 1. Types of dairy products in the research

Type of dairy product	The name of the dairy product	Origin	Technological process
Soft (fresh) cheeses from raw milk	Fresh, soft cow's milk cheese, Fresh cheese of semi-solid consistency from cow's milk, Hurda	Luzani (Podvelezje), Tarcin, Kresevo, Bioca (Ilijaš), Kahrimani (Hadzici), Donji Vukovici (Hadzici), Pazaric, Zavidovici, Travnik, Novi Travnik, Gornja Bukovica (Travnika), Krpeljici (Travnik), Vlasic	Raw milk
Soft (fresh) cheeses from pasteurized milk	Fresh, soft cow's milk cheese, Fresh cheese of semi-solid consistency from cow's milk, Torotan	Nevesinje, Podvelezje	Pasteurized milk
Semi-hard cheeses	Sheep's milk cheese in skin sack, Cow's milk cheese in skin sack, Ripe cow's milk cheese, Semi-hard sheep's milk cheese, Semi-hard cow's milk cheese, Smoked cow's milk cheese, Salty ripe sheep's cheese (Vlasic/Travnik)	Luzani (Podvelezje), Ilijas, Kamsko (Olovo), Zavidovici, Gluha Bukovica (Travnik), Travnik, Novi Travnik, Zavidovici, Visoko, Nevesinje (Podvelezje), Vlasic	Pasteurized milk Raw milk
Butter	Young cow's milk butter	Vlasic	Raw milk
Cream	Ripe cream of sheep's milk, Young cream in skin sack of sheep's milk, Cream in skin sack of sheep's milk, Cream in skin sack of cow's milk, Young cream of cow's milk, Ripe cream of cow's milk	Luzani, Nevesinje (Podvelezje), Vlasic, Ilijas, Tarcin, Kresevo. Gornja Bioca (Ilijas), Gornji Vukovići (Hadzici), Donji Vukovici	Pasteurized milk Raw milk

(*continued*)

Table 1. (*continued*)

Type of dairy product	The name of the dairy product	Origin	Technological process
Skimming cream	Skimming cow's milk cream, Kajmak	Tarcin	Raw milk

2.2 Microbiological Analysis

For microbiological analysis, 25 g of dairy products were weighed after sterile separation of the surface layers of the products. The sample was then comminuted and diluted by dissolving in 225 mL of buffered peptone water and homogenized for 2 min at 200 r/pm. For high-fat dairy products (over 20% of the total weight), a commercial solution of sorbitan monooleate Tween 80 was used, by the fat level (e.g., 40% fat, added 4 g/l) to improve the emulsification flow of the solution. Serial decimal dilutions were then performed to detect *Salmonella* spp. and determining the number of *Listeria monocytogenes* and *Listeria* sp., *Escherichia coli*, coagulase-positive staphylococci, enterobacteria, aerobic mesophilic bacteria, yeasts and molds, and sulfite-reducing bacteria. Analyzes were performed by the procedures described in ISO methods:

- BAS EN ISO 6579–1:2018 Microbiology of the food chain — Horizontal method for the detection, enumeration, and serotyping of *Salmonella* — Part 1: Detection of *Salmonella* spp.
- BAS EN ISO 11290–2:2018 Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and *Listeria* spp. — Part 2: Enumeration method
- BAS ISO 16649–2:2008 Microbiology of the food chain — Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* — Part 1: Colony-count technique at 44 degrees C using membranes and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide
- 21528–2:2018 Microbiology of the food chain — Horizontal method for the detection and enumeration of Enterobacteriaceae — Part 2: Colony-count technique
- BAS EN ISO 4833–2:2014 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 2: Colony count at 30 °C by the surface plating technique
- BAS ISO 21527–1:2008 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and molds — Part 1: Colony count technique in products with water activity greater than 0,95
- BAS EN ISO 15213:2008 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions
- BAS EN ISO 6888–1:2005 Microbiology of the food chain — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) — Part 1: Method using Baird-Parker agar medium

The microbiological laboratory in which the analyzes were performed is accredited according to the requirements of the standard BAS EN ISO/IEC 17025: 2018, with all measures to ensure the quality of test results during the analysis of samples.

In the interpretation of microbiological analysis, we referred to the Guidelines on microbiological criteria for foodstuffs [16] and the Ordinance on microbiological criteria for foodstuffs of Bosnia and Herzegovina [17]. Categorization of samples, the definition of analyzed parameters, and evaluation of analysis results under the maximum allowable number of bacteria for each of the analyzed parameters were performed according to Guidelines on microbiological criteria for foodstuffs of Bosnia and Herzegovina [16].

2.3 Statistical Analysis

Statistical analysis of the obtained data was performed using the basic functions of MS. Excel 2016, Epi Info 6, and Statistica release 7.

3 Results

A total of 62 samples of dairy products were analyzed, of which 35 (56%) samples corresponded to the defined norms of the parameters of applicable legislation, and 27 (44%) samples did not comply (Fig. 1).

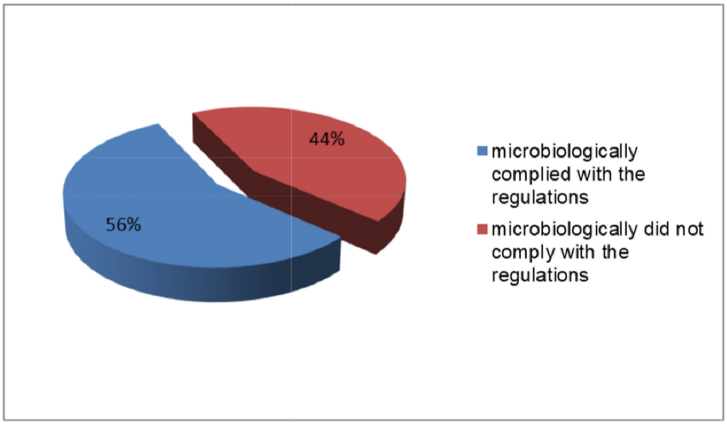

Fig. 1. Graphic representation of the percentage of analyzed samples of dairy products that complied and did not comply with the defined norms of parameters

Of the total number of analyzed samples of dairy products (n = 62), 16 (26%) samples were skimming cream, 1 (2%) sample of butter, 21 (34%) sample of soft, fresh cheese, 22 (35%) sample of semi-hard cheese, and 2 (3%) sour cream samples (Fig. 2).

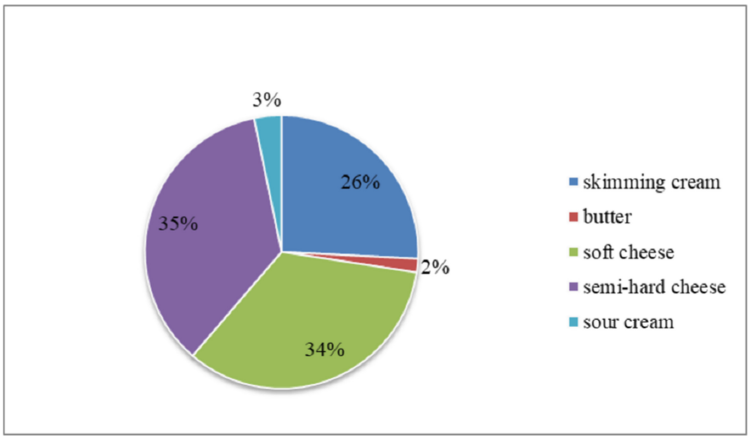

Fig. 2. Graphic presentation of the percentage share of individual dairy product categories in the examined sample

Table 2. Review of research results about the type of individual dairy product categories

	Within the permissible limits		Without the permissible limits		Σ	
	f	%	f	%	f	%
Skimming cream	8	50	8	50	16	25.81
Butter	0	0	1	100	1	1.61
Soft cheese	12	57.14	9	42.86	21	33.87
Semi-hard cheese	15	68.18	7	31.82	22	35.48
Sour cream	0	0	2	100	2	3.23
Σ	35	100	27	100	62	100

When we look at the structure of the samples depending on the type of individual dairy product categories, we see that the largest number of samples was from the semi-hard cheese category 35.48% (n = 22) where 31.82% (n = 7) of the samples were without permissible limits. Butter (n = 1) and soft cream (n = 2) were present in a small number of samples, and all of the samples were without permissible limits (Table 2).

Of the total number of analyzed samples of dairy products (n = 62), a total of 5 (8%) products were produced from pasteurized milk, of which 3 (60%) corresponded and 2 (40%) did not correspond to the defined values of the maximum number of bacteria according to current legislation, and 57 (92%) samples were produced from unpasteurized milk, of which 32 (56%) complied and 25 (44%) did not comply with the applicable standards (Fig. 3).

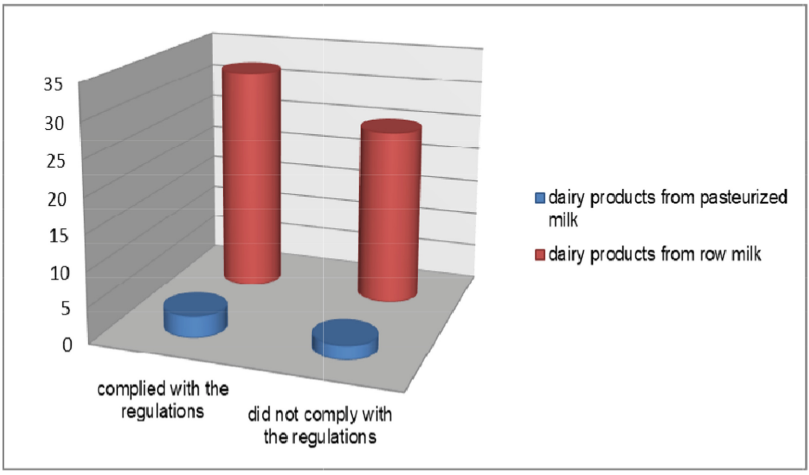

Fig. 3. Graphic presentation of the number of dairy products from pasteurized milk and dairy products from unpasteurized (raw) milk

Of the total number (n = 62) of analyzed dairy products, 50 (81%) samples were from cow's milk and 12 (19%) from sheep's milk. Of the total number (n = 50) of the analyzed cow's milk samples, 28 (56%) samples responded and 22 (34%) did not respond. Of the total number (n = 12) of the analyzed samples of sheep's milk, 6 samples corresponded, and 6 samples did not correspond to the defined parameters of the applicable legislation (Fig. 4).

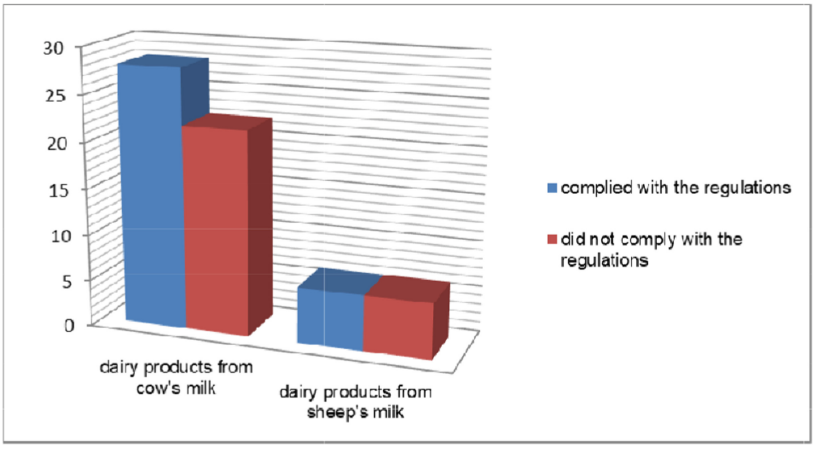

Fig. 4. Graphic presentation of the number of dairy products from cow's and sheep's milk

Of the total number of analyzed samples (n = 62), *Salmonella* spp. was isolated in one sample, and *Listeria* spp. was isolated in 7 samples. *Listeria monocytogenes* were not isolated in any of the analyzed samples. Of the total samples analyzed (n =

62), 59 corresponded to the maximum allowed number of colonies for the *Escherichia coli* parameter, while 13 did not. By the defined reference values for the Coagulase positive staphylococci (CPS) parameter, out of analyzed samples (n = 62), 45 samples corresponded, while 17 did not. Of the 62 analyzed samples, 48 samples corresponded to the defined reference values for the presence of yeasts and molds, and 14 samples did not correspond (Fig. 5).

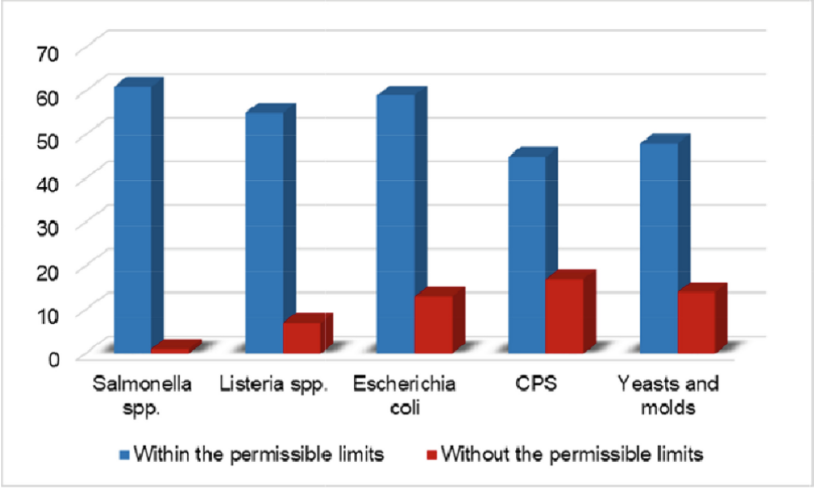

Fig. 5. Graphic presentation of the results of sample analysis by categories of analyzed bacteria.

Out of 21 analyzed samples of young, soft cheese, 15 of them corresponded to the norms of the allowed number of *Escherichia coli* defined by the legislation [16, 17], and 6 samples did not correspond. Of the 16 cream samples, 12 corresponded to the reference values complied with the Guidelines on microbiological criteria and Ordinance on microbiological criteria for foodstuffs [16, 17] concerning the microorganism in question and 4 did not. The analyzed butter sample corresponded, while out of 21 samples of semi-hard cheese, 1 sample did not correspond to the reference values for the analyzed parameter *Escherichia coli*. None of the analyzed cream samples (n = 2) corresponded to the Guidelines on microbiological criteria and Ordinance on microbiological criteria for foodstuffs [16, 17] concerning the microorganism in question (Fig. 6).

Of the 21 soft cheese samples analyzed, 13 met the defined CPS norms, and 8 samples did not. Of the 16 skimming cream samples, 11 matched the CPS reference values and 5 did not. The analyzed butter sample did not correspond to the determined CPS values, while out of 21 semi-hard cheese samples, 6 samples did not correspond to the reference values for the analyzed CPS parameter. None of the analyzed cream samples (n = 2) corresponded to the CPS reference values for this category of dairy products (Fig. 7).

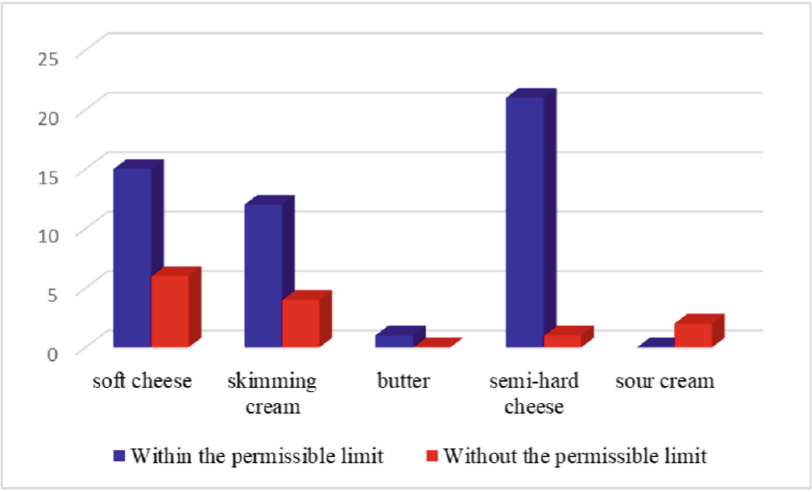

Fig. 6. Graphical presentation of the results of the analyzed samples to the type of individual dairy product categories for the *Escherichia coli* parameter

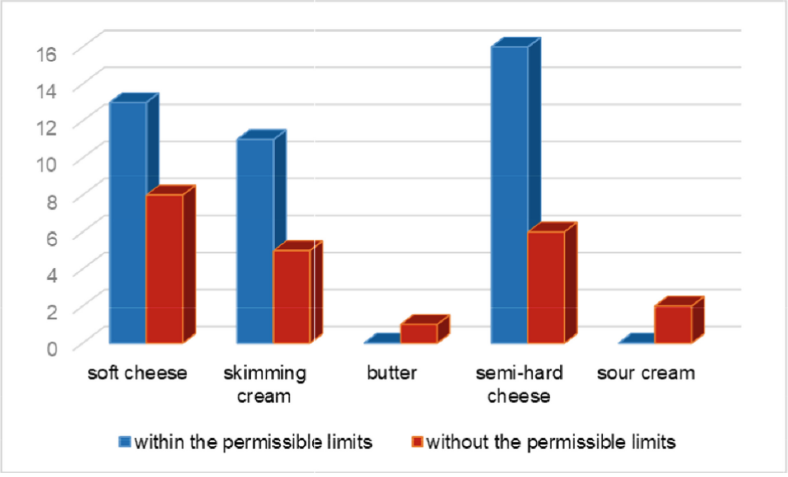

Fig. 7. Graphical presentation of the results of the analyzed samples to the type of individual dairy product categories for the CPS parameter

Out of a total of 62 analyzed dairy products, 20 samples were from the Sarajevo Canton (8 from Hadzici, 4 from Ilijas, and 8 from Tarcin), of which 11 corresponded and 9 did not meet the defined norms. Out of 62 analyzed samples, 20 samples were from the Central Bosnia Canton/Canton of Central Bosnia (2 from Kresevo, 9 from Travnik, and 10 from Vlasic), of which 11 corresponded and 9 did not meet the given categories of defined norms according to the Guidelines on microbiological criteria and Ordinance on Microbiological Criteria for Food. Out of 62 analyzed samples, 10 samples were from

Fig. 8. View of the geographical distribution of the samples

the area of Zenica-Doboj Canton, 3 from Olovo, 1 from Visoko, and 6 from Zavidovici, of which 8 corresponded and 2 did not correspond to the defined values of the monitored parameters. The remaining 11 samples out of 62 analyzed were sampled in the area of Podvelezje in the Herzegovina-Neretva Canton, of which 6 responded and 5 did not respond (Fig. 8).

4 Discussion

The paper investigates the microbiological safety of dairy products (soft fresh cheeses, semi-hard cheeses, cream, individual butter) from cow's and sheep's milk produced by small producers that are not under veterinary and sanitary supervision, in non-original packaging, and sold in places that are not registered for sale and distribution. Our results indicate that out of the total number of samples taken, 44% did not meet the prescribed conditions of microbiological correctness. The study of microbiological correctness of the analyzed samples of milk and dairy products in the period from 2009 to 2013 in the Zenica-Doboj Canton showed that of the total number of samples taken, the prescribed conditions of microbiological correctness were met by 93 samples (75%), while 31 samples were microbiologically defective (25%) [18]. The structure of the samples in this research depending on the type of individual dairy product categories indicates that the largest number of samples was from the semi-hard cheese category 35.48%. According to the brochure "Indigenous dairy products industry in Bosnia and Herzegovina", in the structure of milk processing in Bosnian dairies, liquid dairy products participate with 77.1%, cheese with 19.2%, and other products with 4.7%. The production and consumption of cheese date back several thousand years, which makes it

one of the oldest foods. Most cheeses are named after their origin, but it is often the case that certain cities and regions have become famous for the cheeses that bear their name. At the time of the development of food production, the traditional production of indigenous dairy products, and especially cheese, gained increasing importance. Today, some autochthonous cheeses are produced in BiH, such as Livno cheese, Vlašić cheese, Fat cheese, Boiled cheese, Kalenderovac cheese, Fasting cheese or Torotan, Fresh sour cheese, Dried sour cheese, Hard goat cheese from oil, White goat cheese, Community, Urda or Hurda, Zarice, etc. [10].

The results of the analysis of dairy products of individual producers that are not under the supervision of inspection in FB&H showed that 35 (56%) samples corresponded to the defined norms of the parameters of applicable legislation, and 27 (44%) samples did not comply of the total number of samples (n = 62) (Fig. 1). Some authors consider [19] that cheeses are one of the safest types of food when it comes to their microbiological correctness. On the other hand, the results show that epidemics have been recorded in the world caused by various types of cheese, with a large number of patients, but also fatalities [20].

In this research, shown in Fig. 3, out of 5 (8%) samples produced from pasteurized milk, 3 (60%) met the requirements of applicable legislation and 2 (40%) did not correspond with the Guidelines on microbiological criteria and Ordinance on microbiological criteria for foodstuffs [16, 17]. Out of 62 sampled dairy products, 57 (92%) samples were produced from unpasteurized milk, of which 32 (56%) complied and 25 (44%) did not comply with the applicable legislation (Fig. 4). The results of the work presenting microbiological changes in/on cheeses during production and storage showed that they depend on several factors, such as production technology and type of cheese (pasteurization of milk or unpasteurized milk, application of dairy cultures, acidity, ripening, etc.), physical -chemical properties of cheese, storage conditions, etc. By studying the microbiological quality of soft, semi-hard and hard cheeses during the shelf life presented in terms of pathogenic bacteria, the tested cheeses were in line with the prescribed criteria for *Listeria monocytogenes*, which was also confirmed by recent studies of cheeses produced in our region (crepe, cottage cheese, Livno cheese, Trappist, and hard cheese in olive oil) [21].

Listeria monocytogenes is the most important pathogen in cheese-making, especially in the post-production phase, during storage and distribution, when contamination is possible, during slicing, packaging. The EFSA report [22] shows that on the European market only 0.06% of soft and semi-hard cheeses (3452 cheese samples searched) do not meet the criterion of 100 cfu/g at the end of the shelf life, while the pathogen is present (in 25 g sample) in 0.47% of cheeses. *Listeria monocytogenes* was not isolated in any of the analyzed samples in this research. *Salmonella* spp. was isolated in one sample, and *Listeria* spp. were isolated in 7 samples of dairy products. According to the analyzed parameter of fecal coliform bacteria, *Escherichia coli*, 59 samples of dairy products out the supervision of veterinary and sanitary inspection in FB&H, corresponded to the defined values, and 13 of samples were microbiologically defective. Analysis of the sampled dairy products on the parameter of Coagulase positive Staphylococci (CPS)

showed that 45 corresponded to the defined reference values and 17 samples did not correspond. Of the 62 analyzed samples, 48 samples corresponded to the defined reference values for the presence of yeasts and molds, and 14 samples did not correspond (Fig. 5).

Reports from developed countries indicated that milk and dairy products are implicated in 1–6% of the total bacterial foodborne outbreaks [23], with 39.1% attributed to milk, 53.1% to cheese, and 7.8% to other milk products [24]. In 2013, 2.14% of foodborne outbreaks were attributed to the consumption of cheese and dairy products (11 and 7 outbreaks, respectively) in Europe [25].

A survey conducted in 2008 in Rio de Janeiro and Brazil showed that the typical Brazilian cheeses such as "Minas Frescal" and "Prato" cheese are preferred by 528 (52.8%) of the consumers. Of the total consumers, 764 (76.4%) purchase cheese were from supermarkets, while 236 (23.6%) from open-air markets. Inspected cheese was purchased by 350 (35%) consumers, while 650 (65%) bough it without knowing if they were submitted to the previous fiscalization. Four hundred and thirty (43%) surveyed consumers did not know of any cheese-borne disease that has not been part of surveillance. Overall, educational campaigns must be developed by the Sanitary Surveillance and the Health Agencies to improve the knowledge of the consumer about food safety of cheeses [26].

The aim of research by Congo et al. [27] was to assess the hygienic quality of raw milk used in the manufacture of São Jorge, a Protected Denomination of Origin Portuguese semihard cheese, as well as to ascertain the sanitary conditions prevailing during its processing. Viable counts of *Enterobacteriaceae* and *Micrococcaceae* were accordingly obtained, about 21 independent batches (including samples of raw milk, curd, and cheeses after 1, 3, and 4 months of ripening), from 7 dairy farms. Standard plate counts (log CFU per milliliter or gram) ranged from 6.1 to 8.6 in raw milk, whereas they ranged from 7.0 to 8.0 in 4-month-old cheeses. Viable counts of *Enterobacteriaceae* ranged between 5.9 and 7.0 in raw milk and between 0.0 and 1.3 in 4-month-old cheeses. Species identified within this family encompassed *Klebsiella oxytoca*, *Klebsiella cloacae*, *Klebsiella pneumonia*, *Enterobacter sakazakii*, and *Escherichia coli*. *Klebsiella ornithinolytica*, *Klebsiella terrigena*, and *Serratia odorifera* were detected only in raw milk [27]. Species identified in this research include *Salmonella* spp. (n = 1), *Listeria monocytogenes* (n = 7), *Escherichia coli* (n = 59), and Coagulase positive staphylococcus (n = 45). Analysis of the number of *Escherichia coli* in certain categories of dairy samples in this research, of the 21 soft cheese samples analyzed, 15 met the defined values and 6 samples did not. Of the 16 cream samples, 12 matched the reference values for *Escherichia coli*, and 4 did not. The analyzed butter sample corresponded, while out of 21 samples of semi-hard cheese, one sample did not correspond to the reference values for the analyzed *Escherichia coli* parameter. None of the analyzed cream samples (n = 2) corresponded to the *Escherichia coli* reference values for this category of dairy products (Fig. 6).

Analyses showed that 13 met the defined CPS norms, and 8 samples did not of the 21 soft cheese samples analyzed. Of the 16 cream samples, 11 matched the CPS reference values and 5 did not. The analyzed butter sample did not correspond to the determined CPS values, while out of 21 semi-hard cheese samples, 6 samples did not correspond to the reference values for the analyzed CPS parameter. None of the analyzed cream

samples (n = 2) corresponded to the CPS reference values for this category of dairy products (Fig. 7).

5 Conclusions

The results show the poor microbiological quality of dairy products of individual, small producers that are not under veterinary and sanitary supervision due to pathogenic microflora contamination and indicate shortcomings in hygiene practice during production, distribution or sales.

The microbiological defect of the analyzed samples of cheese and dairy products should be attributed primarily to the poor hygienic quality of fresh raw milk. The impact of the use of inappropriate production equipment and (or) irregularities in its application, especially in the process of heat treatment of milk, then on irregularities and insufficient thoroughness in the implementation of measures for cleaning, washing, and disinfection of equipment and premises as well as insufficient personal care must not be neglected. In addition, the results indicate the need to provide better conditions during the sale of dairy products, regarding the sale in outdoor areas, where refrigerated display cases should be used as in indoor farmers' markets. From the point of view of health and safety of dairy products, it is very important to note that *Listeria* spp. were detected in seven samples, *Salmonella* spp. in one sample, while *Listeria monocytogenes* was not detected.

Good microbiological quality of raw milk is a prerequisite for the good microbiological quality of milk and dairy products. Determining the number and types of microorganisms present in milk and dairy products gives the best insight into the quality of production of a particular producer. In addition, any production of food, including drinking milk and dairy products, implies the legal responsibility of producers for the health of consumers.

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Microwave-Assisted Extraction of Polysaccharides from Brown Algae *Cystoseira Compressa*

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Abstract. Brown algae *Cystoseira compressa* is a source of numerous bioactive molecules including sulfated polysaccharide fucoidan. Due to its anticoagulant, anti-inflammatory and antiviral activities, fucoidan has a potential application in functional foods, cosmeceutical and pharmaceutical products. Fucoidan extraction is conventionally performed in hot aquatic or acidic solutions at high temperatures for several hours. To reduce extraction time, energy and solvent usage advanced extraction techniques such as microwave (MAE) and ultrasound (UAE) assisted extraction are applied. The aim of this paper was to optimize MAE parameters (solvent, solvent to sample ratio, temperature and time) in order to achieve the highest possible polysaccharide yield from *Cystoseira compressa* and to compare it with conventional extraction. First, the effect of different solvents (H_2O, 0.1M HCl, 0.2M HCl, 0.1M H_2SO_4, 0.2M H_2SO_4) and solvent to sample ratios (15:1 and 30:1) on polysaccharide yield were tested. By using 0.2M H_2SO_4, the obtained yield was almost 5 times higher than with H_2O and 30:1 solvent to sample ratio resulted in higher yield than 15:1 ratio. Resulting optimal parameters (0.2M H_2SO_4 and 30:1) were then used to optimize time (5, 10 and 20 min) and temperature (40, 60, 80 and 100 °C) of MAE. Polysaccharide yield increased with temperature increase up to 100 °C while there was no statistical difference ($p \geq 0.05$) between 5 and 20 min. Even though conventional extraction resulted in higher yield (24.47%) then MAE (16.19%), time was reduced from 3 h to 5 min what presents a significant advantage of MAE.

Keywords: Seaweed · Polysaccharides · Microwave-assisted extraction

1 Introduction

Brown algae *Cystoseira compressa* is a source of numerous bioactive molecules including sulfated polysaccharide fucoidan. Due to its anticoagulant, anti-inflammatory and antiviral activities, fucoidan has a potential application in functional foods, cosmeceutical and pharmaceutical products [1]. The extraction process of these polysaccharides includes several complex and time-consuming steps and the correct adjustment of extraction parameters (e.g., time, temperature, solvent and sample to solvent ratio) greatly

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influences the yield, physical, chemical and biochemical properties as well as their biological activities [2]. To reduce extraction time, energy and solvent usage advanced extraction techniques are applied among which microwave-assisted extraction (MAE) showed the greatest potential. Therefore, the aim of this research was to optimize MAE parameters (solvent, solvent to sample ratio, temperature, time) in order to achieve the highest possible polysaccharide yield from *C. compressa* and to compare MAE with conventional polysaccharide extraction.

2 Materials and Methods

2.1 Chemicals

All chemicals and reagents used in this study were of analytical grade. Ethanol, acetone and calcium chloride ($CaCl_2$) were purchased from Gram-mol doo (Croatia), sulfuric acid (H_2SO_4) from Scharlab S.L. (Spain), hydrochloric acid (HCl) from TKI Hrastnik (Slovenia) and absolute ethanol from Carlo Erba Reagents (Italy).

2.2 Algal Material and Preliminary Treatments

C. compressa was harvested from the coastal region of Zadar, Croatia (44°06′26“ N; 15°13′54“ E) from the depth of 0.5 m in April 2018. The algae species was identified by marine biologist Donat Petricioli. Freshly collected algae was initially washed in seawater and then rinsed with distilled water. It was frozen at –60 °C in a ScanCool SCL210P freezer (Labogene ApS, Denmark) and lyophilization process was performed on a CoolSafe lyophilizer, Model: 55–9 PRO, (Labogene, Denmark) for 24 h. The dried algae were milled with an electric mill and the powder was stored at -20 °C until the extraction was carried out.

2.3 Extraction of Polysaccharides

The pre-treatment process was carried out with constant stirring in three steps: first 24 h at room temperature with acetone, then 24 h at room temperature with 80% ethanol and then 4 h at 60 °C with 80% ethanol. After pre-treatment, the dried seaweed (1 g) was extracted in a Start S Microwave Labstation for Synthesis (Milestone, Italy) microwave reactor with H_2O, 0.1M HCl, 0.2M HCl, 0.1M H_2SO_4 and 0.2M H_2SO_4 at 15:1 (mL/g) and 30:1 (mL/g) solvent to sample ratio. Parameters were set as follows: temperature – 100 °C, time –15 min, heating time –5 min, stirring –75%. Solvent and solvent to sample ratio that gave the highest polysaccharide yield were than used to optimize MAE time (5, 10 and 20 min) and temperature (40, 60, 80 and 100 °C). Conventional extraction (CE) was performed under established optimal solvent and solvent to sample ratio for 4 h at 60 °C under constant stirring (400 rpm).

After extraction and vacuum filtration, 1% $CaCl_2$ was added to the supernatant and left for 24 h at 4 °C in order to precipitate alginates. After filtration and centrifugation, crude polysaccharides were precipitated from the supernatant by the addition of 2 volumes absolute ethanol at 4 °C overnight. Crude polysaccharides were recovered by

centrifugation at 5500 rpm for 30 min, dried for 48 h at room temperature and milled in a mortar and pestle to a fine powder. Dried samples were stored at –20 °C.

Crude polysaccharide extraction yield (%PS) was calculated according to Eq. (1) where WP is the weight obtained after ethanol precipitation and WA is the algae weight used in each experiment.

$$\%PS = \frac{WP}{WA} * 100 \quad (1)$$

2.4 Statistical Analysis

Statistical analysis was done using STATISTICA v. 8 software (StatSoft Inc., Tulsa, OK, USA). Dependent variable was %PS while independent variables in the first part of the research were: (a) solvent (H_2O, 0.1M HCl; 0.2M HCl; 0.1M H_2SO_4; 0.2M H_2SO_4), (b) solvent to sample ratio (15:1 and 30:1 mL/g); and in the second part of the research: (a) temperature (40, 60, 80 and 100 °C), (b) time (5, 10 and 20 min). Continuous variables were analyzed by multivariate analysis of variance (ANOVA). Marginal means were compared with Tukey's LSD multiple comparison tests. The significance levels for all tests were $\alpha \leq 0.05$. Descriptive statistics was used to assess grand mean.

3 Results and Discussion

3.1 Effect of Extraction Solvent on Crude Polysaccharide Yield

Big significant difference ($p \leq 0.05$) in %PS can be observed between different solvents (Table 1). The lowest yield (3.85%) was obtained with H_2O, almost twice as much by 0.1M HCl (6.60%), four times higher by 0.1M H_2SO_4 (16.33%) and almost five times higher by 0.2M H_2SO_4 (19.06%). This can be explained by the facilitated polysaccharide extraction due to the cell wall hydrolysis that occurs with the use of acids [3]. Liu et al. [3] also obtained more than two times higher polysaccharide yield by 1M HCl (11.24%) than with H_2O (4.63%) from brown seaweed *Sargassum fusiforme*. It appears that lowering the pH increased the fucoidan yield [4] since 0.2M H_2SO_4 with pH 0.4 was much more effective for fucoidan extraction than 0.1M H_2SO_4 and 0.2M HCl with pH 0.7, 0.1M HCl with pH 1 and H_2O with pH 7. Similar finding was reported by Ptak et al. [4] who achieved a marginally better fucoidan and laminarin yield with 100 mM HCl (pH 2) then with 10 mM H_2SO_4 (pH 4) for seaweed harvested in France but opposite for the ones harvested in Germany.

Another observation regarding the use of different solvents that can be seen on, is that polysaccharides obtained by acids are much lighter in color compared to water extract. Lighter color of polysaccharide extract indicates a higher purity, thus higher quality [5]. Brown color of polysaccharides extracted by H_2O indicates the presence of brown seaweed pigments (fucoxantin, β - carotene, violaxanthin, chlorophyll a and c) trapped in polysaccharides during the extraction process [6] and it correlates with their total polyphenols content [7].

Table 1. Influences of solvent and solvent to sample ratio on crude polysaccharide yield (%PS)

	N	%PS
Solvent		$p \leq 0.05$†
H_2O	2	03.85 ± 0.02^{a}
0.1M HCl	2	06.60 ± 0.02^{c}
0.2M HCl	2	04.54 ± 0.02^{b}
0.1M H_2SO_4	2	16.33 ± 0.02^{d}
0.2M H_2SO_4	2	19.06 ± 0.02^{e}
Solvent to sample ratio (mL/g)		$p \leq 0.05$†
15:1	5	07.38 ± 0.00^{a}
30:1	5	12.73 ± 0.00^{b}
Grand mean	10	10.06

Note. Values with different letters are statistically different at $p \leq 0.05$
* Results are expressed as mean ± SE.
† Statistically significant variable at $p \leq 0.05$.

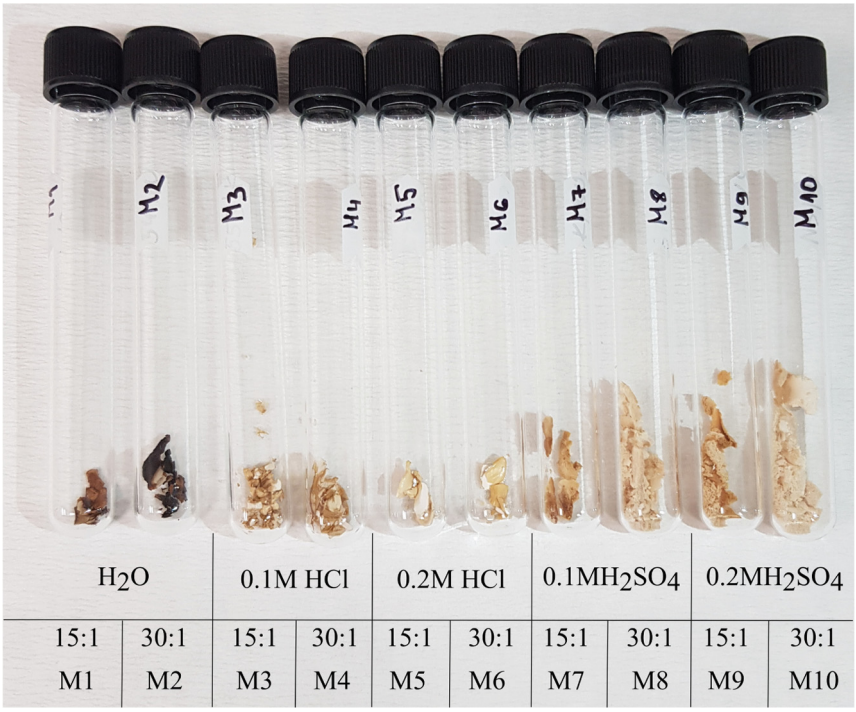

Fig.1. Crude fucoidan obtained from *C. compressa* by microwave-assisted extraction with various solvents (H_2O, 0.1M HCl; 0.2M HCl; 0.1M H_2SO_4; 0.2M H_2SO_4) and solvent to sample ratios (15:1 and 30:1 mL/g)

3.2 Effect of Solvent to Sample Ratio on Crude Polysaccharide Yield

An optimum solvent to sample ratio ensures homogeneous and effective heating. Too high solvent volume causes poor microwave heating since microwave radiation is absorbed by the solvent so additional power is required. On the other hand, too low solvent volume presents mass transfer barrier since bioactive compounds are concentrated in certain regions which limits their movement out of cell matrix [8]. In this research, by increasing the solvent volume from 15 mL to 30 mL, while keeping the constant 1 g of sample, %PS increased from 7.38% to 12.73%. Similarly, by reducing the sample mass from 5 g to 1 g, while keeping the constant 25 mL solvent volume, Rodríguez-Jasso et al. [9] reported fucoidan yield increase.

From previously described results it can be seen that the highest %PS was achieved with 1 g of algal sample and 30 mL of 0.2M H_2SO_4, hence this combination was used for further experiment.

3.3 Effect of Temperature on Crude Polysaccharide Yield

By increasing the temperature from 40 to 100 °C, yield increased from 8.11% to 15.16% (Table 2.). At higher temperature kinetic of chemical reactions becomes higher and faster [5], viscosity and surface tension are reduced so extraction rate increases [9]. Similarly, by increasing the temperature from 80 to 120 °C, in fucoidan and laminarin MAE from *Fucus vesiculosus*, *Fucus serratus* and *Fucus evanescens*, Ptak et al. [4] noted the highest yield increase. Likewise, Rodríguez-Jasso et al. [9] reported that the extraction results were improved when the pressure was increased from 30 to 120 psi, what corresponded to temperature increase from 122 to 172 °C, in MAE from *F. vesiculosus.*

Table 2. Influences of temperature and time on crude polysaccharide yield (%PS)

	N	**%PS**
Temperature (°C)		$p \leq 0.01$†
40	6	08.11 ± 0.02^{b}
60	6	07.68 ± 0.02^{a}
80	6	12.95 ± 0.02^{c}
100	6	15.16 ± 0.02^{d}
Time (min)		$p \leq 0.01$†
5	8	11.32 ± 0.01^{b}
10	8	10.30 ± 0.01^{a}
20	8	11.31 ± 0.01^{b}
Grand mean	24	10.98

Note. Values with different letters are statistically different at $p \leq 0.05$
* Results are expressed as mean ± SE.
† Statistically significant variable at $p \leq 0.05$.

3.4 Effect of Extraction Time on Crude Polysaccharide Yield

Extraction time of 10 min resulted with the lowest yield (10.30%) and even though 5– and 20-min extractions gave significantly ($p \leq 0.05$) higher yields, there was no significant difference between them. Longer extraction usually means higher yield but prolonged exposure to microwaves, even at lower temperature or power, could cause degradation of bioactive compounds so MAE time usually varies from few minutes up to half an hour [8]. Rodríguez-Jasso et al. [9] also noted that MAE time (1–31 min) didn't have a significant effect on polysaccharide yield. However, by looking at interaction between pressure and time they concluded that the use of lower extraction time favored the extraction process since the highest polysaccharide yield was achieved at the highest pressure for 1 min.

According to results of this study, optimal MAE temperature and time that resulted with the highest PS% from brown algae *C. compressa* are 100 °C for 5 min.

3.5 Comparison of MAE and Conventional Extraction

Polysaccharide yield obtained under optimal MAE conditions (H_2SO_4, 1:30 sample to solvent ratio, 100 °C, 5 min) was 16.19% while CE (H_2SO_4, 1:30 sample to solvent ratio, 60 °Cs, 4 h) yield was 24.47% what is statistically significantly ($p < 0.05$) higher. Similar finding was reported by Yuan and Macquarrie [10] who achieved *Ascophyllum nodosum* polysaccharide yield of 16.08% after 15 min MAE and 20.98% after 9 h conventional method. Likewise, in research by Okolie et al. [11] two times higher yield was obtained by 3 h long conventional method (11.9%) than with 15 min MAE (5.71%). Extract yield may correlate with increased amount of bioactive ingredients, but the extracts may also contain high amount of impurities and low amount of the compounds of interest [11]. Even though MAE polysaccharide yield was lower, extraction time was reduced from 4 h to approximately 5 min. This time reduction contributes to significantly lower energy consumption hence MAE is economically and environmentally more efficient.

4 Conclusion

MAE can be considered as potential method to obtain polysaccharides from brown algae *C. compressa*. By using 0.2M H_2SO_4, the obtained yield was almost 5 times higher than with H_2O and 30:1 (mL/g) solvent to sample ratio resulted in higher yield than 15:1 (mL/g) ratio. Crude polysaccharide yield increased with temperature increase up to 100 °C while there was no statistical difference between 5- and 20-min. Conventional extraction resulted in higher yield (24.47%) then MAE (16.19%) but extraction time was reduced from 3 h to 5 min what presents a significant advantage of MAE.

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Response to COVID 19 Pandemic Challenges – Insights from Food Industry in Bosnia and Herzegovina

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Abstract. Multifunctionality and the capability of the food system to address food security, safety, nutrition, quality during the crisis time always play an important role in shaping the quality of life for all. In general, the food system always shows strong resilience during the crisis time, but with a "new" type of threat – the COVID-19 pandemic, the food industry becomes exposed to the different uncertainties putting pressure on quality management systems to adapt and provide a prompt response. Therefore, this study aims to determine whether the Bosnian and Herzegovinian food industry is capable of responding efficiently and what kind of obstacles they are facing during the pandemic time. Besides, the quality/innovativeness of general and food safety protocols, total quality management elements such as leadership, resource management, customer satisfaction, communication with suppliers, work ethics, level of education and training, have to be reconsidered to build up business resilience and capability of the BiH food industry to provide a timely and efficient answer to the challenges of a disruptive global crisis, such as the COVID 19 pandemic. Study results will enrich understandings of the effects of the pandemic on the food industry as well as provide policy/company recommendations for total quality management improvement, emphasizing underdeveloped elements that should and must be prioritized by the top management of the companies in Bosnia and Herzegovina.

Keywords: Food industry · Total quality management · Business resilience · COVID-19

1 Introduction

The recent COVID-19 pandemic influences our way of living, putting strong pressure on global food supply chains, making actors more vulnerable, and reducing the efficiency and performance of business and supply chains (Guan et al. 2020; Ivanov, FAO 2020; Sodhi 2016). Availability, access, utilization are amongst the ones that are affected the most (Cappelli and Cini 2020; Nicola et al. 2020), but we could say there is no specific part of a value chain that is omitted by the negative effects of the COVID-19 pandemic (Gunessee and Subramanian 2020; Paul and Chowdhury 2020). In spite

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of that, food companies are at risk to survive, and their resilience during the crisis period strongly affects food security. Among the challenges, one is related to change in consumer behaviour, especially chaotic buying behaviour (Addo et al. 2020; Bai et al. 2020) that results in the disruption in supply and demand, and of course leads to food waste generation (Burlea-Schiopoiu et al. 2021). Other changes are the result of difficulties to go to supermarkets (Cattivelli and Rusciano 2020) that impact consumers' buying patterns and may give impetus to short food supply chain, homegrown, locally grown food, local shops, or online shopping (Cappelli and Cini 2020; Principato et al. 2020; Shilton 2020; Walljasper and Polansek 2020). Concerns about health aspects, the immune system, and wellbeing in general led to an increase in consumption of organic products, fruits, and vegetables, other healthy products (Ben Hassen et al. 2020), increase in home-cooking, but this comes at the expense of limited physical activities and a more sedentary lifestyle (Arora and Grey 2020). The aforementioned could be summarized in several key ways in which consumer behaviour changed during the COVID-19 pandemic, shift to value and essentials, flight to digital and omnichannel, shock to loyalty, health and "caring" economy, homebody economy (Arora et al. 2020). It should be noted, that food is not a transmission route for COVID-19 (EFSA 2020; FDA 2020; WHO 2020b), but continued activities are undertaken to the potential persistence of the virus on food and its transmission capacity, and therefore some international guidelines are provided (Fig. 1.) For a comprehensive overview of the persistence of coronaviruses on different types of inanimate surfaces as well as the virucidal efficacy of disinfectants against coronaviruses please check Han et al. (2021).

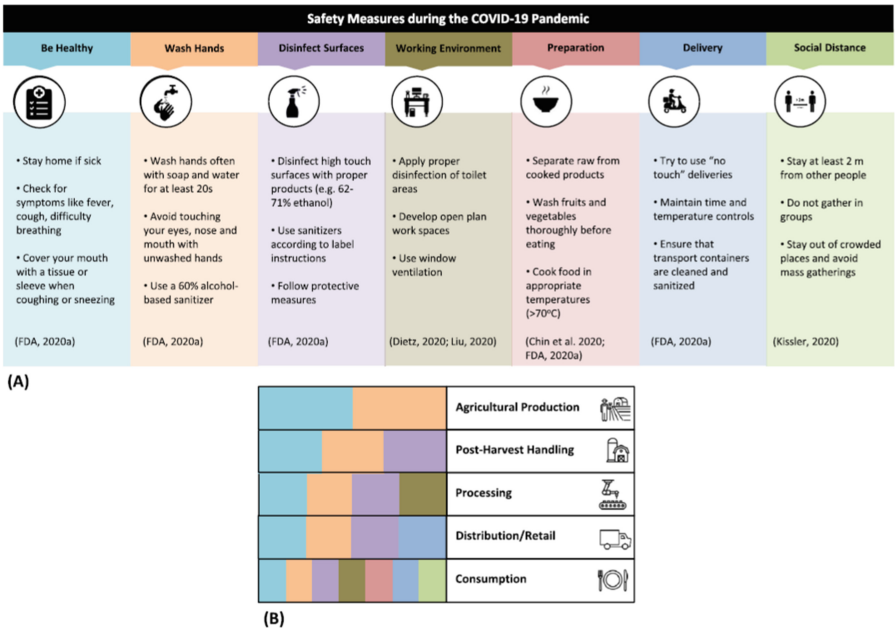

Fig. 1. Safety measures during the COVID-19 pandemic along the food value chain (Rizou et al. 2020)

The complexity of the food value chain strives for a well-planned and urgent response because of the disruption caused by this global pandemic. New challenges arise, to meet the high market demand, protect its workforce, avoiding transportation network disturbances and absenteeism, and maintaining a high level of food safety and consumer confidence (Hailu 2020; Nakat and Bou-Mitri 2021; Weersink et al. 2020). Different countries were differently affected by the COVID-19 pandemic, therefore industries in those countries also were influenced differently. The common outcome was a disturbance in consumers' purchasing behaviour for both essential and non-essential products (Hakovirta and Denuwara 2020; Mollenkopf et al. 2020), while mostly affected are fresh and perishable products (i.e., dairy, fruits, vegetable products) (Burlea-Schiopoiu et al. 2021; Coluccia et al. 2021). Besides, economically-wise, sectors with a greater tendency for export are heavily affected compared to the non-export sectors (ISMEA 2020). Bosnia and Herzegovina as a developing and transitional country is the net importer, therefore it is expected that the food industry sector is strongly affected by the pandemic. Table 1 confirms that the pandemic has had a significant impact on food export and import, resulting in a reduction in export and import (9% and 13%, respectively) in 2020 compared to 2019 for both sectors of vegetables and fruits, as well as in total for the trade of total food in the country.

Table 1. Export and import of fruits and vegetables and total food in Bosnia and Herzegovina for the period 2018–2020 (in thousands BAM)

Category	Year			Absolute change		Relative change	
	2018	2019	2020	2018–2019	2019-2020	2018-2019	2019–2020
Vegetables							
Export_vegetables	35,840	43,361	48,078	7,521	4,717	20.98	10.88
Import_vegetables	86,494	105,801	99,966	19,307	-5,835	22.32	−5.52
Fruits							
Export_fruits	118,285	116,968	122,880	−1,317	5,912	−1.11	5.05
Import_fruits	177,098	200,665	204,938	23,567	4,273	13.31	2.13
Total food							
Export_total_food	11,900,251	11,492,472	10,515,296	−407,779	−977,176	−3.43	−8.5
Import_total_food	19,273,968	19,454,966	16,886,250	180,998	−2,568,716	0.94	−13.2
Trade Balance	*−7,373,717*	*−7,962,494*	*-6,370,954*	*−588,777*	*1,591,540*	*7.98*	*−19.99*

Source: Central Bank (2021) Import and export of goods. Available on: http://statistics.cbbh.ba/Panorama/advanced_en_html.htm, accessed: 10 May 2021

Considering the numerous challenges food industry is facing right now, it remains to see how companies are dealing with the aforementioned limitation and what kind of strategies/actions they undertake to mitigate different risks that occur along their respective value chain. Namely, the that total quality management significantly and positively influence the organizational performances such as efficacy and efficiency (Pankaj et al. 2013), customer satisfaction (Claver and Tari 2008), as well as efficient resource usage (Cetindere et al. 2015).

Foundations for Total Quality as a modern business philosophy has been laid back in the 1980s (Easton and Jarrell 1998) as an attempt of American companies to answer on superior Japanese quality and increase in global competitiveness (David and Strang 2006; Kahreh et al. 2014). Total quality management is an ever-evolving concept, evolving from sole inspection and statistical sampling focus, to focus on organisational quality and focus on customers (Reid and Sanders 2012). However, the modern concept of TQM outlines that TQM is a management approach toward long-term success thorough customer satisfaction where all members of the organisation are involved in process improvement, product improvement, and work culture (ASQ 2021). Most of the definitions identify two aspects or groups of the TQM elements, "soft" (or "philosophical") and the "hard" (or "technical") elements (Ho et al. 2001; Kahreh et al. 2014). Although some research conducted in Bosnia and Herzegovina analyse the level of application of such practice within the food industry sector, identifying that companies are working toward quality improvement and application of modern business philosophies such as total quality management or market orientation (Nikolić et al. 2014; Uzunović et al. 2016; Nikolić et al. 2017), it remains to see how companies perform in such extraordinary circumstances as those caused by the COVID-19 pandemic. Therefore, this paper aims to the literature in this regard by addressing the following research questions:

RQ1: What are the major obstacles that food companies in Bosnia and Herzegovina are facing during times of crisis?

RQ2: How different elements of total quality systems are aligned to cope with ongoing pressures that resulted in a global pandemic?

2 Materials and Methods

The research is based on a food industry survey, conducted in Bosnia and Herzegovina, and utilizing a questionnaire specifically designed for this purpose. The survey was carried out through telephone interviews and social networks, thus resulting in total of 69 respondents (sample structure is presented in Table 2).

An online research tool was developed using the Lime Survey software and the first pilot survey was conducted in January/February 2021, using an online structured questionnaire providing information on the influence of the COVID-19 pandemic over the food business in general, as well as on the implemented TQM elements which enable the companies for prompt and adequate response to imposed pandemic challenges. The questionnaire was divided into six parts: *(i)* basic questions related to the *company characteristics* (e.g., size, type of sector, implemented quality system); *(ii)* questions related to the elements *of quality system responsible* for prompt response on imposed challenges (e.g., documentation for crisis situation, trainings for teams in case of crisis, restrictive measures, hygienic protocols); *(iii)* questions related to the *food safety requirements* (such as hygiene of objects, protection equipment level of stocks, hand/body hygiene, employees awareness, health protocol, etc.); *(iv) development of plans for rapid risk response* (referring to COVID-19 safety recommendations of authorities such as EFSA, FDA, WHO); *(v)* questions related to the *quality management* in general (referring to

elements such as leadership, resource management, measurement and information flow, continuous improvement, management of distribution channels, systems and processes, education and training, work ethics and work environment), and *(vi)* questions related to the most affected *business segments*. The respondents" perceptions were further measured using the 7 levels Likert scale, as suggested in the literature for such type of analysis.

The actual survey was conducted during the period March-May 2021. More than half of surveyed companies are micro and small size companies (53.6%), while processing was core business for 42% of the respondents and milk and meat/fish was business domain for 36.25% from all observed companies. Respondents' structure according to position in companies was 39.1% top managers, 15.9% operational mangers, 39.1% heads of departments of quality control/product development/food analysis, and 5.8% other. Most of the companies are implementing some type quality system (71%), while in addition, 15.9% of the companies have another system such as HALAL, FSSC 22000, OK – certificate of organic production, KOSHER, FAMI QS. Almost 85% of companies from a sample are exporters.

Table 2. Company characteristics (n = 69)

Variable	Frequency (%)			
	Total	Not affected	Affected	Statistical significance Mann-Whitney U test
Respondent profile				
Top management Production Quality control/food safety/product development Other	39.1 15.9 39.1 5.8	17.6 23.5 52.9 5.9	45.0 12.5 37.5 5.0	0.123
Size of the company				
Micro (up to 10 employees) Small (from 10 to 50 employees) Average (51 to 250 employees) Large (above 250 employees)	33.3 20.3 29.0 17.4	23.5 0 52.9 23.5	27.5 25.0 27.5 20.0	0.242
Sector				
Primary production Processing Sales and distribution Other	39.1 42.0 15.9 2.9	35.3 47.1 17.6 0	32.5 50.0 12.5 5.0	0.842

(*continued*)

Table 2. (*continued*)

Variable	Frequency (%)			
	Total	Not affected	Affected	Statistical significance Mann-Whitney U test
Subsector				
Milk	23.2	29.4	25.0	0.859
Meat	11.6	11.8	15.0	
Cereals	5.8	0	10.0	
Fruits and vegetables	18.8	11.8	12.5	
Fish	1.4	5.9	0	
Confectionary	10.1	11.8	10.0	
Beer and alcohol	1.4	0	2.5	
Water and non-alcoholic	0	0	0	
beverages	27.5	29.4	25.0	
Other				
Quality system				
NO	29.0	23.5	25.0	0.970
YES	71.0	76.5	75.0	
HACCP				
NO	58.0	70.6	62.5	0.562
YES	42.0	29.4	37.5	
ISO 22000				
NO	13.0	23.5	12.5	0.300
YES	87.0	76.5	87.5	
BRC				
NO	100	100	100	1.000
YES	0	0	0	
IFS				
NO	18.8	17.6	20.0	0.838
YES	81.2	82.4	80.0	
Global Gap				
NO	8.7	0	10.0	0.180
YES	91.3	100	90.0	
Another quality system	15.9			
Export				
NO	31.9	52.9	32.5	0.242
Up to 25%	24.6	23.5	32.5	
From 25–50%	13.0	5.9	20.0	
From 50–75%	8.7	11.8	7.5	
Above 75%	5.8	5.9	7.5	

Data obtained from the Likert scale were considered as ordinal values with the non-parametric test used. Cronbach α test was used for internal validity and results are above the 0.6 thresholds (Nunnally 1978), A principal component analysis (PCA) was used to identify determinants of quality management systems, while for all tests, the level of statistical significance was set at 0.05. In order to capture most important effects of Covid 19 pandemic, companies were divided on the basis of self-evaluation into two groups: affected and not affected. Mann Whitney U test was used to pinpoint statistically significant difference between two groups.

Table 3. Cronbach α for analysed constructs

Construct	Cronbach α
Total sample	0.984
Level of development of a system to provide prompt response on imposed challenges	0.841
Food safety requirements during a crisis	0.864
Development of plans for quick response during a crisis	0.846
Determinants of the Quality management	0.996
Business segments/value chains that are affected mostly by the pandemic	0.957

3 Results and Discussion

The Coronavirus pandemic affected global supply and demand, seeking prompt response among different stakeholders, governments, the agri-food industry, regulators, and consumers. A response will depend on a wide range of factors, while some authors (Hecht et al. 2019) sum up 10 factors that contribute to organization-level resilience in food supply chains: formal emergency planning, staff training, staff attendance, redundancy of food supply, food suppliers' infrastructure, location, service providers, insurance, and post-event learning. Coronavirus pandemic is more connected to occupational safety and protecting employee's health measures rather than food contamination (FAO 2020; OSHA 2020; Nakat and Bou-Mitri 2021), therefore employee safety is in focus as food safety system is usually developed as response to food legislation requirements. Nevertheless, the food safety issue is not taken for granted, and from the company representative's responses (Fig. 2) you can notice that all elements (suggested by the responsible authorities) are strongly monitored (all elements rated above 5 –important). There are no statistical differences between companies that are affected by the coronavirus and those that are not affected by the coronavirus, clearly identifying that food safety protocols are highly prioritized by the food industry in Bosnia and Herzegovina.

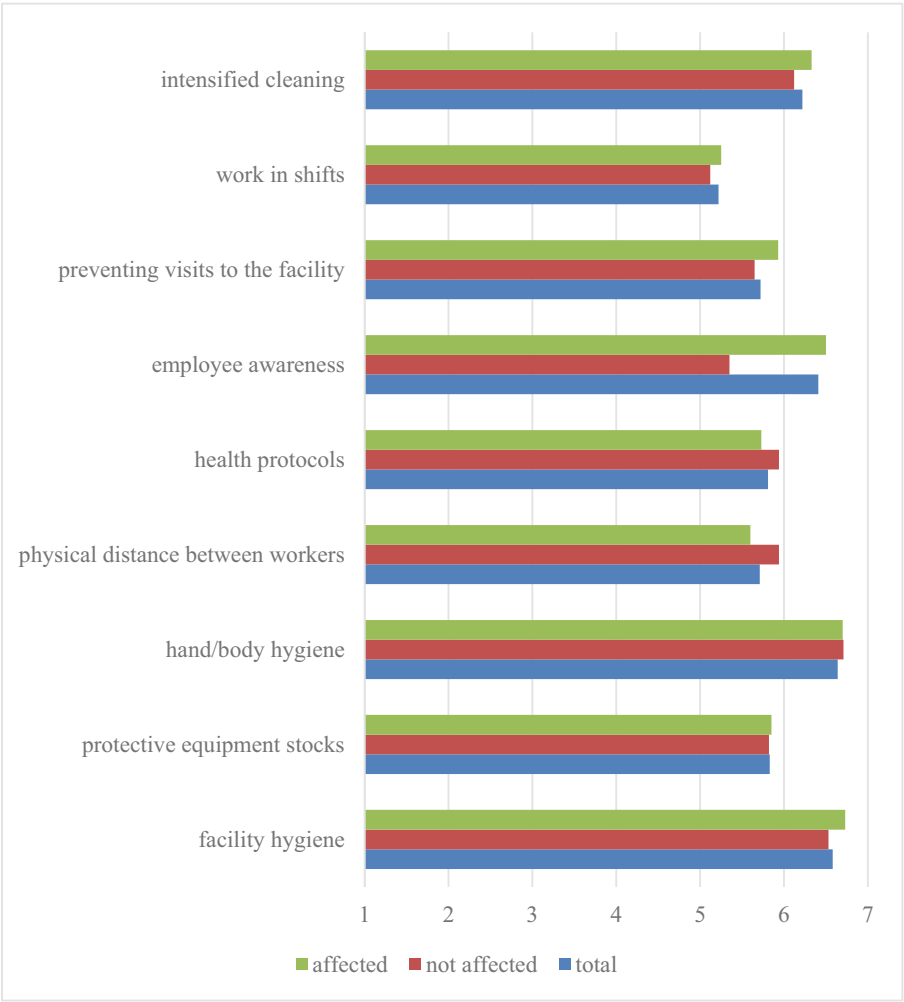

Fig. 2. Evaluation of Food safety requirements importance (1 not important, 7 very important)

Additional analysis tends to identify business segments as well as parts of value chains that are mostly affected by the coronavirus. As previously mentioned, in numerous research it is evident that no business segment nor part of the value chain is not affected by the coronavirus, border closures, lockdown, different interruptions in movements, labor shortages, maintaining physical distance all resulting in supply and demand disturbance (Paul and Chowdhury 2020; Amankwah-Amoah 2020). The flow of information remains of critical importance, where limited activities between different stakeholders reflect a risk of problem and a disturbance, as well as lack of clarity, precision and trust, etc. (Baveja et al. 2020; Gunessee and Subramanian 2020; van Hoek 2020). Research results confirm aforementioned, respondents agree (especially from the affected group) that all parts of value chains, primary production, transport and distribution, food processing,

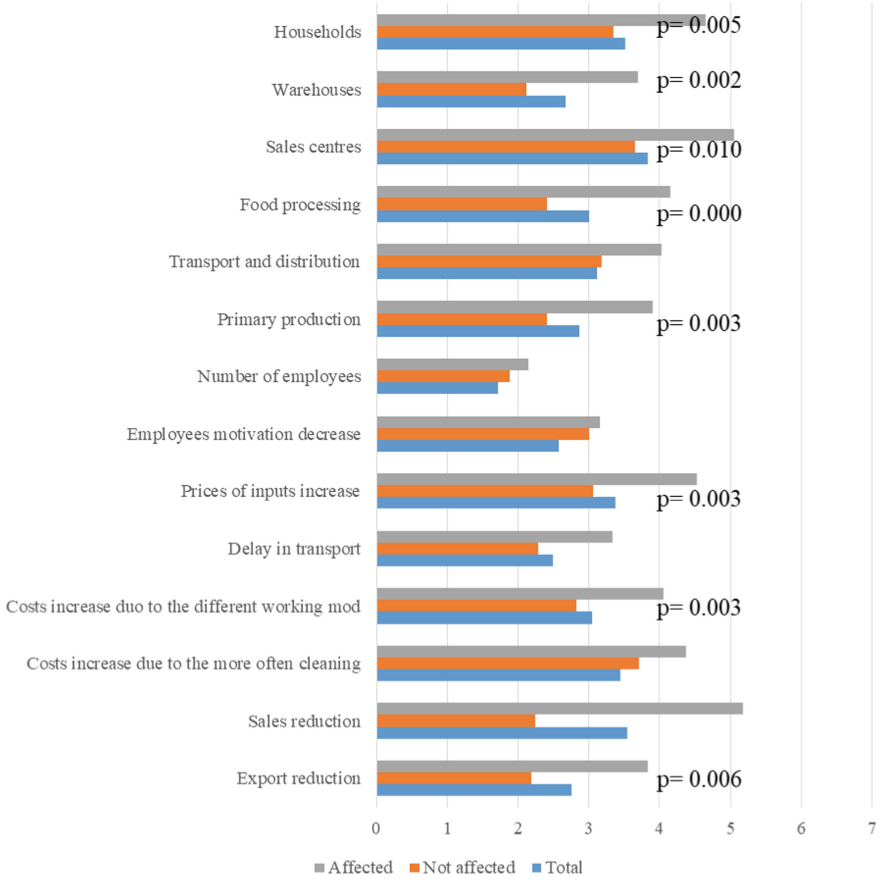

Fig. 3. COVID-19 impact on (a) Company's business activities and (b) part of value chains

sales centres, warehouses, households are impacted by the coronavirus. Among the most affected are sales centres, households, and food processing. Warehouses and primary production are among the least affected, as identified by the respondents. When it comes to segments of business activities, sales are marked as domains mostly affected and second, affected by the coronavirus (Likert scale 4–7, moderate influence, strong, very strong and extreme influence). Beside sale reduction, respondent identifies prices of inputs as the second most affected business domain, and there is statistical significance between affected and not affected companies. The logistical difficulties are important factors driving negative effects of Corona 19 pandemics. So, long lasting value laden connections with suppliers can be a part of strategies to prepare companies for different kind of uncertainties. Also, as a consequence of the COVID-19 pandemic, the cost increase was significant, driven by frequent cleaning and a different working mod (workload). The least affected, from the point of company respondents' view are the number of employees, delay in transport, employee's motivation. Having in mind that more than half of surveyed companies are small, it is not surprising to see preservation

of working places, which is an indicator of their higher resilience. Because companies opt for not reducing the number of employees, the motivation of employees stays the same. One of the possibilities is that during crisis time when a lot of people lost their job, the food industry keeps the level of employees and therefore prevents demotivation amongst the workers (of course, to assess their level of motivation, a survey among the employees would be more accurate than this study where managers are the respondents). Figure 3 depicts how respondents grade the impact of COVID-19 on segments of business activities and different parts of value chains.

The next step was to analyse the level of development of a quality system and practicing measures to provide a prompt response during the coronavirus pandemic. Respondents could choose between tree options of system development: (1) already developed/defined, (2) developed/defined during the pandemic, and (3) plan to develop/define in the future. All results are presented in Table 3. From this table, it is clear that most of the companies already have a quality system structure defined or they develop it during the pandemic. It is a positive sign that most of the companies already develop documentation (plans, procedures, teams) in case of a crisis, procedures for cleaning of facilities in case of a pandemic, as well as they, develop trainings regarding food safety during the pandemic and general trainings. For the majority of companies, the purchase of additional protecting equipment (gloves, masks, etc.) was developed during the pandemic time which is no surprise (Table 4).

In order to identify which plans companies have developed or they intend to develop, we further asked company respondents to identify if they have (1) only developed a plan, (2) plan and documentation developed, and (3) neither plan nor documentation developed, for a different "worse-case" scenario. Table 5 presents the level of development of a system for crisis response. From the obtained result, one may conclude that majority of the companies have both a plan and documentation (average value around 2), but if we look at frequencies it is clear that not all companies have a plan and documentation necessary to respond to crisis. From available options, one that stands out are bioterrorism where almost 60% of the companies do not have either plan or documentation, more than 40% of companies do not have a plan nor documentation in case of traffic accident, and more than 30% of companies do not have plan nor documentation in case of natural disaster. In the case of pandemics and other health crises, more than 26% of the companies do not have plans nor documentation. All aforementioned identify an urgent need for better planning and preparation of several plans in case of a crisis. From the results it can be seen that QMS systems are focused on safety issues (food as well as employee safety), as documentation and plans are developed for the case of water contamination, contamination of ingredients, and situation when a fire occurs. The same applies to environmental pollution and energy cuts. From the results it is obvious that BiH companies do not care for environmental issues.

The last part is dedicated to determinants of the total quality management that enables companies to deal with COVID-19 pandemic challenges. Results from Fig. 4 show that there are no significant differences between affected and not affected companies, or, both "soft" and "hard" skills are graded as important (very important) by the company representatives.

Table 4. Level of development of a quality system to provide prompt response on imposed challenges

QS Element		Total sample		Not affected	Affected	Statistical significance Mann-Whitney U test
		Frequency	Mean	Mean	Mean	Stat. sig
Documentation (plans, procedures, teams) in case of a crisis	1 2 3	52.2 30.4 17.4	1.61	1.41	1.65	0.258
Food safety training for workers in case of a pandemic	1 2 3	30.4 49.3 20.3	1.86	1.82	1.85	0.931
General training in case of a pandemic	1 2 3	20.3 62.3 17.4	1.93	1.82	2.03	0.231
Restrictive measures (i.e. clear procedure and measures) of personal hygiene in case of a pandemic	1 2 3	43.5 47.8 8.7	1.65	1.47	1.7	0.203
Purchase of additional protection equipment (e.g. gloves, masks, chemicals, disinfectants, etc.) in case of a pandemic	1 2 3	34.8 58 7.2	1.72	1.53	1.83	0.05
Procedures for intensifying cleaning processes in case of a pandemic	1 2 3	49.3 40.6 10.1	1.61	1.41	1.7	0.128

Note: 1 – we have developed/defined in current food safety system; 2 – we have developed/defined in food safety system during the pandemic; 3 – We plan to develop/define in food safety system in future

Table 5. Level of development of a system in case of a crisis

Crisis		Frequency	Total sample	Mean (not affected)	Mean (affected)	Stat. sig
Natural disasters	1 2 3	29.0 40.6 30.4	2.01	1.94	2.00	0.794
Bioterrorism	1 2 3	15.9 24.6 59.5	2.39	2.41	2.40	0.875
Fire	1 2 3	15.9 69.6 14.5	1.94	1.88	1.93	0.802
Contaminations (ingredients, packaging)	1 2 3	7.2 76.8 16.0	2.04	2.06	2.05	0.967
Water contamination	1 2 3	17.4 63.8 18.8	1.97	1.94	1.98	0.842
Pandemic or other health crisis	1 2 3	24.6 49.3 26.1	1.97	1.82	1.93	0.706
Traffic accident	1 2 3	29.0 27.5 43.5	2.14	1.88	2.28	0.091
Energy cut	1 2 3	21.7 52.2 26.1	2.00	2.00	1.95	0.753
Environment pollution	1 2 3	18.8 53.6 27.6	2.04	1.76	2.20	0.022

Note: 1 – only plan developed; 2 – plan and documentation developed; 3 – nor plan nor documentation developed

Besides, principal component analysis was done to identify determinants in both groups, within the group "not affected" one "hard" element – (continuous improvement) and two "soft" elements (suppliers' quality management and work culture and environment) were identified. Within the group "affected", three "hard" elements (continuous improvement, systems and processes, and measurement and feedback), and three "soft" elements (suppliers' quality management, work culture and environment t, and leadership) were identified.

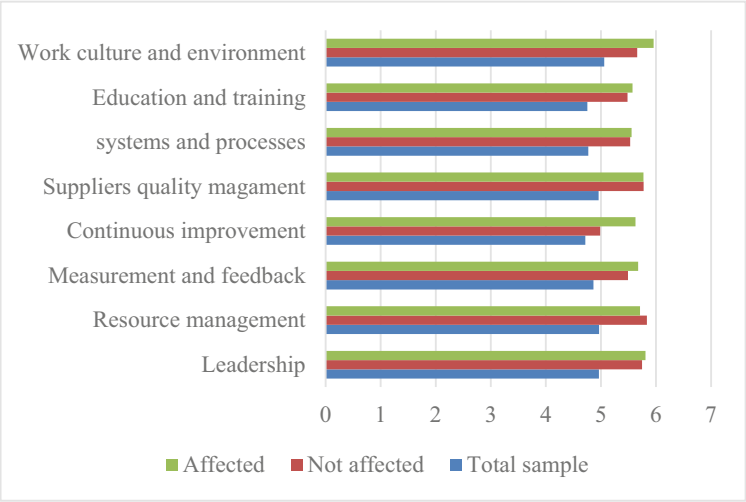

Fig. 4. Average value for TQM elements

The majority of "not affected" companies are middle and large size, with a focus on the domestic market. Therefore, these companies' focus on the three identified QM elements are driven by the legislation and low sophistication of the domestic market that supports price competition as a wise strategy to ensure better market position. Such results confirm that QM elements promoted by the legislation such as food and employee safety system together with QM elements responsible to rapid response (stronger in comparison with affected group) are major driving force behind companies' capability to address unexpected events. According to Djekic et al. (2021) food safety systems in Bosnia and Herzegovina are not fully developed (on-the-way), while ex-ante readiness (plans, documentation etc.) is limited, but present. Same research pointed out higher levels of development of QM elements connected with hygiene and cleaning. This is in line with here presented research results.

However, these results imply the existence of strong leadership, because without it, it is not possible to develop adequate organisational culture, including work culture and environment. The low level of domestic market sophistication is responsible for neglected elements such as process and systems and measurement and feedback. In addition, the difference between those two groups can be rooted in existing research bias. The level of QM elements' development is based on responded self-evaluation and therefore it reflects their attitudes, perception and knowledge regarding the whole system. As it can be seen from Table 2, not a single respondent is top manager in the not affected group. So, this bias has to be taken in account when results are interpreted (Table 6).

Table 6. Determinants of total quality management in food industry in Bosnia and Herzegovina during the pandemic time

NOT AFFECTED 89.51% of variance explained by 6 factors		AFFECTED 86.36% of variance explained by 7 factors	
Factor dimension	Loading	Factor dimension	Loading
Continuous improvement		*Continuous improvement*	
Coordinating body for quality improvement exists	0.655	Coordinating body for quality improvement exists	0.846
Teams for quality improvement are active in all organisational parts	0.712	Teams for quality improvement are active in all organisational parts	0.848
Tools and techniques for quality improvements are used in all organisational parts	0.740	Tools and techniques for quality improvements are used in all organisational parts	0.755
Company improves all products, services and processes	0.777	Company improves all products, services and processes	0.627
Suppliers quality management		*Suppliers quality management*	
Suppliers are chosen based on quality (price is secondary criteria)	0.620		
Company helps suppliers to achieve requirements	0.654	Company helps suppliers to achieve requirements	0.707
Company often do supplier audit	0.802	Company often do supplier audit	0.664
Company work close with suppliers and builds long-term partnership	0.851	Suppliers provide relevant data about the quality and other relevant data	0.823
Work culture and environment		*Work culture and environment*	
Positive work environment is established in all organisational parts	0.703	Positive work environment is established in all organisational parts	0.703
Positive values, such as trust, fairness, and dedication are nurtured by top management	0.909	Positive values, such as trust, fairness, and dedication are nurtured by top management	0.706
Teamwork is well-applied practice	0.914	Teamwork is well-applied practice	0.777
Company promotes employee satisfaction	0.911		
		Leadership	
		Top management ensures that all employees are aware of the company's mission and goals	0.619

(*continued*)

Table 6. (*continued*)

NOT AFFECTED 89.51% of variance explained by 6 factors		AFFECTED 86.36% of variance explained by 7 factors	
Factor dimension	Loading	Factor dimension	Loading
		Top management promotes employees' involvement in quality management and improvement	0.818
		Managers involve and empower employees	0.801
		Clear communication exists between top management and employees	0.681
		Measurement and feedback	
		Consumer satisfaction is measured, systematically and planned	0.640
		Data about quality and consumers are collected and analysed	0.637
		Data about financial performances are collected and analysed	0.718
		Systems and processes	
		Quality system and assurance are implemented	0.882
		Internal system for data collection is established	0.815
		Market data collection system is established	0.705
		Employees involved in different processes known how to analyse those data	0.688

4 Conclusion

Disruptions along the food value chains is evident in most countries, while the size of the disruptions depends on a wide range of factors. Demand disruptions received more attention so far (Shekarian et al. 2020) while supply side have received less attention because it is believed it worked smoothly during the coronavirus pandemic. Recently, after the initial "shock" pass, more research is focused on company performances during the pandemic, where general performances, communication among the stakeholders, food safety costs define resilience of such company (value chain). It is well-known how reliable scientific information, transparency and openness of food safety authorities (trust)

are of crucial importance to reduce misperceptions of food risk (Jonge et al. 2008). Because of this, responsible authorities such as the EFSA, FDA, WHO, FAO provide several guidelines/recommendations for all stakeholders along the food value chain for pandemic and post pandemic behaviour. Multiple sources (Raj et al. 2020; Snow et al. 2021) identify several key elements that make companies (stakeholders) more resilient during the crisis, such as relying on advanced technology, connected/networked, organization in sense of logistic, workloads, safety protocols, emergency protocols, etc. This study shows that companies that participate in research, possess some level of resilience and their rapid response to pandemic situation was positive. Food safety management systems are well implemented, indicating they were able to manage food safety risks and/or prevent food safety emergencies. Quality plans exist, most of them were established before the pandemic, and some of them were developed during the pandemic. Still, some companies do not have several QM elements that shape ex-ante readiness of companies such as quality plans, documentation (procedures, plans) during the crisis situation, plan for food safety training, general training. It means these QM elements connected with ex-ante readiness have to be in focus of QM improvements. This will shift QM systems towards the next development and sophistication phase, to system maturity. However, such system level change requires fast and strong digitalisation and adoption of new modern technologies as well as strong leadership. Such approach promotes companies' capacity to answer changing customer requirements and capacity to become part of attractive food value chain, establishing value-laden connections with suppliers which can be important strategy to decrease different types of risks, including natural and health crises.

When it comes to plans for crisis, most of the companies do have plans in case of ingredient contamination, water contamination or in case of fire, which were developed under the pressure of existing legislation. On the contrary, plans in case of bioterrorism, traffic accidents, natural disasters, environmental pollution, energy cuts, and in case of a pandemic or any other health crisis do not exist, and that significantly reduces the ability of the companies to react during such a crisis.

With Fig. 5, visualization of current situation, quality management system and crisis plan within the analysed companies in B&H food industry is presented. Bottom right corner represents elements that are most developed withing the companies, while top left corner represents elements that are least developed and where future efforts should be done.

When developing a resilient business, food industry companies in the 21st century should and must focus on introducing real-time monitoring and lifecycle product records that will ensure high connectivity between food supply chain trough constant real-time data flow which accessibility will boost transparency, accountability and trust that is necessary for development of value-laden connections that drive chain sustainability na resilience. This will not only serve as an agile and flexible response during the crisis situation but will also accelerate recovery and prepare for the next crisis situation.

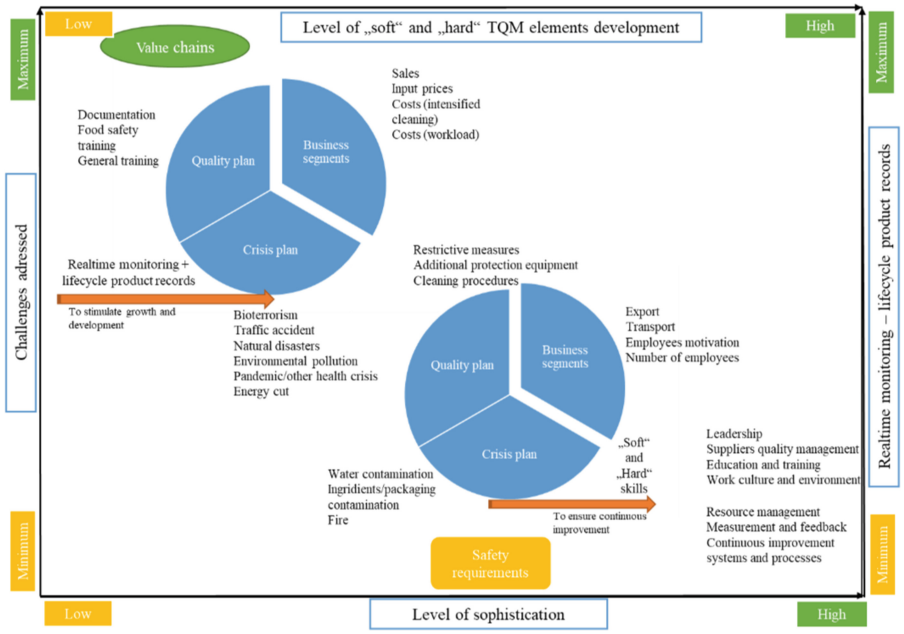

Fig. 5. Current QMS and Crisis plan within the food industry in Bosnia and Herzegovina

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Phenolic Content and Antioxidant Activity Determination in Chamomile (*Matricaria Recutita*) and Sage (*Salvia Officinalis*) Teas

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Abstract. Herbal teas contribute to human health as a dietary source of phenolic compounds, with health benefits thought to be associated with these bioactive compounds. Commercially consumed sage (*Salvia officinalis*) and chamomile (*Matricaria recutita*) teas, purchased in bulk packaging, were used in this research aimed for phenolic content and antioxidant activity determination. Water and ethanol (30:70 v/v) were used as extraction solvents. The extraction was carried out at four different temperatures, 50 °C, 60 °C, 70 °C and 80 °C, respectively. Phenolic content was determinated using Folin-Ciocalteu spectrophotometric method and the pFRAP method was used for antioxidant activity determination. Extraction with ethanol at 80 °C, which was established to be optimum for both teas, resulted in higher phenolic content, as well as higher antioxidant activity, with max TPC in sage teas (1197.08 ± 86.27 mg GAE/100g) and chamomile teas (1133.78 ± 74.04 mg GAE/100g). Antioxidant activity of sage teas was higher than chamomile teas, 550.35 ± 16.56 mg GAE/100g for ethanol extracts and 456.70 ± 30.72 mg GAE/100g for aqueous extracts of sage. Positive correlation was noted between phenolic content and antioxidant activity in sage and chamomile teas. Experimental results indicate that phenolic content can provide substantial antioxidant activity as well as that sage and chamomile teas could be a good alternative as dietary source of bioactive compounds with high antioxidative power.

Keywords: Chamomile · Sage · Teas · Phenolic content · Antioxidant activity

1 Introduction

Sage (*Salvia officinalis* L.) and chamomile (*Matricaria recutita*) teas are consumed in everyday life because of their health-promoting activities and unlimited health benefits due to their antioxidant properties [1]. Numerous studies provide data of tea constituents, which can be related to benefits on human health, such as prevention of cancers and cardiovascular diseases, as well as improvement of immune system [2, 3]. Tea extracts exhibit potent antioxidant power due to extensive range of phenolic compounds [4], mainly flavonoids, which are good scavengers of free radicals [5].

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Chamomile (*Matricaria recutita*) is a member of the *Asteraceae* family and is known to be used in various forms of preparations, with the most popular form being herbal tea which has been used in treatment of inflammation, menstrual disorders, ulcers etc. [6]. Chamomile is widely represented by two known varieties: German (*Matricaria chamomilla*) and Roman (*Chamaemelum nobile*), with hollow, bright gold cones of the flowers arranged with disc-shaped or tubular florets [7]. Flavonoids and phenolic acids have been identified as major secondary metabolites in chamomile and main constituents of chamomile flowers were found to be apigenin, querzetin, lueolin and their glucosides [8], rutin trihydrate, ferulic acid, chlorogenic acid and apigenin-7-O-glucoside [9]. Mekinić et al. [10] reported rosmarinic, gallic, cinnamic, caffeic and protocatechuic acids in chamomile extracts.

Sage (*Salvia officinalis* L.) is a member of mint *Lamiaceae* family, with approximately 240 genera and 7000 species [11]. It is a perennial, evergreen subshrub, native to the Mediterranean region and mostly gathered from the wild [12]. Besides its role as good diuretic, hemostatic, wound-healing plant, sage tea has been recognized as excellent medicine for sore throat and mouth cavity [13, 14]. Bakkali et al. [15] identified two classes of secondary metabolites in sage: phenolic compounds and terpenoids. Aleksovski and Sovova [16] reported camosol, rosmarinic acid and luteolin-7-O-beta glucopyranoside, as the most abundant phenolic compounds in sage, which contribute to its antioxidant power [17], together with salvianolic acid K and ferrulic acid [18]. In addition to this activity, sage shows anti-diabetic, anti-inflammatory and antimicrobial activities [19–21] and it has a role in preventing cardiovascular diseases [22].

The objective of this study was to evaluate phenolic content and antioxidant activity in sage and chamomile teas in bulk packaging, extracted at four different temperatures and with two extraction solvents using spectrophotometric assay.

2 Material and Method

2.1 Samples

Chamomile (*Matricaria recutita*) and sage (*Salvia officinalis*) teas were purchased from local markets in Sarajevo during April, 2015 in bulk packaging and from different manufacturers. In the case of sage, leaves were used for analysis and for chamomile, flowering parts were the material for analysis.

2.2 Extraction

For the extraction of chamomile and sage polyphenols ethanol (30:70 w/w) and distilled water were used. Extraction was carried out at four different temperatures (50°, 60°, 70° and 80 °C.). Chamomile flowers and sage leaves were weighed and 2 g of homogenized plant material was extracted with 40 mL of extraction solvent (ethanol/water mixture 30/70, v/v and distilled water, respectively) for 15 min with reflux (condenser). After cooling, the extracts were filtered through a Whatman no.40 filter paper (Whatman International Ltd, Kent, UK) and filtrates were adjusted to 50 mL with ethanol or distilled water.

2.3 Spectrophotometric Determination of Total Polyphenols

Total phenolic content was determined according to protocol described by Ough and Amerine [23] with slight modifications, using Folin-Ciocalteau assay. The method is based on the colorimetric reaction between phenolic compounds and the Folin-Ciocalteau reagent. 1mL of extract was transferred into a test tube, then 2.5 mL of Folin-Ciocalteau reagent (1:10) was added to each sample. After 5 min, 6.5 mL of saturated sodium carbonate (Na_2CO_3) was added and the mixture was incubated in a water-bath at 50 °C for 30 min, protected from the light. After incubation, absorbance was measured at 600 nm using UV-VIS spectrophotometer (Cary 1E UV-VIS spectrophotometer, Agilent Technologies, USA). Quantification of total phenol content was made against a Gallic acid calibration curve, which was prepared by diluting stock solution of Gallic acid with extraction solvents to yield 15 to 150 mg/L of total phenolic compounds. Results were expressed as means ($N = 3$) $\pm$ SD.

2.4 Determination of Antioxidant Activity

Antioxidant activity of chamomile and sage tea extracts was determined using pFRAP (potassium ferricyanide reducing power) method described by Meng et al. [24]. 1mL of tea extract was transferred to a test tube and 1 mL of $K_3[Fe(CN)_6]$ aqueous solution was added. After 5 min, 1 mL of $FeCl_3$ and 7 mL of distilled water were added and the absorption of blue complex between phenolic compounds and $K_3[Fe(CN)_6]$ was measured at 700 nm using a UV-VIS spectrophotometer (Cary 1E UV-VIS spectrophotometer, Agilent Technologies, USA). The quantification of total phenol content was made against Gallic acid calibration curve.

2.5 Statistical Analysis

Each analysis was performed in triplicate and expressed as mean $\pm$ standard deviation (SD). Two-way ANOVA and Tukey's test were used to compare significant differences in total phenolic content and antioxidant activity.

3 Results and Discussion

The content of phenolic compounds and antioxidant activity are presented in Table 1 and Table 2. Total phenolic content and antioxidant activity were calculated on the basis of Gallic acid and expressed as equivalents of Gallic acid of dry material.

All sage and chamomile extracts shoved high phenolic content, as well as antioxidant activity, which can be related with excellent health benefits of these two plants. However, the type of extraction solvent and temperature of extraction had high impact on content of phenolic compounds and antioxidant activity. In this research, distilled water and 30% ethanol solution were used for extraction of chamomile and sage polyphenols.

Results showed that extraction with 30% ethanol resulted with higher phenolic content in both chamomile and sage tea extracts. Total phenolic content (TPC) in sage extracts varied between 356.37 ± 48,48 and 1197.08 ± 86.27 mg GAE/100g dw, depending on the solvent and temperature of extraction. TPC in chamomile extracts varied between 275.63 ± 11.76 and 1133.78 ± 74.04 mg GAE/100g dw. Highest phenolic content was found in the sage extracted in ethanol at 80 °C, and the lowest TPC was found in the aqueous chamomile extract at 50 °C.

Table 1. Total phenolic content of investigated sage (*Salvia officinalis*) and chamomile (*Matricaria recutita*) teas

Plant	Extraction solvent	Temperature of extraction (°C)	TPC (mg GAE/100g dw)
Sage (*Salvia officinalis*)	Ethanol (30:70, v/v)	50	511.70 ± 10.42 [c]
		60	727.69 ± 14.70 [c]
		70	1127.88 ± 104.52 [b]
		80	1197.08 ± 86.27 [a]
Sage (*Salvia officinalis*)	Water	50	391.68 ± 43.68 [c]
		60	356.37 ± 48.48 [c]
		70	582.09 ± 9.51 [b]
		80	834.42 ± 16.01 [a]
Chamomile (*Matricaria recutita*)	Ethanol (30:70, v/v)	50	533.58 ± 13.08 [c]
		60	636.34 ± 14.41 [c]
		70	913.39 ± 69.63 [b]
		80	1133.78 ± 74.04 [a]
Chamomile (*Matricaria recutita*)	Water	50	277.71 ± 5.2 [c]
		60	275.63 ± 11.76 [c]
		70	444.08 ± 18.47 [b]
		80	648.45 ± 30.92 [a]

Results expressed as mean ± SD (N = 3); Different letters (a-c) in the same row represent statistically different results between temperature of extraction and extraction solvent within the same herbal tea.

As it can be seen from Table 1, increasing the temperature of extraction increased the phenolic content in chamomile and sage tea extracts, which is in accordance with previous studies [25]. The reason for better extractability at higher temperatures could be due to increased solubility, but extraction at temperatures higher than 90 °C could lead to a decrease in the content of phenolic compounds [25]. Same group of authors showed that extraction at above 63 °C decreased content of polyphenols, which can be explained with possible degradation of phenolic compounds and the reason for that could be hydrolysis,

redox-reactions and polymerization [26]. Total phenolic content in sage extracts measured by Dent et al. [26] at 60 °C and 90 °C ranged between 5170.62 to 6278.12 at 60 °C in aqueous and 30% ethanol solution, respectively. However, aqueous extracts of sage and chamomile at 60 °C showed lower content of phenolic compounds than at 50 °C , which can be described with polarity of water molecules and reduced capability to dissolve polar molecules at higher temperatures [27]. According to Sotiropoulou et al. [9], who studied chamomile, aqueous extracts at 80 °C showed higher total phenolic content and at 25 °C phenolic compounds could not be detected at all. Our research can be correlated with previous data reported in case of chamomile extracts at different temperatures. Data showed decreasing of TPC in order 80° > 70° > 60° > 50 °C in ethanol and aqueous extracts of chamomile and sage teas, which is in accordance with published data [27]. On the other side, different studies showed higher total phenolic content in aqueous chamomile extracts obtained at 100 °C than at 80 °C [1]. All sage samples showed higher content of phenolic compounds in both ethanol and aqueous extracts in comparison with chamomile extracts (Table 1.), which is in accordance with previous researches [27], so it can be said that total phenolic content depends on the origin and type of tea, plant phenolic type (phenolic acids or flavonoids) and is affected with temperature of extraction, as well as type of solvent used for extraction [28, 29]. In our research, distilled water and 30% ethanol were used as extraction solvents. Obtained results showed that solvent had significant impact at TPC at higher temperatures of extraction. Naczk et al. [30] reported role of solvent polarity as a key factor for increasing phenolic solubility. Dent et al. [26] showed that recovery of phenolic compounds was dependent on type of solvent used, polarity of solvent and solubility of phenolic compound in extraction solvent. Amount of water in extraction system made with organic solvents has been recognized as factor that had influence on extraction of polyphenols. Data reported for polyphenols extracted from medicinal herbs with different aqueous methanol solutions showed better extractability with lower organic solvent content [31]. Influence of temperature was observed in previous studies showing strong influence of polar groups of solvent on solubility depending on type of phenolic compound, indicating that the presence of side chains, conjugated bonds, glucose moiety in molecules influence the efficiency of polyphenols extraction [32].

Antioxidant activity of chamomile and sage tea extracts was measured in terms of reducing ability of extracts against pFRAP reagent. Obtained results are presented in Table 2. Our samples showed good antioxidant activity and excellent power in reducing Fe^{3+} to Fe^{2+}. Highest antioxidant activity has been obtained in sage ethanol extracts at 80 °C (550.35 ± 16.56 mg GAE/100g dw) and lowest antioxidant activity has been detected in aqueous chamomile extracts at 50 °C (191.76 ± 0.78 mg GAE/100g dw). Antioxidant activity has followed the same pattern as total phenolic content and extraction at 80 °C has been established to be optimal for both herbal teas. Satiropoulou et al. [9] reported maximum antioxidant activity of aqueous chamomile and sage extracts at 80 °C and these authors also showed that besides temperature of extraction, time of extraction and type of solvent also have huge influence on the evaluation of beneficial properties of herbal teas.

Table 2. Antioxidant activity of investigated sage (*Salvia officinalis*) and chamomile (*Matricaria recutita*) teas

Plant	Extraction solvent	Temperature of extraction (°C)	AA(mg GAE/100g dw)
Sage (*Salvia officinalis*)	Ethanol (30:70, v/v)	50	437.67 ± 19.50 [c]
		60	463.62 ± 18.18 [b]
		70	505.02 ± 5.98 [b]
		80	550.35 ± 16.56 [a]
Sage (*Salvia officinalis*)	Water	50	288.37 ± 2.69 [c]
		60	345.80 ± 49.19 [b]
		70	368.27 ± 11.3 [b]
		80	456.70 ± 30.72 [a]
Chamomile (*Matricaria recutita*)	Ethanol (30:70, v/v)	50	218.04 ± 0.94 [c]
		60	240.87 ± 1.54 [b]
		70	287.87 ± 7.42[b]
		80	303.02 ± 2.49 [a]
Chamomile (*Matricaria recutita*)	Water	50	191.76 ± 0.78 [c]
		60	201.42 ± 3.60 [b]
		70	257.14 ± 16.4 [b]
		80	248.64 ± 5.54[a]

Results expressed as mean ± SD (N = 3); Different letters (a-c) in the same row represent statistically different results between temperature of extraction and extraction solvent within same herbal tea.

Mechanism of antioxidant action is complex; they act in several mechanisms so numerous different methods have to be used in order to evaluate antioxidant activity. Depending on the nature of the antioxidant molecule and reaction that their action is based on, methods can be separated as electron transfer or hydrogen atom transfer reactions [33]. Electron transfer reactions measure the ability of an antioxidant molecule to reduce a compound and hydrogen atom transfer reactions gave information about the ability of antioxidant molecule to react with free radicals and neutralize their presence. Mekinić et al. [10] showed that antioxidant activity of chamomile extracts ranged from 21.7 for FRAP (Mm Fe^{2+}) method to 313.3 for DPPH IC_{50} (mg GAE/L) method with 228.7 and 76.0 mg GAE/L for ABTS IC_{50} and CHEL IC_{50}, respectively. Due to a well-known fact that reducing ability is usually in dependence on the content of bioactive compounds, it can be said that extracts with high phenolic content exhibit high antioxidant activity. Our research showed strong correlations between TPC and pFRAP activity for both chamomile and sage tea extracts (Table 3.).

Table 3. Correlations between total phenolic content and antioxidant activity in sage (*Salvia officinalis*) and chamomile (*Matricaria recutita*) teas

Plant		TPC	pFRAP
Sage	TPC	1	0.862**
(*Salvia officinalis*)	Pfrap	0.862**	1
Chamomile	TPC	1	0.894**
(*Matricaria recutita*)	Pfrap	0 .894**	1

**. Correlation is significant at the 0.01 level (2-tailed)

4 Conclusion

Experimental results indicate that sage (*Salvia officinalis*) and chamomile (*Matricaria recutita*) tea extracts provide substantial phenolic content and antioxidant activity and could be an important dietary source of phenolic compounds and exhibit antioxidant power. Binary system of extraction solvents (ethanol: water) showed better efficiency in extraction of polyphenol compounds compered to water. A positive correlation was noted between total phenolic content and the ability of antioxidant molecules to reduce the pFRAP reagent. This research contributes to better understanding of biological activity and beneficial properties of chamomile and sage herbal teas.

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Physicochemical Properties of Heat-Treated Sheep Meat Under Different Processing Conditions

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Abstract. The influence of processing (boiling/smoking) through analyzing physicochemical and sensory properties on the quality characteristics of the final meat products was examined. Heat treatment of cooking and smoking was carried out in controlled conditions, under different processing regimes (temperature 55–75 °C) and cooking time (1 h and 47 min).

Our studies have shown that certain experimental groups/treatments have been isolated (four treatments with different temperature and treatment time). Treatment in the technological process of boiling/smoking with elevated temperatures and prolonged boiling time based on the obtained values for physicochemical and sensory properties proved to be favorable and optimal. The content of water, ash, organic acids, salts, nitrites, hardness, pH value, color and sensory properties were examined in four treatments.

The results show that the tested components and properties that contributed most to the overall variability of the four boiled sheep meat combinations in each of the main components (ash, organic acids, hardness, red color a*, nitrites) were highest in the experimental group with boiling temperature up to 75 °C and time 1 h and 47 min.

Industrial production showed to be more desirable compared to traditional production in controlled conditions in terms of physical and chemical properties and ratings for overall sensory quality where evaluators gave ratings in high percentages for boiled sheep meat (83.66%–96.33%).

Keywords: Sheep meat · Boiling · Physicochemical properties · Sensory properties

1 Introduction

During the preparation, processing and production of meat products, various technological procedures are applied. The choice of procedures, which will be applied during the processing of meat, is influenced by the traditions and habits of consumers, region, season, the type of animal whose meat is used and the types of products to be obtained. During meat processing, various technological operations are implemented: reception

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and preparation of animals for slaughter, slaughter-bleeding, cooling, boning, cutting, salting and pickling, heat treatment, packaging and storage. The properties of meat, indicators of quality (texture, juiciness, color and smell of processed meat) depend on the type, composition, muscle properties, method, temperature and heat treatment time [1]. The chemical composition of sheep meat is extremely important, because it provides basic information about the quality, price and energy value of meat [2, 3]. Fresh sheep meat contains about 65% water, 15% fat, 18% protein, the other nutrients (carbohydrates, vitamins and minerals) are in trace amounts [4, 5]. Estrada-Solis and coworkers [6] analyzed physicochemical components of sheep boiled meat. Dutra and coworkers [7] explained the technological process and quality characteristics of cooked sheep meat ham. The test results showed significant differences in chemical composition (water, protein, fat and ash content), water pH activity and different treatments consistency. The chemical composition and color of Cres lamb meat were examined by Vnučec and coworkers [8]. Who found differences in the composition and color of Cres lambs and lambs of some Mediterranean breeds. Teixeirao et al. [9] examined the influence of the processing regime on the water content of sheep meat and determined an average value of 36.7%. Various studies have been performed on digestion process influence analyzing some physicochemical and sensory properties of sheep meat products such as litter [10, 11] and smoked sheep meat [12]. During the processing and heat treatment of sheep meat, there is a change in chemical composition, parameters such as color, hardness and pH value, in both raw and heat-treated meat. The color of smoked meat is also influenced by pigments of brined meat, pH value (acids stabilize the color) and the way of smoking [5, 13, 14]. This influence was obtained in studies by measuring the color in fresh and heat-treated meat [9, 15–17]. Considering the aforementioned research and existing knowledge, this paper investigates the physicochemical properties and sensory characteristics of sheep meat heat-treated by boiling under different processing regimes (temperature and time) in controlled industrial conditions.

2 Materials and Methods

2.1 Sample Preparation

Sheep meat from Pramenka breed was used for research. The animals kept freely and were fed exclusively on grass (green fodder-clover grass mixtures) on the mountain pastures of Bosnia and Herzegovina. Twelve (12) well-fed females, aged 9 to 12 months, were isolated from the same herd. During 4 weeks, i.e. every 7 days, bleeding was performed on 3 heads, which were used for sampling (cooling for 24 h at temperature of 1 °C). After cooling (temperature in the middle of the thigh reached <4 °C), the thighs were separated from the shank from which the femur and the excess connective and fatty tissue were removed. The thigh is cut into pieces of regular shape (length 20–25 cm, width 4–5 cm and thickness 3–4 cm). Shaped pieces of meat were brined. The brine mixture consisted of sodium chloride (2.5%) and nitrite salt (0.3%), as well as spices, pepper and garlic. The pickling process was carried out under controlled conditions: temperature 2–4 °C, duration 10 days. From each leg (obtained from the 12 sheep) 24 pieces of meat were formed, which were divided into 4 experimental groups formed according to the conditions in which the meat is treated during the heat treatment. From

each experimental group, 24 pieces of meat were taken and heat-treated by cooking (thermodynamic chamber for smoking, roasting and cooking - manufacturer Atmos Maurer, Germany).

The technological boiling processing was carried out in thermodynamic chambers with the change and monitoring of the stated parameters whose values are shown in Table 1.

Table 1. Heat treatment parameters used during thermal treatments process of chicken meat

Thermal treatments		Temperature (°C)	Time (hours, minutes)	Air flow (m^3/min)	Relative humidity (%)
Experimental group I (EG I)	I drying	65	20 min	7.1	74–76
	I smoking	70	5 min	7.1	74–76
	Boiling	72	30 min	7.1	74–76
	Rosting	75	10 min	7.1	74–76
	II smoking	70	15 min	7.1	74–76
Experimental group II (EG II)	I drying	65	20 min	9.7	78–82
	I smoking	65	5 min	9.7	78–82
	Boiling	72	35 min	9.7	78–82
	Rosting	75	10 min	9.7	78–82
	II smoking	65	20 min	9.7	78–82
Experimental group III (EG III)	I drying	65	20 min	9.7	78–82
	I smoking	60	5 min	9.7	78–82
	Boiling	72	40 min	9.7	78–82
	Rosting	75	10 min	9.7	78–82
	II smoking	60	25 min	9.7	78–82
Experimental group IV (EG IV)	I drying	65	20 min	12.7	82–86
	I smoking	55	5 min	12.7	82–86
	Boiling	72	42 min	12.7	82–86
	Rosting	75	10 min	12.7	82–86
	II smoking	55	30 min	12.7	82–86

2.2 Physico-chemical and Sensory Analysis

pH value was determined according to reference method BAS ISO 2917:2007. The reference method (BAS ISO 1442: 2007) was used to determine the water content in the tested meat samples. Determination of total ash content in the tested samples was performed by the reference method BAS ISO 936:2007. The reference method BAS ISO 1841–1:2007 was used to determine the sodium chloride content. Determination of

nitrite concentration was carried out by the reference method BAS ISO 2918:2007. For determination and quantification of the total phenol content in meat products, the Folin Ciocalteu method was used as described in Naveena et al. [23].

Total acids are expressed as a percentage based on the consumption of NaOH solution factors. After grinding and mixing the samples with distilled water, the samples were filtered and 20 mL of the filtrate was taken. Filtrates to which the indicator was previously added, was titrated with a solution of NaOH (sodium hydroxide), concentration 0.1 mol/L, until the indicator changed color from the first excess drop of alkali.

The hardness of sheep meat products was determined using a Warner Bratzler platform knife (Knife Blade HDP/BSK), which is used to measure the force required to cut the material under test. The instrument used is a TA. XT Plus Texture Analyzer (Stable Micro Systems, Godalming, UK) with a 25 kg cell.

A colorimeter (Minolta Chroma Meter CR-400, Konica Minolta Inc., Osaka, Japan) was used to determine the color parameters. Measurements were performed in D-65 illumination with a standard shelter angle of 2 °C. The color characteristics are expressed in CIE L*a*b*, based on three coordinates over which the color of the samples is defined: L* (color brightness), a* (proportion of red (+a*) or green color (−a*)) and b* (proportion of yellow (+b*) or blue (−b*)). Sensory analysis of boiled sheep meat products was performed by a group of 6 trained evaluators. A point system of analytical descriptive tests with a scale from 0 to 5 was used for grading, where each grade represents a certain level of quality [19].

2.3 Statistical Analysis of Results

The results of the analyzed samples are shown as the average repetition value ± standard deviation. One-way analysis of variance (ANOVA) and multiple comparisons (Duncan's post-hoc test) were used to estimate significant difference in data at the significance level of $p < 0.05$. Descriptive statistical analysis, the mean, standard deviation (SD) and 95% confidence interval for the estimated mean were calculated for all measurements (statistical program IBM SPSS, Statistics 26, IBM Corp., Armonk, NY, USA).

3 Results and Discussion

Tables 2, 3 and 4 show the results of the physicochemical and sensory properties in four experimental groups sheep meat products. Sheep meat products were obtained by boiling in industrial conditions under different technological regimes (temperature, time) during the heat treatment process.

In the final products obtained by boiling, the highest water content was measured in EG III ($\bar{x} = 60.01 \pm 9.88$), and the lowest EG IV ($\bar{x} = 50.52 \pm 12.05$), the boiling time was 1 h and 47 min, temperature 55–75 °C, relative humidity 82–86%. One-factor analysis of variance of different groups showed that there was no statistically significant ($p > 0.05$) effect of treatment on water content in samples (Table 2).

Table 2. Chemical properties of the tested components in samples of final products from sheep meat obtained by boiling process in different technological conditions

Chemical properties	Experimental group (EG)	Mean ($\bar{x}$)	S. D.	Min.	Max.	Sig.
Water %	EG I	51.19	4.58	45.18	56.31	0.330
	EG II	58.69	6.63	49.58	64.12	
	EG III	60.01	9.88	45.25	65.64	
	EG IV	50.52	12.05	35.03	63.84	
Total ash %	EG I	3.79	0.34	3.28	4.02	0.005
	EG II	3.71	0.68	2.73	4.29	
	EG III	3.68	0.98	2.63	4.82	
	EG IV*	5.57	0.58	5.19	6.44	
Oganc aids %	EG I	0.83	0.10	0.70	0.95	0.002
	EG II	1.06	0.11	0.97	1.20	
	EG III	0.99	0.19	0.73	1.18	
	EG IV*	1.44	0.22	1.25	1.75	
Sdium chloride %	EG I	4.13	0.84	3.49	5.37	0.002
	EG II	3.90	0.40	3.49	4.46	
	EG III	3.48	0.56	2.81	4.18	
	EG IV*	5.86	0.80	5.19	7.04	
Nitrite mg/kg	EG I	14.93	0.75	14.00	15.86	0.000
	EG II	15.86	0.68	15.02	16.70	
	EG III	19.11	0.99	17.89	20.33	
	EG IV*	23.88	0.54	23.21	24.55	
Phnols mg/kg	EG I	267.09	4.84	261.16	273.03	0.616
	EG II	268.81	1.94	266.43	271.19	
	EG III	271.90	0.96	270.72	273.09	
	EG IV	266.49	11.14	252.85	280.14	

Legenda: SD-Standard deviation, Min- Minimum, Max-Maximum, *Average differences significant

The highest mean ash content ($\bar{x} = 5.57 \pm 0.58$) was measured in EG IV, and the lowest ($\bar{x}$=3.68 $\pm$ 0.98) in EG III. One-factor analysis of variance showed that there was a statistically significant difference ($p = 0.05$) in the mean values of ash content between EG IV ($\bar{x} = 4.74 \pm 0.47$) and other groups with statistical significance.

The lowest average value of organic acid content ($\bar{x} = 0.83 \pm 0.10$) was determined in EG I, and the highest ($\bar{x} = 1.44 \pm 0.22$) in EG IV. One-factor analysis of variance revealed a statistically significant difference between EG IV ($\bar{x} = 1.47 \pm 0.22$) and other groups. The highest NaCl content in samples of boiled products was $\bar{x} = 5.8 \pm 0.80$ and was measured in EG IV, while the lowest ($\bar{x} = 3.48 \pm 0.56$) was measured in EG III.

One-factor analysis of variance revealed a statistically significant difference between EG IV ($\bar{x} = 5.86 \pm 0.80$) and other groups.

When determining the nitrite content, the lowest value ($\bar{x} = 14.39 \pm 0.75$) was obtained in EG I, and the highest ($\bar{x} = 23.88 \pm 0.54$) in EG IV, which stood out as statistically significant ($p = 0.05$) in relation to other experimental groups. The highest average values of total phenols content ($\bar{x} = 271.90 \pm 0.96$) in boiled sheep meat products were showed in EG III. One-factor analysis of variance of different groups showed that there was no statistically significant ($p > 0.05$) effect of treatment on phenol content in samples. The results of comparing the mean values of the measured hardness show the highest average value ($\bar{x} = 2.89 \pm 0.16$) in EG IV, and the lowest ($\bar{x} = 1.71 \pm 0.23$) in EG I. There is a statistically significant difference between the mean values between the EG IV group ($\bar{x} = 2.89 \pm 0.16$) compared to all other groups.

Table 3. Physical properties of tested components in final sheep meat product samples

Physical properties	Experimental group (EG)	Mean ($\bar{x}$)	S. D.	Min.	Max.	Sig.
Hardness	EG I	1.71	0.23	1.48	1.94	0.000
	EG II	2.54	0.21	2.24	2.69	
	EG III	2.40	0.12	2.30	2.56	
	EG IV*	2.89	0.16	2.74	3.10	
Lightness L*	EG I*	44.88	3.51	41.47	49.12	0.019
	EG II	38.63	3.04	34.42	41.66	
	EG III	38.76	2.09	36.96	41.79	
	EG IV	39.04	2.02	37.01	41.36	
Color a*	EG I	11.94	1.47	10.25	13.30	0.010
	EG II	12.01	1.20	10.30	12.98	
	EG III	11.89	1.01	10.63	13.12	
	EG IV*	14.85	0.95	13.83	16.14	
Color b*	EG I*	20.55	2.04	18.49	23.30	0.001
	EG II	15.08	1.44	13.20	16.73	
	EG III	15.87	0.86	14.97	16.76	
	EG IV	17.47	0.74	16.52	18.18	
pH value	EG I	6.06	0.00	6.07	6.07	0.146
	EG II	6.11	0.03	6.16	6.15	
	EG III	6.08	0.06	6.19	6.18	
	EG IV	6.04	0.03	6.09	6.09	

Legenda: SD-Standard deviation, Min- Minimum, Max-Maximum, ANOVA- One-factor analysis of variance, * Average differences significant at $p = 0.05$

The highest value of the color light content ($\bar{x} = 44.88 \pm 3.51$) was measured in EG I, and the lowest ($\bar{x} = 38.63 \pm 3.04$) in EG II. One-factor analysis of variance showed a statistically significant difference ($p = 0.05$) in the proportion of color brightness (L*) between EG I and other groups. The highest value of the red color content (a*) ($\bar{x} = 14.85 \pm 0.95$) was measured in EG IV, which stood out as statistically significant in the experimental groups. The highest average value of yellow content (b*) ($\bar{x} = 20.55 \pm 2.04$) was measured in EG I, and the lowest in EG II ($\bar{x} = 15.08 \pm 1.44$).

One-factor analysis of variance revealed a statistically significant difference ($p = 0.05$) in the average values of the yellow color content (b*) between EG I and other groups. The highest pH value in samples of boiled products ($\bar{x} = 6.11 \pm 0.03$) was measured in EG II, and the lowest pH value ($\bar{x} = 6.04 \pm 0.03$) was measured in EG IV. One-factor analysis of variance did not reveal a statistically significant difference in the results of average pH values.

Table 4. Parameters of sensory evaluation descriptive indicators of sheep meat samples obtained by boiling (evaluators O1 to O6)

Statistical parameter		O_1	O_2	O_3	O_4	O_5	O_6
EG I	Mean ($\bar{x}$)	97.66	80.00	65.33	90.66	90.00	74.33
	S.D.	3.299	0.00	9.17	11.14	7.25	10.20
	Min.	100.00	80.00	75.00	100.00	100.00	83.00
	Max.	93.00	80.00	53.00	75.00	83.00	60.00
EG II	Mean ($\bar{x}$)	91.00	50.00	78.66	94.00	90.33	87.33
	S.D.	6.48	0.00	4.64	6.48	5.31	6.12
	Min.	100.00	50.00	85.00	100.00	97.00	95.00
	Max.	85.00	50.00	74.00	85.00	84.00	80.00
EG III	Mean ($\bar{x}$)	96.33	76.66	77.33	95.66	95.3	88.00
	S.D.	2.62	12.47	3.68	6.128	2.35	6.16
	Min.	100.00	90.00	82.00	100.00	97.00	95.00
	Max.	94.00	60.00	73.00	87.00	92.00	80.00
EG IV	Mean ($\bar{x}$)	95.66	83.66	85.33	93.33	96.33	92.33
	S.D.	4.18	16.85	6.23	9.42	0.94	5.55
	Min.	100.00	98.00	92.00	100.00	97.00	100.00
	Max	90.00	60.00	77.00	80.00	95.00	87.00
Sig.		0.00	0.204	0.009	0.036	0.423	0.00

Legenda: EG – experimental group, O(1–6)-Evaluators, SD-Standard deviation, Min-Minimum, Max-Maximum

The sensory properties of the final product related to texture, such as softness on chewing, ease slicing, appropriate hardness and elasticity are very important from the product quality point of view and are directly related to the boiling and smoking process.

Results for sheep meat samples (Table 3) showed that the highest number of evaluators (4 out of 6) gave the highest marks to samples of sheep meat products (EG IV) obtained by the cooking process under the following conditions: cooking time 1 h and 47 min and a boiling temperature of 65–75 °C. Compared to other experimental groups, these were the most intensive processing conditions (Table 1).

The highest water content determined by samples of boiled sheep meat products was in EG III ($\bar{x} = 60.01\%$, SD = 9.88). The obtained values for the proportion of water in boiled meat were slightly lower than the values (66%) found in lamb meat cooked at 80 °C [10]. The presented results indicate a higher concentration of ash in boiled products compared to the values (1.05%) in boiled lamb obtained by Teixeira and coworkers [9].

The presence of organic acids is mentioned in the studies of Marusic et al. [17] who found the concentration of examined ham between 0.6 and 0.9%. The values obtained in our paper (0.11%) were similar to the values presented in this study.

In this paper, slightly higher values of sodium chloride content in samples of boiled and sheep meat products were determined in relation to the data published by Prica et al. [20]. The value of 3.06%, the mentioned authors [20] published for boiled sausages, while in smoked products the value was 3.44%. These results were also higher in relation to the values of salt content published by Kovacevic and coworkers [21].

Further, analyzing the samples after heat treatment by the boiling process, lower values of nitrite content were recorded. Lower values of nitrite content in heat-treated sausages and semi-durable meat products are a consequence of heat treatment [22]. Pleadin and coworkers [23] found higher values of nitrite (44.83 mg/kg) in heat-treated products. Results showed that the amount of added nitrite decreases to 30% of the added value after heat treatment carried out in the experimental part of this paper.

Comparing the values of the obtained results for the light color L* in the final products of sheep meat with the results of research by other authors [9, 22], great agreement can be observed. The lowest average values in boiled sheep meat (38.63) are similar to the values presented by Teixeira and coworkers [9] in sheep meat products (27.83), and the values that Marušić and others [17] found in samples of Istrian ham (31.6–34.7).

During the boiling process, the proportion of red color decreases (a*), because a larger amount of lactic acid is formed, which denatures myoglobin and nitrosylmyoglobin, which can be related to the results obtained in this paper. The maximum values of red (a*) and yellow (b*) in sheep meat were similar to those presented by Teixeira and coworkers [9]. The measured pH values in the boiled products (6.11) are similar to the values presented by Dutra et al. [7].

4 Conclusions

Based on the test results of the physicochemical properties of heat-treated sheep meat under different cooking regimes, it can be concluded that the tested processing procedures (boiling/smoking) had an impact on the product quality parameters. Conditions of higher boiling and smoking temperatures (controlled conditions) resulted with more intensive concentration of dry matter content, increase in total ash content, organic acid and sodium chloride concentrations. In conditions of higher boiling and smoking temperatures there is a decrease in phenol content in finished products samples, although the

values obtained in the specified treatment do not affect the treatment seclusion and have no statistical significance. Tested meat boiling procedures do not affect the pH value of finished products, which means that treatments and values of technological parameters (temperature and time) did not have a significant impact on the pH value of finished products. In terms of hardness testing (cutting force), the tested meat boiling procedures have a positive effect on the measured values in the samples of finished products improving these parameters. In this research examined meat boiling procedures positively affect the values of color parameters L*, a* and b* (CIE - Lab system) in the finished products.

The examined meat boiling procedures affect the sensory evaluation for the overall quality of finished sheep meat products. The highest percentage value of the assessment for the total sensory quality for samples of boiled finished products was for the treatment of EG IV (boiling temperature 55–75 °C, cooking time 107 min).

Treatment in experimental group IV with elevated temperatures (55–75 °C) and prolonged cooking time (107 min) based on the obtained values for physicochemical and sensory properties proved to be optimal and favorable for the technological process of cooking sheep meat.

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Nutritional Advantages of Barley in Human Diet

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Abstract. Cereal grain is one of the oldest components of human diet. Barley was used for preparation of bread until XVI century throughout Europe. The introduction of other cereals led to tremendous decrease of barley usage and cultivation. Due to the rediscovery of high nutritional value recently, there has been a growing interest for use of barley as human food. The high content of soluble dietary fibre present in barley has boosted the status of barley as a food ingredient. Products with new functional and nutritional properties are a precondition for a higher acceptance of barley, for instance as products with a high content of dietary fibre. Soluble fibres form a gel like substance, delay gastric emptying and also retain water. Cellulose is beneficial for digestion efficiency and it also binds to other micronutrients, and toxins such as bile acids. Lignin contains different kind of chemicals, such as ferulic acid, coumaric acid, vanillic acid, vanillin, syringaldehyde and furfural. Hemicelluloses are important components of dietary fibre, which exhibit strong sorptive properties for heavy metals, increasing health benefits and value of food. β-glucan decreases cholesterol levels and has potential cancer-protecting properties and controls blood glucose levels. Human consumption of whole grains (e.g. barley, oat, brown rice, buckwheat, bulgur, millet, maize) has significant role in controlling weight, preventing the risks of gastrointestinal disorders including cancer, vascular and coronary diseases, and type II diabetes.

Keywords: Human diet · Dietary fibre · β-glucan

1 Introduction

Cereal grains (which include wheat, rice, barley, maize, rye, oats and triticale) represent one of the oldest components of human diet. Cereals are grown on 40% of arable land worldwide, providing over 50% of energy and same amount of protein consumed on Earth [1]. Cereals are considered staple food in developing countries, and invaluable source of essential macronutrients and micronutrients. Grain of these cultivated plants is a rich source of minerals, which make up crude ash. The importance of crop production is related to growing areas, yields per hectare and quantities produced [2]. From the second half of XX, and beginning of XXI century crop production has been driven mainly by a significant increase in yields per unit land area [3].

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Barley (Hordeum vulgare L.) is one of the ancient grain crops cultivated and used worldwide [4]. First evidence of barley cultivation dates back to about 10,000 years ago [5], as the crop was first domesticated about 8000 BC. From its centre of origin, barley has been widely spread and cultivated all over the world. Barley crops reached Egypt in 5000 BC, Mesopotamia in 3500 BC, finally reaching Northern and Western Europe in 3000 BC. Hebrews, Greeks, Romans and Europeans until XVI century mainly used barley for bread production. Introduction of other cereals in human nutrition, foremost wheat, followed by rice, and maize resulted in significant decrease of barley use and cultivation [6]. Nevertheless, barley remains one of the most important cereals, as fourth on the list regarding quantity produced and growing areas. Average growing area is about 50 million hectares and the total annual grain production is more than 140 million tons in the world. Due to the richness of genetic diversity, barley is one of the most adaptable cereal crops to different global climate conditions. Barley can be grown where other cereals, such as maize, wheat, and rice cannot be cultivated, reaching arctic and subarctic zones in the North and subtropical regions in the South [7, 8].

A great variability of barley is grown today, with different morphological traits, such as two-rowed and six-rowed genotypes, hulled or hullless genotypes of various colors, including black, blue, purple and yellow. Cereal grains are a rich source of proteins, carbohydrates, fat, phospholipids, vitamins, minerals and other nutrients. Over 70% of seed dry weight is made up of carbohydrates, which makes it the major components of cereals. Major part of carbohydrates is classified as hemicelluloses, followed by sugars and starch. Chemical composition and quality of barley grain depends on cumulative effect of genetic background, i.e. cultivar, weather conditions and agricultural practices [9, 10]. The high content of soluble dietary fibre present in barley has boosted the status of barley as a food ingredient [11]. Besides that, barley has some of the most unique phytochemical properties that usually don't occur in other cereals, such as the presence of all eight tocopherols (α-, β-, γ-, and δ-) and tocotrienols [12]. Functional foods and products with new nutritional properties, such as a high content of dietary fibre are a precondition for an increase of barley use in human diet [13, 14].The aim of this work is to highlight the importance of barley in human nutrition and influence on human health.

2 Barley for Food and Health

The most important uses of barley grain are livestock feed, raw material for alcohol and starch production, and food [15, 16]. Barley produced in Western Balkan countries is mainly used as animal feed [17]. Although barley is not widespread in human diet due to its poor baking quality and taste it has been used for a long time to improve bakery products, especially in flour-based confectionery products due to its nutty taste and aroma [18–21]. High contents of β-glucan are identified as shortage in brewing industry, and low levels of β-glucan in malt are required [22]. Although there is a significant variation in barley quality used as feed, test weight is usually the only quality parameter specified for feed of ruminants [23]. High content of β-glucan in grain is only identified in poultry feed due to the "sticky droppings" phenomenon.

Barley remained a major staple crop in several regions in the world: highlands of Central Asia, Andean and Baltic countries, parts of North Africa and Near East, and the

Horn of Africa. In these regions, leave the poorest farmers, depending on low productive systems and living conditions. In developed countries, barley is used as human food in low quantities. However, in the last two decades popularity of barley increased as the foods containing barley rediscovered. Countries with the highest consumption of barley as food are China (4 million tons), followed by USA (2.9 million tons), Russia, Germany, Morocco, Ethiopia and Saudi Arabia (1 million to 1.3 million tons). China, the country with the biggest consumption of barley as food, most of it is consumed in Tibet (56% of the total food production) feeding about 2.1 million people. Saudi Arabia and Morocco have the highest average consumption (more than 35 kg per capita), followed by Germany and Ethiopia (around 14 kg per capita), UK, USA, and Russia (about 10 kg per capita). India has the lowest average annual consumption (0.7 kg per person) [24].

In Tibet Autonomous Region, roasted barley flour – Tsangpa, major staple foodstuff and chhaang, an alcoholic beverage are both produced from hullless barley. Barley is an ingredient of many traditional dishes in many other countries such as Kasha in Russia and Poland, miso in Japan, and sattu or popped barley in India. After rice, barley is the second most important food crop in Korea. Barley is consumed as pearled grain in soups, ground grain in cooked porridge and flour in flat bread in countries of the West Asia and North Africa. Much less, barley is used in Western countries in breakfast cereals, soups, stews, porridge, bakery blends, and for baby foods [25–27].

Barley flour is becoming more important in the last decade due to its valuable content of bioactive compounds [28]. Barley products used in traditional food preparation can be classified as whole grain, whole roasted grain, cracked grain, raw-grain flour (fine and coarse), roasted grain flour (fine and coarse). Products made of barley grain are used for preparation of bread, pasta, baby food and even rice extenders, showing better cooking properties [29, 30]. Roasted barley can be used as coffee-substitute. Very popular product in Europe is 'Barley coffee'. Caffe d'orzo, barley coffee mixed with milk, is commonly used as a breakfast drink for children in Italy. Flour obtained from sprouted grains retained 87% of the initial β-glucan content and higher levels of ascorbic acid, riboflavin, and phenolics compounds compared to non germinated grains [31, 32].

2.1 Nutritional Value of Barley

Barley is a rich source of dietary phenolic compounds, which can be found free or bounded to fibre. The main compound in the free phenolic fraction of barley grain is flavanols, especially catechin, procyanidins and prodelphinidins. Stored proteins in barley grains belong to two solubility classes – globulins diluted in salt solution and prolamins diluted in alcoholic solution. About 10 to 20% of the total protein content of barley grains consists of globulins [33]. Nutritional quality of barley proteins is moderate; barley is rich in prolamin storage proteins (hordeins) and deficient in lysine. There is correlation between protein content and essential amino acids; higher the protein content, lower the content of essential amino acids, lysine in particular [34].

Recent increase of interest in food containing barley is attributed to the high content of soluble fibre linked to hypocholesterolemia and hypoglycaemia in non-insulin-dependent diabetes [35]. Nutritional value of barley received attention in recent years and initiated interest in including barley in human diet [24]. Whole grain barley is rich in dietary fibre (14.8 g/100 g) and contains satisfactory levels of other biologically active

compounds and minerals such as calcium, iron and zinc [36]. Significantly higher protein content (10–20%) is found in barley compared to maize (9.5%) and wheat (14%) [37]. Protein found in barley contain essential amino acids, and moreover these proteins are characterized by desirable functional properties (such as elasticity, water holding, and emulsifying capacity) resulting in barley grain as an ideal component of food supplements [38, 39]. Barley is an important source of dietary fibre, particularly β-glucan and antioxidant polyphenols [40–42]. Nutrients classified as antioxidants, such as vitamin E, have proven benefit against free radicals, with a major protective role and are invaluable part of human diet. Barley is the only grain crop proved to contain all eight isomers of vitamin E [43, 44]. The content of vitamin E and antioxidant capacity of barley depend on genotype, and thus breeding for higher antioxidants content is crucial for use of barley as functional food [45].

2.2 Dietary Fibre

Cereals are major and excellent sources of dietary fibre (DF) and the content and composition of dietary fibre is directly proportional to the quality of a cereal and cereal products. Crude fibres are a component of dietary fibre. DF consists of numerous components each with different physical and chemical properties and effect on human organism [46]. DF is composed of carbohydrate polymers with ten or more monomeric units, which cannot be hydrolyzed by the endogenous enzymes in the small intestine of humans [47, 48]. Crude fibre consists of cellulose, lignin and partly hemicelluloses. Although high amounts of dietary fibre could decrease absorption of nutrients in intestines, they are essential part of human nutrition as they stimulated normal peristalsis [49].

DF is categorized into soluble and insoluble fibre. This categorisation is based on the digestion of fibres by floral bacteria in the intestine, dissolution capability in water, and other chemical properties such as retention of water [37]. β-glucan, a soluble cell wall polysaccharide (1 → 3), (1 → 4) is an important component characterized by high viscosity in aqueous media. The soluble and insoluble components of DF demonstrate different characteristics depending if they are hydrated, swollen, or catalysed by enzymes, changing their structure and physical properties. Carbohydrate chain of fibre does not dissolve in water, fermentation is limited, and they retain water. Soluble fibre forms are a gel like substances, resulting in delayed gastric emptying and retaining water. Although insoluble and soluble DF has different effects in intestines of human organism, both contain components that have equal effect. An example is uronic acids that reduce postprandial concentration of blood glucose level [50]. Fibres of major cereal crops, wheat, triticale and barley consist mainly of insoluble fibre, while husked oats grain are more abundant in soluble fibre content [37].

Lignin, hemicelluloses and cellulose are neutral detergent fibres (NDF). Insoluble carbohydrate fraction are the most abundant structural components in plant cells. The concentration of NDF and energy concentration in feed are negatively correlated. The NDF content in small grain cereals varies from 11% DM in triticale to 40% DM in husked oat [37]. Husked oat grain has also the highest content of acid detergent fibre, hemicelluloses and acid detergent lignin [37].

Cellulose, similar to other fibres, has beneficial effects on digestion efficiency and binds to other micronutrients, and toxins such as, bile acids. Lignin contains numerous

other chemicals, such as ferulic acid, coumaric acid, vanillic acid, vanillin, syringaldehyde and furfura [51]. Lignins are located in cell walls. Bile to cellulose, and cellulose acts like catalyst, stimulating polyesterification of bile acid, inactivating it and thus reducing the faecal toxicity [52]. Depending on a fraction origin, cellulose and lignin bind heavy metals, but not as efficiently as hemicelluloses. Hemicelluloses are a mixed polysaccharide and prime compound of plant cell walls, accounting up to one-third of the total dry plant biomass [53]. Hemicelluloses present an important component of DF, which pectin demonstrate strong sorptive properties of heavy metals. High hemicelluloses concentration acts beneficially, expanding and absorbing water in human intestine [54, 55].

2.3 β-Glucan Content

The genetic analysis of β-glucan is a precondition for breeding barley with desirable malting properties and low β-glucan content. Germination of barley grain is the main process during malt production, and these analyses must include synthesis of β-glucan and degradation of β-glucan by β-glucanases [56]. Three genes encoding β-glucanases are known, but are not considered important for breeding of barley varieties used as human food since barley products and β-glucan extraction is based on unmalted grain [57]. Regarding β-glucan content in barley grain, it is reported that it is controlled by a simple additive genetic effect.

Pleiotropic effects of the waxy-starch allele at the granule-bound starch synthase locus facilitated breeding of barley for high content of β-glucan in grain [58]. Positive correlations between waxy starch and grain β-glucan content was found [59–61] and majority of barley varieties developed for human consumption are waxy. Another characteristic of waxy-starch varieties is lack of hulls. Hulless varieties have shown to have increased β-glucan content due to elimination of the so-called "diluting" effects of the hull [60]. Hullless varieties are preferred varieties for human food uses as dehulling is not a desirable property. However, hullless varieties have lower yield potential and lower vigour, resulting in value of hullless barley varieties at the market same as for barley used as feed [62].

β-glucan is a major carbohydrate of cell walls of cereal grains. Oats and barley are peculiarly rich in β-glucan continent [63]. β-glucan is a polysaccharide composed entirely of glucose units linked together in polymer chain. Higher β-glucan levels are found in oats compared to barley grains [64]. β-glucan and arabinoxylan are the two major constituents of grain endosperm cell walls in barley, while β-glucan levels are lower in the hull and outer layers of the grain [65]. β-glucan has shown to decrease cholesterol level, some initial studies show cancer-protective properties and controls fluctuation of blood glucose level [66]. The highest β-glucan content (3.9–5.7%) was found in oat grains (Table 1) [37]. Wheat grains, just as in triticale, contain much lower concentration of β-glucan than barley, oat or rye (Table 1). The smallest amount of β-glucan has been found in rice (0.4–0.9% DM). Although high β-glucan content in human food is generally considered desirable, lower β-glucan content in fodder and malting barley is preferable. Consumption of food containing whole grains (e.g. barley, oat, brown rice, buckwheat, bulgur, millet, or maize) has important role in controlling weight [67, 68], preventing the risks of gastrointestinal disorders including cancer [69], vascular and coronary diseases,

and type II diabetes [70, 71]. USFDA (United States Food and Drug Administration) [72] approved claims for some foodstuff containing soluble fibre derived from oats stating beneficial effect on cardiovascular system. EFSA (European Food Safety Authority) [73] scientific panel concluded that scientific evidence supports the following two-part statement: "Oat β-glucan has been shown to lower/reduce blood cholesterol. Lower blood cholesterol may reduce the risk of coronary heart disease". Thus, as evidence suggest, the intake of β-glucan is beneficial for decreasing coronary heart disease risk. Inclusion of β-glucan in the diet (3g β-glucan per day) supports reduction of cardiovascular heart disease risk related to reduce cholesterol levels [37]. Considering the accepted health statements related to effects of β-glucan and LDL-cholesterol levels in blood, presence of a wide range of tocols and phenolic compounds with proven health benefits, it is of special interest to support and develop novel foodstuff containing oat and barley grain or flours [37, 74].

Table 1. β-glucan content (%) in cereal grains [37]

Item	Range
Barley	3.2–4.6
Oat	3.9–5.7
Rye	0.7–1.5
Triticale	0.5–1.0
Rice	0.4–0.9
Wheat	0.5–1.1

3 Conclusion

Cereals are the main source of carbohydrates in the human diet, providing energy and proteins to a lower extent. Whole grains are abundant in vitamins, minerals (zinc, iron, copper, manganese, phosphorus, potassium, calcium and magnesium) and fats. Before wheat and other cereals barley was an important human food crop for many millenia. In recent times small quantity of barley has been used in the food production. The chemical composition of the barley grain provide a possibility for a wider range of barley uses in the production of foods. The positive effect of certain barley components on human health, such as soluble plant fibres beta-glucans, makes barley a crop beneficial for health and provides its place in the functional food production. This paper provides a overview of properties, health benefits, genetic background and recent advances of nutritional advantages of barley in human diet, with particular reference to the importance of β-glucan. β-Glucan is a useful functional ingredient, which can provide numerous health benefits for humans – reduce the risks of colorectal cancer, high serum cholesterol, obesity, non-insulin-dependent diabetes and cardiovascular diseases such as coronary heart disease and hypertension. Breeding programs should focus on the development of new varieties

of high nutritional value, high levels of β-glucan, vitamin E and antioxidants that are beneficial to human health, as well as varieties with improved sensory characteristics suitable for various purposes in the food industry.

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Investigation of the Development Possibilities of a Liquid Sourdough to be Used in the Production of Sourdough Bread

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Abstract. Bread has been one of the basic foods since ancient times. It is considered that the first breads were sourdough breads produced by uncontrolled fermentation with joint activity of lactic acid bacteria and yeasts found naturally in flour and water. Sourdoughs are categorized under 3 main groups as Type I, II and III depending on their production technologies. In this research, it was aimed to develop Type II semi-liquid sourdough, to examine the changes of this sourdough during the storage and to investigate the possibilities of using it in sourdough bread production.

A liquid sourdough was formed by using ripe sourdough prepared from collected sourdoughs from Isparta province. The dry matter and the viscosity of it was determined as 21.75% and 1.38 Pa.s, respectively. After the fermentation for 15 h, it was found that its total titration acidity was 0.75% in terms of lactic acid, the pH value was 3.65, the number of lactic acid bacteria and yeast were 9.31 log cfu/g and 5.72 log cfu/g, respectively. The liquid sourdough, stored in refrigerator in one month, was still suitable for bread production with 6.83 log cfu/g of lactic acid bacteria and 7.35 log cfu/g of yeast. Analytical and sensory properties of this sourdough breads were detected to be quite acceptable.

In conclusion, it has been evaluated that the obtained liquid sourdough can be stored well in +4 °C with the addition of 4% salt, used in producing good quality sourdough bread and it will be more suitable for industrial usage.

Keywords: Type II sourdough · Lactic acid bacteria · Yeast · Sourdough bread

1 Introduction

Bread has been one of the basic foods consumed by humans since ancient times and will continue to be so in the future. A wide variety of breads are produced in the world, as the recipe and technology of bread production can vary according to countries and even regions within the country. It is evaluated that bread variation is sourced from factors such as the economic situation of the community, the traditional grains grown and its suitability for bread making, the importance of bread in traditional nutrition and the culture and lifestyle of the people. It is estimated that the oldest known form of bread is

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flat bread such as Indian chapatti, Mexican tortilla and Middle Eastern pita [1, 2]. It is also considered that the first breads were sourdough breads produced by an uncontrolled fermentation as a result of the joint activity of lactic acid bacteria and yeasts found naturally in flour and water [2, 3].

Sourdough bread, a traditional bread with high nutritional value, good sensory properties and a long shelf life, is produced using sourdough that is fermented dough as a result of the symbiotic relationship of lactic acid bacteria and yeasts found in the natural microflora of flour and water. A wide variation of sourdough breads has been developed according to the natural microflora in cereals, the flavour preferences and production facilities in different countries and also its regions [4]. In the last century, the use of sourdough has decreased as a result of the discovery and widespread use of bakery yeast (*Saccharomyces cerevisiae*) as a starter culture [5].

Sourdough microflora contains a wide variety of lactic acid bacteria and yeasts. In dough produced using sourdough, lactic acid bacteria are responsible for the sour taste while yeasts are responsible for the gas-forming capacity [6–8]. The ratio of lactic acid bacteria and yeast in a good sourdough is accepted as approximately 100:1, while lactic acid bacteria can vary between 1–3 $\times$ 10^9 and yeast 1 $\times$ 10^6–5 $\times$ 10^7 cfu/g [9].

Lactic acid bacteria are a group of bacteria that produce lactic acid by using carbohydrates as homo- and heterofermentative [10, 11]. Yeasts in the microflora of sourdough must be resistant to stressful environmental conditions such as low pH, oxygen restriction, high carbohydrate concentration and the presence of lactic acid bacteria [12].

L. sanfranciscensis, *L. plantarum*, *L. brevis* [7], *L. fermentum*, *L. reuteri*, *L. rossiae*, *L. alimentarius, L. paralimentarius* [13], *L. johnsonii*, *L. acidophilus*, *L. delbrueckii* and *L. lactis* [14] are high number and widely defined lactic acid bacteria in sourdough. The species of *Weissella*, *Pediococcus* and *Leuconostoc* genus are also found in sourdough, although they are not as dominant as the species belonging to the *Lactobacillus* genus [15]. During fermentation, while lactic acid bacteria become dominant, their number can increase to 10^9 and the number of yeast remains at 10^6–10^7 cfu/mL. *Saccharomyces cerevisia* yeast is widely available because it is tolerant of low pH and high osmotic pressure in sourdough yeasts [16]. *S. cerevisiae*, *S. exiguus*, *Pichia norvegensis*, *Candida krusei* and *C. milleri* are the most common yeast species in sourdough [17–19].

It has been reported that in Turkey, sourdoughs are generally produced by using wheat flour and lactic acid bacteria such as *L. brevis*, *L. plantarum*, *L. acidophilus*, *L. sake*, *L. amylophilus* and *L. acetotolerance* that they are widely found in Turkish type sourdough [20].

During sourdough fermentation, exopolysaccharides, organic acids, alcohols, volatile compounds and various enzyme activities are formed as a result of the lactic acid bacteria and yeast activity. These contribute to the increase of dietary fibre content, the improvement of the textural and sensory properties, the extension of the shelf life, and the breakdown of phytic acid which reduces the bioavailability of minerals in sourdough bread, respectively [9, 21–23]. The aroma of sourdough originating from the diversity of its microflora is one of the most important sensory properties of bread and also plays an important role in consumer preference [18, 24].

Today, with the developing technology, the sourdough technique has also been developed and has been applied in various ways according to the needs. Sourdoughs are categorized under 3 main groups as Type I, II and III depending on their production technologies [25].

Traditionally, the first sourdough is obtained by resting the dough, which is formed by kneading of flour and water by hand, in warm conditions (27–32 °C).

During this resting, the dough is **spontaneously fermented** by microorganisms in the natural microflora of flour and water, especially as a result of the symbiotic activities of lactic acid bacteria and yeasts, and turned into sourdough.

A piece of this sourdough is taken and is used to inoculate the next dough, which is formed by using about twice of the flour and water. Back-sloping inoculation is repeated 5–10 times after every 6–24-h fermentation to ensure good adaptation of microorganisms to the dough ecosystem and natural selection of sourdough strains.

Thus, the first sourdough is being produced and then sustained by daily using. This first sourdough is also known as Type 0, mother, sponge, starter, head, ferment and yeast [12].

Type I sourdough is separated dough from the previous sourdough bread production for using as yeast in the next sourdough bread production. This sourdough is used in ratio of 10–25% of the dough as yeast in this technic and it is mostly preferred by small enterprises.

Type II sourdough is semi-liquid dough prepared with high dough yield and it is mostly used in industrial enterprises because of easily being processed.

Type III sourdough is powdered sourdough from Type II with various drying and milling techniques. This sourdough is mostly used in increasing acidity in bread production and situations where it is difficult to store yeast [26].

Unlike the last century todays, due to the developing in the awareness of healthy nutrition of people, the consumption of sourdough bread and consequently the industrial use of Type II semi-liquid sourdough has increased because of its advantages. The semi-liquid sourdough has been used not only in the production of sourdough bread but also in the production of other cereal products such as pizza, cakes and crackers [9]. Industrially, the use of semi-liquid sourdough provides easy control of fermentation conditions, obtaining late staled bread with uniform and fine pores, reduced production costs, and less labour, area and time usage [4, 5, 27]. In addition, it is considered that the widespread usage of semi-liquid sourdough in industry is due to its ease of use such as being easily transported through pipes, suitable for doping and not forming dust [4, 28].

Many types of lactic acid bacteria have been isolated in Type II sourdough. Among these, the most common obligatory homofermentative species are *L. acidophilus*, *L. johnsonii*, *L. farciminis*, *L. delbrueckii* and *L. amylovorus*; heterofermentative species are *L. fermentum*, *L. sanfranciscensis* and *L. reuteri*. In addition, *L. brevis*, *L. pontis*, *L. panis*, *L.* frumenti and *Weissella spp.* have also been found in Type II sourdough [6, 15, 29–32].

While producing Type II semi-liquid sourdough, 10% to 70% of the total flour that will be used in bread making can be used. During the production, the pH value of the environment in the first 24 h of fermentation can rapidly decrease to 3.5 as a result of the activity of lactic acid bacteria and thus, the metabolic activities of microorganisms slow down [33]. Since this situation causes negative effects especially on yeasts, it might require the addition of commercial baker's yeast to the dough in the production of sourdough bread using semi-liquid sourdough [16]. Microorganism activities in the produced semi-liquid sourdough are stabilized by slowing down with applying one or more of the processes such as cooling, pasteurization and salt addition, and the semi-liquid sourdough stabilized in this way is used by storing it in the cold.

In this research, it was aimed to develop Type II semi-liquid sourdough from Type I sourdough which is a natural source of microflora, to examine the changes of this sourdough during the shelf life and to investigate the possibilities of using it in sourdough bread production.

2 Material and Methods

2.1 Material

In the research, 5 sourdough samples were collected under aseptic conditions from 5 well-known bakeries in Isparta province of Turkey, placed in sterile sample transport containers, brought to the laboratory in the cold chain on the same day and placed in the refrigerator. Wheat flour with the extraction yield of 85%, water, salt and fresh yeast used in sourdough and bread production were obtained from the market in accordance with the relevant communiques of the Turkish Food Codex. Chemical and microbiological substances were used in analytical grade in the analyses.

2.2 Methods

Preparation and Storage of Semi-liquid Sourdough (Type II)

Each collected sourdough sample was mixed with equal amounts of flour and water, and back-slopped at 30 °C every day throughout 3 days for microflora activation.

A representative sourdough was formed by taking equal amounts (5 × 10 g) of 5 different activated sourdough and combining them with 50 g of flour and 50 mL of distilled water. This representative sourdough was back-slopped at 30 °C for 7 days with equal amounts of sourdough, flour and water, every 24 h in the first 3 days and every 12 h in the last 4 days, and thus ripe sourdough (Type I) was prepared.

To prepare semi-liquid sourdough with a dough yield of 400, 96 g of the ripe sourdough was taken and mixed with 112 g of flour and 432 mL of water. This mixture was fermented at 30 °C for 15 h and thus a liquid sourdough (Type II) was obtained. After adding 4% of salt to this semi-liquid sourdough, it was stored in refrigerator conditions (+4 °C) for a month.

Processes were followed by taking samples every 3 h during the fermentation of semi-liquid sourdough and every week during the storage. Every sample was analysed for determining the total titratable acidity, pH value, total lactic acid bacteria and yeast number.

Sourdough Bread Production by Using Semi-liquid Sourdough

By using a freshly prepared liquid sourdough, 4 different liquid sourdough ratios of 0, 10, 20 and 30%; and 4 different final dough fermentations as 45, 90, 135 and 180 min were used to produce sourdough bread in 4 × 4 factorial design. In the bread formulation; 2% of commercial yeast, 1.5% of salt, 65% of water and liquid sourdough, the amount of which is determined in the experiment design, were used based on 100 g of flour. In the production of bread, the calculation of other components was made using 700 g of flour to obtain 15 roll breads. In the calculation of the water and salt ratio, the amount of water and salt that would come from the liquid sourdough was also considered.

The dough components were weighed in the required amount and mixed in the mixing bowl. Then, the liquid sourdough and 40 °C of water was added on the mix. Kneading took place in a dough maker (Kitchen Aid, USA) for 10 min at 120 rpm. For the mass fermentation of the kneaded dough, it was covered with a damp cloth and kept at 25 °C for 30 min. After the mass fermentation stage, the dough was partitioned as equal pieces and rolled. These rolled dough pieces were placed on a tray, covered with a damp cloth and left for intermediate fermentation for 1 h. After the intermediate fermentation, degassing was done in the rolled dough pieces and left to the final fermentation for a period in accordance with the experimental design. The fermented doughs were baked at 225 °C for 24 min.

Statistical Methods

The research was carried out in two replicates and the analyses were performed in parallel. All statistical calculations were conducted with the SPSS statistical program. The significance was evaluated with variance analysis and Duncan multiple comparison test was applied to the factors. The results were arranged in tables as mean ± standard error.

Analysis of Sourdough and Semi-liquid Sourdough

Dry Matter

Dry matter contents of sourdough samples were determined by keeping the sample in an oven at 105 °C until they reach a constant weight [34].

PH Value and Total Titratable Acidity

After 10 g of sample was homogenized in 90 mL of distilled water, the pH value was measured using a pH meter (HI 2210, Hanna, Leighton Buzzard, UK) [17].

Total titratable acidity (TTA) was performed by using supernatant used in pH analysis. It was titrated with 0.1 N NaOH solution in the presence of phenolphthalein indicator until the colour change occurring. TTA was calculated as %lactic acid on the used volume of base solution [34].

Viscosity
The viscosity analysis of the liquid sourdough was carried out using falling spheres according to Stokes' law and the viscosity was calculated as *Pa.s* with the following equation. In the equation, μ is the viscosity, r is the radius of the sphere, ρ_s is the density of the sphere, ρ_{sd} is the density of sourdough, g is the acceleration of gravity and v is terminal velocity of the sphere.

$$\mu = \left[(2/9) \times r^2 \times (\rho_s - \rho_{sd}) \times g)/v\right]$$

Microbiological Analysis

Sample Preparation
After 10 g sample was weighed into 90 mL Ringer's solution (1/4), it was homogenized, and the 10^{-1} dilution was formed. Serial dilutions were prepared in test tubes containing 9 mL of sterile Ringer's solution by taking 1 mL of the previous dilution, and these serial dilutions were used in microbiological analysis [35].

Yeast Enumeration
To determine the number of total yeasts, 0.1 mL of the dilutions were spread on the PDA agar which contains 50 μg/L oxytetracycline against the bacterial growth, the petri dishes were incubated at 25 °C for 5 days, and the colonies were counted [35, 36].

Lactic Acid Bacteria Enumeration
To determine the number of total lactic acid bacteria, 0.1 mL of the dilutions were spread on the MRS agar which contains 50 μg/L sikloheksimit against the yeast growth. Petri dishes were incubated for 3 days at 30 °C in anaerobic desiccator containing oxygen holding kits (Anaerocult Darmstadt, Germany) and colonies were counted [37, 38].

Analysis of Sourdough Bread

Specific Volume
The weight and volume of the breads were measured 2 h later after baking. Bread volume was detected according to the principle of replacement with rapeseed and the weight of bread was determined with a scale. The specific volume of bread samples was calculated by dividing the volume value to the weight of bread and expressed as cm^3/g [34].

Colour Analyses
The colour analysis of breads was carried out 2 h later after bread production. A bread slice (3 cm) removed from the middle of the bread and the crust and crumb colour of the bread was measured with a colour determination device (Chroma meter CR-400 Conica Minolta, Japan) on these slices as L^*, a^* and b^* values [34].

Textural Hardness Analyses
The hardness analysis of breads for tracing staling was performed 2, 24, 48 and 72 h later after bread production. A bread slice (3 cm) removed from the middle of the bread and it was used measuring of hardness. The analysis was performed using a texture analyser device (TPA, TA Plus, England) and a 50 mm diameter cylinder probe, 1.7 m/s test speed, 40% compression ratio and 10 g trigger force [39].

Sensorial Analyses
Sensorial analysis of bread was carried out 2 h later after bread production by graduate students of the food engineering department trained in sensory analysis. The odour, volume, crust color, crumb color, pore structure, sourness and overall characteristics of sourdough breads were evaluated respect to 5 point hedonic scale.

3 Results and Discussion

3.1 Collected Sourdough Samples

Chemical and microbiological analyses results of 5 sourdough samples collected from various regions and bakeries of Isparta province are given in Table 1. When the analyses results were evaluated as average, it was determined that dry matter, TTA, pH value, total lactic acid bacteria and yeast number were 52.63%, 0.95%, 3.68, 3.76 log cfu/g and 4.14 log cfu/g, respectively.

As reported in the sourdough literature, the dry matter value, TTA, pH value, total number of lactic acid bacteria and the number of yeast can have been 53.21–57.33% [40], 0.93–1.48%, 3.8–4.9 [41], 9.10 log cfu/g and 5.25 log cfu/g [42], respectively. It was evaluated that the research findings of collected sourdough samples were compatible with the literature.

Table 1. Some chemical and microbiological properties of the collected sourdough samples

Sourdough sample	Dry matter (%)	TTA (%Lactic acid)	pH	Lactic acid bacteria (log cfu/g)	Yeast (log cfu/g)
1	54.32	1.26	3.67	2.81	4.43
2	45.08	0.76	3.74	3.70	4.32
3	51.91	0.90	3.52	3.90	4.14
4	56.68	0.90	3.65	4.24	3.95
5	55.14	0.95	3.81	4.15	3.86
Mean	52.63	0.95	3.68	3.76	4.14

3.2 Ripe Sourdough Samples (Type I)

The chemical and microbiological analyses results of the ripe sourdough sample obtained by mixing and ripening the first collected sourdough samples in equal amounts are given in Table 2. The chemical and microbiological status of the obtained ripe sourdough sample, especially the ratio of lactic acid bacteria and yeast numbers of approximately 100:1, was considered as an indicator of the success of the ripening process of sourdough. In a survey research conducted by Yağmur [43] on sourdoughs collected from different regions of Turkey, it has been reported that the pH value was between 3.77–5.44, the lactic acid bacteria number was between 6.71–9.16 log cfu/g and the yeast number was between 5.27–8.08 log cfu/g of the samples. In the same research, pH, lactic acid bacteria and yeast number for sourdough of Isparta province were reported as 4.04, 7.85 log cfu/g and 6.68 log cfu/g, respectively.

Table 2. Some chemical and microbiological properties of the ripe sourdough (n = 2)

Sourdough sample	Dry matter (%)	TTA (%Lactic acid)	pH	Lactic acid bacteria (log cfu/g)	Yeast (log cfu/g)
Ripe sourdough	46.21	1.07	3.77	8.46	6.53

3.3 Semi-liquid Sourdough (Type II)

The changes in acidity and microbiological properties of semi-liquid sourdough produced using ripe sourdough are given in Table 3. According to the results of variance analysis, it was determined that the fermentation time had a statistically significant ($p < 0.01$) effect on the pH, TTA and microorganism numbers of the fermented semi-liquid sourdough. It was determined that TTA value increased from 0.31% to 0.75% due to organic acids formed during 15 h of fermentation, and as a result, the pH value significantly decreased from the value of 4.74 to 3.65. Lactic acid bacteria number increased approximately 1.3 log cfu/g unit and the yeast number decreased approximately 2.1 log cfu/g unit during fermentation. It was evaluated that the lactic acid bacteria could only increase 1.3 log cfu/g unit because of competing with each other during the fermentation period and the low pH value of the environment, while the number of yeast decrease 2.1 log cfu/g unit because of being affected negatively by the high acidity and low pH conditions created by the lactic acid bacteria.

In a research, the lactic acid bacteria number of sourdough produced with various starter cultures was reported to ranged from 7.9 to 9.6 log cfu/g [44]. In a research formed the liquid sourdough with a dough yield of 400, it was stated that the pH value decreased from 5.98 to 3.12 [43].

After 15 h fermentation of semi-liquid sourdough produced using ripe sourdough, it was determined that the viscosity was 1.38 *Pa.s* and the dry matter content was 21.75%. In a study conducted by Wehrle, Arendt [45], it was reported that the dry matter content of liquid sourdough ranged from 19.27% to 32.94% and the viscosity value was 70 *Pa.s* at 1 Hz.

Table 3. Some chemical and microbiological properties of the semi-liquid sourdough

Fermentation time (h)	TTA (%Lactic acid)	pH value	Lactic acid bacteria (log cfu/g)	Yeast (log cfu/g)
0	$0.31^{e} \pm 0.02$	$4.74^{a} \pm 0.10$	$8.04^{d} \pm 0.09$	$7.84^{a} \pm 0.23$
3	$0.39^{d} \pm 0.01$	$4.45^{b} \pm 0.06$	$8.35^{c} \pm 0.07$	$7.37^{b} \pm 0.06$
6	$0.48^{c} \pm 0.02$	$4.01^{c} \pm 0.03$	$8.41^{c} \pm 0.04$	$6.86^{c} \pm 0.85$
9	$0.63^{b} \pm 0.01$	$3.83^{d} \pm 0.03$	$8.57^{c} \pm 0.02$	$6.52^{d} \pm 0.04$
12	$0.63^{b} \pm 0.02$	$3.71^{d} \pm 0.04$	$8.82^{b} \pm 0.03$	$5.92^{e} \pm 0.02$
15	$0.75^{a} \pm 0.02$	$3.65^{d} \pm 0.00$	$9.31^{a} \pm 0.11$	$5.72^{e} \pm 0.05$
Sign., n = 2	**	**	**	**

3.4 Storage of Semi-liquid Sourdough

The acidity and microbiological properties of the semi-liquid sourdough which is stored in refrigerator conditions (4 °C) for 1 month are given in Table 4.

It was determined that the storage time had a statistically significant effect on pH value ($p < 0.05$) and TTA ($p < 0.01$), lactic acid bacteria and yeast number ($p < 0.01$). TTA increased by 0.66% and as a result, the pH value decreased from 4.52 to 3.57 in the semi-liquid sourdough during 1-month storage in refrigerator. It was evaluated that this situation was sourced from the organic acids produced by the lactic acid bacteria even under hard conditions such as low temperature and high saltness. The lactic acid bacteria and yeast numbers decreased 2.3 and 3.1 log cfu/g unit with storage, respectively. These decreases sourced from the stopping of microorganism growth, and also the death of them due to high acid and salty environment.

Table 4. Some chemical and microbiological properties of the semi-liquid sourdough stored in refrigerator

Storage time (week)	TTA (%Lactic acid)	pH	Lactic acid bacteria (log cfu/g)	Yeast (log cfu/g)
0	$0.40^{d} \pm 0.18$	$4.52^{a} \pm 0.19$	$9.12^{a} \pm 0.00$	$7.35^{a} \pm 0.02$
1	$0.68^{c} \pm 0.22$	$3.68^{b} \pm 0.01$	$8.73^{ab} \pm 0.22$	$7.23^{a} \pm 0.00$
2	$0.81^{bc} \pm 0.07$	$3.73^{b} \pm 0.00$	$8.22^{ab} \pm 0.25$	$6.16^{b} \pm 0.02$
3	$0.94^{ab} \pm 0.18$	$3.68^{b} \pm 0.00$	$7.98^{b} \pm 0.48$	$5.17^{c} \pm 0.02$
4	$1.06^{a} \pm 0.01$	$3.57^{b} \pm 0.04$	$6.83^{c} \pm 0.01$	$4.13^{d} \pm 0.13$
Sign., n = 2	**	*	**	**

3.5 The Effect of Semi-liquid Sourdough Ratio and Dough Fermentation Time on Physical Properties of Sourdough Breads

Some physical properties of sourdough breads produced using different semi-liquid sourdough ratio and different final dough fermentation time are given in Table 5.

According to the results of variance analysis; specific volume values of sourdough breads were not significantly affected by the semi-liquid sourdough ratio ($p > 0.05$) but were affected by the fermentation time ($p < 0.05$). Although the semi-liquid sourdough ratio added in an increasing rate does not have a statistically significant effect on the specific volume values of breads, it has been determined that the specific volume values of the breads have increased descriptively from 2.58 to 2.97 cm^3/g with the increasing sourdough ratio. It was evaluated that it was caused by the expansion of volatile metabolites produced by microorganisms on baking. It was determined that the specific volume of the breads decreased from 3.06 to 2.37 cm^3/g as the final dough fermentation time increased from 45 to 180 min. This decrease might have been sourced from the gas formed during the prolonged fermentation could not be kept in the dough due to the weakening of gluten by fermentation.

In a research, the specific volume of sourdough bread produced using various starter cultures were measured and it was stated as between 3.4 and 4.0 cm^3/g [44]. In another research, sourdough bread was produced with the fermentation of various microorganisms at 30 and 40 °C for 16 h and the average specific volume of these breads was reported to be 2.14 cm^3/g [46].

The semi-liquid sourdough ratio did not affect any colour value ($p > 0.05$) of the breads statistically and the fermentation time only significantly affected ($p < 0.05$) the a^* value of crust colour. It was evaluated that as a result of the increasing usage of free glucose with the extended period of dough fermentation, a lower level of Maillard reaction occurred on baking and thus the a^* value, which is an indicator of redness, decreased from 17.21 to 13.92 in the crust. It was determined that the average L^*, a^* and b^* colour values of the produced sourdough breads were 69.16, 15.04 and 35.90 in the crust and 71.18, 2.53 and 15.73 in the crumb, respectively. In a research, the sourdough bread was produced by using liquid sourdough with 400 dough yield and the average L^*, a^* and b^* colour values of the sourdough breads were 57.44, 6.95 and 18.05 in the crust, and 69.74, −0.69 and 10.83 in the crumb, respectively [43].

3.6 The Effect of Semi-liquid Sourdough Ratio, Dough Fermentation Time and Bread Storage Time on Textural Hardness Value of Sourdough Breads

Textural hardness value and its temporal changes of sourdough breads produced using different semi-liquid sourdough ratio and different final dough fermentation time are given in Table 6.

Table 5. Some physical properties of sourdough breads produced using different semi-liquid sourdough ratio and different final dough fermentation time

Sourdough ratio (%)	Specific volume (g/cm^3)	Colour, ΔE	
		Crust	Crumb
0	$2.58^a \pm 0.17$	$45.51^a \pm 1.59$	$29.04^a \pm 1.24$
10	$2.88^a \pm 0.17$	$46.312^a \pm 0.73$	$27.45^a \pm 1.43$
20	$2.70^a \pm 0.12$	$46.56^a \pm 0.57$	$27.67^a \pm 1.76$
30	$2.97^a \pm 0.09$	$43.51^a \pm 0.48$	$27.55^a \pm 1.22$
Sign., n = 8	–	–	–
Fermentation time (min.)			
45	$3.06^a \pm 0.13$	$46.78^a \pm 0.65$	$27.25^a \pm 1.07$
90	$2.87^a \pm 0.09$	$44.76^a \pm 0.66$	$27.75^a \pm 1.13$
135	$2.82^a \pm 0.12$	$44.44^a \pm 1.46$	$29.00^a \pm 1.86$
180	$2.37^b \pm 0.12$	$45.50^a \pm 1.03$	$27.72^a \pm 1.53$
Sign., n = 8	*	–	–

It was determined that semi-liquid sourdough ratio ($p < 0.01$), dough fermentation time ($p < 0.05$) and bread storage time ($p < 0.01$) had a significant effect on the textural hardness value of bread samples. When force is applied on bread, the force measured at the point where the breaking first occurs is defined as hardness. It has been evaluated that the increased fermentation time decreases the hardness due to the thinning in the crust of bread caused by the fermentation, and the increasing semi-liquid sourdough ratio also affects the hardness of the bread by reducing the pH value of the dough and accelerating the starch retrogradation in the breads. When the hardness of the produced breads was measured at the 2^{nd}, 24^{th}, 48^{th} and 72^{nd} h after baking, the values of 642.70, 1320.73, 1655.08 and 1875.88 g were determined, respectively. It was evaluated that the increase in the hardness of breads due to the aging time was mainly due to the staling that occurred because of starch retrogradation.

In a research, it was reported that the hardness value of sourdough breads produced using various starter cultures was between 2113–5765 g (20.73–56.54 N) [47]. In another research, the hardness value of sourdough breads was determined to be between 2381 and 5621 g [48]. Crowley et al. [49] stated that the bread hardness decreased with the increase of sourdough ratio and aging time, and the hardness values were 17832–13373, 16807–12819, 12444–9809 and 9739–9220 g, respectively.

Table 6. Textural hardness value and its temporal changes of sourdough breads produced using different semi-liquid sourdough ratio and different final dough fermentation time

Sourdough ratio (%)	Hardness (g)
0	$1145.83^{c} \pm 71.57$
10	$1190.75^{c} \pm 98.41$
20	$1422.36^{b} \pm 125.42$
30	$1743.45^{a} \pm 160.25$
Sign., n = 32	**
Fermentation time (min.)	
45	$1520.77^{a} \pm 118.75$
90	$1181.04^{b} \pm 89.86$
135	$1475.50^{a} \pm 160.20$
180	$1317.08^{ab} \pm 114.46$
Sign., n = 32	*
Storage time (min.)	
2	$642.70^{c} \pm 31.26$
24	$1320.73^{b} \pm 62.68$
48	$1655.08^{a} \pm 88.59$
72	$1875.88^{a} \pm 149.99$
Sign., n = 32	**

3.7 Sensory Properties of Sourdough Breads

Sensory properties of sourdough breads produced using different semi-liquid sourdough ratio and different final dough fermentation times are given in Table 7. Sensory properties of sourdough breads were not significantly affected by the semi-liquid sourdough ratio and dough fermentation time ($p > 0.05$). It was determined that the sensory properties of sourdough breads were evaluated as average 3.95 for volume, 3.87 for crust colour, 4.12 for crumb colour, 4.10 for porosity, 3.58 for sourness, 4.06 for odour, and 3.89 points for general taste.

In a research, it was reported that after the sourdough with 200 dough yield was fermented for 3 h at 26 °C, this sourdough was used the production of bread and the average sensory values were determined as 2.97 for colour, 1.41 for odour and 0.96 for sourness according to the 5 point hedonic scale [50].

Table 7. Sensory properties of sourdough breads produced using different semi-liquid sourdough ratio and different final dough fermentation time

Sourdough ratio (%)	Volume	Crust colour	Crumb colour	Porosity	Sourness	Odour	Overall
0	$4.06^{a} \pm 0.11$	$3.95^{a} \pm 0.16$	$4.21^{a} \pm 0.09$	$4.03^{a} \pm 0.08$	$3.51^{a} \pm 0.19$	$3.51^{a} \pm 0.06$	$3.75^{a} \pm 0.11$
10	$3.72^{a} \pm 0.24$	$4.00^{a} \pm 0.10$	$4.18^{a} \pm 0.14$	$4.17^{a} \pm 0.11$	$3.75^{a} \pm 0.17$	$3.75^{a} \pm 0.10$	$4.08^{a} \pm 0.19$
20	$4.02^{a} \pm 0.26$	$3.97^{a} \pm 0.18$	$4.13^{a} \pm 0.14$	$4.20^{a} \pm 0.13$	$3.37^{a} \pm 0.11$	$3.37^{a} \pm 0.11$	$3.85^{a} \pm 0.12$
30	$4.00^{a} \pm 0.15$	$3.56^{a} \pm 0.22$	$3.96^{a} \pm 0.10$	$4.00^{a} \pm 0.13$	$3.72^{a} \pm 0.14$	$3.72^{a} \pm 0.10$	$3.90^{a} \pm 0.18$
Sign., n = 8	–	–	–	–	–	–	–
Fermentation time (min.)							
45	$3.77^{a} \pm 0.30$	$3.85^{a} \pm 0.17$	$4.13^{a} \pm 0.16$	$4.16^{a} \pm 0.09$	$3.50^{a} \pm 0.14$	$3.50^{a} \pm 0.12$	$4.08^{a} \pm 0.16$
90	$4.11^{a} \pm 0.10$	$3.96^{a} \pm 0.12$	$4.22^{a} \pm 0.08$	$3.92^{a} \pm 0.09$	$3.72^{a} \pm 0.13$	$3.72^{a} \pm 0.07$	$3.88^{a} \pm 0.10$
135	$3.96^{a} \pm 0.17$	$3.75^{a} \pm 0.26$	$4.02^{a} \pm 0.11$	$4.15^{a} \pm 0.15$	$3.68^{a} \pm 0.15$	$3.68^{a} \pm 0.11$	$3.60^{a} \pm 0.17$
180	$3.96^{a} \pm 0.17$	$3.92^{a} \pm 0.13$	$4.11^{a} \pm 0.13$	$4.17^{a} \pm 0.11$	$3.45^{a} \pm 0.21$	$3.45^{a} \pm 0.10$	$4.01^{a} \pm 0.13$
Sign., n = 8	–	–	–	–	–	–	–

4 Conclusion

In the research, the possibilities of forming liquid sourdough from a natural microflora source, storing this liquid sourdough and using it for bread production were investigated. A ripe sourdough with a ratio of 100:1 lactic acid bacteria and yeast number was able to formed by using collected sourdoughs from Isparta province.

A liquid sourdough with a dry matter of 21.75% and a viscosity of 1.38 *Pa*.s was produced using this ripe sourdough. At the end of the fermentation of this liquid sourdough for 15 h, it was determined that the total titration acidity in terms of lactic acid was 0.75%, the pH value was 3.65, the number of lactic acid bacteria was 9.31 log cfu/g and the number of yeast was 5.72 log cfu/g.

It was determined that this liquid sourdough, which was stored for one month under refrigerator conditions after the fermentation was stopped with the addition of 4% salt, was still acceptable as a suitable sourdough for sourdough bread production with 6.83 log cfu/g of lactic acid bacteria and 7.35 log cfu/g of yeast. Analytical and sensory properties of sourdough breads produced with this liquid sourdough were found to be quite acceptable.

As a result, it has been concluded that a liquid sourdough can be developed from ripe sourdough that it can be stored well in the refrigerator with the addition of 4% salt, used in producing analytically and sensorially good quality sourdough bread, and it was evaluated that this liquid sourdough will be more suitable for industrial usage.

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Analysis of the State and Possibilities of Development of Geographical Indications for Herzegovina Cheese in Skin Sack

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Abstract. Although it cannot be compared to the most developed European cheese-making countries, cheese-making in Bosnia and Herzegovina has a long tradition. One of the most famous indigenous cheeses is *Herzegovina cheese in skin sack*, where the first data go back to the distant past, some 650 years ago, which by "age" places it with the known types of cheese. The aim of this paper is to determine the extent to which *Herzegovina cheese in skin sack* has special properties derived from the value of its ingredients, method of production and processing, climate from which it comes, and whether it meets the prescribed standards for registration of designations of origin or geographical origin.

The subject of the research were agricultural farms, but also legal entities - dairies engaged in the production of *Herzegovina cheese in skin sack* in the traditional way. The nutrition of animals, the geographical origin of raw materials that participate in the production process, the technological process of production, microbiological and physico-chemical tests of raw milk and finished products were monitored. The analysis of the results obtained in these tests concluded that *Herzegovina cheese in skin sack* has a recognizable, unique quality and uniqueness that is closely related to the geographical area from which it originates and retains the traditional method of production passed down for generations in that geographical area. Also, conclusions were reached regarding the fulfillment of the conditions of *Herzegovina cheese in skin sack* in terms of registration of these quality labels.

Keywords: Cheese in skin sack · Geographical indications (GI)

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1 Introduction

In the international market, there is a great competition in the placement of food products. In this struggle for competitiveness, indigenous products are increasingly valued, i.e. those whose special characteristics derive from the value of their ingredients, the method of production and processing, and the climate from which they come. Indigenous products also help promote the national gastronomic and tourist-catering offers.

The territory of Bosnia and Herzegovina is known for its specific food products or traditional gastronomic specialties. Indigenous food products in many ways "tell" the story of a rich tradition, experience, special life and customs of many generations. Given that the production of food products with designations of origin and geographical indications has come to life in the European Union and that products with these characteristics are welcome on the tables in Europe, the question arises as to the chances of reviving and protecting the production of various food products in Bosnia and Herzegovina. Products that are specific to certain areas.

Although it cannot be compared to the most developed European cheese-making countries, cheese-making in Bosnia and Herzegovina has a long tradition. One of the most famous indigenous cheeses is *Herzegovina cheese in skin sack*, where the first data date back to the distant past, some 650 years ago, which, according to its "age", classifies it as a well-known type of cheese. The oldest data date back to 1379, and they say that, "if the local population does not bring meat and cheese to Dubrovnik, there is neither meat nor cheese in Dubrovnik." The same source provides data (Filipović-Fabijanić 1977; Sarić et al. 2007; Radić 2008) that until the end of the 16th century, Dubrovnik was given cattle in unlimited quantities from Herzegovina, including cheese in bags in quantities of up to 800 kg. The oldest Vlach law in the Turkish text in the defter for the Braničevo subasluk from 1487/8. on the giving of one katun to the sandžak-beg (one tent, one cheese, three ropes, six ulars, *cheese in sack* and one ram) shows that it must have been a gift from the katun, that is, from the elder of the katun (Bijeljac and Sarić 2005). According to the same, it can be seen that in the Middle Ages, the Vlach katuns played a significant role, where sheep were mostly raised, because the best natural conditions existed for them. Therefore, it is not surprising that cheese is significantly present in traditional dairy products, which is prepared in sheep's skin and delivered to Herzegovinian squares or Dalmatian towns on donkeys. Filipović-Fabijanić (1977) states that *Sir iz miha* (Rakitno) i *Sir iz mješine* (Površ) produced not only for domestic use but also for the market. It was taken from Rakitno for sale to Imotski and Posušje, and from Površa to Trebinje and Dubrovnik. Cheese in skin sack is traditionally produced from whole or skimmed cow's, sheep's and goat's milk, or a mixture of cow's and sheep's and cow's and goat's milk (Bijeljac 2004). There are basically two basic technologies. One involves whole milk as a base, curdling with rennet, processing of pears, and ripening in bags (bellows). The second type is cheese that is obtained after milk collection, i.e. the production of sour cream or cream (the latter is a more common case), where acid coagulation occurs, followed by processing of pears and ripening in skins (Sarić 2009). In addition to the type of milk and the method of production, the content of individual ingredients varies a lot depending on the breed, diet, climatic conditions, manner of keeping and animal health (Brenjo et al. 2012).

2 Objective and Methods of Work

The survey, conducted during 2019 and 2020, sought to determine how well a *Herzegovinian cheese in sack* qualifies for registration with a designation of origin or geographical indication.

The production of *Herzegovinian cheese in sack* in traditional production on farms and industrial production in dairies was carried out. The feeding of the animals was tested, from which milk is produced for the production of this cheese. The method used was a non-standard questionnaire, drawn up on the basis of a preliminary analysis of the area under consideration, the review of existing studies and the reports on scientific research (Brenjo et al. 2012; Sarić 2009; Filipović-Fabijanić 1977).

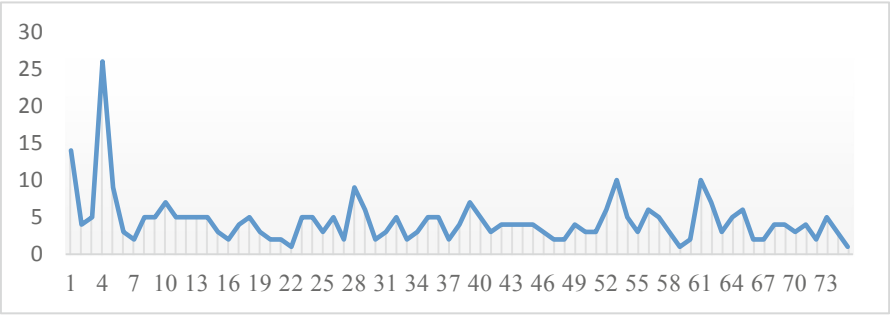
The task was to examine raw materials entering the production process and taking their samples from the producers (individuals and legal entities) engaged in the production of Herzegovina cheese from animal skin sack in a traditional manner. The basic raw materials entering the production of this cheese are: milk, starter cultures of selected micro-organisms, table salt, rennet, and lysozyme and calcium chloride in industrial production. The analyses were conducted following accredited methods according to the standards BAS EN ISO/IEC 17025: 2006 for conducting tests on physical, chemical and microbiological characteristics of milk and milk products. When determining the relationship of the quantity of milk fat and protein as well as casein and milk fats, the calculation for milk fat and protein was used while in the case of casein ratio (80% - the average value of casein in total proteins) was used for the calculation of casein and milk fat. The cheese samples have been taken from different stages of maturation and microbiological (10 sample), physico-chemical (23 sample) and nutrient analysis (3 sample) with two different geographical areas of production have been carried out (eastern and western Herzegovina). The definition of demarcation of the geographical area of production was carried out in collaboration with members of associations of producers, dairies, representatives of local communities, the competent entity ministries of agriculture and the Food Safety Agency of BiH.

3 Results of Work and Discussion

3.1 Animal Nutrition for Milk Production for *Herzegovinian Cheese in Sack*

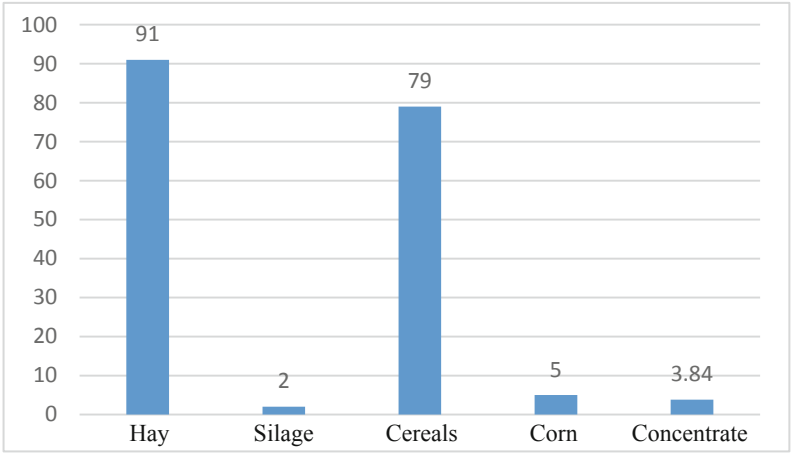
In the production of *Herzegovinian cheese in sack*, examination of the diet of dairy cows was performed on 75 farms. All farms are engaged in milk production, which is processed into dairy products, where cheese in sack dominates. Because of production of Herzegovinian cheese in sack is dominant, and from year to year the amount of cheese from goat's and sheep's milk decreases, the tests were done exclusively on cow's milk producers. The average number of people, on 75 surveyed agricultural holdings, who are permanently involved in the operation of the farm is 2.01, while 1.60 persons per farm are occasionally included. The test results showed that the average number of dairy cows is 4.26/agricultural holdings (AH) with a tendency to increase, if the general conditions for milk production are improved, to 7.43 dairy cows. The capacities of facilities for storage of hay and cereals are, if we take the current number of milking animals, at a satisfactory level and for hay on average for all surveyed farms it is a maximum of 20.44

t/AH, and for cereals 10.94 t/AH. All facilities for milking and storage of animal feed are located on the AH. The number of milking animals on AH ranged from 1 to 26 (Graph 1).



Graph 1. Number of milking animals per AH, in milk production for *Herzegovina cheese in skin sack*

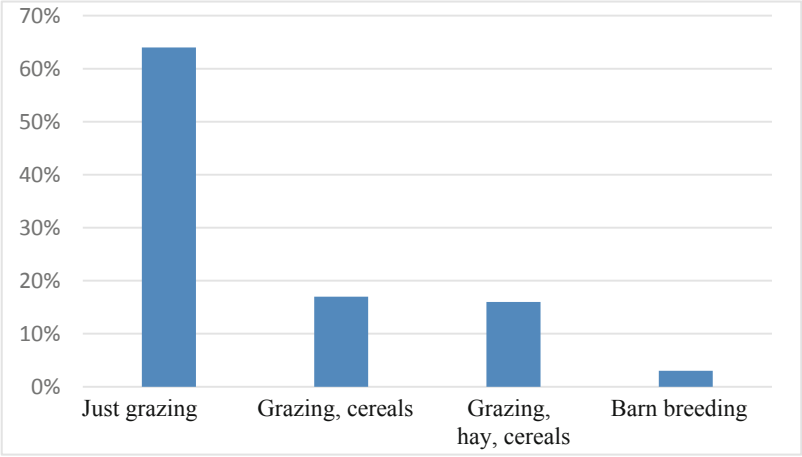
Animal feed is mostly provided on the AH itself. As shown in Graph 2, 99% of hay comes from AH, and the rest is procured from the same area, mostly from the first neighbors. Cereals (79.00%) are grown on AH while corn, due to the high elevation space (AH are located at over 850 m above sea level and the only crop that can be grown is silage corn) is sourced from local agricultural pharmacies originating from Bosnia and Herzegovina or Serbia.



Graph 2. Animal feed with AH (%) in milk production for *Herzegovina cheese in skin sack*

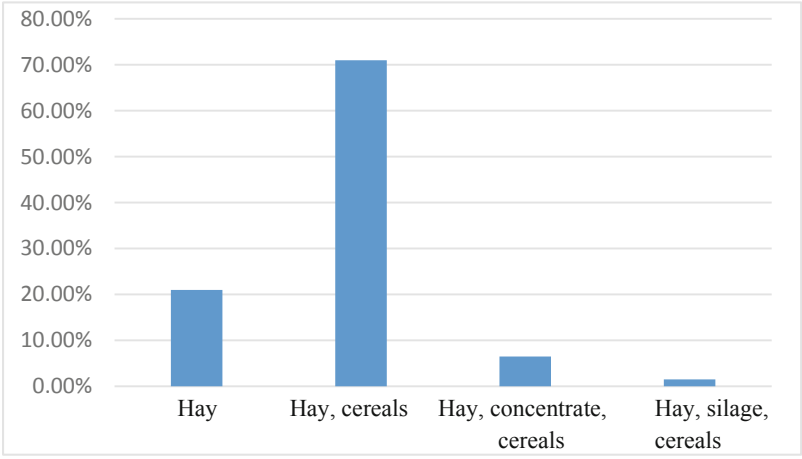
The diet of dairy cows, based on the tested AH in the production of milk for *Herzegovina cheese in skin sack*, is mainly based on the traditional method. The summer feeding season of dairy cows, shown in Graph 3, consists of grazing 97.00% and stable

breeding 3.00%. Almost two thirds of AH (64.00%) in the summer season use only grazing for animal nutrition, 27.00% a combination of grazing and cereals whereas 16.00% of AH in addition to grazing and cereals feed their animals with hay as well.



Graph 3. Livestock meal in the grazing season of musk deer

Winter livestock rations of dairy cows, Graph 4, in most cases (71.00%) consist of hay with a daily addition of cereals. One fifth of the animals or 21.00% whose diet was tested are fed exclusively with hay, while 6.50% use concentrates in addition to hay and cereals. Silage was used by only one AH, which is 1.50%.



Graph 4. Livestock meal in the winter season of milking animals

3.2 Technological Process of Production Herzegovina Cheese in Skin Sack

Herzegovina cheese in skin sack is produced from cow's and sheep's whole milk, and mixtures of these types of milk in different proportions, which come from the territory of Herzegovina. Cheese is produced throughout the year, but the proportion of certain types of milk varies. This cheese is industrially produced in three dairies from pasteurized cow's milk, standardized to fat content. Dairies are located in three municipalities of Herzegovina (Nevesinje, Bileća and Posušje). Milk intended for its production should originate exclusively from the defined area of Herzegovina. The purchase of milk is allowed, which must also be from the described area. The milk must come from animals whose diet consists exclusively of grazing, fresh fodder and hay of first-class quality, all from the described area of Herzegovina. *Herzegovina cheese in skin sack* is characteristic due to ripening in animal skins, which are made of sheep skin. *Herzegovina cheese in skin sack* on agricultural farms (small producers) is produced from raw, skimmed and milk with non-standardized milk fat content. Milk is obtained from one or two milkings (evening and morning) and must be processed within 24 h of the first milking. If the milk is processed within 12 h, it can be stored at a temperature of 8–12 °C. Milk to be processed after 12 h must be cooled to a temperature of 4 °C. The milk must be cooled to the appropriate temperature within 2 h. Raw milk is filtered through multilayer gauze, cloths or filters. The curdling milk is heated to a temperature of 31–35 °C. For inoculation of milk, possibly, milk or whey from previous production can be used. Only liquid animal rennet (dairy as well as AH) is used for coagulation. In the past, cattle breeders used exclusively natural rennet produced on their own farm to produce *Herzegovina cheese in skin sack*. Therefore, it can be said that the tradition of rennet production existed, but due to the growing supply of industrially produced rennet, it almost disappeared. On the other hand, in addition to the dramatic decline in the number of sheep and goats in recent decades, many shepherds and goats often slaughter their lambs and kids while they are still suckling milk. This calls into question the quality of naturally derived rennet using rennet of own lambs/kids. Despite the fact that the production of rennet on their own farm is economically viable, given the rising prices of natural rennet, the fact is that fewer and fewer farms produce it and it is difficult to obtain. Perhaps the reason is, in addition to those already mentioned, that the production of natural rennet on the farm is sensitive and demanding, and requires additional engagement, working hours and the necessary knowledge and production conditions. When buying cheese, cheese producers, especially small ones on AH, should pay attention to the expiration date, because it is a biological, easily perishable material. For that reason, it is recommended to use rennet in smaller packages, which the manufacturers generally adhere to. The use of other additives in the production of *Herzegovina cheese in skin sack* is not practiced. The cheese mass is roughly cut and then mixed intensively. The cheese mass is placed in cloths and then the lump is wrapped in cloths and squeezed under pressure to form the shape of a cake. Drained forms (lumps) are stacked, whole or broken, in plastic buckets or bags and stored until inserted into the skin sack. When inserted into the sack, the form is broken or cut into pieces measuring 8–10 cm, salted with about 3% salt and inserted into the sacks.

The sack is prepared by washing and shaving the skin after slaughtering the animal. The preparation of the skin requires a special technique to avoid damaging it because the entry of air through the damaged skin would spoil the cheese. The bag is turned over, salted, the openings are tied, inflated and air or smoke-dried. Only undamaged skin may be used for production of *Herzegovina cheese in skin sack.* To disinfect all the openings on the skin, they are smeared with vinegar or traditional brandy (plum brandy or grape brandy). After the drying process is completed, the skin is blown out and stored in an oven or refrigerator. Before use, the skins should be soaked in water to soften, and after it can be washed with vinegar (Nevesinje), brandy (Posušje and Široki Brijeg) or white wine (Kupres). The skins can also be prepared by soaking in whey after which they are washed with water. Also, it is allowed to use unshaven skins in areas where it is traditional (Posušje, Tomislavgrad, Kupres and Široki Brijeg). The unshaven skin is prepared by washing and air-drying it after slaughtering the animal, and then drying in smoke. After drying, the inside of the skin is washed with water and then with brandy. Cheese is placed in the bags prepared in this way. When filling the skins, the cheese is well compacted so that no spaces are filled with air. After filling, the bag is closed and tied. The cheese matures for a minimum of 2 months and a maximum of 8 months, provided that the skin sack does not open (anaerobic conditions). During ripening, it is necessary to specially nurture (maintain) the sacks by turning and wiping at the beginning of ripening every day, and later less often. Maturation must be on wooden boards (fir and ash boards), in dark rooms used for this purpose. The cheese that ripens in the sacks develops an extremely strong taste and aroma. Precisely for that reason *Herzegovina cheese in skin sack* possesses a strong and spicy taste as a result of anaerobic maturation in animal skin (Fig. 1).

Fig. 1. *Herzegovina cheese in skin sack* (Brenjo and Saric 2020)

3.3 Raw Milk for Cheese Production

As prescribed by the legislator (Rulebook on Raw Milk 2011), raw milk for the production of *Herzegovina cheese in skin sack* was tested for milk fat, protein, fat-free dry matter and freezing point as well as the number of somatic cells/ml and the number of

bacteria/ml. Testing the quality of milk is a very important area of the dairy industry. Impure and counterfeit milk can be, to a greater or lesser extent, harmful to processing and consumers. For this reason, the producers of *Herzegovina cheese in skin sack* pay a lot of attention to the quality of raw milk. The summary results of the parameters examined are given in Table 1.

Table 1. Quality of raw milk for production of *Herzegovina cheese in skin sack*

	Min	Max	Number of analysis/tests	Average	Standard deviation	Coefficient of variation
Milk fat (%)	2.95	5.87	93	4.09	0.609491	14.88%
Proteins (%)	2.75	4.19	93	3.39	0.338905	9.99%
Fat-free dry matter (%)	8.17	9.82	93	8.94	0.373723	4.18%
Freezing point (^{O}C)	−0.622	−0.530	100	−0.552	0.011649	2.11%
Somatic cell/ml	6.604	1.826.170	91	166.507	243.511	146.25%
Number of bacteria/ml	4.899	958.948	93	56.197	125.030,9	222.49%

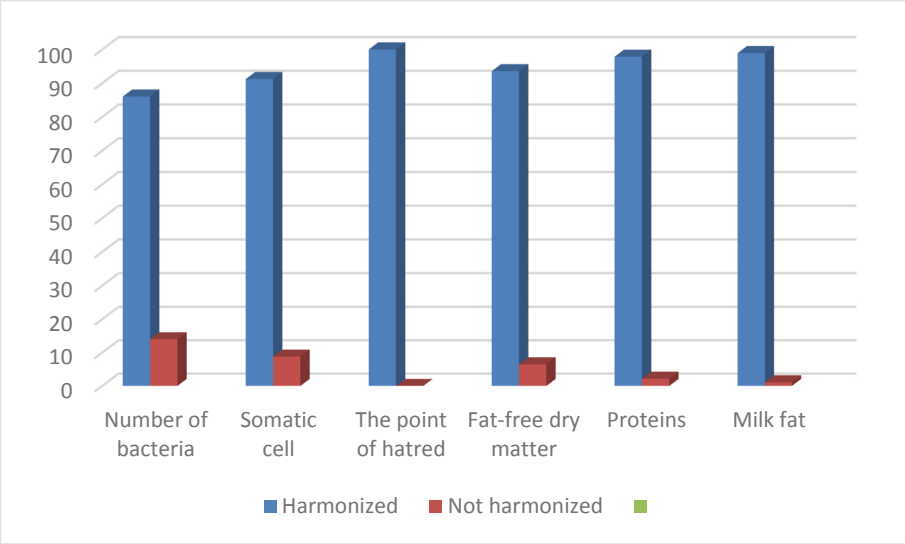
The summary table shows the minimum, maximum values and mean value of the tested samples as well as the standard deviation and coefficient of variation.

It is known that milk fat, as it can be seen in Table 1 in this text, is a component of milk whose amount in dry matter is subject to the greatest variations. For the production of Herzegovina cheese in skin sack, the content of dry matter without fat, showed an average of 8.94%, a minimum of 8.17% and a maximum of 9.82%. The coefficient of variation showed the uniformity of the sample and was 4.18%.The percentage of added water in the sample with the minimum content of dry matter without fat (8.17%) was 3.89% of added water.

The average freezing point per 100 analyzes of raw milk was −0.552 °C, the minimum −0.622 °C and the maximum −0.530 °C. All samples were compliant with applicable regulations while the coefficient of variation was 2.11%.

Of the total number of samples, 27% did not meet the current legislation for raw milk quality. The most non-compliant samples were on the number of bacteria/ml (13.97%), the number of somatic cells/ml (8.79%), the content of dry matter without fat (6.45%), protein (2.15%), milk fat (1.07%) while all tested samples in terms of freezing point values were in accordance with the prescribed norms (Graph 5).

Out of a total of 93 analyzed samples of cow's milk for the production of *Herzegovina cheese in skin sack*, the average value of milk fat is 4.09% (Table 1), the minimum 2.95% and the maximum value 5.87%. Only one sample (2.95% m.m.) did not meet the applicable regulations regarding the minimum milk fat content in raw milk. The coefficient of variation in the tested samples for mixing fat was 14.88%, which is considered a



Graph 5. Quality compliance of raw milk (%)

homogeneous sample. By researching the influence of the region on the chemical composition of milk, Dozet (1976) found that the highest milk fat content was in the milk of cows from the mountain region (4.27%), whereas the lowest content was in milk of cows from the sub-Mediterranean region (3.70%). Tests of milk quality for the production of *Herzegovina cheese in skin sack* in this paper were done in hilly and mountainous areas and the mean value of milk fat of 4.09% would be equal to the mean value of the mountainous and sub-Mediterranean region determined by Dozet (1976).

The average protein content was 3.39%, the minimum 2.75%, and the maximum 4.19%. Two samples did not comply with the regulations and their protein content was 2.75%. The coefficient of variation was 9.98%, which shows that the aggregate sample is homogeneous. The average protein content of 3.39% obtained is probably lower than the annual average of the surveyed farms due to the fact that the samples were taken in December, and the protein content in milk is usually lower in the winter months, due to poor basic bulk feed (hay, straw), corn or grass silage).

The content of casein and fats and their mutual relationship is especially important in cheese-making. The degree of transition to cheese, and thus the impact on yield, depends on the quantity and properties of the listed ingredients, as well as on the correctly applied technology (Martini et al. 2008). Only rennet casein is interesting for *Herzegovina cheese in skin sack*. By the way, the main role of casein in the food industry is in cheese production, since most proteins and fats pass from milk to cheese, and casein passes completely. The first process in the production of cheese, including *Herzegovina cheese in skin sack*, is the coagulation of proteins, namely casein, which is made by adding animal rennet that contains proteolytic enzymes. The resulting curd is pressed and squeezed to separate the whey, and then cut into cubes which are later processed in an appropriate manner (Šćurić 1991). Milk contains about 3.40–3.50% (30–35 g/l) of protein, which makes up about

28% of dry matter. This suggests that milk is a protein-rich food. On average, sheep's milk contains the most protein, about 4.60%, while cow's milk about 3.50% (Bylund 2003), which coincides with the values obtained in this research (average protein content 3.39%).

The average ratio of milk fat to protein in this study is 1.21, which is the average ratio according to literature sources (USDA-AIPL 2004). In modern cheese-making, an effort is made to standardize the ratio of the amount of casein and milk fat in milk, in order to ensure a typical structure and consistency and maximum yield (e.g. for the production of Cheddar type cheeses, the casein-fat ratio should be 0.70). Too high fat content in cheese milk can make it difficult for whey to separate during curd processing, but too low a content will not give the desired characteristics and consistency of cheese, which will be chewier with flaws in taste (Dozet et al. 1996). In the tests within this work, the ratio of casein and milk fat was 0.66.

From all the above, it can be seen that this is a very important indicator and that each type of cheese should have a harmonized ratio of protein and fat that is specific to a given type of cheese, and especially the ratio of casein and fat (Table 2).

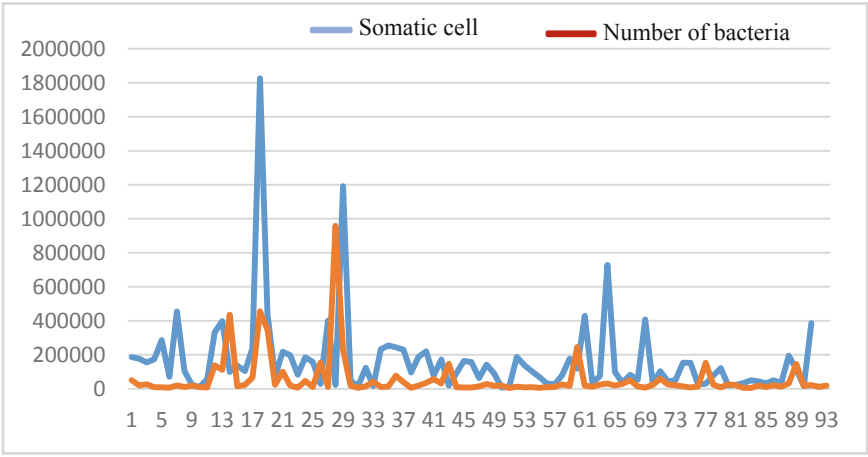
Table 2. The ratio of the amount of milk fat and protein as well as casein and milk fat

	Number of analyzes	Min	Max	Average
Milk fat %	93	2.95	5.87	4.10
Proteins %	93	2.75	4.19	3.39
RATIO		1.07	1.40	1.21
Casein %	93	2.20	3.35	2.71
Milk fat %	93	2.95	5.87	4.10
RATIO		0.81	0.57	0.66

The number of somatic cells and bacteria/ml is shown in Graph 6, the coefficient of variation in somatic cells was 146.25%, which shows that the aggregate sample was heterogeneous. As for bacteria/ml, its value of 222.49% was high, which is a feature of an extremely heterogeneous aggregate sample.

Characteristics of Herzegovina cheese in skin sack

Five samples of *Herzegovina cheese in skin sack* from whole cow's milk and 5 samples of cheese from skin (Torotan) made from skimmed cow's milk respectively were tested for *Coagulase-positive staphylococci* and *Listeria monocytogenes* were tested. All tested cheese samples were made from milk that was subjected to heat treatment during the technological process of production. There were no positive results on the presence of the parameters examined.



Graph 6. Number of bacteria and somatic cells/ml of milk of individual samples

Table 3 shows the results of statistical processing of chemical tests on *Herzegovina cheese in skin sack* for the parameters examined: Water content, fat, protein, fat in dry matter, ash and salt. The water content varied from 36.00% to 45.00% with a mean value of 39.57%. Based on the results obtained for water content, it can be concluded that cheese samples were taken at different stages of cheese ripening and that this is why there are such deviations. If we take into account that the minimum time that the cheese should be held in the skins is 60 days, the fact is that after 90 days there is a significant drying of the cheese in the sacks, especially in the summer. The content of milk fat in the dry matter of cheese varied from 41.78% to 64.38% with an average content of 50.67%. The cheeses tested c, with the average content of milk fat in the dry matter of cheese obtained, based on the provisions of the Rulebook on Dairy Products and Starter Cultures (2011), are classified as full-fat cheeses. The coefficient of variation ranged from 5.88% for water, 11.14% for the fat and fat content in dry matter, while it was over 20% for ash and 26.41%.

Table 4 presents data on the content of milk fat, protein, milk fat in dry matter and salt in cheese from the sacks from partially skimmed milk, also from the territory of Herzegovina, for a better overview of the difference between *Herzegovina cheese in skin sack* from the whole cow milk and the sack cheese from partially skimmed cow milk - *Torotan*,. This difference is, as expected, visible in terms of milk fat content and milk fat content in dry matter. However, *Torotan* cheese may contain milk fat in dry matter in an amount that classifies it as fat cheese (Rulebook on Dairy Products and Starter Cultures 2011), but this is still a rare case and depends on how much milk for cheese production is skimmed. According to the data from the literature, AH mainly produces *Torotan* cheese with a lower fat content in dry matter (below 25%) and thus it belongs to the semi-fat cheeses. Of the three tested samples of *Torotan* cheese, based on the proportion of milk fat in the dry matter, two were categorized as semi-fat cheeses and one as fatty cheese.

Table 3. Statistical processing of chemical parameters *Herzegovina cheese in skin sack*

	Min	Max	Number of analyzes	Average	Standard deviation	Coefficient of variation
Fat (%)	25.40	37.60	20	30.61	3.41	11.14
Dry matter (%)	45.09	64.00	20	60.03	4.00	6.66
Water in non-fat matter (%)	49.24	63.06	20	56.77	4.12	7.25
Proteins (%)	16.93	28.14	20	23.39	3.95	16.90
Fat in d.m. (%)	41.78	64.38	20	50.67	6.06	11.95
Ash (%)	2.98	6.10	18	4.04	0.97	23.96
Salt (%)	1.87	5.46	19	3.41	0.91	26.41

Table 4. Chemical composition of cheese *Torotan*

Sample	Fat (%)	Proteins (%)	Fat in dry matter (%)	Salt (%)
Cheese in skin sack - *Torotan*	15.00	28.00	26.00	4.00
Cheese in skin sack - *Torotan*	9.26	33.35	18.73	4.40
Cheese in skin sack - *Torotan*	7.53	36.70	13.45	5.05

The nutritional values of *Herzegovina cheese in skin sack* made from cow's milk were examined from two different geographical areas of Herzegovina, east and west, as well as the nutritional value of sack cheese made from partially skimmed cow's milk. The indication of nutritional value in Bosnia and Herzegovina is an obligation that cheese producers, before placing it on the market, must emphasize on the declaration from the end of 2016 (Rulebook on Information to Consumers About Food 2013). Mandatory nutrition declaration contains: Energy value; the amount of fat, saturated fatty acids, carbohydrates, sugars, protein and salt.

The results showed that *Herzegovina cheese in skin sack* from the geographical area of eastern Herzegovina has an energy value of 1,529 kJ/368 kcal whereas the same parameter for the cheese from the area of western Herzegovina was 1,380 kJ/330 kcal. *Torotan* cheese has an energy value of 1048 kJ/251 kcal. The energy value and the amount of nutrients in cheese are expressed in 100 g of product.

3.4 Geographical Indication

Research on indigenous cheeses in order to protect origin or geographical origin has long been relevant in the EU and so has been the issue of safety, especially in the production of indigenous cheeses. The main purpose of protection is to differentiate in relation to similar products of lower quality with constant emphasis on product specificity and raising the value of the brand, which all together contributes to better placement and higher price compared to competitors' cheeses.

It is characteristic of *Herzegovina cheese in skin sack* that, despite its limited quantity, it has a recognizable, unique quality and uniqueness that is closely related to the geographical area from which it originates as well as the traditional way of production passed down to generations in that geographical area. It is this recognition with the development of logos, packaging, slogans, web presentation and other marketing that can build a strong brand from this cheese that will be the driving force of the economy of the region.

The data obtained are very important for proving the geographical origin of raw materials. Based on the data processed as they refer to the diet and origin of raw materials as well as cheese production and depending on the proposed designation (origin or geographical origin), a unique form can be designed for all producers for the animal nutrition phase and it would include:

- Defined animal nutrition,
- Instructions for proper nutrition (meal dosing, meal quantity, keeping records of meals, safe keeping and keeping declarations and accounts for animal feed, as well as other purchased raw materials),
- Instructions to manufacturers on how to keep all this information.

The traditional technology of cheese production is based on sheep's unpasteurized milk, whereas today it is also produced industrially, mainly from cow's, pasteurized milk. Regulations on the protection of designations of origin or geographical indications do not interfere with this issue, provided that the manufacturers agree on the specification of the production of a particular product. The product specification includes several essential factors that manufacturers must agree on before applying for registration of a mark. They are as follows: The name protected as a designation of origin or geographical indication, the description of the cheese; a clear definition of the geographical area; proof that the cheese originates from a defined geographical area; a description of the production method and details linking the quality or characteristics of the cheese to the geographical environment; the link between the specific quality, reputation or other characteristics of the cheese and the geographical origin from which it can be sourced. The largest cheese producers had the opportunity to attend various trainings on quality policies and the importance of protecting their products. They have no problem with the name and designation they want to register - designation of origin (PDO), precisely defined geographical area of production (administrative border of Herzegovina, east and west), production technology is agreed, cheese can be prepared from unpasteurized or pasteurized sheep's, cow's milk or their mixtures with a ripening period of at least 60 days and there is a clear link between the specificity of the cheese and the geographical area (Fig. 2).

Fig. 2. Geographical area of production of *Herzegovina cheese in skin sack*

4 Conclusions

At a time of accelerated globalization and European integration, there is a real possibility of losing the traditional values of an area or region. In these processes, there is increasing competition where regional recognition is less and less pronounced. Based on the presented results for *Herzegovina cheese in skin sack*, from the discussion of these results it can be concluded:

- that the diet of dairy cows for the production of *Herzegovina cheese in skin sack* is mainly based on the traditional method and in a large part animal feed is provided on the AH,
- that out of the total number of raw milk samples, 27% did not meet the legislation for raw milk quality.
- that in the **technological process of production** *Herzegovina cheese in skin sack* is produced from cow, raw sheep milk, and mixtures of these types of milk in different proportions, which come exclusively from the territory of Herzegovina,
- that cheese is produced throughout the year, industrially in three dairies and in AH, and that cow milk cheese dominates,
- that the milk must come from animals whose diet consists exclusively of grazing, fresh fodder and hay of first class quality, all from the described area of Herzegovina,
- hat only animal rennet is used for coagulation and that any use of other additives is prohibited,
- that the cheese ripens in dark rooms and lasts a minimum of 2 months and a maximum of 8 months,
- that the content of milk fat in the dry matter of *Herzegovina cheese in skin sack* ranged from 41.78% to 64.38% with an average content of 50.67%, which classifies it as a full-fat cheese,

- that despite the fact that its quantities are limited, it has a recognizable, unique quality and uniqueness that is closely related to the **geographical area** from which they originate as well as the traditional way of production that is passed down to generations in that geographical area,
- that, taking into account the prescribed rules in the EU and BiH on geographical indications of food products, *Herzegovina cheese in skin sack* of cow, sheep milk and their mixtures meets all the conditions for registration of the **designation of origin**.

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Some Important Aroma Active Compounds in Apple Distillates

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Abstract. Aroma active compounds are all compounds that are present in food in concentration above their threshold. That means they active contribute to aroma sensation of food. In apple distillates there are huge number of volatile aroma compounds. Some of them are present in very small quantities but contribute to distillates aroma profile. In this research some of important aroma active compounds in apple distillates were measure by GC/MS method. Behaviour of benzaldehyde, phenethyl alcohol, furfural, ethyl benzoate and 2-phenethyl acetate in apple distillates were investigated depending on distillation technique used and time of aging in oak barrels (6, 12 and 18 months). From those investigated aroma active compounds dominant value show benzaldehyde and phenethyl alcohol. The average content of benzaldehyde was significant higher in apple distillates obtained by column system (14,82 mg/l) then in alembic pot (4,34 mg/l). Also, concentration of benzaldehyde increased during the maturation. The average content of phenethyl alcohol was higher in alembic distillates (12,93 mg/l) in ratio to column distillates (11 mg/l). The concentration of others investigated aroma compounds was significantly lower and mostly dependent on distillation technique used.

Keywords: Apple distillates · Aroma active compounds · Alembic · Distillation column

1 Introduction

Fruit spirits are characterized by a richness of aromatic compounds that belong abroad chemicals groups: alcohols, carbonyl compounds, terpenes etc. They are present in low concentration but are crucial for the quality and sensory acceptability of the drinks [1–5]. Aroma compounds in fruit spirits originated from the fresh fruits [2, 6–9], and are derived from each successive stage of the production process: fermentation [10–13], distillation [13–19] and maturation [20–26]. Also types of container during stored of final product effect on aroma compounds [27]. Study of Matias-Guiu [28] shows that composition of bottled spirits depends on storage conditions.

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However, all aroma compounds present in the spirits don't have the same effect on the overall sensation of the spirits. Their influence depends on their concentration but also on their individual intensity or aroma potency. The potency is dependent by thresholds concentrations of aroma compounds. Only aroma compounds released in sufficiently high concentrations, above their detection thresholds, will be detected by olfactory receptors [29]. Value of aroma potential of some compounds is depended by odour activity values (OAVs). OAV is ratio concentration of compounds found in matrix to its odour threshold. The contribution of aroma compounds to the overall aroma depends on the value of its odor activity value. It is considered that all compounds that have OAV $>$ 1 contributes to the overall aroma of the food matrix and they are aroma-active compounds [29].

In this research some of the important aroma active compounds (benzaldehyde, phenethyl alcohol, furfural, ethyl benzoate and 2-phenethyl acetate) in apple distillates were measured with an aim to investigate effect of distillation technique used and time of aging in the oak barrels on their behaviour.

2 Material and Methods

2.1 Material

Two apple varieties, Idared and Pink Lady, were used for the spirits production. A total of 1 tone of apple was used in this study. The ratio of Idared and Pink Lady in the mixture was 57:43. The pH of Idared mash was 3.4 and extract was 13°Brix and the pH of Pink Lady mash was 3.5 and extract was 14°Brix. See in [30].

2.2 Methods

Apple Destillate Production

The all collected apples were crushed and shredded by the rotating rollers with stainless steel teeth. The pH of mash was corrected with addition of previously calculated quantities of diluted solution of sulphuric acid to lower pH to 3. Fermentations were performed using commercial *Saccharomyces cerevisiae* var. *bayanus*, wine yeast (Oenoferme - Freddo, Geisenheim, Germany). Fermentations were conducted in the two stainless steel fermentation vessels of 6000 L and were carried out at 19 $\pm$ 3 °C. The fermentation was considered completed when the extract was below 4.5° Brix. It lasted 26 days. Fermented mash was divided into two parts for distillation process. Distillation was carried out on two distillation devices: alembic copper pot still (A) and batch distillation column (C). In alembic pot distillation was conducted as double. A 25 first distillations were conducted giving 870 L of raw distillate with 22% vol of alcohol. During the second distillation three fractions were cut. Only heart fraction was subjected for the analysis in this research. The heart fractions were filled into new oak barrels and maturation of distillates were lasted 18 months. Each 6 months the samples were taken for the chemical

analysis. The whole procudere of apple distillates production and maturation of distillate was described in Spaho et al. [30].

Chemical Analysis

Sample Preparation - A 100 ml of apple distillates was diluted with 100 mL distilled water followed by the addition of 15 mL of dichloromethane, 1 mL of internal standard (methyl ester of 10-undecenoic acid, 2 mg/mL in dichloromethane), 10 g sodium chloride and continuously extracted on vortex for 3 min. The dichloromethane extract was dried over anhydrous magnesium sulfate, and concentrated under nitrogen flow to final volume of 1.5 mL.

GC–FID-MS analysis- GC/MS analysis of aroma compounds were analyzed on Agilent 7890A equipped with a 5975C mass selective detector (MSD) and a FID connected by capillary flow technology through a two-way splitter. Agilent column 19091S-433 (30 m $\times$ 250 μm $\times$ 0.25 μm film thickness) with non polar liquid phase HP-5MS (Phenyl Methyl silx) was used. Helium was used as carrier. The injection volume was 1 mL (split 50:1). The injector temperature was 250 °C. Non polar column temperature was programmed linearly in the range of 60 °C to 240 °C at a rate of 3 °C/min, then at a rate of 35 °C/min to 310 °C with final 8 min hold.

Identification of volatiles were done by comparison of retention times of authentic standards in GC-FID chromatograms. The concentration of the aroma compounds was determined using the peak area of internal standard methyl ester of 10-undecenoic acid, and calculated according to the procedure reported in Ivanović et al. [31].

Statistical analysis- Two-way analysis of variance (ANOVA) was performed to establish whether a significant difference ($p < 0.05$) existed between the mean (three repetitions) concentrations of the compounds in the analysed sample depending on the distillation technique used and ageing time. The ANOVA was followed by Tukey HSD test to verify the statistical difference at the 0.05 significance level. Principal component analysis was performed for the visualisation of all experimental variables in order to determine the differentiation between the distillation technique employed and the ageing time. All statistical analyses were performed with the statistical package StatBox 6.7 (Grimmersoft, Paris, France).

3 Results with Discussion

In the samples of apple distillates over the hundred compounds have been identified. Some of them are present in paper of Spaho et al. [30]. In this paper five compounds that are present in higher concentration (furfural, benzaldehyde and phenethyl alcohol) or they are known as aroma contributor (2-phenylethyl acetate and ethyl benzoate) [4, 32] were investigated.

3.1 Results of GC–FID-MS Analysis

The mean concentrations of three repetitions for each aroma compounds in studied apple distillates were presented in Table 1.

Table 1. Means of aroma compounds in mg/L and standard deviations (±) in apple distillates obtained by using alembic (A) and column devices (C) after 6, 12 and 18 months of maturation

Aroma compounds	A 6	A12	A18	C6	C12	C18	Effect of Distill.	Effect of Matur.	Effect of Dist. × Matur.
Furfural	2.60 ± 0.89	2.60 ± 0.45	3.83 ± 0.62	0.90 ± 0.36	0.90 ± 0.24	2.90 ± 0.70	***	***	ns
Benzaldehyde	1.70 ± 0.17	5.37 ± 1.10	5.97 ± 1.96	13.10 ± 2.38	14.47 ± 2.7	16.90 ± 2.76	***	**	ns
Phenethyl alcohol	12.77 ± 3.92	16.07 ± 0.85	10.92 ± 2.19	8.60 ± 1.42	14.03 ± 2.47	10.37 ± 1.93	*	*	ns
Ethyl benzoate	0.47 ± 0.12	1.23 ± 0.51	2.83 ± 0.85	1.57 ± 0.75	1.53 ± 0.06	4.00 ± 1.20	***	***	ns
2-Phenylethyl acetate	0.43 ± 0.25	0.37 ± 0.12	0.27 ± 0.06	0.73 ± 0.21	0.60 ± 0.1	0.63 ± 0.25	**	ns	ns

Results of two-way ANOVA: factor Influences on * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns-no influence.

Among investigated compounds the most abundant is phenethyl alcohol, whose concentration was ranged from 8.6 to 16.07 mg/L depending on distillation technique used and time of maturation in oak barrel. Concentration of benzaldehyde was especial high in column samples ranged 13.1 to 16.9 mg/L. while in the alembic samples values were much lower. The concentrations of furfural ranged from 0.9 to 3.83 mg/L followed by ethyl benzoate (ranged from 0.47 to 4.0 mg/L) and 2-phenylethyl acetate that was showed the lowest values, ranged from 0.27 to 0.73 mg/L (Table 1). However, the contribution to overall aroma of fruit spirits does not depend only on concentration of present compounds. It is very important to know aroma potential of individual compounds. In Table 2 was showed a threshold of those five compounds express in ppm in different type of matrix and their OAVs. OAV was obtained by considering the concentration in the samples and odour threshold of each compounds. According to OAV values (Table 2) the highest contribution to overall aroma of apple distillates have ethyl benzoate followed by benzaldehyde. Furfural and phenethyl alcohol showed the similar influence to sensory profile of apple spirits. The concentration of 2-phenylethyl acetate was to low in order to consider it as aroma active compounds.

Table 2. Threshold and OAVs of five aroma compounds

Aroma compounds	Threshold in ppm	Refernecese	OAV
Furfural	0.77	Tamura et al. [33]	>1 in all cases
Benzadehyde	2.0 in beer	Miller [34]	>2 in most cases
Phenethyl alcohol	7.5 in spirits	Miller [34]	>1 in all cases
Ethyl benzoate	0.06	Buffery [35]	>7 in all cases
2-Phenylethyl acetate	0.7 in Spirits 34% vol	Miller [34]	<1 in most cases

In order to investigated the effect of different distillation technique used and different time of maturation in oak barrels two-way ANOVA was applied. Since both factors had effect (Table 2) the means are tested using pairwise multiple comparisons (Tukey HSD test) and results were showed in Fig. 1 A and B. The distillation in alembic pot was affected to the significantly higher concentration of furfural and phenethyl alcohol in apple distillates compared to distillates obtained by column distillation (Fig. 1-A). It is in accordance with results of Lukić et al. [36]. As states Spaho [37] the probable reason for this is heating the alembic during double stage of distillation by direct flame. Many authors find a similar concentration of furfural in fruit spirits [38, 39]. It is typical component of tail fraction [14]. The content of furfural was significantly increased after 18 months of maturation achieving the higher value 3.36 mg/L (Fig. 1-B). The increase of furfural during aging were published by other authors [40, 41]. The pentose from wood hemicellulose can be converted into furfural during aging by action of acids [41] and also, thermal modification of wood during barrel making, lead to an increase of furfural in wood task [42].

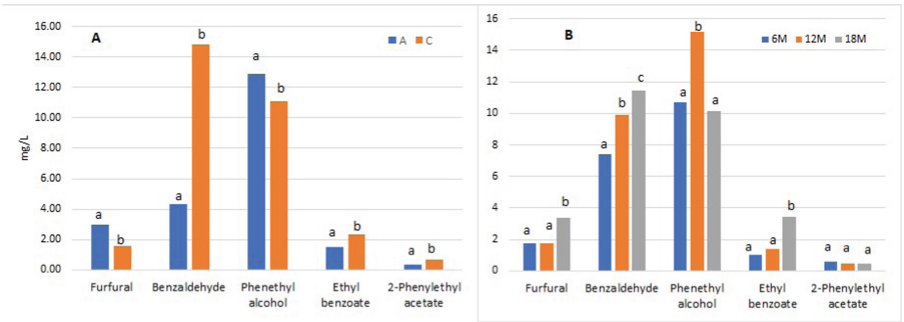

Fig. 1. Significant groups (a, b, c) between the distillation technique used (A) and between time of maturation distillates in months (B) using pairwise multiple comparisons (Tukey HSD test) after a two-way ANOVA ($p < 0.05$).

Benzaldehyde is important aroma active compounds especially in the stone fruit spirits because it originated from amygdaline present in fruit seeds and stones. It contributes to bitter almond, marzipan, cherry flavour in spirits [43]. The statistically significant differences between benzaldehyde contents in analogous heart fractions obtained folowing alembic distillation (4.35 mg/L) and column distillation (14.81 mg/L) were observed (Fig. 1 A). It is acording with results of Balcerek et al. [14], although Cortes et al. [44] Arrieta-Garay et al. [45] reported no differences in content of benzaldehyde existed depending on distillation technique used. A possible explanation for the benzaldehyde behaviour in distillation lie in its better solubility in higher alcohol solutions than in lower. Despite the high boiling point (178 °C), benzaldehyde will tend to distil more when ethanol distils. Since in batch column distillation with reflux the content of alcohol was more increased then in the distillation with alembic pot, obviously that ethanol carries a significant amount of benzaldehydes with it. Benzaldehyde has tail character [15, 37, 46]. Considering changes during maturation, the concentration of benzaldehyde

was significantly increased during aging time (Fig. 1-B). Since oak wood does not contain benzaldehyde consequence it was not extracted from wood staves. The increase of benzaldehyde over time of maturation was caused by evaporation of distillate.

Phenethyl alcohol, are formed during fermentation due to yeast activity, according to the Ehrlich pathway [4]. It is one aromatic alcohol and has a rose-like odour [37]. Its mean value was the highest among analysed component in this study. The statistically significant ($p < 0.05$) differences between phenethyl alcohol content in distillates obtaind by alembic pot and batch column distillation was recorded. Mean value of distillates obtaind in alembic pot was 12.91 mg/L while mean value for the column distillation was 11.09 mg/L (Fig. 1-A). This aromatic alcohol more intensive distils at the end of hart fraction and accumulates in the tail fraction [47]. Regarding effect of aging (Fig.1-B) content of phenethyl alcohol was significant higher after 12 month of distillates maturation in oak barrels. This is due faster evaporations of alcohol during summertime (in this study summertime was coresponded to twelf months of aging). Aged distillates had a lower content of phenethyl alcohol in compared to unaged distillates [4].

Although ethyl benzoate was present in the lower quantity in ratio to above mentioned compounds, it had the highest OAV (Table 2). This mean that is expected its notable contribution to overall aroma profile of distillates. Alembic distillate has significant lower mean levels of ethyl benzoate (1.51 mg/L) in compared to the distillates from column (2.37 mg/L) owing to high rectification. During distillation ethyl benzoate was increased in tail fraction [47]. Content of ethyl benzoate was higher in apple brandy maturated in vessel with different kind of wood chips compared to the control [32]. In this study ethyl benzoate showed the significantly higher content after 18 months of aging (3.42 mg/L) in compared to other two observed time.

A concentration of 2-phenylethyl acetate in the samples of apple distillates was low. Since this compound was present below its threshold concentration it not contributes to the overall aroma impression of distillates. It is interesting that this compound was more concentrated in fermented pear mash than pear distillates [4]. The same authors [4] observed the content of 2-phenylethyl acetate was higher after maturation unlike of results in this study where the content of 2-phenylethyl acetate slowly decrease during maturation period.

3.2 Principal Component of Analysis

A principal component analysis with all studied aroma compounds was carried out on the set of samples: the distillates from the different distillation devices and different time of maturation in the oak barrels (Fig. 2).

Figure 2 shows how components behave differently in the samples which were obtained with alembic pot and batch column devices and aged over the time in the oak barrels. It is clear that the distillation technique was crucial for separating the samples according to their content of benzaldehyde and phenethyl alcohol. Thereby, apple distillates obtained with alembic pot were more characterized by flower smell while apple distillates from column devices were more distinguished by more intensive aroma of almond. The aging effect was not the basis for separating the samples in according to content of any compounds.

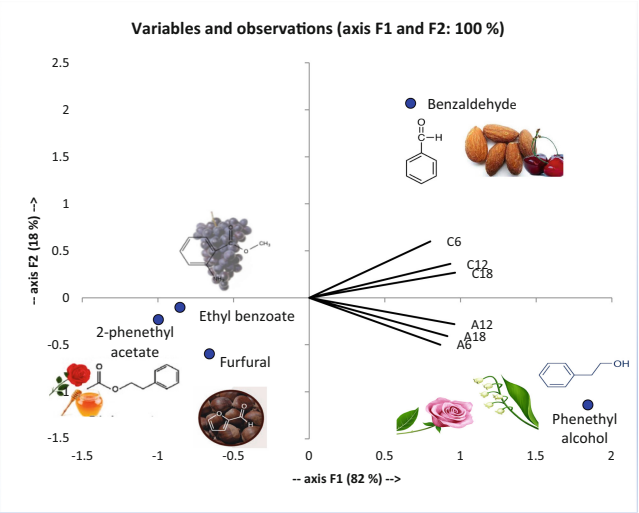

Fig. 2. Principal component analysis with apple distillates obtained by different distillation technique (alembic and column) and aged over 6, 12 and 18 months in oak barrel.

4 Conclusion

A concentration of furfural, benzaldehyde, phenethyl alcohol and ethyl benzoate were present above their threshold, so they are aroma active compounds in apple distillates. They contribute to the overall aroma impression. Unlike the above-mentioned components, 2-phenylethyl acetate has no significant effect on the aroma of apple distillates due to its low presented concentration. Alembic distillation enhanced content of furfural and phenethyl alcohol while distillation in batch column devices increased content of benzaldehyde, ethyl benzoate and 2-phenylethyl acetate. Maturation of apple distillates in oak barrel over 18 months was affected on a constant increase of benzaldehyde. The content of furfural was increased after 18 months of aging owing to extraction and acid hydrolysis of pentose from wood hemicellulose. Ethyl benzoate has similar behaviour as furfural while the content of 2-phenylethyl acetate slightly increased over time of aging. Behaviour of aroma compounds during maturation was more caused by evaporation of distillates than by directly influences of barrel, with exception of furfural.

Conflicts of Interest. The authors declare no conflict of interest.

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Influence of Exploitation of Peatland on Quantity and Quality of Organic Matter in Histosol

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Abstract. Peatlands have been identified as hotspots in the global carbon cycle because exploitation has brought them from carbon sequestration sites to CO_2 emitting sites. The aim of this paper is to analyse how the changed hydrological situation after the continuous drainage and tillage as well as peat body excavation affected the emission of CO_2-C and the quantity and quality of organic matter in Histosol.

Peatland Ždralovac is situated in two depressions in Livno's karst field in Bosnia and Herzegovina. Variation of levels of water table in peatland Ždralovac in period 2011–2014 was monitored in system of piezometers by separated hydrological zones depend of way of use peatland. In the average year with a dry summer period (2012), in northern „virgin" non-drained part Veliki Ždralovac with a deep Histosol > 100 cm, CO_2-C emissions was 5.210 kg ha^{-1} a^{-1} according by Renger et al. 2002. The adjacent belt shallowly drained peatland to remove the surface layer of peat, with a deep Histosol > 100 cm too, has a higher rate of CO_2-C emissions 5.765 kg $ha^{-1}a^{-1}$. The Histosol in southern Mali Ždralovac, which is converted for agricultural production and with reduced depth to only 20 cm, has a CO_2-C emission rate of 2.200 kg ha^{-1} a^{-1}.

Exploited peatland compared with native condition differ in particular in the quantity and quality of soil organic matter and morphology of surface horizon in Histosol. With only 31–43% organic matter, shallow Histosol converted for agricultural production is classified as organic soil and not as peat, with a characteristic muck layer formed after drainage in combination with tillage.

Keywords: Peatland · Exploitation · Mineralization

1 Introduction

Peat stocks have been singled out as a significant source of organic matter and as a key indicator for assessing the role of soil in the global carbon cycle [12]. Although peatlands themselves occupy about 4% of the global land area, they store about 30% of the global amount of C soil [19].

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Karst fields are important production landscapes characteristic of the Mediterranean region. Livanjsko polje is one of the largest karst fields in the world situated in southwest Bosnia and Herzegovina. It covers an area of about 400 km^2 (65 km × 6 km) with an altitude of about 700 m. It can be divided into three separate hydrographical parts: the peatland Ždralovac in the northwest of the field, the middle part of the field from which water is collected and drained into the Buško Blato hydro accumulation in the southeast (the third part of the field) and used for energy production in the power plant Orlovac in Croatia. Along the edges of the field rise mountain ridges built of carbonate rocks that border it from the northeast and southwest. The whole field is practically completely flat with bottom from impermeable carbonate clay and slightly sloping to the southwest. Along the perimeter of the field, most often in the higher hypsometric levels there are springs and estavels from which field is fed with water, and in its lower hypsometric positions there are abysses/sinkhole in which water sinks in underground. It is intersected by river flows and ravines with variable flow that end at the entrance to the sinkholes [23]. In the wet period when the inflow of water from springs and estavels into the field is greater than the runoff of water into the sinkhole, water retentions are made around the sinkhole so that combine with the flooded peatland two thirds of the field takes on the appearance of a large shallow lake [22].

For centuries, people have used peatland in the traditional way: easy grazing of cattle with young grass and reeds, summer field crops in higher positions in the field and reed felling and peat and peat-coal extraction mainly for their own needs. Recently, work on peat extraction from the central part of the Ždralovac peatland with deep Histosol has been intensified. Further, the area of the southern part of the peatland with shallow Histosol has been converted for use in agricultural production by building a hydro melioration system with controlled shallow drainage, what is changed hydrological condition in peatland. In this way, the boundaries of aquatic and terrestrial ecosystems were changed, water table levels were reduced and the terrain dried up what causes the loss of organic matter by mineralization and as a result peat compaction and subsidence and CO_2-C emissions into the atmosphere occurred.

In this paper the aim is to analyse the state of Histosol in changed hydrological condition after many years of exploitation and C-CO_2 emissions from the peatland surface. In accordance with the set goals, it is necessary to perform the following tasks:

- in the context of the peatland use way and related hydrological conditions to single out different zones on peatland,
- analyse the climate data
- set up a series of piezometers by zones and monitoring water table,
- using the mathematical model of Renger et al. [20] to quantify the impact of mineralization on peat subsidence and C-CO_2 emissions,
- sampling Histosols by hydrological zones and analyse the physical and chemical properties of Histosol,
- calculated organic carbon stocks (kg m^{-2}) in Histosol in this study compared with the stocks of C in Histosol 45 years ago [8], before intensification of exploitation peatland

It is especially interesting that the obtained results of C-CO_2 emissions on an annual basis using Equation Renger et al. [20] will be compared with the calculation of carbon

losses in Histosol after 45 years of peatland drainage for peat extraction and use in agriculture. Namely, the obtained results of quantification of organic carbon in Histosol in this study will be compared with the amounts of C in Histosol 45 years ago (1967) in the work of Bogdanović et al. [8] when peatland exploitation has not yet increased.

2 Material and Method

Research area in this paper is peatland Ždralovac (3.615 ha), located in the north western part of Livanjsko polje, formed in two shallow landscape depressions Veliki Ždralovac (deep Histosol) and Mali Ždralovac (shallow Histosol) as a geogenous fen type peatland, fed by groundwater from springs (Bastašica) and estavels Bastasi that has been in contact with limestone and mineral soils, and with dominant vegetation reeds and sedges. Main drainage channel is Ždralovački channel which ended in sinkhole Kazanci. Within the set goal climate, hydrological and pedological researches were carried out.

2.1 Climate Analyses

Precipitation and temperature data from meteorological station Livno are analysed in the series from 1961–2014 and in years of research 2011–2013 [14].

2.2 Hydrological Research

Altitude and border of surface water table in Veliki Ždralovac and water retention around swallow-hole Kazanci were measured by geodetic instruments.

Measurement of water table levels in peatlands Veliki and Mali Ždralovac was performed in series piezometers (CP Control Points 1–11) in period 2011–2014.

The total of 11 monitoring control points has been defined by hydrological zones:

1. Zone 1 - 856 ha; piezometers 1, 2 and 3; deep Histosol in the non-drained northwest „virgin“ part of peatland Veliki Ždralovac; with characteristic wetland vegetation sedge and reed;
2. Zone 2 -137 ha; piezometers 4, 5 and 6; a narrow strip between virgin zone 1 and the peat excavation zone 3; deep Histosol whom shallow surface peat layer (25 cm) was excavated 25 years ago; with shallow drainage channels 50 cm depth; and with changed vegetation which consist from two tree-lined avenues of stunted white poplar and bushy vegetation, between which is meadow vegetation.
3. Zone 3 - 640 ha; piezometers 7, 8 and 9; the active peat excavation sites whit drainage channels
4. Zone 4 - 1093 ha; piezometers 10 and 11; peatland Mali Ždralovac with shallow Histosol converted in reclamation area “Table” with grid of drainage channels and water retention around sinkhole (abyss) Kazanci (Fig. 1)

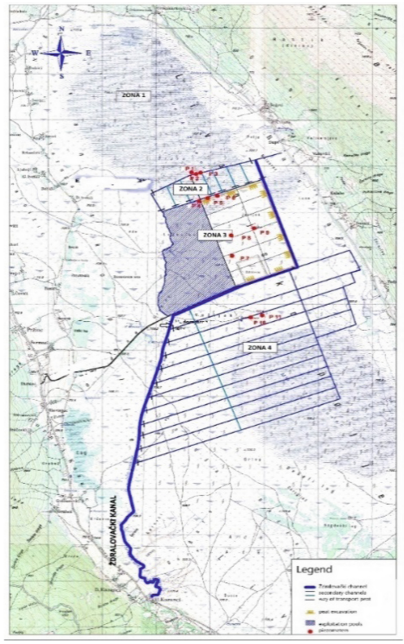

Fig. 1. Selected hydrological zones in peatland: thicker blue line - main drainage Ždralovački channel which drainage all peatland and ended in sinkhole Kazanci on south; blue line greed of drainage channels in the reclamation zone 4 named "Table", and in zone 3 of excavation peat in the central part of peatland; red points - series of piezometers by zones

2.3 Pedological Research

Pedological profiles are opened in period (2012–2014) nearby piezometers (control points) by hydrological zones: Profile 1 in Zone 1 with non-drained virgin Histosol; Profile 2 in zone 2 with shallow drained histosol; Profile 4 in zone 4 with peatland used in agriculture with mineralized muck-layer. In zone 3 peat body is excavated (Figs. 2, 3 and 4).

Fig. 2. Profile 1

Fig. 3. Profile 2

Fig. 4. Profile 4

Peat samples from different depths of Histosol (layer depth 10–20 cm) were taken in three replicates in distributed conditions and in rings (100 cm^3 volume) for analysis of its physical (Bulk density, Specific gravity, Porosity, Water holding capacity) and chemical characteristics (pH, ratio organic i mineral part of peat) by ATSM standard methods [3–6].

The concentration (%) of total carbon (C) and total nitrogen (N) in the peat sample was determined by dry incineration in an elemental analyser (CHNS elemental analyser: CHNS Elementar Vario EL III, Hanau, Germany).

2.4 Calculation Peat Subsidence and CO_2-C Emission

Renger et al. [20] developed a subsidence equation for 4 types of minerotrophic peatlands in northern Germany that are used as grasslands (from less mineralized peat to more strongly mineralized peat). The regression equation mainly refers to subsidence due to mineralization in places where peat has been drained for years. Peat subsidence regression equation for a minerotrophic peatland of the less mineralized type is used in this paper and calculated by the following equation:

$$\mathrm{DPT} = 0.147 \cdot \mathrm{SWT} - 0.0006 \cdot \mathrm{SWT}^2 + 0.05 \text{ where}$$

DPT = reduction of peat thickness (mm/year), and
SWT = mean summer groundwater level (cm below the surface).

Renger et al. [20] also developed regression equations for CO_2-C emissions, also for 4 types of minerotrophic peatlands used as grasslands, which corresponds to their peat thickness reduction equation. CO_2-emission (kg $ha^{-1}a^{-1}$) regression equation for minerotrophic peatland of the less mineralized type is:

$$\mathrm{CO_2\text{-}C_{Renger}} = 121 \cdot \mathrm{SWT} - 0.482 \cdot \mathrm{SWT}^2 - 121 \text{ where}$$

$$\mathrm{CO_2\text{-}C_{Renger}} = \mathrm{CO_2\text{-}C\ emission}\left(\mathrm{kg\ ha^{-1}a^{-1}}\right)$$

3 Results and Discussion

The results will be presented as an analysis of climate data, variation of groundwater levels by zones, calculation of peat subsidence and CO_2-C emissions, as well as presentation of Histosol properties by isolated zones and comparison of carbon stocks with stocks calculated from Histosol research 45 years ago.

3.1 Climate

According to First national report in accordance with the framework of climate change [13] the area of Livanjsko polje was concluded to be in the region of increase of average annual air temperature in the range of 0.6 to 0.8 °C in the last 100 years. These changes are

manifested in the more frequent occurrences of temperature extremes, above the absolute maximum, especially in the period 2000–2012. In the same report the precipitation in this wider area shows an increase of 2% and can be said to stagnate but noticeably increase the intensity of the particular rain while simultaneously extending the non-rainy periods. The first phenomenon results in increased risk of flooding, and the second drying of the surface layer in the summer period (Table 1).

Table 1. Climate data for the years research 2011.-2013. and in the series1961–2014 from meteorological station Livno [14].

Month	Precipitation (mm)				Temperature (°C)			
	2011	2012	2013	Average 1961–2014	2011	2012	2013	Average 1961–2014
I	41	37	164	97	0,8	-0,9	2,2	- 0,1
II	21	102	151	86	1,9	-4,6	1,3	1,0
III	88	1	204	91	4,5	7,0	4,4	4,4
IV	48	177	116	99	10,5	9,1	11,0	8,6
V	86	102	132	78	14,1	13,4	13,3	13,5
VI	82	35	99	87	18,5	20,7	17,3	16,9
VII	115	34	28	52	19,7	23,1	21,1	19,3
VIII	21	0	79	66	20,8	22,2	21,0	18,7
XI	28	194	102	97	17,7	17,0	14,3	14,4
X	82	212	81	115	9,3	11,0	11,3	9,9
XI	57	102	166	154	4,4	8,4	6,8	5,1
XII	140	202	44	132	2,8	0,8	1,7	0,9
Anual	808	1197	1365	1155	10,4	10,6	10,5	9,4

The year 2012, during which water table variations were monitored is characterized by the average amount of precipitation but the dry summer VI, VII and VIII months, while the rainy 2013 is characterized by annual precipitation more than average. In 2014, a major flood was recorded.

3.2 Monitoring Groundwater Level in Piezometers in 2012 and 2013 Years

It should be noted that subsidence and C-CO_2 emissions from zone 3 - peat extraction zone were not calculated because the peat body was removed (Table 2).

Floods in drained zones 2, 3 and 4 usually begin in late November or December, and mostly end in March-April. However, in the area of the non-drained zone 1 (piezometers 1, 2 and 3) which is closest to the north west area of springs and estavelas which feed peatland with water, and where is kept the body of peat (deep Histosol), the flooding period is still extended for another three months, meaning up to July. Dry bottom was recorded in non-drained Zone 1. in 2012 in summer period in duration of 45 days. The

Table 2. Water table depths (cm) in piezometers from the ground surface by zones.

		ZONE 1			ZONE 2			ZONE 4	
Year	Piezom. depth / Date	P1 135 cm	P2 124 cm	P3 100 cm	P4 180 cm	P5 105 cm	P6 142 cm	P10 25 cm	P11 26 cm
2012	Nov.-Mar	flood							
	12.04.	-10*	-13	-10	26	28	-10	-0	19
	23.04.	-8	-11	-8	13	18	-7	3	-
	08.05	-6	-9	-6	24	33	3	5	-
	23.05.	-3	-4	-3	28	35	-1	12	-
	11.06.	0	-3	-1	33	36	3	15	-
	12.07.	55	60	64	83	79	91	dry**	20
	05.08.	dry	dry	dry	110	dry	124	dry	dry
	21.08.	dry	dry	dry	113	dry	126	dry	dry
	05.09.	dry	dry	dry	113	dry	126	dry	dry
	21.09.	dry	dry	dry	107	94	120	dry	dry
	08.10.	90	94	90	109	96	121	-	-
	06.11.	82	80	79	100	84	107	20	
	29.11.	1	0	3	28	34	23	0	
	06.12.	-10	-10	-10	-10	-10	-10	-6	
	20.12	flood							
2013	Jan-Feb.	flood							
	28.02.	-10	-10	-10	-10	20	-10	-6	
	16.04.	-16	-16	-16	-16	9	-16	-9	
	26.05.	-6	-6	-6	7	20	1	0	
	18.06.	-6	-6	-6	4	15	-3	4	
	06.07.	-3	-3	-3	33	46	20	-	
	21.07.	-3	-3	-3	39	55	26	-	
	06.08.	-	20	-	62	93	38	-	
	22.09.	-	55	-	67	97	42	-	
	22.10.	-	40	-	32	56	14	-	
	08.11.	-	33	-	34	50	39	18	
	22.11.	flood							

* negative numbers indicate the period of flooding, piezometers still be approached
** dry bottom in piezometers

flooding period is also extended in the western part of zone 2 around piezometer 6, which is also located not far from the northwestern estavelas Bastasi area, which feeds this part of the peatland with water, as noted Vlahinić in your research 43 years ago [21].

Zones 2 and zone 3 represent the areas where the deepest peat is recorded and where the wetland depression is the deepest, so that depression is a kind of collector for peat groundwater, so that during the measurement there was no recorded dry bottom in piezometers although the depth of the water table was in the range of 110–126 cm. The exception is the piezometer 5 in zone 2 with dry bottom in that period, but which is placed on a slightly higher ground, which is, figuratively speaking, some kind of a ridge in this depression with deep peat.

Zones 3 and zone 4 with greed of drainage channels are, in a certain way, unique water body with the water retention formed around Kazanci in wet season, in which, by the system of connected vessels, variation of water table i.e. flooding period are identical and have seasonal character. Duration water retention Kazanci in dry 2012 was from 25.12.2011 until 12.04.2012, and in rainy 2013 from 20.12.2012. until 22.05.2013.

3.3 Emission CO_2-C and Peat Subsidence

In this paper, the mathematical model Renger et all. [20] was used to estimate CO_2-C emissions. Due to the impossibility of direct measurement of captured greenhouse gas from emissions in the field from peatland surfaces under different type of use (non-drained part of peatland, drained part, peat extraction part, reclamation part) this model was used due to similar conditions in peatland Veliki Ždralovac like in Renger et al. research [20] (drained minerotrophic peatlands used as grasslands, the less mineralized type) and because we have a data about fluctuation ground water level in 2012 and 2013 years. It should be noted here that peat losses due to mineralization according to the equation of Renger et al. [20] apply to all categories of minerotrophic peatlands except for those areas that were cultivated for agricultural purposes. It was not originally planned to calculate CO_2-C emissions and subsidence for the reclaimed zone 4 – "Table" because it is a highly mineralized and shallow peat, in fact, according to its characteristics, it is classified as organic soil, which is also converted for use in agriculture. However, taking into account the fact that reclamation zone 4 have not been cultivated since 1990, i.e., the 24 years and that they are covered with mostly grass vegetation, and that we have data about summer groundwater level in 2012, the equation Renger et al., 2002, was subsequently used to approximately calculate subsidence and CO_2-C emissions on these areas.

According to Renger and all. regression equations [20] peat subsidence and C-CO_2 emissions are calculated according to the mean summer groundwater depth - SWT. In this paper is calculated two values of SWT, one for vegetation or growing season of reed from IV-IX month with some period of flood as we can see in Table 2, and second SWT for summer season from VI-XI month with flood in rainy 2013 year only in non-drained zone 1. The total duration of the vegetation period is calculated from the day when the average air temperature reaches approximately 10 °C, and when at the same time the soil temperature is around 5 °C. A total of five months in the period May-September has a temperature higher than 10 °C. In first calculation we will take the readings from piezometers for vegetation season from 23 April – 21 September for 2012 year, and 16 April – 22 September for 2013 year. In that period flood is recorded only in non-drained zone 1, duration 50 days in dry 2012 and 97 days in rainy 2013 year. In second calculation SWT for summer season are used the readings from piezometers in period 11. Jun – 21 September for 2012 dry year without flood, and 18. Jun – 22 September for 2013 year, with flood in zone 1 for 34 days.

The mean groundwater depth was calculated over the mean values between the two measurements in piezometers as follows: add two adjacent level of water table readings from the ground surface (cm) and divide by 2, then multiply the obtained value by the number of days between the two measurements; then add up all the multiplications thus obtained and divide by the total number of days for the vegetation or summer season.

2012 is considered an average year because with an annual rainfall of 1.197 mm it is at the level of an average annual rainfall of 1.155 mm, but at the same time this is a year with a dry summer period. In this context, 2013 was considered rainy because with an annual rainfall of 1.365 mm higher than the average.

Table 3. Subsidence of peat and CO_2-C emission by hydrological zones and by years.

Zone	SWT for growing season (cm)	SWT for summer season (cm)	Subsidence of peat (cm a^{-1}) [20]		Emission CO_2-C ($kgha^{-1}a^{-1}$) [20]	
			Growing Season	Summer Season	Growing Season	Summer Season
	23.04. – 21.09.2012	11.06. - 21.09.	2012 average year with dry summer			
Z 1	57	85	0,65	0,82	5.210	6.682
Z 2	66	89	0,71	0,84	5.765	6.830
Z 4	19	20	0,30	0,32	2.200	2.505
	16.04. -22.09.2013	18.06.-22.09.	2013 rainy year			
Z 1	12	20	0,17	0,28	1.262	2.106
Z 2	33	51	0,42	0,60	3.347	4.796

It is understandable that the calculated mean values groundwater depths and consequent subsidence and CO_2-C emissions are higher in the summer season than in the growing season due to the absence of floods, less precipitation and higher evapotranspiration.

In general, the subsidence and CO_2-C emission are higher in the dry 2012 than in the rainy 2013 year. Furthermore, in both dry and rainy years, mineralization is higher on drained deep peatlands in zone 2 compared to nearby non-drained deep peatlands in zone 1 or on drained shallow peat in reclamation zone 4 - Table.

The calculation was not made for reclamation zone 4 - Table in rainy 2013, because during the summer period, until 26 May this part of the peatland was flooded with water retention formed around sinkhole Kazanci and after that level groundwater in zone 4 was not recorded.

In the non-drained zone 1, in 2012 with dry summer, CO_2-C emissions are about 4 times larger than in rainy 2013, calculated by SWT for growing season. At the same time, in zone 2 with shallow drainage channels, a CO_2-C emission is higher in dry 2012 than in rainy 2013 less than 2 times. This smaller difference in CO_2-C emissions between rainy and dry years in this zone 2 is because the drainage channels during the rainy year lowered the average groundwater level, creating conditions for more intensive mineralization in rainy year too.

In 2012 dry year, CO_2-C emission in zone 2 with shallow drainage channels is only slightly higher than in the nearby untouched part of Veliki Ždralovac in zone 1 which

is still affected by drainage in the rest of the peatland and there one cannot speak of an intact "virgin" peatland.

According to [16] when wetland soils dry out, higher rates of aerobic degradation result in soil subsidence, e.g., 2–3 cm per year.

According to [20] in places where peat has been drained for years, mineralization in deeper, less mineralized peat is in the range of 2.600–7.000 kg CO_2-C ha^{-1} a^{-1}, just as in zone 1 i zone 2 in Veliki Ždralovac, while mineralization in shallow highly mineralized peat is from 2.200–4.900 kg CO_2-C ha^{-1} a^{-1}, as is the case in reclamation zone 4 - Table.

3.4 Histosol by Hydrological Zones

In this chapter would be presented physical and chemical characteristics Histosols in non-drained zone 1 by Profile 1, drained zone 2 with Profile 2 and abandoned reclamation zone 4 – "Table" by Profile 4.

Non-drained Zone 1 Profile 1

Profile 1 is opened in untouched part of peatland Veliki Ždralovac, away from the nearest channel in zone 2 approximately 100 m. The vegetation consists of sods of sedges *Carex spp.* and reeds *Phragmites spp* (Table 4).

Table 4. Profile 1, Physical and chemical characteristics Histosol in non-drained zone 1.

Depth (cm)	pH H_2O	Organic content (%)	Mineral content (%)	Bulk density (BD) (gcm^{-3}	Specific gravity (gcm^{-3})	Porosity (P) (%)	Water retention capacity (%)	C (%)	N (%)
0–10	6,9	78,1	21,9	0,21	1,07	80,8	301,3	36,7	2,9
10–20	6,8	81,2	18,8	0,19	1,23	84,8	334,3	39,5	2,8
20–35	6,6	84,2	15,8	0,13	1,08	87,8	540,3	42,7	2,3
35–50	6,7	84,8	15,2	0,12	1,07	88,4	639,3	43,4	2,3
50–65	6,5	75,8	24,2	0,14	1,13	87,8	580,2	37,7	2,3
65–80	6,2	37,3	62,7	0,22	1,51	85,4	370,2	20,4	1,4
80–100	6,2	30,9	69,1	0,34	2,18	84,6	233,5	16,0	1,2
Clay	8,7	6,5	93,5	0,87	2,73	68,1	74,0	8,8	0,1

The classification of peat according to pH (6,9–6,2) here is weakly acidic peat by full depth [6]. In general, the body of Histosol can be divided into two layers according to the degree of mineralization, upper 0–65 cm lower mineralized layer and deeper 65–100 cm strongly mineralized layer. These two layers further can be divided into two more sublayers according to the same criteria. In this context strong mineralized layer 65–100 cm with mineral content in range 62,7–93,5% belong organic soil and not peat layer [15, 17].

Higher values of specific gravity indicate a higher degree of degradation and a higher proportion of the mineral part [1, 10] and BD values in the dry state are higher in more decomposed peat [7]. The highest values of water retention have the weakest mineralized layer of peat at a depth of 20–65 cm. The difference in mineralization explains and much lower concentration of C and N in the deeper more mineralized layer.

Drained Zone 2 – Profile 2

Profile 2 is opened between untouched part of peatland and excavated zone. That narrow belt is used from excavated only surface pet layer 25–30 cm depth and for reed cut in 1970s. In the belt are active shallow drainage channels (Table 5).

Table 5. Profile 2 Physical and chemical characteristics Histosol, drained zone 2.

Depth (cm)	pH H_2O	Organic part (%)	Mineral part (%)	Bulk density (BD) (gcm^{-3}	Specific gravity (gcm^{-3})	Porosity (P) (%)	Water retention capacity (%)	C (%)	N (%)
0–10	6,63	80,3	19,7	0,20	1,24	83,3	296,5	37,3	3,3
10–20	6,68	81,3	18,3	0,20	1,31	84,6	285,6	41,5	2,6
20–35	6,75	79,3	20,7	0,19	1,28	85,1	362,2	39,9	2,3
35–50	6,79	76,8	23,2	0,19	1,30	85,0	418,0	38,2	2,2
50–65	6,63	67,7	32,3	0,15	1,25	87,6	549,8	33,1	2,1
65–80	6,55	53,2	46,8	0,21	1,53	86,5	387,1	26,8	1,8
80–100	6,38	45,2	54,8	0,20	1,37	85,1	401,4	23,0	1,6
100–120	6,30	37,5	62,5	0,27	1,64	83,2	292,3	18,3	1,3

Related to rate of mineralization this Profile could be divided in to layer too. Upper less mineralized layer is some shallower (0–50 cm) then in Profile 1 (0 - 65 cm). Deeper layer is more mineralised with 32–62% of mineral part. In generally, bulk density and specific gravity in drained profile 1 are higher and water retention is smaller than in non-drained profile 1 what indicated more mineralized peat.

4 Reclamation Zone 4 – "Table" Abandoned from 1990 – Profile 4

Especially low peatlands are unusually grateful for reclamation because good regulation of hydrological conditions and rational agri-techniques can convert them into much more fertile soil than mineral soil [18]. Area of 1093 ha shallow peat in south zone of peatland was 1970 converted for use in agricultural purpose and named Table. There is built system of drainage channel with water gates which are controlled shallow drainage which is allow capillary wetting rhizosphere layer. From 1990 this reclamation zone is abandoned and water gates are devastated, drainage is uncontrolled and in summer period

Table 6. Profile 4, Physical and chemical characteristics Histosol in abandoned reclamation zone 4 –"Table".

Depth (cm)	pH H_2O	Organic content (%)	Mineral content (%)	Bulk density (BD) (gcm^{-3}	Specific gravity (gcm^{-3})	Porosity (P) (%)	Water retention capacity (%)	C (%)	N (%)
0-10	6,9	43,7	56,3	0,33	1,73	81,0	229,8	20,5	1,6
10-20	7,2	31,1	68,9	0,53	2,18	75,8	133,1	13,9	1,1
Clay	–	–	–	0,87	2,73	68,1	74,0	–	–

shallow peat have no water so that agricultural production could not be re-established (Table 6).

The depth of peat is 0,2 m, with 31–43% organic matter which classifies it in the organic soil with characteristic muck layer [2] occurred after drainage combined with tillage. Values of bulk density of these layers are in the range 0,33 - 0,53 g cm^{-3}, specific gravity 1,73–2,18 g cm^{-3} and the water capacity are in the range 130–230% what indicate strong mineralised layers.

4.1 The Difference in Organic Carbon Stock in this Research and Before 45 years

As the research of Bogdanović et al. [8] conducted at the Livanjsko polje peatland 45 years ago and before drainage intensification, also contains data on peat properties at different depths, it is possible to quantify data on C content in their studies and compare them with the carbon content calculated in this study, and further, compare it with the CO_2-C emission estimates in the model used in this paper. The calculation of the difference in carbon stocks before and after drainage will be performed especially for deep Histosol in the untouched part of Veliki Ždralovac – zone 1 and especially for shallow Histosol in the reclamation area – zone 4. In deep peat in the untouched part of the peatland, will be used data from Profile 4 from the research of Bogdanović et al. [8] depth 140 cm, and data from this work will be used for Profile 1 depth 100 cm (2012). Profile 1 from this study was estimated as more suitable for comparison, because in zone 2 the surface layer of peat of 25 cm was removed (excavated), and also in favour of this choice is the fact that the depth of Profile 1 should be added and calculated subsidence for 45 years of approximately 30 cm (Table 3) due to the mineralization of deep peat, so that the assumption that the depth of profile 1 before drainage was about 130 cm (100 + 30 = 130), which, true, only approximately corresponds to the depth of Profile 4 of 140 cm from Bogdanović's [8] research (Table 7).

We see that according to this calculation, the difference in carbon stocks between these two profiles is 26,1 kg C m^{-2} (83 – 56,9 = 26,1). When divide this value by the number of years between these two analyses, which is 45, we get an average annual carbon loss rate of 0.58 kg C m^{-2} annual (26,1 / 45 = 0.58) or 5.800 kg C ha^{-1} a^{-1}. The value thus obtained is found between the calculation of CO_2-C emissions by Renger et al.

Table 7. Calculation of organic carbon stocks before and after drainage in untouched zone 1 in deep Histosol.

Profile 4, Bogdanović et al. 1967y. [8]				Profile 1, this study 2012 y.			
Depht (cm)	Bulk dens. (kgm^{-3})	C (%)	C (kgm^{-2})	Depht (cm)	Bulk dens. (kgm^{-3})	C (%)	C (kgm^{-2})
0–20	200	41,5	16,6	0-10	210	36,7	7,5
20–30	175	36,7	6,4	10-20	190	39,5	7,4
30–60	150	31,9	14,4	20-35	130	42,7	8,5
60–90	120	39,5	14,2	35-50	120	43,4	8,1
90–110	155	37,15	11,5	50-65	140	37,7	7,8
110–140	190	34,8	19,8	65-80	220	20,4	6,8
				80-100	340	16,0	10,8
Total			83,0	Total			56,9

[20] from the untouched part of the peatland of 5.210 kg CO_2-C $ha^{-1}a^{-1}$ or 6.682 kg CO_2-C $ha^{-1}a^{-1}$ (Table 3).

In the same way is estimated the difference in carbon stocks in shallow Histosol in reclamation zone 4. As per the formula of Renger et al. [20] the subsidence of shallow peat in reclamation zone 4 for 45 years is about 14 cm (Table 3), it can be assumed that the depth of peat in Profile 4 analysed in this paper (depth 20 cm) before drainage was about 34 cm. Based on this, we will compare the stocks of C (kg m^{-2}) of one such shallow Histosol in Profile 1 from Bogdanović et al., 1967, with the stocks C in Profile 4 (2013) in this study, to obtain an estimate of the annual loss rate of C mineralization in reclamation part of peatland Table.

Table 8. Calculation of organic carbon stocks (kg m^{-2}) in zone 4 –Table in shallow Histosol before and after reclamation (in this study 2012).

Profile 1, Bogdanović et al.,1967 [8]				Profile 4, zone 4, this study, 2012			
Depth (cm)	Bulk dens. (kg m^{-3})	C (%)	C (kg m^{-2})	Depth (cm)	Bulk dens. (kg m^{-3})	C (%)	C (kg m^{-2})
0–15	240	32,5	11,70	0–10	330	20,5	6,74
15–35	220	28,5	12,54	10–20	530	13,9	7,31
Total			24,24	Total			14,05

According to this calculation, the difference in carbon stocks between these two profiles is 10,19 kg C m^{-2} (24,24–14.05 = 10,19). Analogous to the first calculation, when this value is divided by 45 years between these two analyses, an average annual level of carbon loss of 0,2266 kg C m^{-2} annual (10,2/45 = 0,2266) or 2.266 kg C

$ha^{-1}a^{-1}$ is obtained. It is almost equal to the calculation of CO_2-C emissions by Renger et al. [20] for shallow peat, more mineralized, with groundwater depth below 30 cm, where it is 2.200 kg C ha^{-1} a^{-1} or 2.505 kg C ha^{-1} a^{-1} (Table 8).

5 Conclusions

According to Ranger et al. regression equation [20] in the average 2012 year with a dry summer period at non-drained the „virgin" part Veliki Ždralovac (zone 1) with deep Histosol mean groundwater depth was in range 57–85 cm and average annual peat subsidence is in range 0,65 – 0,82 cm and CO_2-C emissions in the range 5.210–6.682 kg $ha^{-1}a^{-1}$. Adjacent belt also with deep histosol that is shallowly drained (zone 2) has slightly deeper mean groundwater depth (66–89 cm) than in zone 1, resulting in a greater annual peat subsidence of 0,71–0,84 cm, just like the rate of CO_2-C emissions which is in the range of 5.765–6.830 kg $ha^{-1}a^{-1}$, as a result to continuous drainage in this zone. The shallow peat (20 cm) in the reclamation zone 4 "Table", with a mean ground water depth of 19 cm, has an average annual subsidence of peat 0,28 cm and the rate of CO_2-C emissions of 2.200 kg/ha/year. In general, the subsidence and CO_2-C emission are higher in the dry 2012 than in the rainy 2013 and in drained deep peat compared to nearby non-drained deep peat or on drained shallow peat in reclamation zone 4. The smaller difference in CO_2-C emissions between rainy and dry years in drained zone 1 with deep Histosol compared with that difference in non-drained zone 1 is because the drainage channels in zone 2 during the rainy year lowered the mean groundwater level, creating conditions for more intensive mineralization. Varying levels of water table monitored in piezometers in deep peat in non-drained "virgin" part of Veliki Ždralovac – zone 1, showed a dry bottom as phreatic minimum in the summer of 2012 for a 45 days period. Further, CO_2-C emission in zone 2 with shallow drainage channels is only slightly higher than in the nearby untouched part of Veliki Ždralovac in zone 1 which implies that undrained zone 1 is still affected by drainage in the rest of the peatland and there one cannot speak of an intact "virgin" peatland.

Most represented type of soil in non-drained zone 1 and drained zone 2 is Histosol (nH-G) with depths of peat body usually from 0, 8-1, 2 m, which are in generally divided in two layers that are distinctive by the level of humification. In the upper lower mineralized layers (0–65 cm) with 75–84% organic matter, bulk density is lower (in range 0,12–0,21 gcm^{-3}) and water capacity is higher (in range 300–640%), than in more mineralized deeper layer with 31–53% organic matter. The result of compaction and mineralization of drained peat in zone 2 is reflected in the increased bulk density and reduced total porosity and retention capacity compared with Profile 1 in non-drained part of peatland. Generally, Histosols are characterized by slight acid reaction in all zones and at all depths. Further, Histosols are characterized by a high content of total N (1,2–3,3%), and C/N ratio ranges between 11,3 and 19,2. In zone 4 (peatland converted into agricultural land), depth of peat is 0,2 m with 31–43% organic matter. Drainage and tillage converted the top layer of peat into a strong mineralized muck layer.

Calculation of difference in organic carbon stocks in Histosol (kg m^{-2}) in this research and research 45 years ago before drainage, confirms the calculation of carbon losses using the formula of Renger et al. [20] on CO_2-C emissions from peatland based on the depth of the mean summer groundwater.

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Residues of Commonly Used Insecticides in Peach Fruits

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Abstract. For the control of *Grapholita molesta* (Busck), one of the most important peach pests, insecticides from various chemical classes are used. Besides pyrethroids and organophosphates, currently, insecticides with a shorter preharvest interval (PHI) and more convenient ecotoxicological properties, such as diamides and spinosyns are registered. However, even if they are applied in accordance with good agricultural practice, the pesticides may leave residues, which require determination of their residual behavior, as well as, persistence. The aim of this study was to assess the dissipation dynamics and half-life (DT_{50}) of the commonly used insecticides for the control of *G. molesta* in peach (chlorantraniliprole, cyantraniliprole (diamides), deltamethrin (pyrethroids), indoxacarb (oxadiazine) and spinetoram (spinosyns)). Above mentioned insecticides were applied at the recommended rate, according to the standard methods (EPPO). A QuEChERS extraction method followed by HPLC-DAD analysis was developed and validated according to SANTE/12682/2019. Based on the obtained results, the half-lives for chlorantraniliprole and cyantraniliprole were 3.15 and 2.5 days, respectively. In this study, the content of deltamethrin and indoxacarb, at the MRLs, were obtained after ten and seven days of the treatment, where DT_{50} was 1.58 and 4.62 days, respectively. After the third and fourth day of the application, the level of spinetoram was below MRL, while obtained half-life was 2.55 days. Results obtained in this study showed that residues of all analyzed insecticides were below the MRL, at the end of the prescribed PHIs residues.

Keywords: Peach · Residues · Insecticides · Dissipation dynamic

1 Introduction

Peach is one of the most cultivated stone fruits. According to Byrne et al. [1], it ranks third in the world in terms of production compared to other continental fruit species, following apple and pear. Along with the increased growth of peach farming areas, the problems and concerns related to its growing are getting more serious. Orchards plant protection is a specific and challenging discipline that aims to protect plants from pests and diseases, while reducing their number to a minimum, producing healthy fruits, and prolonging plants' longevity. Compared to the other fruits, peach has a relatively short

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lifespan but achieves high yields, which requires that its protection should be detailed and carefully planned. Among the harmful insects that lower the quality and yield quantity, the following species stand out: *Grapholita molesta, Anarsia lineatella, Cetonia aurata, Platynota ideeusalis, Myzus persicae, Synanthedon exitiosa* etc. [2].

Grapholita molesta (Busck) stands out as one of the most harmful and widespread pests in peach orchards. To preserve a healthy orchard, it is required to implement integrated control [3]. In the early spring, *G. molesta* interferes with terminal growth and damages fruits in mid-summer. *G. molesta* has more generations per year which depends on the temperature and usually varies from six to seven. The second and third generations are highly harmful and cause most of the damage [4]. In addition to direct damages, there are very significant indirect damages in the form of reduced fruit quality, which leads to large economic losses. The control of this insect relies heavily on the application of insecticides from different chemical classes [5]. Besides pyrethroids and organophosphates, nowadays insecticides with a shorter pre-harvest interval (PHI) and more convenient ecotoxicological properties, such as anthranilic diamides and spinosyns are registered. Anthranilic diamides can be considered as a nearly new class of insecticides that manifest remarkable good control which is achieved through a novel mode of action - the ryanodine receptor. Anthranilic diamides act by selectively activating the ryanodine receptor which is places in the endoplasmic reticulum which leads to paralysis of the muscle contraction and cessation of the insects feeding [6]. The first commercialized insecticide belonging to anthranilic diamides was chlorantraniliprole, which showed great activity on pests from the order Lepidoptera. The second insecticide was cyantraniliprole with display excellent cross-spectrum activity against a wide range of insects [7]. Pyrethroids are synthetic organic insecticides synthesized on the model of pyrethrin, an active substance that naturally occurs in chrysanthemum flowers. These substances are widely used as commercial insecticides. The insecticidal effects are based on the activity of the lipophilic keto-alcoholic esters of two acids, chrysanthemum and pyrethroid [8]. Pyrethroids' mechanism of action is based on their effect on increasing permeability to sodium ions at the level of the Na-channel membrane of arthropod nerve cells. Additionally, pyrethroids increase the spontaneous release of the neurotransmitter GABA. Among pyrethroids, deltamethrin is one of the most commonly used insecticides in the world [9]. The development of the oxadiazines that belong to the new class of pyrazoline-type insecticides has led to the finding of a new insecticide, named indoxacarb. Nowadays it is successfully used as one of the most effective insecticides in crop protection, especially in orchards. Spinosyns are a chemical group of insecticides synthesized on the model of *Sacharopolispora spinosa* metabolites. The mode of action of spinosyns differs from all the mechanisms of insecticidal action described so far. Although not fully studied, spinosyns are known to act as allosteric modulators of nicotine acetylcholine receptors (nAChR) [10]. However, numerous researches have shown that spinosyns also act on γ-aminobutyric acid (GABA) receptors, which makes their mode of action unique [11]. Spinetoram is a new compound from the chemical group of spinosyns with insecticidal action and consists of two relatively close components, XDE-175-J and XDE-175-L. It was synthesized to control butterfly larvae, leaf miners and thrips on various crops [12].

Considering that control of *G. molesta* is mainly carried out by using chemical plant protection products (PPP), an anti-resistance strategy must be implemented. This can be achieved by applying insecticides with different modes of action, combined or individually. From the aspect of food safety, the correct and timely application of plant protection products is extremely important, as well as the continuous control of pesticide residues present in agricultural products, with special attention to products for nutrition. Also, in order to avoid an increased amount of pesticide residues, we strive for maximum optimization of selection and application of currently available pesticides, as well as research of new toxicologically more favorable compounds, all to achieve high efficiency and economy in plant protection, whit the goal of obtaining safe products. Intensive use of pesticides in fruit production may lead to their accumulation at levels higher than MRLs [22]. Thus, assessment of the pesticides dissipation rate after the application is the most important step in the process for the determination of its behavior in agricultural products and for calculation of PHIs. The dissipation rate could be influenced by different parameters, such as climate conditions, pesticide characteristics, type of formulation, way of application, etc. By monitoring the dissipation dynamics, the last moment for the application of pesticides before harvesting is determined, which represents the PHI.

Due to all mentioned, it is highly important to conduct dissipation dynamics experiments in different agroecological conditions. This study was conducted in order to evaluate the dissipation dynamics and half-life (DT_{50}) of the commonly applied insecticides belonging to different chemical classes. Those are active substances used for the control of *G. molesta* in peach orchards.

2 Material and Methods

Field experiments were conducted near Novi Sad (Vojvodina, Serbia), in the 7 years old peach orchards. PPPs based on the analyzed insecticides were applied at the recommended rate (Table 1), according to the standard EPPO methods [13], when the peaches were in the BBCH 75 phenophase.

Table 1. Insecticides

Insecticides	g a. i./l	Formulation	Applied concentration
Chlorantraniliprole	200	SC	0.2 l/ha
Cyantraniliprole	100	SE	0.6 l/ha
Deltamethrin	25	EC	0.5 l/ha
Indoxacarb	150	EC	0.3 l/ha
Spinetoram	250	WG	0.2 kg/ha

With the aim of analyzing insecticide residues, fruit samples were collected after drying of the deposit, and in equal intervals, until the end of the pre-harvest intervals (PHIs).

A single residue method based on QuEChERS [14] extraction method (see Fig. 1), followed by HPLC-DAD, was used for the analysis of insecticide residues in peach fruits. The method was validated according to SANTE/12682/2019 criteria [15], through linearity, precision, accuracy, matrix effect and limits of detection as well as quantification.

Fig. 1. Determination of insecticide residues in peach fruits – extraction and clean up procedure

3 Results and Discussion

High-performance liquid chromatography equipped with a diode array detector was applied for the quantification of insecticide residues [16, 17]. Values of the validation parameters completely fulfilled SANTE/12682/2019 criteria (Table 2).

Table 2. Validation parameters.

Insecticides	Linearity	Precision (%)	Accuracy (%)	Matrix effect (%)	LOD (mg/kg)	LOQ (mg/kg)
Chlorantraniliprole	0.9999	0.97	79.3–95.1	110.2	0.02	0.05
Cyantraniliprole	0.9998	0.57	86.3–93.9	114.3	0.01	0.03
Deltamethrin	1.000	1.39	96.7–106.5	99.92	0.014	0.042
Indoxacarb	0.996	0.27	83.3–91.6	102.39	0.006	0.02
Spinetoram	0.995	1.23	81.2–95.91	97.95	0.01	0.03

The validated methods were used for the analysis of insecticide residues in treated peach fruit samples. Dissipation dynamics were evaluated through the amount of insecticide residues over time. The dissipation dynamics of the analyzed insecticides in peach

fruits were in accordance with the first-order kinetic equation, $C_t = C_0e^{-kt}$, where C_t (mg/kg) is the residue after time t (d), C_0 (mg/kg) is initial residue, and k is dissipation rate constant. This method was primarily adopted to clarify the relationship between residues and time. This model is widely utilized for describing the pesticides fate in soil, as well as in plants [18].

Half-life (DT_{50}) is described as the time it takes for an amount of a pesticide to be reduced by half through degradation (based on initial residue levels after application). The dissipation rate was calculated by using constant value (k) and the equation $DT_{50} = \ln 2/k$ (Table 3).

Table 3. Dissipation dynamics of the analyzed insecticides in peach fruits.

Insecticides	MRL (mg/kg)	Regression equation	R^2	Half-life DT_{50}, days
Chlorantraniliprole	1.0	$C = 2.32e^{-0.22t}$	0.932	3.15
Cyantraniliprole	1.5	$C = 1.46e^{-0.28t}$	0.964	2.5
Deltamethrin	0.15	$C = 1.23e^{-0.44t}$	0.861	1.58
Indoxacarb	1.0	$C = 2.21e^{-0.15x}$	0.852	4.62
Spinetoram	0.3	$C = 0.63e^{-0.27x}$	0.947	2.55

The highest level of cyantraniliprole residues in the peach fruits was straight away after deposit drying (1.46 mg/kg), but still below the EU MRL of 1.5 mg/kg. The residues level of chlorantraniliprole, one hour after the application was 2.32 mg/kg, while the amount of 1 mg/kg was achieved seven days after the treatment. Based on the results obtained in these studies, the calculated half-lives for chlorantraniliprole and cyantraniliprole were 3.15 and 2.5 days, respectively.

PPPs based on deltamethrin and indoxacarb have been intensively used for the control of *G. molesta* in peach fruits for the past few decades. In this study, their content at the MRLs (0.15 mg/kg and 1 mg/kg) were obtained ten and seven days after the treatment. Using obtained results, calculated half lives were 1.58 days and 4.62 days.

The dissipation dynamic of semi-synthetic insecticide, spinetoram, was also evaluated. It is a novel insecticide with favorable ecotoxicological properties. The maximum level of spinetoram residues in peaches was determined after drying the deposit (0.63 mg/kg). In the sample collected on the first day after treatment, the average content of spinetoram residues was 0.54 mg/kg, with a loss of 14.29%. In the sample collected two days after the treatment the amount of spinetoram was additionally reduced to 0.41 mg/kg, indicating that another 65% of the initial amount of this compound remained. In the samples collected between the third and the fourth day after the application, the amount of spinetoram was at the level of MRL (0.3 mg/kg). Seven days after the application of insecticides, i.e., after the PHI prescribed in the EU, the presence of spinetoram in the sampled peach fruits could not be detected, which means that the residues were below the detection limit (LOD = 0.01 mg/kg). Based on the obtained results, a degradation curve ($y = 0.63e^{-0.271}$, $R^2 = 0.9471$) and half-life (DT_{50}) were constructed. By using insecticides based on spinetoram in the amount of 0.3 kg/ha, the half-life in peach

fruits brings out 2.55 days. In sum, results obtained in this study showed that at the end of the prescribed PHIs (7 d - cyantraniliprole and indoxacarb, 14 d - chlorantraniliprole, deltamethrin, spinetoram) residues of all analyzed insecticides were below the MRL.

There is a lack of studies researching the behavior of cyantraniliprole and chlorantraniliprole in crops, with only a few results regarding indoxacarb, deltamethrin, and spinetoram residues in peach fruits. In the previous studies [19], indoxacarb residues (indoxacarb + R enantiomer) in peach fruits after the application of PPP in the recommended rate in Greece were 0.12, 0.13, and 0.18 mg/kg. Moreover, PPP based on indoxacarb applied in the amount of 0.075 kg/ha in Italy, left the residues between 0.16–0.06 mg/kg. The analysis of indoxacarb and spinetoram A residues in peach samples collected on the market showed their presence in the amount of 4.3 μg/kg and 9.2 μg/kg, respectively [20].

4 Conclusions

Given the obtained results achieved in this study, as well as the high efficiency of applied insecticides [21], novel generation compounds, such as anthranilic diamides and spinosyns, can be successfully used for the protection of peaches against *C. molesta*. Better ecotoxicological properties and specific mechanism of action of chlorantraniliprole, cyantraniliprole, and spinetoram significantly contribute to the delay of the resistance occurrence. The level of residues of these insecticides in peaches, determined after PHI, makes their application acceptable from the aspect of food safety.

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Get Ready for Industry 4.0 – Tool to Support Food Value Chain Transformation

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Abstract. According to Conijn et al. (2018) current consumption or degradation of most of the food-related resources (e.g., land, freshwater, fossil energy, and nutrients) exceed their global regeneration rate. At the same time, along the food supply chain, 30% of total production is lost. The majority, 46% to 65% of total food waste is generated on the consumer level (Annosi et al. 2021). The main drivers behind waste generations are overproduction due to market uncertainties and consumer behavior. Those drivers reflect the inadequate structure, power distribution, and management of food supply chains, shaped by the fragmented, altered, and slow flow of information needed to make decisions aiming for improving efficiency and effectiveness of the value chains. There is an urgent need for food system/food value chain transformation to keep food production under the planetary boundaries. The different technology known under the common name Industry 4.0 is seen as a promising solution, enabler of a needed transformation of food value chains. Although the benefit of this technology is well known in practice, the rate of its adoption is very low, especially in emerging and transitional economies. Both researchers and experts are focused on technological and technical solutions, profitability, and fragmented application on one chain entry point (e.g., logistic), while neglecting research and deeper discussion about new business opportunities opened up by the Industry 4.0 ability to provide mass customization and ability to support resilience and trust (quality and safety), needed in time of crisis such as COVID-19 pandemic. The main objectives of this discussion are to bridge this gap and to underlay technological ability to promote short value chains development through global, distributed chain networks as a frame in which each food chain will act as an individual and as a part of longer (even global) food chain.

Keywords: Industry 4.0 · Business resilience · Food value chain · Change management · Mass customization

1 Introduction

The agrifood system is very complex (Farmery et al. 2021), multifaced web of resources, process, activities, people and institutions (Mausch et al. 2020), extended across many

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different phases and multiple locations (Tijan et al. 2019), incorporating flow of knowledge, data/information and goods originated from agriculture, forestry and fishery (Tefft et al. 2017). It is a very complex and vivid system with multiple power dimensions (Mustafa et al. 2021) that reflects and responds to social, economic, political and environmental conditions. The capacity to be resilient, provide business opportunities, decent/attractive jobs, access to sustainable, nutritional, affordable, safe, quality and sufficient food, feed, fiber and fuel to all stakeholders, is the main direct output of agrifood systems (Zhaoa et al. 2019). Therefore, an agrifood crosslinked and the value-added network has implications for the quality of life on the Planet, and therefore it is critical to ensure this interrelated complex system running smoothly and successfully (Zhaoa et al. Zhao et al. 2019). In literature (Agri)food system, (Agri)food value chain, (Agri)food supply chain are terms that are used interchangeably, but they refer to the same concept that is presented in Fig. 1.

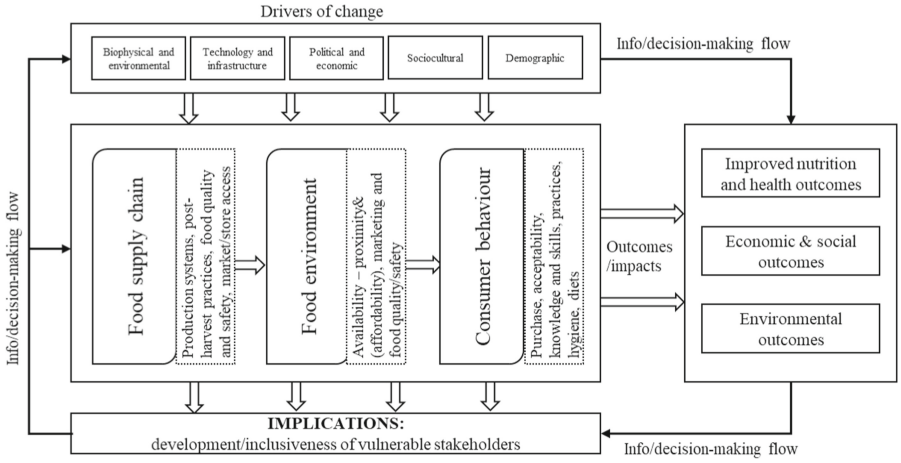

Fig. 1. Concept of agrifood system (based on Mausch et al. 2020)

It has to be outlined that agrifood system relies on experience-based heuristic methods (Braun et al. 2018), so it is reluctant to new emerging techniques, technologies as well as disruptive business models (Mausch et al. 2020). Linear agrifood system organized under the principle "extract-make-dispose" is highly vulnerable and with low capacity to anticipate the need to change to address multifaced, interconnected and very complex challenges that can be roughly divided into three clusters: a) challenges related to sustainability of current system; b) everchanging end-user consumption patterns that reflect societal challenges; and c) low capacity of new, emerging technology adoption which deepens digital gap, and which in turn inhibit so needed re-conceptualization at the system level (Fig. 2).

Challenges related to (un)sustainability of the current highly globalized agrifood system decrease its capability to provide complex and interlinked outputs (Fig. 1) that not only shapes the rate of climate change, environment health, but social and economic wellbeing of each and every person and whole Planet. The current highly globalized

agrifood system is unsustainable and recognized as responsible for ongoing air, soil and water pollution (Sun et al. 2017; Leurent and Abbosh 2018; Farmery et al. 2021), with a high environmental footprint. Agrifood systems account for 70% to 90% of freshwater consumption (Richter and Bokelmann 2016; Leurent and Abbosh 2018; Charania and Li 2020), 25% of GHG (greenhouse gas) emission (Sun et al., 2017; Leurent and Abbosh 2018) and 75% of global ammonia emission mainly from the use of fertilizers and animal waste (Sun et al. 2017). Agrifood emission is likely to increase 15% to 20% by 2050 (WEF 2020a). Such unsustainable food production patterns are responsible for 70% of the reduction of biodiversity by 2050 (Lezoche et al., 2020) and loss of 10 to 15 million hectares of cropland annually, caused by fertilization, pesticides, deforestation and overirrigation (Leurent and Abbosh 2018). In addition, more than 30% of food is wasted across the food value chain, reaching 179 to 290 kg per capita annually in developed countries (Annosi et al. 2021), while increased food waste generation is expected to occur (an increase of 42% in the period 2016–2020 is predicted in EU according to Richter and Bokelmann (2016)). At the same time, due to the inefficiency of the Agrifood system to distribute food, 812 million people are facing food deprivation (Smitha and Floro 2020; WEF 2020a). Different types of uncertainty (weather, market, behavior, etc.) drive both overuse of agricultural inputs (according to Richter and Bokelmann (2016) 2/3 of chemicals in China are overused) and food overproduction shaping an ongoing increase in food waste generation. At the same time, there is a growing need to increase production to feed a permanently growing population that will reach 9,8 billion in 2050 (Charania and Li 2020). Such population growth will request at least a 60% to 90% increase in food production (Richter and Bokelmann 2016; Sun et al. 2017), while at the same time the agrifood system faces both trends of the aging rural population and lack of workforce in rural areas globally. So, the agrifood system is undergoing slow and mostly not-recognized radical transformation to balance food security and quality with sustainability, decreasing environmental footprint and increasing productivity, efficiency while offering attractive jobs. This has to be accounted for all agrifood actors: farmers, processors, distributors, input producers, customers, but also non-governmental sector, expert/business/research community and governments. Currently this is not a case because the awareness of current situation and ongoing change is still low, which contributes to the complexity of existing problems.

Very important challenges connected with everchanging end-user consumption patterns that reflect societal, environmental and economic challenges are not fully recognized by relevant stakeholders, especially acters of agrifood system. According to WEF (2018), in many countries, consumption on average accounts for 80% of GDP, which means that consumption patterns shape business models and the whole economy. So, the shift from mass production to mass customization/individualization is occurring in all sectors of the global economy (Leng et al. 2020). Due to the low level of transparency and traceability, food fraud has become a multimillion industry (Soona and Manning 2019), covering 7% of world trade (Spink et al. 2013), affecting public health and consumer trust. Therefore, consumers increasingly request different sets of reliable information about food authenticity, production and distribution processes, business accountability (Kayikci et al. 2020; WEF 2020c) and environmental performances, especially SDG (Sustainable Development Goals) performances. Due to the

misperceptions of food risk, consumers are willing to either pay premium prices for safety and quality guarantee (168.7% according to Santeramo and Lamonac (2020)) or to become part of alternative food initiatives such as different types of local food (short) supply systems (Tefft et al. 2017), which becomes increasingly attractive (Bhawana and Race 2020) and able to re-establish value-laden connections which empower both customers and producers/farmers (Lioutasa and Charatsari 2020). However, driven by ever-increasing public and policy makers' pressure, the globalized agrifood system has to find a way to increase collaboration and connectivity between all stakeholders by providing reliable and timely lifetime product information to decrease power asymmetry, changing the current production pattern toward mass customization to ensure sustainability. Therefore, the agrifood system is usually seen as a source of problems (Fanzo et al. 2020; Torky and Hassanein 2020) that needs innovative, non-traditional solutions that relies on profound change in the way we think, work and live. The necessary radical change in economic and social structures/institutions behind consumption and production patterns (Belaud et al. 2019) has been started and it is driven by new Industry 4.0 technology in attempt to reconcile economic, environmental and social aspects.

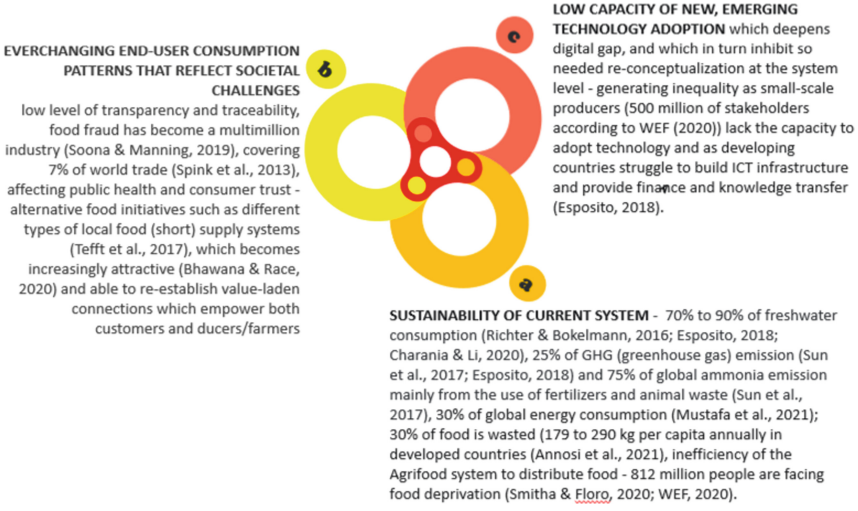

Fig. 2. Challenges of current agrifood system

It is very important to discuss **challenges regarding agrifood system capacity of new emerging technology adoption**. For several decades, the agrifood system has been active in rapid automatization, digitalization and digital innovation (Akyazi et al. 2020; Lezoche et al. 2020; Tian et al. 2020). However, the agrifood system has been recognized as a slow adopter of technology (Braun et al. 2018; Duong et al. 2020). According to WEF (2019), investment in agricultural technology lags far behind other sectors' technological investments and is disproportionately concentrated in certain markets. Investment in technology for the agrifood system in developing countries accounts for only 25%, while 75% of agricultural value-added is generated across developing countries. In addition, 765 million people in rural areas lack access to electricity and less than 50% of the

global population uses the internet (WEF 2019). Such situation deepens and widens the existing digital gap, generating inequality as small-scale producers (500 million of stakeholders according to WEF (2020a)) lack the capacity to adopt technology and as developing countries struggle to build ICT infrastructure and provide finance and knowledge transfer (Leurent and Abbosh 2018). Such situations point out the need to build a mindful approach to digitalization and use of technology because technology is not context neutral (Klerkx and Rose 2020). It is very important not to raise unrealistic expectations regarding new technology to be "silver bullet", to solve each and every problem. Use of the new technology will produce expected improvements and benefits only if it is used to transform the current agrifood system (Lajoie-O'Malley et al. 2020). If it is used just to reproduce the existing system it will become the main drive of problems, not solutions. Those challenges are still underrecognized which can dramatically affect the ability of the food system to provide enough safe, quality and accessible food.

The emerging technology under the common name Industry 4.0 has potential to transform the challenges into opportunities (Lioutasa and Charatsari 2020), providing potential for future development of the agrifood system by decreasing different types of uncertainty and limiting opportunistic behavior (Leng et al. 2020). Up-to-date technological advances prove to be environmentally sustainable, but also socially sustainable from the workforce point of view (Bianco 2016). The application of emerging technologies increases availability and accessibility of reliable information across the whole agrifood system, which in turn increases connectivity and trust among people and institutions "unlocking" the capacity to address very complex challenges by strengthening sustainability through the development of attractive jobs. The deeper emerging technology adoption brings deep, radical changes across the whole agrifood system reshaping structure, role, business and social context, values and means of production opening up opportunities to adopt new business models. So, the agrifood system is on the way to evolve from labor-intensive to technology native system (Charania and Li 2020).

Due to the complex implication of emerging technology application, researchers and expert community brought into focus technical aspects and partial, fragmented solutions that have immediate, easy to understand implication for transparency, food safety and quality, cost-cutting, increased profitability, new business opportunity and improved sustainability through decreasing transport, improving logistics and warehouse and productivity. It means, researchers thinking about technology issues have been focused on profitability, while the capability to create added value is neglected. Up to date literature focus has been on "are", while "do" has been neglected (Lioutasa and Charatsari 2020), meaning there is not too much research providing a holistic view of what can be accomplished by emerging technology. Such an approach fails to raise strong awareness of the digitally enriched agrifood system that has been established, so people, businesses and institutions have to embrace change to survive. Therefore, this paper aims to synthesize results available in literature to explain the shape and structure of future "Technology enriched agrifood system" with a hope of informing future research and practice, considering strategic framework to generate added value. We hope that this simplified bigger picture will inspire small farmers and SMEs to anticipate and embrace change initiating new business models. In addition, preconditions, barriers and some negative implications will be discussed as well. This paper is prepared with the hope to trigger

productive discussion about ways to transform current, unsustainable agrifood system into a future system that is carbon neutral, resilient to different types of shocks, inclusive, pro-small holders, transparent, accountable and able to distribute culturally appropriate nutrition, providing attractive jobs, generating value-added and business opportunities while respecting planetary boundaries.

In attempt to simplify very complex picture of the future technology enriched agrifood systems which is shaped by different, new, multifaced, interconnected trajectories of radical, but still not sufficiently recognized, undergoing transformation process, the first part of this paper (introduction) frames the research question, while in the following part (research method) the approach to develop answers is presented. The results of literature review are presented within part New emerging model of "Technology enriched Agri-Food system" which allows simultaneous consideration of three main aspects that models agrifood transformation: operational change (challenges), new business models (opportunities) and finally, impacts and implications. It points out main important aspects and characteristics of transformation that has to be accounted for and deeply understood by farmers, managers, NGOs, researcher, academia, policy makers and supranational organizations. Started discussion is finalized by conclusions in which some future research trajectories are mentioned.

2 Research Method

Research presented here was conducted in two phases that rely on systematic literature review which according to Nicholson et al. (2021) help to first frame the research question (set a scene) and then provide opportunity to synthetize research outputs to develop mindful answers. The first phase shed light on ongoing change within the Agrifood system driven by a different set of emerging technologies commonly called Industry 4.0, setting the stage for modeling the future – "Technology enriched Agrifood system". In the second phase, a new "Technology enriched Agrifood system" has been presented as a simplified representation of the bigger picture informing business and researchers about the ongoing radical, deep process of change enabling all agrifood actors to understand it and start changing by adopting emerging technologies to use emerging business opportunities. So, research is motivated by two main principles of the contemporary world: (i) An ounce of prevention is worth a pound of cure (Spink et al. 2016); and (ii) Simplicity is the ultimate sophistication (WEF 2021). It means that research is dedicated to small farmers and SMEs who are struggling to plan (Annosi et al. 2021) and anticipate a need to embrace change and adopt new business models.

The first part of the research was a systematic literature review (SLR) which is a method to select the most relevant and high-quality studies from previous literature (Zhaoa et al. 2019). SLR is according to Forcina and Falcone (2021) better approach than traditional one because it is applied through a replicable, scientific, and transparent process. In this way the risk of research bias or non-critical evaluations is reduced significantly. The SLR has been carried out through the three stages, that involve 'planning the review', 'conducting the review' and 'reporting and dissemination' which are suggested by few authors (Tranfield et al. 2003).

Review is focused on the period from 2019 to 2021 and uses "Agriculture 4.0" and "Food system and Industry 4.0" as keywords to undertake search through Sciencedirect, which is recognized as the most relevant. In total 5850 papers were reviewed within two stages: (i) rapid-review searching through summary to select duplication and papers that are either pilot studies or literature reviews and (ii) full-text review resulted in the inclusion of few most relevant papers outside of the search period. In this phase a search through the World Economic Forum (WEF) database of developmental reports was also conducted. Finally, 89 papers and WEF reports had been selected.

Based on SLR results, the second phase shed light on the simplified systematic and holistic paths of ongoing change driven by emerging technologies but fueled by new opportunities to generate value +, underpinning the distinct nature of companies, providing extended customer satisfaction, while in the same time addressing pressing challenges, such as sustainability. The final result is a model of the future "Technology enriched Agrifood system" that is created based on the framework (Fig. 3) and assumptions regarding capabilities of the technology-enriched supply chain (Fig. 4).

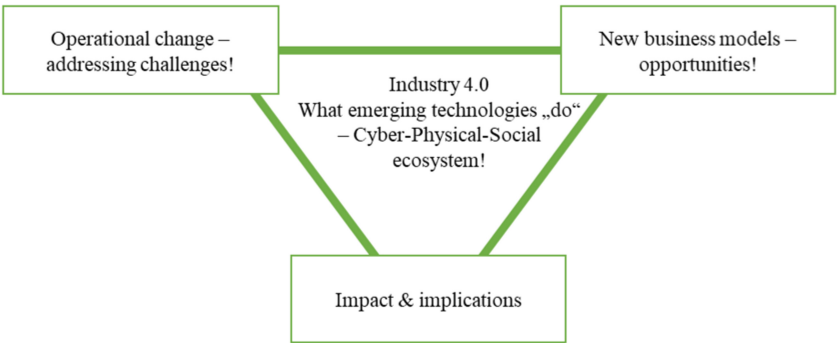

Fig. 3. Frameworks to present a future agrifood system model - simplified bigger picture

The model is based on an integrated multifaceted approach to digital transformation (Fig. 4) and on identified trends relevant to the business to expand its scope of anticipation and to support stakeholders' decision-makers to succeed in the digital-led business model. This framework will ensure a description of how digital transformation will change the way business, collaboration and transformation take place in a new technology-enriched agrifood environment, and how digital transformation impacts across all levels of supply chain and company departments. It will enable a better understanding of innovative technologies, methodologies and tools used to enrich and improve strategies, processes and activities of the company.

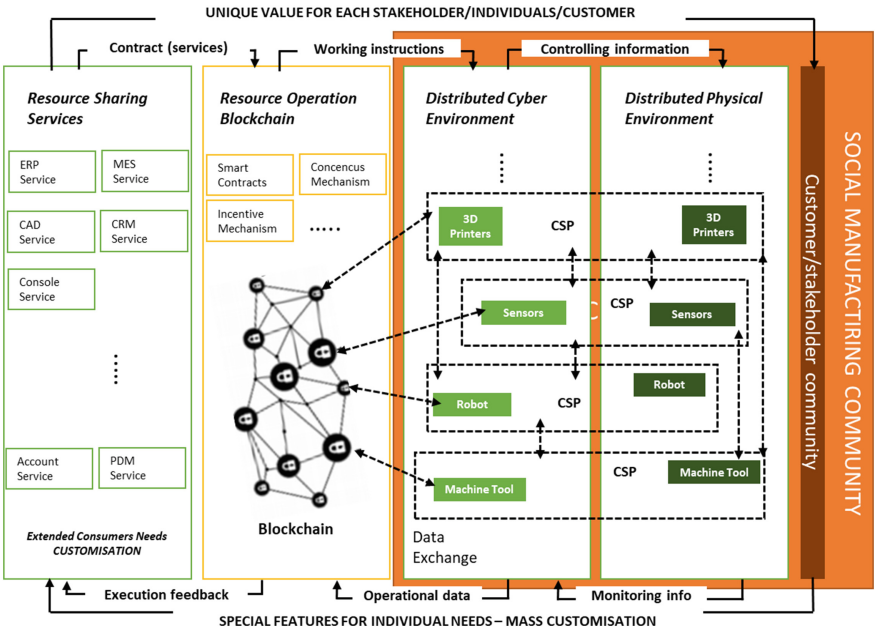

Fig. 4. "Technology enriched Agri Food system" unlocking mass customization (adapted from Farmery et al. 2021; Yu et al. 2020; Zareiyan and Korjani 2018; Leng et al. 2019)

3 New Emerging Model of "Technology Enriched AgriFood System"

The here presented discussion is based on systematic literature review results and structured to provide deep "panoramic view" that simultaneously considers discovered challenges (operational changes) and opportunities (new business models) driven by Industry 4.0 technology along with clearly stated impacts and implications. The way discussion is structured points out complexity of this radical transformation and facilitate understanding of main drivers, characteristics and implication of undergoing change that will shape way we do business, make decisions, live and enjoy life. Change is coming, but for all food system actors it is important to anticipate its consequence and prepare in order not to be "wiped off the face of the earth". Therefore, first we will discuss general characteristics and consequences of Industry 4.0 technology application. After that changes and opportunities as well as some radical innovations in everyday operations along whole value chain will be presented. Finally, implications and impact of Industry 4.0 technology "enrichment" as well as change in social and economic structures will round up explanation that provides "panoramic view" of Technology enriched Agrifood system.

3.1 General Characteristics and Consequences of Industry 4.0 Application

Currently, the world is moving towards a new era focusing on the digital transformation that involves adoption and future improvement of the Industry 4.0 technologies, applications, and solutions that are transforming the production capabilities of all industries, including the Agri-Food domain (Charania and Li 2020; Lim et al. 2021) to change contemporary challenges into the new business opportunities through communication, information and intelligence (Bai et al. 2020) enhancing data-driven processes making it more informed, efficient, profitable, secure, safer, environmentally friendly, sustainable and resilient (Lezoche et al. 2020). Industry 4.0 refer to cutting edge technologies (Zareiyan and Korjani 2018) that connect cyber and physical objects with the main agenda to enhance the level of data generation, usage and information integration across the supply chain (Esmaeilian et al. 2020) wich results in creating an engaging interactive automated activities (Sestino et al. 2020) focused on intelligent, anticipative, self-organising, self-structuring business processes allowing value generation and innovative services (Esmaeilian et al. 2020) to improving quality of life for all.

According to Bai et al. (2020), Industry 4.0. technologies can contribute to (i) economic dimension (reduced set-up times, shorter lead times, reduced labor and material costs, decreasing logistics effort, such as order delay, damage to goods, errors, and multiple data entries (Tijan et al. 2019), while increasing production flexibility, productivity and customization); (ii) ecological dimension (managing system to work smarter minimising waste (Sestino et al. 2020), allowing reuse, recycling, or remanufacturing and reducing energy and resource consumption through detection and data analysis across production and supply chain processes, decreasing the environmental footprint, (Lioutasa and Charatsar 2020); (iii) social dimension offering attractive jobs, while smart and autonomous production systems can support employee health and safety, by taking over monotonous and repetitive tasks; resulting in higher employee satisfaction and motivation (Bai et al. 2020).

Industry 4.0 encompasses two groups of emerging technologies: physical (eg. additive manufacturing, or sensors and drones), and digital technologies (modern information and communication technologies, such as cloud computing, blockchain, big data analytics, and simulation) (Bai et al. 2020). The industry 4.0. technologies are fast-growing, while some of them (Internet of Thing (IoT), artificial intelligence, 5/6G networks, serverless computing, Blockchain, Robotics, Biometrics, 3D printing, Augmented Reality/Virtual Reality, and Drones) are among the top ten emerging technologies in 2019 according to the Computing Technology Industry Association (Esmaeilian et al. 2020). According to Akyazi et al. (2020), those digital technologies are seen as key enabling technologies (KETs) which facilitate a new phase of automation allowing (partial) transfer of autonomy, intelligence and autonomous decisions to machines (Tijan et al. 2019). A more detailed explanation of Industry 4.0 most prominent technologies is provided in the Annex 1.

Industry 4.0 is focused to ensure a constant flow of trustful real-time product lifecycle data and preventing tampering and single point failure through offering fault-tolerance, immutability, trust, transparency and full traceability (Zhao et al. 2019), developing an authentic chain of records of the food ecosystem (Kayikci et al. 2020) trough application of mathematical principles of asymmetric cryptography, which allows users to make

deals with partners (Leng et al. 2020) ensuring anonymity while keeping accountability (Tijan et al. 2019). This way, adoption of innovative Industry 4.0 technologies diminishes information asymmetry, which paves a path to resolving major challenges, such as traceability, trust, and accountability in the food industry (Kayikci et al. 2020). This emerging technology allows building of distributed peer-to-peer network of actors within the supply chain enabling efficient sharing of all resources, unlocking mass customization through the empowerment of customers and engaging all stakeholders, including administration and academy to radically change the business paradigm, but also the way we live. Therefore, according to (Zareiyan and Korjani 2018) manufacturing is not anymore about where to locate production, but it is about changes in customer demand, resilience in the supply chain, and cost factors. It means both companies' and economies' future depend on effective use of data that drive stakeholder value through the development of new forms of collaborations (WEF 2020b) that are authentic and constructive without a hierarchical approach. The adoption of emerging technologies enables smooth and smart governance of complex, changing and interconnected economic systems that are flexible and adaptable with high transformative potential (Fielke et al. 2019).

However, according to Bai et al. (2020) Industry 4.0 technologies also bring many challenges and limitations to society (reduced employment, information security issues, data complexity, electronic wastes, and poor quality can prevail) and it seems to work in large scale organizations whereas SMEs, especially in developing nations, struggle to achieve sustainability effectively (Yadav et al. 2020). It will impact working paces through the process of servilisation where most of the centralized physical factories are replaced with more cloud-based decentralized locations, requesting different skill sets, flexibility, decision-making capabilities from working forces (Esmaeilian et al. 2020).

Although imperfect, Industry 4.0 technologies are evolving, providing low-cost, easy-to-use systems and therefore they will potentially provide tremendous innovation and competitiveness growth to improve industrial system sustainability (Bai et al. 2020) and develop potential to benefit all 17 United Nations Sustainable Development Goals (Yadav et al. 2020).

3.2 Operational Change – Addressing Challenges of Agrifood System Transformation

The current Agrifood System, through adopting the technology will evolve into a distributed sparkled peer-to-peer network promoting fast, accessible continuous flow of data/information coming from trusted sources, verified by blockchain consensus mechanisms (51% of nodes have to verify it). It will enclose multiple actors (individual subjects), different groups, but also a plethora of parallel short/local/regional food chains into one highly collaborative cyber-physical-social system. The plurality and diversity of agrifood system actors make it an inclusive decentralized agrifood community, that will become an open collaborative platform engaging all (especially customers) in building new customised/individualised products, while the actor's resources can be shared across a network. It is a new innovative structure promoting the use of smart contracts (software algorithms) for each and every transaction along the product life cycle (even after consumption - extended producers' responsibility (CGS 2021)) enabling development of product life cycle immutable records that diminish the role of intermediates (banks, lawyers, etc.), risk of censorship and decrease transaction costs. In such a way, each

actor can remain transparent, accountable, but anonymous in the same time. A continuous flow of "free and accessible" real-time data together with big data technology and artificial intelligence will decrease all types of uncertainties and information asymmetry across Agrifood systems enabling optimisation, boosting trust and collaboration instead of hierarchical coordination. Way digital solutions will induce system-level changes making it flexible and adaptable, able to act and react, so the system becomes auto organising, self-optimizing, self-structuring and self-nurturing network. It will change relations, norms and the way the business is done (see Fig. 4).

The empowerment of engaged customers and local communities through trustful, accessible real-time lifecycle product information will pressure Agrifood system to evolve adopting technology to address complex and interconnected challenges to increase economic, environmental and social sustainability. So, permanent system evolution will be driven by both faster adaptation of different digital solutions and by strengthening customer interest in food provenience, authentication and alternative food system, resulting in numerous very distinctive local/regional decentralized peer-to-peer networks of short agrifood systems. At the same time, a new global agrifood system will arise connecting on different levels both autonomous food actors and these new, agile short/local agrifood systems into one distributed peer-to-peer network/community able to share resources, knowledge to offer highly customised products and services, as it is presented at the following figures. The new "Technology enriched Agrifood system" will be a net of interconnected, interrelated short food chains, food hubs (groups) and individual actors. In other words, it will be a connected system of value-laden systems (Lezoche et al. 2020).

New structure of emerging Agrifood system and digital solutions (immutable product lifecycle transaction records) will enable the shift of trust from the organisation (peer) to analytics, automated smart contract (Esmaeilian et al. 2020), so it will be possible to remain anonymous, but still highly accountable (Tijan et al. 2019; Annosi et al. 2021) as well as to provide accessible truthful information to all actors. Such an approach boosts trust supporting a new distributed collaboration paradigm (Leng et al. 2019), necessary to deliver individualised products on acceptable costs and diminished negative impacts. At the same time, such structure without centralized source of power and increased capacity to monitor and control operations (blockchain smart contracts) will diminish different entrance barriers for small and middle-size food producers, processors and distributors, which are very relevant for food and nutrition security (Tisenkopfs et al. 2020). At the same time, this AgriFood system enables a more even distribution of benefits, which decreases profitability margin pressure for small producers, while enabling them to invest in future technology adoption. This means that adoption of digital solutions will radically change relations between actors generating and harmonising common values and goals promoting a highly inclusive collaborative community able and willing to provide public goods increasing food accessibility and affordability for all.

Constant flow of immutable real-time data, IoT, big data technology, machine and deep learning, and AI boost system capabilities to predict (better planning, optimised resource use), to control (capability to identify and correct problems) and to harmonise and optimise all processes along and across the whole agrifood system "from stable to table". Technology solutions encompass real-world and corresponding digital models

into new and complex physical-cyber-social ecosystems with the capability of self-organising, self-structuring (Charania and Li 2020), and the ability to make instant decisions without or with minimal human interaction (Lim et al. 2021). In such way, system becomes more context-sensitive and able to anticipate even unexpected changes, but also enables us to be better prepared in the cases of crisis and disruption. This will strengthen the "Technology enriched Agrifood system" resilience (Tefft et al. 2017; Annosi et al. 2021).

Transparency and traceability as a major driver behind the prevention of customer trust system omissions, including food fraud, will be ensured in an almost effortless and seamless way through a robust tracking system (Lim et al. 2021) developed within the process of technology adoption. Better coordination and the ability to share resources shift the paradigm in the way transactions are made, decreasing the role of intermediates (Kamble et al. 2020), speeding up the delivery process through significantly shorter routes affecting energy dependence and carbon footprints. As a result, the need to transport goods and the cost of operating the supply chain will be drastically decreased from the current 2/3 of the final cost of goods (Kamilaris et al. 2019). System capability to track and trace all costs, especially cost of positive and negative externalities will enable all parties to report environmental footprints, which will in turn raise capability to detect and share best practice and to promote innovations while creating knowledge to raise a better understanding of the whole system. According to CGS (2021), corporate standardized environmental impact is very high on EU, UN, G20's and individual countries policy agenda to find solutions for activating multiregional emissions trading systems, which currently include only a few key participants preventing the generation of substantial welfare gain. It means, emerging "Technology Agri Food system" capability to measure what matters to society, customers and business will induce development of sustainable consumption and production patterns addressing contemporary and future challenges. This It means that the new "Technology enriched agrifood system" has high self-nurturing potential that shifts all actors toward more sustainable development trajectories. In addition, implementation of public policies promoting sustainability can be more precise, targeting specific actors, businesses with low levels of sustainability performances (taxation etc.) and promoting good practices. Therefore, the mentioned digital solution focused on strengthening traceability and transparency can be a soft alternative to the classical way of regulating the modern food market (Charlebois et al. 2016).

New physical-cyber-social ecosystems promoting system prediction capabilities are the main tool to decrease uncertainty at very different levels of agrifood system preventing overuse of inputs and resources as well as overproduction, which is mainly responsible for the generation of food and other types of waste and emissions across the system (Pachayappana et al. 2020). Application of "Technology lead initiatives" together with system "prediction capabilities" will dramatically decrease food waste, which in turn reduces the need to engage new land/water and other resources protecting natural habitat and biodiversity. "Technology enriched Agrifood system" development trajectory will accelerate systemic and radical improvements while promoting sustainable and accountable production and consumption to stay within Planetary boundaries (Conijn et al. 2018). Therefore "Technology enriched agrifood system" will be equipped to address the biggest global challenges - sustainability and climate change (Table 1).

Table 1. Summary of methods and approaches for "Technology enriched Agri Food System" decision-making processes (adapted from Lezochea et al. 2020)

Agriculture supply chain domain	Recommended key/relevant decision-Making methods
Risk management	Multi-Criteria decision-Making & Interpretive Structural Modelling, Hazard analysis and critical control points, Chain Traceability Critical Control Points
Collaboration	Supply Chain Collaboration Index, Policymaking
Governance	Transaction cost economics, vertical integration, product development and diversification
Cold chain management	Fuzzy interpretive structural modelling, Structural self-interaction matrix, RFID Technology, Time–temperature data loggers, Microbiological analysis
Globalisation	Six T's, surveys, Define–Measure–Analyse–Improve–Control, Inspections
Information and communication technologies	ISO 22000, Radio Frequency Identification, Critical Control Points, Food Traceability System, ITC hubs, Production planning, five-point Likert scale, ANOVA, surveys, ORACLE database management, EDI, iterative design steps a proof of concept, Cordys, IBM Websphere, SAP Netweaver, Microsoft Biztalk, B2B
Logistics	Two-phase solution approach, Capacity analysis, mixed integer program, multi-objective optimization, two-echelon location–routing problem, Genetic Algorithms, sustainable supply chain network design, multi-objective mixed-integer programming, triple bottom line, Particle swarm optimization, System of Quality Safety Control, ERP Systems, Automated Information Systems, Digital control systems
Short food supply chain	Policy reports analysis, labelling, Marketing Challenge regulations, resilience analysis, information sharing, vertical integration, interviews
Sustainability of ASC	Conceptual models, Food supply chain economic analysis, labour productivity, data analysis, life cycle assessment

3.3 New Business Models – Opportunities for Development

Adoption of digital solutions will generate higher levels of trust and new forms of collaboration (WEF 2020b) turning the agrifood system into an open platform to boost the exchange of knowledge and ideas with all stakeholders to generate value (Zylbersztaj 2017; Zareiyan and Korjani 2018). The pioneering industry-academy-stakeholder open platforms are designed to capitalise on the ability to share resources and knowledge among all actors and utilisation the local expertise to provide richer stakeholder experience to advance transformative capacity making the system more sustainable and resilient (Tefft et al. 2017). New data-driven business paradigms empower customers to become an active part in value creation and product and service customisation. Such customer pressure together with increased customer demand to be informed (WEF 2021) about all food system activities raise a sense of community (Annosi et al. 2021) promoting self-authentic products as a basis for extended customer satisfaction (Soona and Maning 2019) at the point of purchase or consumption. Emerging technology together with the possibility to share and optimise resources across Agri Food network advance the influence of customer behaviour patterns such as the adoption of a healthier diet, reducing waste, and valuation of more sustainable and healthier food products (WEF 2020a). Strong customer pressure along with increased customer demand to be informed (WEF 2021) about all food system activities raise a sense of community (Annosi et al. 2021). Such an approach boosts innovation and provides new attractive business and job opportunities. Therefore new business models, defining how resources will be used for value creation (Esmaeilian et al. 2020), shaped upon concepts, methods and tools of the digital environment support a few separate development trajectories: (i) **Servitization or "manufacturing as a service"** is a business model that focuses extended customer and shareholder experience offering integrated product-service experience striving for distinct added values for customers, local economies, environment and business. Another example of servilisation is Software-as-a-Service (SaaS) whereby software applications are made available on-demand in exchange for a subscription fee enabling end customers to nearly eliminate the need to plan and maintain an IT infrastructure (Charania and Li 2020). (ii) **Social manufacturing** is another business model in which open design platforms allow broad participation in designing and producing products. They use capabilities embedded in online community platforms to co-manufacturer products. All stakeholders are engaged while customers are taking the lead role. The sense of community is built as an added value that enables sharing of knowledge, good sustainable practices and decreasing uncertainty which is allowing smooth and smart operations in minimising wastage. (iii) **Community-based platforms** for sharing economy that includes sharing or collaborative consumption as peer-to-peer exchanging, giving, or sharing consumer goods, enabled through community-based online platforms. More sustainable consumption and production opens up an opportunity for such business model. It is underpinned by "green customer behaviour", but also by new legislations regarding environmental impacts. According to Esmaeilian et al. (2020) examples of such platforms are food banks, accommodation sharing in the tourism industry (e.g., Airbnb, Couchsurfing), ridesharing for mobility (e.g., Zipcar, Uber), peer-to-peer employment markets

(e.g., TaskRabbit, PeoplePerHour), resource sharing in waste disposal, and production-consumption and the ICT industry (e.g., Freecycle, Peerby). **(iv) Cognitive manufacturing** is according to Leng et al. (2020), a business model where artificial intelligence, data analytics, and deep learning technologies are combined for cognitive configuration and operation of logistics, equipment, and quality management. In cognitive manufacturing, the data mining process analyzes the context of equipment operation and worker motion captured by massive sensors, making it possible for advanced decision support, including process monitoring, fault diagnosis, and trend prediction. It envisages the open business model offers opportunities for small and medium enterprises to fulfill various customer needs in an innovative crowdsourcing manner. Example of such opportunities are scalable blockchain-based cross-enterprise framework to achieve the secure sharing of manufacturing knowledge and resources in open manufacturing ecosystems, thereby enabling the manufacturer to provide flexible, high-quality, and efficient services. The increasing gap between farmer's expectations and the ability of the government led extension services has created a big business opportunity that provides knowledge and information shaped digital-based techniques, such as ultrasound, digital photographic techniques, light sensors, high-resolution radar images, high-resolution X-ray computed tomography, stereo vision and LIDAR sensors; have innumerable applications in agriculture (Lezoche et al. 2020). **(v) Circular economy** – According to Martín et al. (2017) this concept is used to convert an open-end system into a circular system when the relationship between resource use and waste is considered. Applying the first law of thermodynamics, where the planet is seen as a closed system. Thus, circulating matter and energy in the economic system would reduce the number of inputs and limit the increasing entropy. A circular economy involves the adoption of sustainable standards at the company level, an increase in the responsibility of producers and consumers, the use of renewable technologies, as well as more sustainable, clean and stable policies and tools. According to Esmaeilian et al. (2020) model better balance between the economic aspect and the environmental and social aspects of sustainability through the application of the principle "Regenerate, Share, Optimize, Loop, Virtualize, and Exchange" (ReSOLVE). This approach aims to keep products/materials in use, by design, for as long as possible to capture their maximum value, e.g., sustainable supply chains and post-production consumption (WEF 2020c). The circular economy is a certain trajectory for the AgriFood system striving to secure the sustainable production or extraction of nutraceutical components from food waste, using emerging technologies (Galanakis 2013, 2020).

3.4 Impact and Implications

The new business models are working. The WEF (World Economic Forum), 2015 presented 31 practice applications undertaken by responsible corporations achieving revenue uplifts of 5–20% for responsible products, overall supply chain cost reductions of 9–16%, brand value increases of 15–30% and carbon gas reductions of 13–22%, among other benefits. In addition, the costs of deep decarbonization across supply chains are surprisingly low and result in an increase of only 1–4% on end-consumer prices. So, new business models are a real opportunity to ensure more sustainable development trajectories for emerging "Technology enriched Agri Food System". Adoption of both Industry 4.0 technology and new business models' approach will allow an increase of capability to

generate value-added and improve business performance while raising new sustainable competitive advantages (Sestino et al. 2020). A synergistic sweet spot of "Technology enriched Agri-food System" stakeholder collaboration across the globe should not promote a combination of over-represented technology and underrepresented nature. It has to be shaped by the guiding principles of 3S (smart sustainable and safe) guiding principles. As it is presented on the Fig. 5. Technology enriched Agrifood system develops capability of actors to benefit from the change transferring challenges into the opportunities. But, trust, co-option (instead competition), untraditional solutions, creativity and full engagement of all stakeholders are needed to "bear fruits" from deep radical food system transformation bearing.

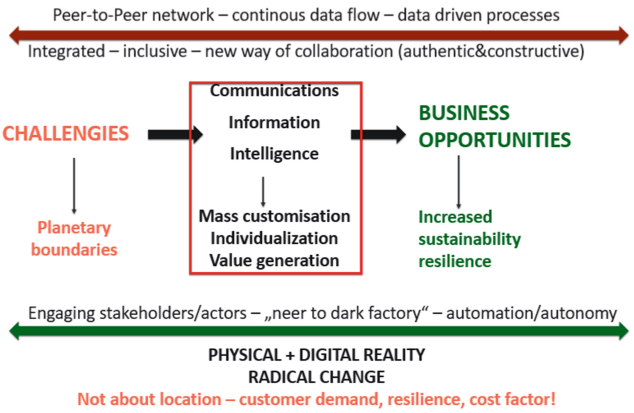

Fig. 5. Transformation of challenges into the opportunities enabled by Industry 4.0 technologies

Technology adoption brings complex, interrelated and interconnected system levels and individual benefits. However, emerging technology is connected with a set of disadvantages that impacts both sustainability and resilience of new emerging "Technology enriched Agri Food system". Those disadvantage are connected with: (i) lack of trust (Esmaeilian et al. 2020) and difficulties to coordinate with partners that are different in size, mindset, resource compatibility and involvement (Annosi et al. 2021), (ii) privacy, ethics and security of data/informations which requires international alliance to develop new crossborder rules that encompass the digital economy to create a truly decentralized data economy providing content that is safe, trusted, and user empowered (Duong et al. 2020); (iii) standardisation of data format, technology, compatibility ports, interfaces and infrastructure and absence of government regulations to provide internationally optimised standards to boost connectivity and diffusion of smart technology (Davidson et al. 2017; Braun et al. 2018; Glass et al. 2018; Karimanzir et al. 2019; WEF 2019; Akyazi et al. 2020; Bai et al. 2020; Kayikci et al. 2020; Lioutasa and Charatsari 2020; Lezoche et al. 2020), (iv) accessibility and affordability that is shaped by relatively high cost and lack of specific skills like managing complexity, complex information processing and abstraction decreasing capability of SME and small farmers to become part of Agri Food chain (Davidson et al. 2017; Glass et al. 2018; Akyazi et al. 2020; Bai et al. 2020; Lezoche et al. 2020; Annosi et al. 2021); (v)high environmental footprint, shaped by energy consumption (Braun et al. 2018; Zhao et al. 2019), (vi) missing innovative

cooperation partners such as universities or start-ups and funding programs by the government (Glass et al. 2018), (vii) capability to plan adoption and to maintain technology due to systemic complexity of cyber-physical-social system (Lioutasa and Charatsari 2020) that require sophisticated knowledge, constraint upgrade of technical solutions and generate high energy costs (Kamilaris et al. 2019; Bai et al. 2020).

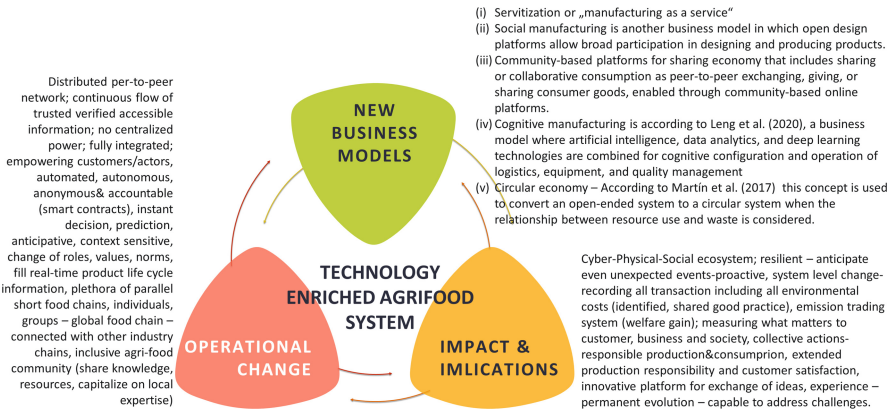

Fig. 6. Technology enriched Agrifood system – opportunities, challenges and implications

4 Conclusions

Contemporary global Agri food system is very complex encompassing a plethora of different interconnected and interrelated actors but increasingly disaggregated with increasingly stringent national laws (Leng et al. 2020) and highly centralised, hierarchical, monopolistic, asymmetric and opaque, while transaction complex and paper-heavy settlement processes relying on so accountable, but very powerful intermediates which increase cost, but decrease transparency making business activities vulnerable to fraud (Kamilaris et al. 2019; Zhao et al. 2019). Such a situation results in serious trust problems, which make the system highly inefficient and reluctant to change and outside of Planetary boundaries, or better to say according to Conijn et al. (2018) current consumption or degradation of most of the food-related resources (e.g., land, freshwater, fossil energy and nutrients) exceed their global regeneration rate. At the same time, digitalization and adoption of new emerging Industry 4.0 technologies are neglected (Esteki et al. 2019; Duong et al. 2020). Agri food system has to be transformed, but it requires a multipronged approach and effective multistakeholder collaboration (WEF 2019) to boost digital solution adoption, which is seen as a most promising tool to transform complex and interconnected challenges into the window of new business opportunities. Therefore, the adoption of technology must be seen as a driver transforming the way business is done and promoting an endless number of innovative business opportunities.

Emerging innovative Industry 4.0 technologies promote new ways of doing business and push agrifood system transformation towards emerging "Technology enriched Agrifood systems" which rely on robust information tracking systems aiming to generate a

continuous flow of real-time trustful data/information easily accessible by all actors. The synthesis of systematic literature research results is presented as "panoramic view" of "Technology enriched Agrifood system" which integrates challenges opportunities and implications in one meaningful model (Fig. 6) drawing-up paths of change.

Fig. 7. Agri Food System improvements contribute to the advancement of SDG's (WEF 2019)

Such approach connects individual actors, groups and parallel short/local/regional supply chains into distributed peer-to-peer network that promote a new way of responsible collaboration and trust, which in turn raises the capability to make instant decision and transaction which lead to harmonisation and optimisation of all processes decreasing uncertainty which is the main driver of overproduction and overuse of resources. The innovative structure of "Technology-enriched Agri Food System" is a highly transparent and traceable cyber-physical-social system led by end-user extended satisfaction which unlocks mass customisation through open-architecture products supporting share of knowledge and resources which promotes responsible production and consumption. This new modern emerging "Technology enriched Agri Food System" that is capable of self-optimisation, self-structuring and self-nutrition, strives to achieve: (i) high level of sustainability and resilience (driven by policy and legislation pressure), (ii)increased productivity and efficiency while optimising externalities (driven by scarcity of resources and change of customer behaviour pattern), (iii) promoting inclusiveness and enabling small farmers and SME to have partner position within the system, (iv) high level of benefits redistribution across and between all actors. In short, system-level change is about building a sense of community and collaborating to integrate and promote climate-agri-food system - health nexus, which rely on innovative open responsible research and innovation platforms necessary to support a company's capacity to understand and anticipate a need to be a changed to stay in business. In such a way "Technology-enriched Agri Food System" contributes to SDG as it is shown in Fig. 7.

The existing digital gap is the main obstacle to the transformation of the Agri Food System towards reality previously assumed to be inoperable (WEF 2018). Aplication of Industry 4.0 technology is still limited to few innovative firms within developed economies (Klerkx and Rose 2020). Therefore digital infrastructure has to be developed as a common good by the international alliance to connect 4.1 billion people in the world who are currently deprived of access. It should be approached in a non-legal but economic sense because digitalisation opens up many hidden opportunities which utilisation will

bring benefits to all and move forward the whole system and empower new business models (Mausch et al. 2020) and data-driven innovation. Therefore it is necessary to introduce a new business paradigm that is about moving from profit maximization to value optimization (CGS 2021).

Currently, there is an extraordinary opportunity and responsibility to reorient our market economies to intentionally serve the interests of societies (CGS 2021) to build an urban-rural continuum that will within "Technology enriched Agri Food System" offer an attractive and exciting working environment for urban working force addressing one of the most important challenges, aging population and lack of skillful working force in rural areas.

Annex 1

Annex 1. Definitions of various Industry 4.0 technologies (Zareiyan and Korjani 2018; Tijan et al. 2019; Bai et al. 2020; Kayikci et al. 2020; Sestino et al. 2020; Forcina and Falcone 2021)

Technologies	Definitions
Additive manufacturing (3D printing)	Is a manufacturing technology that creates three-dimensional (3D) solid objects using a series of additive or layered development frameworks. It is for those with ambitions to expand into new markets, companies, or individuals that are looking to bring more value to their consumers, innovators, researcher, and scholar in the manufacturing industry. It enables open product architecture
Artificial intelligence	Is an area of computer science that emphasizes the creation of intelligent machines that work and react like humans. It enables the system to become active (not only reactive) and to anticipate and predict the future
Augmented reality	Is a type of interactive, reality-based display environment that takes the capabilities of the computer-generated display, sound and other effects to enhance the real-world experience. Such technology establishes a new human-machine interaction, through hi-tech systems ever more sophisticated and smarter
Autonomous robots (Robotics)	Are used to replicate human actions in manufacturing
Big data and analytics	Refer to the strategy of analyzing large volumes of data that are used when traditional data mining and handling techniques cannot uncover the insights and meaning of the underlying data. They can be defined as structured data such as organizational databases, and unstructured data generated by new communication technologies such as the IoT, as well as images, videos, audio (Lansley and Longley 2016). It generates torrents of data by connecting and monitoring people. By leveraging Big Data, businesses can make more effective decisions

(*continued*)

Annex 1. (*continued*)

Technologies	Definitions
Blockchain	Is a distributed database that maintains a complete, distributed and non-tampering continuously growing list of records using new encryption and authentication technology and network-wide consensus mechanism. A selection of smart contracts can be included in the blockchain platform to facilitate secure communication between the users and machines. It is primarily recognised for secured crypto money transactions (Crosby et al. 2016) but can deal with asset ownership, acceleration of transaction time, cost reduction, and reduced risk of fraud
Cloud	Refers to any IT services that are provisioned and accessed from a cloud computing provider. Thanks to a digitalized environment, it is possible to manage and perform tasks more efficiently, protecting operating profits and cash flows
Cobotic systems	Is a robot intended to physically interact with humans in a shared workspace
Cybersecurity	Refers to preventative methods used to protect information from being stolen, compromised, or attacked
Unmanned aerial vehicle (Drones)	Is aircraft without a human pilot onboard, and commonly known as a drone
Global Positioning System (GPS)	Is a technical marvel made possible by a group of satellites in Earth's orbit that transmit precise signals, allowing GPS receivers to calculate and display accurate location, speed and time information to the user
Industrial Internet of Things	Is the various sets of hardware pieces that work together through the internet of things connectivity to help enhance manufacturing and industrial processes
Mobile Technology (eg. Mesh networks)	Is the wireless communication technology integration based on the wireless devices. A mesh network is typically a local network in which the hosts are interconnected directly and cooperate to route data between clients, addressing the low data-rate devices powered by batteries, typically used in home automation applications. Mesh network protocols include Bluetooth Mesh Networking, Wi-Fi EasyMesh, Thread, and Z-Wave. Mesh networks self-organize and self-configure making them fault-tolerant and resilient

(*continued*)

Annex 1. (*continued*)

Technologies	Definitions
Nanotechnology	Also now referred to as molecular nanotechnology, is the particular technology to control individual atoms and molecules for the fabrication of macroscale products
RFID (radio frequency identification), NFC (near field communication)	Refers to data-capture technologies that use wireless communication between an object (or tag) and interrogating device (or reader) to automatically track and identify such objects RFID, NFC is used for issues related to the safety of products, storage places, transports, environment and operators
Sensors and actuators	Is a device that responds to a physical stimulus (such as heat, light, sound, pressure, magnetism, or a particular motion) and transmits a resulting impulse (as for measurement or operating a control)
Simulation	Refers to technologies that use the computer for the imitation of a real-world process or system
Software-as-a-Service (SaaS)	Whereby software applications are made available on-demand in exchange for a subscription fee enabling end customers to nearly eliminate the need to plan and maintain an IT infrastructure

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Dietary Habits During the COVID-19 Lockdown in Bosnia and Herzegovina's COVIDiet Study

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Abstract. The aim of the paper is to evaluate dietary habits of adults living in Bosnia and Herzegovina prior and during the COVID-19 lockdown. Participants (N = 1.507) completed a 44-items online survey designed for the assessment of their general food habits and consumption frequency of selected foods using the validated 14-items Mediterranean diet (MedDiet) as a reference of a healthy diet with the addition of socio-demographic characteristics, weight gain and physical activity. Survey was distributed using social media during the first lockdown, in the spring of 2020.

Survey respondents were mostly: females (76.71%), people living in a family home (89.91%), with higher education degree (76.38%), aged between 36 to 50 years old (44.39%) and being 37.96% of 21 to 35 years old.

Consumption of olive oil (81.95%), vegetables (70.07%), red meat (68.75%), carbonated drinks (63.17%), legumes (85.47%), fish (80.16%), non-homemade pastries (62.24%), wine (74.72%), intake of fried foods (69.61%) remained the same as usual but higher than 60%.

Chicken and turkey meat were preferred over red meats (70.94%). Cooked vegetables were consumed 2 and more servings by 49.04% of participants and 36.63% consumed 1–1.9 servings. 52.62% had one meal outside the home prior lockdown and more than half (53.88%) cooked more during the lockdown. Proffered cooking method was stew (72.06%) and oven (19.04%) but also 58.79% consumed fried food 1–3 times a week using sunflower oil for frying (72.33%). Around 54% decreased the intake of fast food during the lockdown while 47.05% ate more than usual. However, snacking remained the same for near 60% of participants. 37.49% maintained physical activity and 31.59% decreased physical activity during the lockdown. 54.68% did not gain any weight. The BMI of participants showed that 54.35% had normal weight, 33.51% has overweight and 10.68% were obese.

We assume that intake of the most of food remained as usual because in the time of conducting the survey, pandemic has just started and prior to lockdown people bought the food they usually consume and has a longer shelf life. Positive dietary changes are presented in cooking methods where stew and oven are mostly

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chosen and the decerase in the intake of fast food. We also look positively at the fact that snacking and alcohol consumption have remained the same due to the risks posed by lockdown, and in relation to mental strain and stress. Recommendation is to conduct survey again, one year after pandemic started.

Keywords: COVID-19 · Lockdown · Dietary habits · Bosnia and Herzegovina

1 Introduction

The COVID-19 pandemic expended from Wuhan, the capital city of Hubei Province in China to a growing number of countries [1] and it was followed by the "closure" of people around the world to reduce the spread of the infection. Measures to close business entities (except for food stores, pharmacies, gas stations and hospitals) occurred on March 12, 2020 in the Federation of Bosnia and Herzegovina. All the employed, where possible, were sent to work online and schools and universities were transformed into online schedule. Because adults over 65 are vulnerable group and children were suspected to be carriers, in mid-March, their movement was restricted. The restriction lasted until April 24, when the Crisis Headquarters of the Federation of Bosnia and Herzegovina issued an order allowing young people under the age of 18 and those over the age of 65 to move at certain and different times [2].

People from this territory are still under the influence of the war (1992–1995): food shortages, police hour, restriction of movement, closing borders. These pandemic restrictions resulted in panic buying of long-shelf groceries which are mainly carbohydrates and conserved foods. Still with something we can call post war syndrome, people were scared and anxious because of invisibile enemy and unknown circumstances which leads to emotional eating [3, 4] and along to that, being closed in homes, watching TV more than usual which is associated with snacking energy-dense food [5]. Limited movement means limited physical activity which with previously mentioned is predictor to obesity.

In this context, the COVIDiet_INT project has been established to estimate the impact of COVID-19 related lockdown measures on eating habits among the adult population. COVIDiet INT [6] is an international, crowd-sourced online study translated in 16 languages and conducted in 19 European plus 4 non-European countries. Cross-sectional data from this project have been presented with respect to the effect on the Spanish population [7], as well as in a comparative paper [8]. Study conducted in Denmark, Germany and Slovenia [9] showed that, depending on the type of food, 15–42% of study participants changed their consumption frequency during the pandemic, compared to before. The food categories with the highest rates of change were frozen food, canned food, and cake and biscuits; among the food categories with lower rates of change were bread, alcoholic drinks, and dairy products. People across all three countries shopped less frequently during lockdown and there was an overall reduction in the consumption of fresh foods, but an increase in the consumption of food with a longer shelf life in Denmark and Germany. Some people were decreasing and others increasing their consumption frequencies, demonstrating that the pandemic had different impacts on people's lifestyles and food consumption patterns.

The aim of this paper is to evaluate dietary habits of adults living in Bosnia and Herzegovina prior and during the COVID-19 lockdown assessing general food habits and consumption frequency of selected foods.

2 Materials and Methods

2.1 Study Design and Participants

A cross-sectional study (COVIDiet) was carried out among adults living in Bosnia and Herzegovina who were encouraged to participate in the present study without any exclusion criteria further than the age (>18 years old). Participation in the online questionnaire was entirely voluntary and anonymous. The study was conducted in agreement with the Declaration of Helsinki, and all data were collected anonymously and recorded according to the Spanish Organic Law of Personal Data Protection (LOPD) 15/1999. Since questionnaire is anonymous and no personal data are collected, no informed written consent was requested. However, participants were informed about the objective of the research and they were asked for permission to use and publish the data from the study before starting the questionnaire. This study was approved by the Research Ethics Committee of the University of Granada (1526/CEIH/2020). The questionnaire was open for B&H population from April 14, as soon as we found out about this research and joined the team from Spain and the April 24 was set as the end of the research. Accordingly, all of this data was collected in 10 days.

2.2 Instruments and Variables

A questionnaire was made according to food recommendations of EFAD [10] and WHO [11] during COVID-19 and Mediterranean Diet as the most adequate diet for cardiovascular and cognitive health. A questionnaire containing 44 items was designed for the assessment of data about consumption frequency of selected foods (mainly related with the MedDiet), general food habits and socio-demographic characteristics. In particular, the questionnaire was self-administered and divided into three main sections distributed as follows. Socio-demographic information items i.e., sex, age, place of residence, country, dependent children, and level of education were included. Furthermore, the weight was requested. Fourteen items with reference to the MedDiet pattern based on the validated PREDIMED MedDiet Adherence Screener (MEDAS) [12], were incorporated in the second section. Participants were also asked to answer about changes in their usual dietary habits during the confinement, i.e., way and frequency of cooking, snacking, alcohol intake or type of oil employed for frying, among others. In this case, all questions were also designed to know if participants increased, decreased or maintained their habits during the COVID-19 outbreak confinement. Additionally, participants were also asked whether they perceived that their physical activity and body weight had changed since the confinement started.

To try to cover the whole B&H territory and to reach the greatest number of participants through mobile phones, tablets and computers, the questionnaire was created using the Google Forms tool and was distributed using instant messaging apps e.g., WhatsApp, Viber, social media such as Facebook and emails through snowball sampling.

2.3 Statistical Analyses

Statistical analysis was carried out with SAS University Edition (2021). Student's t-test or Kruskal-Wallis test (for continuous normal or non-normal distributed data, respectively), and Chi-squared tests (for categorical data) were used to evaluate differences in means or proportions by these variables.

3 Results and Discussion

A total of 1507 adults living in Bosnia and Herzegovina completed questionnaire. For this paper, results are summarized in two tables representing socio-demographic characteristics of participants and consumption frequency of 14 foods during confinement as well as physical activity (Table 1).

Table 1. Socio-demographic characteristics of participants

Variable		Percent
Sex	Female	76.71
	Male	23.29
Place of residence	Family home	89.91
	Alone	7.83
	Shared flats	1.86
	Student's residence	0.40
Education	Primary school	3.12
	Secondary school	20.50
	Faculty degree, master, PhD	76.38
Age	<20	2.06
	20–50	37.96
	35–50	44.39
	51–65	14.60
	<65	1.00
BMI	Underweight	1.46
	Normal	54.35
	Overweight	33.51
	Obese	10.68

Survey respondents were mostly: females (76.71%), people living in a family home (89.91%), with higher education degree (76.38%), aged between 36 to 50 years old (44.39%) and being 37.96% of 21 to 35 years old. The BMI of participants showed that 54.35% had normal weight, 33.51% has overweight and 10.68% were obese. Results

among Spanish population [7] included 7514 participants and showed about 71% of the participants were females, 53.6% were from the south of Spain and the majority attained a graduate (46.4%) or postgraduate education (31.5%). There were few participants in the youngest (3%) and oldest age groups (6%), whereas 92% of them were aged 31–65 years. Participants with higher adherence to the MedDiet were more likely females, those living in family homes, in the mid-age groups (51–65 y) and with higher educational level i.e., university or postgraduate students. In the study conducted among Danish population [13] the sample population (N = 2,462) was well-distributed in terms of territorial coverage over the Danish regions. A prevalence of women (71.1%) and respondents with higher education was observed (88.5% completed higher education). These figures are virtually identical to those reported by Rodríguez-Pérez et al. [7], and likely reflect the fact that women and university educated respondents are more likely to voluntarily participate in food and health studies (Table 2).

Table 2. Changes in consumption of food and dietary habits during confinement

Consumption of olive oil	More	9.42
	Less	8.63
	The same	81.95
Consumption of vegetables	More	24.48
	Less	4.45
	The same	70.07
Consumption of fruits	More	37.89
	Less	4.51
	The same	57.60
Consumption of meat	More	6.37
	Less	24.88
	The same	68.75
Consumption of carbonated, sugary beverages	More	5.84
	Less	30.99
	The same	63.17
Consumption of legumes	More	10.29
	Less	4.25
	The same	85.47
Consumption of fish	More	10.02
	Less	9.82
	The same	80.16

(*continued*)

Table 2. (*continued*)

Consumption of commercial (non-home made) pastries	More	19.18
	Less	18.58
	The same	62.24
Consumption of home-made pastries	More	30.72
	Less	13.21
	The same	56.07
Consumption of wine	More	4.05
	Less	21.23
	The same	74.72
Consumption of snacks	More	28.33
	Less	11.28
	The same	60.38
Consumption of fast food	More	2.32
	Less	53.95
	The same	43.73
Proffered meat	Chicken, turkey	70.94
	Veal, pork, beef	29.06
Consumption of cooked vegetables	2 or more	49.04
	1–1.9	36.63
	0–0.9	14.33
Outside meals before confinement	1	52.62
	2	18.38
	3	7.76
	4	21.23
Cooking more	More	53.88
	Less	2.12
	The same	43.99
Proffered type of cooking	Fried	7.561
	Oven	19.04
	Microwave	0.20
	Griddle	1.13
	Stew	72.06
Proffered oil type	Olive oil	20.77
	Sunflower oil	72.33
	Other	6.90

(*continued*)

Table 2. (*continued*)

Eating more	Yes	47.05
	No	52.95
Weight during confinement	More	23.56
	Less	54.68
	The same	21.77
Physical activity	Decreased	19.64
	Increased	31.59
	The same	37.49
	I don't practice	11.28

Consumption of olive oil (81.95%), vegetables (70.07%), red meat (68.75%), carbonated drinks (63.17%), legumes (85.47%), fish (80.16%), non-homemade pastries (62.24%), wine (74.72%), intake of fried foods (69.61%) remained the same as usual but higher than 60%. In Germany and Denmark was increase in the consumption of food with longer shelf life [9].

Chicken and turkey meat were proffered over red meats (70.94%). Cooked vegetables were consumed 2 and more servings by 49.04% of participants and 36.63% consumed 1–1.9 servings. 52.62% had one meal outside the home prior lockdown and more than half (53.88%) cooked more during the lockdown. Proffered cooking method was stew (72.06%) and oven (19.04%) but also 58.79% consumed fried food 1–3 times a week using sunflower oil for frying (72.33%). Around 54% decreased the intake of fast food during the lockdown while 47.05% ate more than usual. However, snacking remained the same for near 60% of participants. Danish paper [13] reports that the food categories that were most notably affected by the lockdown were a higher intake of both commercial (21.1%) and (especially) homemade pastries (38.1%), and a higher intake of alcohol and carbonated beverages while we have more consumption of home-made pastries (30.72%). Consumption of wine (74.72%) and snacks (60.38%) remained the same at the highest percentage. In the Spanish study [7] the majority cooked in a similar way than before the confinement and used the griddle as the main technique for cooking (44.4%). Eating small amounts of food between meals (snacking), the intake of fried foods and fast-food were also similar than before the COVID-19 confinement, and 63.7% of participant declared not to have been eating more during the confinement. Around 73% of participants kept their intake of fried foods as before the COVID-19 confinement, which meant that nearly 39% of them continued consuming fried foods 1–3 days a week and around 37% less than 1 time per week. The majority of the participants (68.4%) used olive oil for frying.

37.49% maintained physical activity and 31.59% decreased physical activity during the lockdown. In the Danish study [13] almost half of the sample (47.7%) reported having exercised less and 29% increased the physical activity. In our study more than half of the sample (54.68%) did not gain any weight and almost 30% of Danish population reported gaining weight during the lockdown [13].

4 Conclusions

Results in this paper are not made with the adherence to MedDiet, only frequencies of reported changes in food intake and dietary habits during confinement. We assume that intake of the most of food remained as usual because in the time of conducting the survey, pandemic has just started and prior to lockdown people bought the food they usually consume and has a longer shelf life because of war related experience. Positive dietary changes are presented in cooking methods where stew and oven are mostly chosen and the decerase in the intake of fast food. We also look positively at the fact that snacking and alcohol consumption have remained the same due to the risks posed by lockdown, and in relation to mental strain and stress. Recommendation is to analyse results with the adherence to MedDiet and to conduct survey again, one year after pandemic started.

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Nitrogen Balance on Small Dairy Farms in Central Bosnia Region

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Abstract. Due to negative ecological aspects of livestock nutrition/production, in many countries worldwide farmers are encouraged to quantify and adjust nutrient balance (particularly nitrogen and phosphorus) on their farms. Differences between managed inputs and outputs is a good indicator of environmental sustainability of livestock production. Ideally, N balance on the farm should be close to zero; however, in practice, due to gaseous losses, nitrate leaching and soil stock changes, this target cannot be achieved. The investigations were conducted on five small dairy farms in the central Bosnia region. The average number of animals per farm was 13.2, ranging from 8 to 23. Average milk production was 82060 kg/year. All of the farms grew feed (mainly forage) on the farms that were fed to their cows. Average land size for animal feed production was 10.66 ha. The farmers bought almost all of the concentrates on the market and imported to the farm. All inputs (animals, feed, fertilizers) and outputs (milk, animals, manure) were used to calculate the balance. The major inputs in the farms were feed while milk was the main output accounting for 78.30% of all inputs and 77.99% of all outputs, respectively. Whole farm nitrogen balance ranged from 2.31 to 6.51. The main reason for considerably high whole farm nitrogen balance is low nitrogen utilization efficiency caused, apart from variability in protein digestibility, by unbalanced ration for animals. Thus, the best way to reduce the whole farm nitrogen balance is to maximize conversion of nitrogen from feed to milk using balanced rations as well as feeds with better nitrogen (protein) digestibility

Keywords: Nitrogen · Balance · Dairy farms · Environment

1 Introduction

Expansion in consumption of animal products and growth of the livestock sector has contributed, and continues to contribute, to increased pressures on ecosystems and natural resources – soil, air, water and biodiversity. Environmental pollution by nitrogen, in addition to having adverse effect on human and animal health, contributes to global warming, formation of acid rains, and eutrophication. Taking the forms of NH_3, N_2O and N_2, nitrogen is released to the air, while nitrogen in the form of NO_3^- and NH_4^+ enters into and pollutes surface and ground water.

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In order to avoid a harmful, even catastrophic environmental impact, the cycle of nitrogen must be retained within specific environmentally acceptable limits. The dairy farms produce a significant share of nitrogen environmental emissions, particularly when the milk production is intensive. In terms of animal production, the greatest losses of nitrogen through feces and urine were found at the dairy farms [1].

The expected increase in milk production by 2050 from 664 million tons, as much as it was in 1999, to 1077 million tons [2] gives rise to concerns and focuses the public attention to the dairy sector and nitrogen use efficiency (NUE) in the sector. The purpose of improving NUE is to reduce nitrogen losses from milk production, while avoiding a negative impact on economic sustainability of the farm [3].

The nitrogen balance, identified as the difference between the imported and exported nitrogen on farms (farm-gate N) is a good indicator of potential nitrogen losses into the environment [4–6]. Proper management and more effective use of nitrogen on the farms requires identification of main sources of nitrogen import to the farms, as well as the guidelines on how to mitigate its negative environmental impact [7]. Registration of input and output of nitrogen and other nutrients at farms enables recognition of the main sources of nitrogen and assessment of potential environmental burden, performance of the whole farm system, as well as the comparison among various production system [8].

The concept of the nitrogen balance means to achieve "zero farm balance" by implementing appropriate practices, i.e., balancing the nitrogen inputs and outputs at the farms. In practice, the zero balance is hard to achieve, and nitrogen losses are always present in some form. Borderline values of surplus nutrients, including nitrogen, that are acceptable from ecological aspects and economical sustainability of farm, have not yet been clearly identified. The main reason for this is great variability in how the animals are kept and how the milk is produced, specific characteristics of the farm location, specific characteristics of the animal feeding, etc. [9]. Farms that have high nitrogen losses, where the difference between the nitrogen input and nitrogen output from the farm is significant, constitute a potential environmental risk. Nitrogen is imported to the farm in purchased feed, purchased fertilizers, purchased animals, precipitation, by way of irrigated water and through fixation by legumes. The main nitrogen outputs include animal products, crops and manure. Speaking of milk production, a great number of studies suggest that the main source of nitrogen at farms is the imported by animal feed. Also, large losses of nitrogen are a result of inadequate animal feeding by, first of all, poor quality fodder and rations that do not suit the nutritional needs of the animals.

The purpose of this paper is to determine the farm-gate nitrogen balance on the dairy farms located in Central Bosnia, and to establish to what extent the animal feed impacts the resulting balances.

2 Materials and Methods

Over a period of one year, investigations were conducted at five selected dairy farms in the area of Central Bosnia. The basic information on the farms (number, category and breeds of animals, size of farm land area, quantities of bought and produced animal feed, structure of the animal rations, plant and animal production, fertilizers bought and sold (organic and mineral), animals bought and sold, methods of preparing the manure, etc.)

were obtained through a survey. Samples of fodder and milk were taken from the farms, and photo-documentation was made.

Farm-gate N balance was calculated as the difference between the sum of all annual N inputs (animals, fodder, fertilizers) and N outputs (milk, animals, manure) from the farm. The nitrogen balance takes into consideration only the nitrogen that crosses the farm borders and does not include nitrogen recycling within the farm. Inputs, outputs and nitrogen balance are expressed as total in kilograms and per farm area unit (kg/ha).

Nitrogen is imported to the farms in the form of animal feed (concentrate), fertilizers and animals. The nitrogen import by feed (concentrate) is calculated multiplying the imported quantity of concentrate by concentration of nitrogen. For calculation of nitrogen content of mineral fertilizers (kg) used were the values declared by the manufacturer.

The nitrogen output in milk is calculated by multiplying the determined nitrogen content in milk by the quantity of produced milk. The nitrogen export through animals is calculated by multiplying the total live weight of sold and died animals by nitrogen content in animal weight. For nitrogen content (kg N/kg live weight) in live weight of calves the value of 0.029 was used, and for dairy cows the used value was 0.024 kg of N/kg live weight [10].

Nitrogen Use Efficiency (NUE) was calculated as follows: (i) at the farm level, as a ratio between the nitrogen exported from the farm (in the form of products) and the nitrogen imported to the farm; and (ii) in milk production, as the ratio between the quantity of nitrogen in the produced milk and the quantity of consumed nitrogen.

The feed samples (forages and concentrate mixtures) were taken from the farm and analysed for the purpose of determining the content of nitrogen, crude protein, crude fibre, ether extract, ADF and NDF content and intake.

The nitrogen content in fodder was determined using the Kjeldahl method. Multiplication of the nitrogen content by factor 6.25 provided the content of crude protein (CP) in the fodder. Content of crude fibre (CF) was determined by cooking the samples in a 1.25% solution of H_2SO_4 for a period of 30 min, filtering it and cooking again with 1.25% of KOH, for another 30 min. After removing the base by washing the sample with water and acetone, the samples were dried at 105 °C, and after drying, burned at 550 °C. Content of crude fat (ether extract EE) was determined by Soxhlet method, where the petrol ether was used as organic solvent to extract the crude fat. Content of crude ash (Cash) was determined by burning the samples at the temperature of 550 °C over 4 h. Content of dry matter (DM), organic matter (OM) and nitrogen-free extract (NFE) was determined via calculations. Contents of acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined using the Van Soest method.

Energy content of forage feedstuffs and diets was calculated on the basis of crude nutrient and ADF content [11]:

(i) NEL, Mcal/lb = 1,0876 − (0,0127 * ADF) for grasses and mixed forage,
(ii) NEL, Mcal/lb = 1,044 − (0,0119 * ADF) for legumes,
(iii) NEL, Mcal/lb = 1,044 − (0,0124 * ADF) for corn silage,
(iv) NEL, Mcal/lb = 0,9265 − (0,00793ADF) for grain, and
(v) NEL, Mcal/lb = 0,866 − (0,007ADF) for concentrate mixtures.

Energy content expressed in Mcal is converted to MJ multiplying by 4.184.

Animal nutrient requirements were calculated according to the [12]. Milk samples were taken in spring and autumn, and kept at the temperature of −20 °C. Nitrogen content of milk was determined using the Kjedlahl method, using the factor 6.38 for determining the protein content of milk.

3 Results

The number of dairy cows at investigated farms ranged between 8 and 23. Predominating breeds were Holstein Friesians and Simmental, and only at one farm, the Brown Swiss breed was found. Annual production of milk at Farm 1 was 48000 kg, Farm 2 – 154300 kg, Farm 3 – 85000 kg, Farm 4 – 69000 kg, and Farm 5 – 54000 kg. Average annual milk production per head went from 5866 kg at Farm 3, up to 6700 kg at 2. Land area of the farms were: 13 ha (Farm 1), 10 ha (Farm 2), 5.5 ha (Farm 3), 15.4 ha (Farm 4) and 10.42 ha (Farm 5).

In mixed farming (livestock-crop) system, nitrogen balance is the function of many variables, including imported and exported animal feed, fertilizers, animals and other products containing nitrogen as well as practice with manure management and crop production inside farms' boundaries. Whole-farm nitrogen balance expressed as kg of N per year and N output/input ratio is shown in Table 1.

Table 1. Average whole farm N balance per farm

Variable	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Area, ha	13	10	5.5	15.4	10.42
N inputs, kg					
Imported feed	389.29	2202.35	1301.94	1288.90	810.62
Imported animals	0	0	0	0	0
Imported fertilizer	916.50	610.50	630.00	1107.75	796.50
Total N input, kg	1305.79	2812.85	1931.94	2396.65	1607.12
Total N input, kg N/ha	100.38	281.28	351.26	155.63	154.23
N outputs, kg					
Animals	56.70	178.20	113.40	81.00	64.80
Milk	216.40	631.09	367.97	287.04	239.30
Crop	0	0	0	0	0
Manure	292.50	0	0	0	236.25
Total output, kg	565.60	809.29	481.37	368.04	540.35
Input i – output i, kg	740.19	2003.56	1450.57	2028.61	1066.77
Input/output : 1	2.31	3.48	4.01	6.51	2.97
Total N output kg N/ha	43.50	80.93	87.62	23.90	51.85
N balance (input-output), kg N/ha	56.88	200.36	263.64	131.72	102.38
Nitrogen use efficiency (output /input, %)	43.33	28.77	24.94	15.35	33.61

Nitrogen Inputs
Nitrogen inputs to the farms ranged from 100.38 kg N/ha (Farm 1) to 351.26 kg N/ ha (Farm 3). The most significant source of imported nitrogen at all farms (except Farm 1) was the animal feed, while the second most significant source of nitrogen was the imported mineral fertilizer. Import of nitrogen through animals has not been seen in any farm over the period of one year. As the bedding, the straw produced at the farm had been used, so the nitrogen from the bedding was not calculated as nitrogen input. Expressed in percentages, the N input by concentrated mixtures ranged from 29.81% (F1) to 78.29% (F2). The largest nitrogen input by imported fertilizer was found at Farm 1, and amounted to 70.19% of the total nitrogen input. In the other farms (2, 3, 4 and 5), the nitrogen input by fertilizers amounted to: 21.71%, 32.61%, 46.23% and 49.55%, respectively.

Nitrogen Outputs
Main sources of exported nitrogen from farms were milk, sold animals, but also the sold manure, which was registered in two farms. The largest quantity of nitrogen was exported from farms through milk, and it ranged from 38.20% (F1) to 77.99% (F4). By sold animals, farms exported between 10.02% and 23.62% (F4) of nitrogen. From two farms, 43.72% and 51.71% of total exported nitrogen was exported by manure sold. In absolute values, the nitrogen output ranged 23.90 kg N/ha (F4) to 87.62 kg N/ha (F3).

Nitrogen Balances
At all investigated farms, nitrogen balances were positive, and ranged from 56.88 kg N/ha (F1) to 263.64 kg N/h (F3). At the other farms (2, 4 and 5), the nitrogen balance per hectare amounted to: 200.36 (F2), 131.72 (F4) and 102.38 (F5). Nitrogen use efficiency, i.e. the percentage of nitrogen exported from the farms ranged from 15.35 % (F4) to 43.31% (F1).

4 Discussion

The best balances and the greatest outputs from farms 1 and 5 are result of, first of all, export of some of manure from the farms, and a significant quantity of nitrogen exported through manure. On the other hand, the sale of a small number of animals from the farm, and use of the manure within the farm are a cause of unfavourable balance, i.e. the nitrogen use efficiency at the Farm 4. The fact that the farms disposed of a relatively large land area (15.4 ha) mitigated such whole farm nitrogen balance to an extent when expressed per area unit.

Milk production at the farms, which ranged between 5866 kg (F3) and 6700 kg (F2), is primarily a consequence of quality of the fodder, but also of the unbalanced rations, i.e. the imbalance between energy and protein contents in the rations. The voluminous fodder (hay, corn silage, grass-clover mixtures) was grown at the farms thanks to the available land area, while the concentrated fodder was purchased from outside. Conversion of dietary nitrogen into nitrogen in milk (NUE) ranged from 23.52 % at Farm 3, to 27.31% at Farm 1 (Table 2).

Table 2. Nutrient intake and nitrogen use efficiency on the investigated farms (all values were expressed as daily average per cow)

	Farms				
	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Land area, ha	13	10	5.5	15.4	10.42
Milk yield, kg/day	20	22	20	20	20
N in milk, g/day	98.12	89.98	93	83.2	87
Energy intake, NEL, MJ/day*	111.53	116.9	91.79	97.29	119.39
Crude protein intake, g/day*	2245	2169	2471	2209.23	2069
N intake, g/day*	359.2	347.04	395.36	353.48	331.04
N use efficiency in milk production (NUE), %**	27.31	25.93	23.52	23.54	26.28

* the value is the average of intakes of nutrients in wintertime and summertime period
** estimated as relative difference between N in milk and N consumed

According to [13], nitrogen use efficiency in milk production ranges from 20% to theoretical maximum of 45%, while the rest is excreted in urine or faeces in relatively equal quantities. Higher values of NUE indicators are indicative of better conversion of nitrogen from feed into milk protein, and lower excretion of nitrogen into the environment. Otherwise, low NUE may be an indicator of protein deficit in the rations and consequently, lower milk production.

NUE content above 35% is indicative of optimal content of protein in the ration. Any additional decrease of NUE by 5% suggests lesser or greater protein imbalance, where the NUE below 20% shows that the efficiency of using the protein from feed for production of milk protein is poor, and excretion of nitrogen into the environment is high [14, 15].

Taking this into consideration, nitrogen use efficiency at farms 1, 2 and 5 may be assessed as good, while at the other farms, the identified NUE value is indicative of improper feeding of the cows, i.e. of poor balance of energy and protein in the rations, or increased protein content of rations. Main characteristics of rations fed to lactating animals in summer and winter are shown in Tables 3 and 4.

Protein content in rations for dairy cows is one of the most significant factors determining the level of NUE. According to [16] the increase in dietary protein of 14.8 to 19.4% in dry matter of animal feed does not lead to increased milk production; however, the efficacy of use of nitrogen in milk production is reduced, resulting with increased excretion of nitrogen into the environment. On the other hand, for every percent of reduction of nitrogen in animal feed, the NUE increased on the average by 2%, leading to lesser excretion of nitrogen. Findings of the study by [17] suggest that increase in dietary protein from 13.5% to 19.4%, the percentage of use of nitrogen in milk production went down from 36.5% to 25.4%.

Table 3. Characteristics of summer time rations for lactating cows

Parameters	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
DM intake, kg	17.22	15.80	15.76	16.54	17.52
DM intake from forage, % of total DM intake	61.67	63.37	66.50	73.40	64.84
DM intake from concentrates, % of total DM intake	38.33	36.63	33.50	26.60	35.16
Crude protein intake, g/day	2394.00	2249.20	2460.90	2359.90	2077.20
Energy intake, NEL, MJ/day	98.07	105.35	83.77	97.12	102.80
NDF intake, g/day	5481.60	5740.15	5957.20	6355.20	6236.80
NDF intake, % of DM	31.83	36.34	37.80	38.42	35.60
ADF intake, g/day	4189.80	4151.55	4289.40	4675.60	4584.40
ADF intake, % of DM	24.33	26.28	27.22	28.27	26.17
Energy potential of rations in milk production, kg	20.78	26.06	18.60	23.21	25.18
Protein potential of rations in milk production, kg	23.90	22.18	24.70	23.49	20.13

Table 4. Characteristics of winter time rations for lactating cows

Parameters	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
DM intake, kg	18.10	16.79	17.19	15.96	19.80
DM intake from forage, % of total DM intake	73.26	58.07	56.49	69.67	62.22
DM intake from concentrates, % of total DM intake	26.74	41.93	43.51	30.33	37.78
Crude protein intake, g/day	2096.00	2088.85	2481.40	2058.56	2061.70
Energy intake, NEL, MJ/day	125.41	128.42	99.81	117.40	136.54
NDF intake, g/day	7599.20	5711.80	5986.60	6514.00	7344.40
NDF intake, % of DM	41.98	34.02	34.83	40.81	37.09
ADF intake, g/day	4983.00	3718.50	3992.10	4304.40	4854.00
ADF intake, % of DM	27.53	22.15	23.22	26.97	24.52
Energy potential of rations in milk production, kg	33.00	34.04	24.15	30.23	36.85
Protein potential of rations in milk production, kg	20.36	20.27	24.95	19.91	19.95

Low values of NUE at Farm 3 may be explained by great imbalance of protein and energy in the rations of lactating cows. The surplus of protein in summertime feedings enables production of additional 6.1 kg of milk, what has not been achieved due to insufficient consumption of energy (Table 3).

Inefficient use of nitrogen in synthesis of milk is related to the digestibility of the diet, what in turn depends on the content of fibres, and ratio between carbohydrates and proteins in the rumen, as their imbalance slows down the synthesis of the microbial protein, and consequently, causes large losses of nitrogen in the form of ammonia. Rations with high NDF content (around 40% in dry matter) or high content of protein digestible in rumen (around 13.24% in dry matter) contribute, according to [18, 19], to lower efficacy of nitrogen use. The above facts may explain the low efficacy of use of nitrogen at Farm 4, as the large portion of dry matter comes from forages both in the summertime (74.4%) and in the wintertime (69.67%) rations, compared to the total consumed dry matter, has led to high consummation of NDF (Tables 3 and 4), and consequently to poorer digestibility of organic matter in the rations, including crude protein. In addition, the feeding practice of using high protein feed in order to ensure maximum milk production may also be the reason for low efficacy of nitrogen use. If a ration contains adequate quantities of energy, the protein content of 17% in DM is quite sufficient to provide for the requirements of cows in early lactation for milk production. Other characteristics of the rations, including the content of ADF and energy, also have a significant negative correlation with NEU [20] and this is an additional argument that points to the imbalanced diets as the main factor in uncontrolled loss of nitrogen, and thus relatively unfavourable nitrogen balance at the level of investigated farms (whole farm nitrogen balance).

Namely, increasing the content of energy in the rations by 1 Mcal (4.184 MJ) leads to reduced NUE of 6.67% [20]. The investigated summertime rations at Farms 2 and 5, and wintertime rations at all investigated farms, had significantly higher energy potential comparing to protein content for milk production.

5 Conclusion

Low level of practical implementation of the postulates of ration balancing at the investigated farms has led to the extensive use of energy feedstuffs without balancing the energy and protein contents in the rations. The attempts to use less expensive components of the rations resulted with high share of forages, and consequently with increased consumption of fibres, which has additionally decreased the use of nitrogen in milk production. Such uncontrolled losses of nitrogen, coupled with production practices at certain farms where the manure remained within the farm (from which additional nitrogen is lost without control), has resulted with unfavourable nitrogen balances on the farms 2. 3 and 4 (the ones that had not sold the manure).

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Development of Food–Grade Controlled Delivery Systems by Microencapsulation of Polyphenols with Health Benefits

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Abstract. Polyphenols have gained attention as functional compounds in food and pharmaceutical/cosmetic industry, as potent agents against oxidative stress, therefore, their application in different products and consumption may contribute to the prevention of several degenerative diseases such as neurodegenerative and cardiovascular diseases, inflammatory disease, cancer, and diabetes. The effectiveness of polyphenols depends on conserving the stability, bioactivity and bioavailability of the active ingredients. However, in general, these compounds are very sensitive and have low bioavailability in human body. The unpleasant taste of most polyphenols also limits their application in food products. The utilization of encapsulated polyphenols can overcome some of the above mentioned limitations. Five polyphenols (rutin, gallic acid, epigallocatechin gallate (EGCG), resveratrol and curcumin) were microencapsulated by a spray drying process. The more soluble polyphenols (EGCG, rutin and gallic acid) were microencapsulated directly by a spray drying technique. Curcumin and resveratrol were microencapsulated considering a two steps approach: emulsification followed by spray drying. The microparticles were characterized (SEM and laser granulometry) and evaluated in terms of controlled release. The Weibull model was applied to the experimental release profiles obtained in simulated conditions. The encapsulating agents used in the preparation of the microparticles influenced the type of particles obtained and their characteristics. The release was total, varying the release times with the polyphenol encapsulated and with the encapsulating agent used. The encapsulation efficiency was around 100% for the gallic acid, rutin and EGCG microparticles. Then, the polyphenol microparticles prepared by a spray drying process have revealed a great potential for the encapsulation and protection of sensitive bioactive compounds for food-related applications.

Keywords: Microencapsulation · Polyphenols · Spray drying

1 Introduction

In the last years, there has been growing interest in including natural bioactive compounds into functional food products and nutraceuticals, for their potential additional or even synergistic health benefits [1].

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The consumer's interest in healthier food products, containing bioactive and natural ingredients, generates demand for new products, and new technologies, that allow the incorporation of compounds with good properties and benefits in a practicable way in food products [2, 3]. Related to this interest for new products containing bioactive compounds, it arises the term "nutraceuticals" that can be explained as the food items, as a total or a part, which possess some nutritional value with also medicinal properties [4]. Several types of bioactive compounds have been shown to exert exceptional biological activities (i.e. anticancer), including polyphenols [5].

The fundamental reason for this interest in polyphenols is the recognition of their special properties, namely the antioxidant characteristics, and their role in the prevention of several diseases associated with oxidative stress, such as: cancer and neurodegenerative, cardiovascular and inflammatory diseases [6–9]. They also show promising effects in the prevention and treatment of others diseases, like: obesity, diabetes, autoimmune diseases, inflammatory bowel disease, colorectal tumors and other chronic diseases [10].

Several plants, rich in polyphenols, were used in the traditional medicine by different civilizations and during centuries, considering the existence of health benefits [11]. In this moment, there are more than 8000 varieties of polyphenols, including flavonoids, anthocyanins, phenolic acids, tannins and other phenolic compounds, and they can be categorized into different groups and subgroups (Fig. 1) [1, 12].

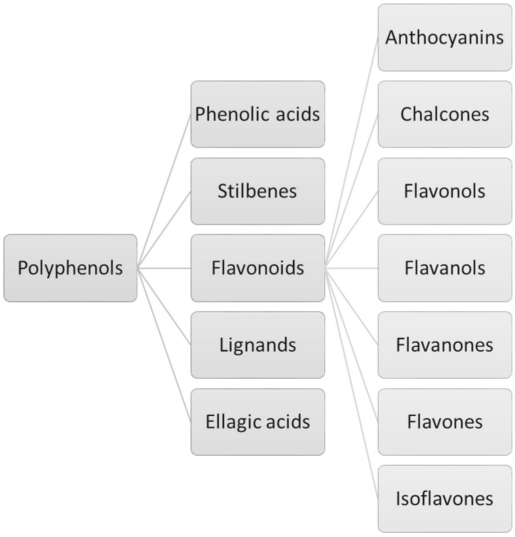

Fig. 1. Classification of the different groups of polyphenols.

The polyphenols are typically stable and active when present in plants or part of plants [1, 13]. However, they are susceptible to degradation and oxidation after being extracted, which is a big challenge for the food industry, namely during processing foods and food products storage [14, 15]. Several other limitations have been associated to these biocompounds, due to their low oral bioavailability, rapid catabolism and excretion, low

water solubility, and low stability in environmental, processing and gastrointestinal conditions [12]. These compounds can be degraded if exposed to light, enzymatic activities, oxygen, adverse temperature and pH conditions, metal ions and water, which leads to an alteration of their beneficial properties [3, 16].

Therefore, the effectiveness and beneficial activity of polyphenols depends on preserving their bioactivity, stability, and bioavailability [17]. The utilization of encapsulated polyphenols instead of free polyphenols can overcome the weaknesses of their instability, reactivity with other compounds, oxidation, alleviate unpleasant tastes or flavors, as well as, increase the solubility, the bioavailability, the bioaccessibility and half-life of the compound *in vivo* and *in vitro* [1]. So, the microencapsulation allows the protection of the active compounds by their incorporation into a protective matrix, preserving the biochemical functionalities of these substances. The characteristics of the encapsulating materials utilized for creating a protective barrier/matrix can influence the characteristics of the final product, such as: the size, shape, and structure of the particles [18].

There are several microencapsulation techniques as well as options of encapsulating agents [1, 19, 20]. In the present work, considering the possibility of using polyphenols in food and pharmaceutical applications, the encapsulation of the different polyphenols was made by a spray-drying technology (Fig. 2). This decision was taken considering the industrial context (simple, with an easy scale up, rapid, continuous and economical process) [11].

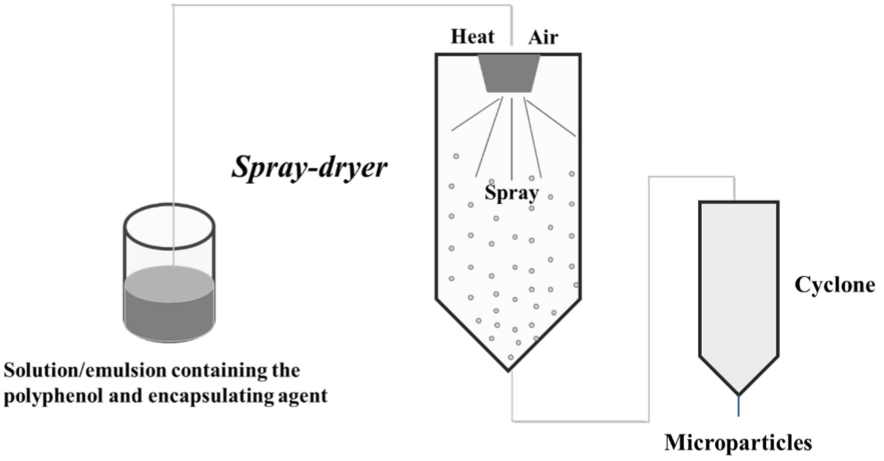

Fig. 2. Scheme of the spray drying process.

This work studies the production of 5 different polyphenols microparticles (rutin, gallic acid, epigallocatechin gallate (EGCG), resveratrol and curcumin) such that they can be incorporated in food or pharmaceutical products in order to improve their benefits in human health. The polyphenols were selected considering 2 flavonoids and 3 non-flavonoids polyphenols:

Rutin is a flavonoid, more specifically a flavonol and it is abundantly found in plants, such as buckwheat, tea, passion flower, and apple. Rutin is considered a vital nutritional component of food stuff. Rutin has demonstrated a number of pharmacological activities, including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective [21].

Gallic acid is a phenolic acid more specifically a hydroxybenzoic acid. It has several properties that are beneficial for health, such as anti-inflammatory, antifungal, antiviral, hypotensive, antibacterial, immunomodulatory and anticarcinogenic properties [22].

Epigallocatechin gallate (EGCG) is a flavonoid and more specifically a catechin, and it is the most abundant and the most active polyphenol of green tea leaves. EGCG produces various biological effects, including: antitumorigenesis, antimutagenicity, free radical scavenging activity and antimicrobial activity against pathogens. It plays also an important role in protecting the Human body against cardiovascular disease, and other chronic conditions [23].

Resveratrol is a polyphenol from the stilbenes family. It is found naturally in the skin of red grapes, berries, red wine, peanuts and other peanut products in low amount. Resveratrol is related with health benefits, namely related to: prevention of cancer, neurodegenerative diseases heart disease,, diabetes and other biological activities, such as antioxidant, anti-viral and anti-inflammatory properties [7].

Curcumin is a yellow-orange polyphenol and it is one of the active ingredient of turmeric. It is recognized by the pharmacological properties related to anti-inflammatory, antioxidant, anticholesterolemic, antidiabetic, antihepatotoxic, anticarcinogenic, antivenom, antidepressant and neuroprotective activities [10, 11, 15].

Considering the reasons previously presented it is important to microencapsulate these polyphenols to benefit as much as possible from their advantages. The microencapsulated delivery systems will give protection and will also provide a controlled release mechanism, improving their compatibility in the food matrix and increasing their bioavailability. So, depending on the type of polyphenol studied, different approaches in the microencapsulation process were performed. The result obtained with the 2 different microencapsulation methodologies were compared. The first group, which included the most soluble polyphenols (rutin, gallic acid, and epigallocatechin gallate (EGCG)), was microencapsulated directly by a spray drying technique, the second one was microencapsulated considering a two steps approach emulsification followed by spray drying (curcumin and resveratrol). Microparticles' characterization and the application of mathematical models were also an accomplished aim in this work. With a properly designed controlled-release system, the active compounds are released at the desired site and time, and at a desired rate. The use of mathematical models to predict in vivo bio-performance is an advantage in the development of new formulations and products.

2 Material and Methods

2.1 Materials and Solutions

Reagents with high purity and pharmaceutical grade were selected for this work. Epigallocatechin gallate (EGCG), Rutin, gallic acid, resveratrol and curcumin were purchased from Sigma-Aldrich.

Different encapsulating agents were used: modified chitosan, arabic gum, and sodium alginate. Modified chitosan was provided from China Easter Group (Dong Chen) Co. Ltd.. Arabic gum from acacia tree was acquired from Fluka, Germany. Sodium alginate was purchased from Sigma-Aldrich, USA. Coconut oil was from Sigma-Aldrich (China).

The encapsulating agents solutions were prepared at room temperature with deionized water, according to previous works. Different solutions of the biopolymers with a concentration of 1% (w/V) were prepared and placed under stirring for 2 h at a speed of 1200 rpm.

Emulsions were prepared with coconut oil and curcumin/resveratrol and arabic gum 10% (w/V) using a homogenizer (CAT, Germany). The coconut oil was chosen considering the solubility of curcumin and resveratrol in this oil and also because this oil can be used as a food matrix.

2.2 Preparation of the Microparticles - Spray-Drying Process

Depending on the characteristics of polyphenol, namely solubility, a different procedure in the microencapsulation method was performed.

The first group of polyphenols (rutin, gallic acid, and EGCG), which included the most soluble polyphenols, was microencapsulated directly by a spray drying technique, the second one (curcumin and resveratrol) was microencapsulated considering a two steps approach: emulsification followed by spray drying. The aqueous phase of emulsions was composed by the arabic gum solution. The encapsulating agent (arabic gum) was dissolved in ultra-pure water. In turn, the oily phase was composed by the active compound (curcumin or resveratrol) prepared in coconut oil. All the emulsions were prepared under stirred conditions (20000 rpm for 10 min), using a homogenizer. In all the cases the microparticles were produced in a Mini Spray Dryer B-290 from BÜCHI (Flavil Switzerland).

For the spray-dryer microencapsulation experiments, the biopolymer solutions were mixed with the ones of bioactive compounds to obtain the feed solutions/emulsions. The feed solutions/emulsions were spray-dried under conditions selected in previous studies done to achieve the most feasible parameters of the spray-dryer equipment [1, 7, 11].

At the end of the test, the dried powders, obtained from the spray-dryer, were collected and stored in conical tubes, sealed and covered with aluminum foil, at 4 °C, before further analysis.

2.3 Characterization of the Microparticles - Scanning Electron Microscopy

Scanning Electron Microscopy, SEM, was performed at Centro de Materiais da Universidade do Porto (CEMUP), with an equipment Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M equipment (Eindhoven, The Netherlands).

2.4 Size Characterization of the Microparticles

Microparticles size distribution was evaluated by laser granulometry using Coulter LS 230 (Coulter, Miami, FL) according with the methodology developed in previous studies.

Ethanol was used as dispersant of microparticles in order to prevent their agglomeration during measurement. The size distributions and the mean sizes of microparticles (volume and number distributions) were determined for three 30 s runs.

2.5 Controlled Release Studies and Kinetic Models

Considering the different types of microparticles prepared, the controlled release studies were made in 2 different dissolution media (deionized water or coconut oil). Microparticles prepared directly by spray drying (rutin, gallic acid, and epigallocatechin gallate) were tested in deionized water. Microparticles prepared considering a two steps procedure, emulsification plus spray drying (curcumin and resveratrol), were evaluated in coconut oil.

In all the cases, spectrophotometric methods were selected to confirm the presence of the bioactive compounds (rutin, gallic acid, epigallocatechin gallate (EGCG), resveratrol and curcumin) in the microparticles and for the evaluation of the release profiles done in a dissolution media (deionized water or coconut oil).

The different bioactive compounds release from the microparticles were measured by absorbance analysis using a UV/VIS spectrophotometer (SPEC RES+, Sarspec, Porto, Portugal), at the maximum absorbance wavelength for each compound. The microparticles were placed in a cuvette (CV10Q3500F, Thorlabs), on top of 4 mL of deionized water or coconut oil, with stirring at room temperature.

The release profiles were evaluated in a continuous mode (intervals of 30 s). The release profiles were evaluated for the microparticles immediately after spray-dryer production.

The Weibull model was adjusted to the release profiles. The Weibull model (Eq. 1) is more adequate model for analyzing the release profiles of matrix type drug delivery, which is normally the case of microparticles produced by spray drying [1]. In the equation: M_∞ is the dissolution/release (%) at infinite time, M_t is the dissolution/release (%) at time t (min), and t_0 the lag-time (min) of the dissolution (normally $t_0 = 0$). β represents the shape parameter of the curve and τ_d the time (min) when 63.2% of M has been dissolved/released.

$$M_t = M_\infty \left[1 - e^{-\left(\frac{t-t_0}{\tau_d}\right)^\beta} \right] \tag{1}$$

3 Results and Discussion

3.1 Microencapsulation

Microparticles using different methodologies, with different bioactive compounds and different biopolymers (encapsulating agents) were prepared (Table 1). The product yield (quantity of powder recovered, considering the quantity of raw materials – encapsulating and active agent – used) ranged from 26% to 66%. Microparticles prepared with a combination of methods (emulsification + spray drying) presented lower products yields. The presence of coconut oil decreases the product yield. Similar results were already

described in the literature. For instance, Gonçalves et al. [24] observed a clear decrease on the product yield of arabic gum carrier-based microparticles (from 2.1 to 5.2. times) when the microparticles contained coconut oil instead of being composed only by the encapsulating agent.

Table 1. Microparticles prepared with the different bioactive compounds, different biopolymers as encapsulating agents and using different microencapsulation methodologies.

Polyphenol encapsulated	Method	Encapsulating agent	Product Yield (%)
EGCG	Spray drying	Alginate - 1% (w/V) Arabic gum - 1% (w/V) Modified chitosan - 1% (w/V)	49 66 48
Rutin	Spray drying	Modified chitosan	34
Gallic acid	Spray drying	Modified chitosan	36
Curcumin	Emulsification + spray drying	Arabic gum - 10% (w/V)	29
Resveratrol	Emulsification + spray drying	Arabic gum - 10% (w/V)	26

3.2 Scanning Electron Microscopy (SEM)

Microparticles prepared with the different polyphenols (rutin, gallic acid, epigallocatechin gallate (EGCG), resveratrol and curcumin) were analyzed by scanning electron microscopy (SEM). The morphology of the spray dried particles is affected by factors as the drying kinetics and the composition of the formulation. So, at the first stages of the spray drying process, the surface of the atomized drops starts to dry developing a skin or crust, then occurs bubble nucleation. These bubbles increase and burst outward over the surface occurring inflate-deflate cycle. This process will be responsible for the final surface of microparticles [1].

Microparticles prepared in all the assays, with the different polyphenols, were spherical and presented a smooth or rough surface depending on the methodology and of the encapsulating agent used.

Microparticles prepared with the same bioactive compound (EGCG) and different encapsulating agents presented on SEM analysis, different morphologies (Fig. 3). Microparticles prepared with gum arabic have an indented surface, with multiple holes, the modified chitosan microparticles have a very smooth surface and the sodium alginate microparticles present a regular and smooth surface although they present also some holes.

On the other hand, microparticles prepared with the same encapsulating agent and with different bioactive compounds (EGCG, Rutin and Gallic acid) present equal morphology. In Fig. 4 it is presented the case of microparticles prepared with modified chitosan and different polyphenols.

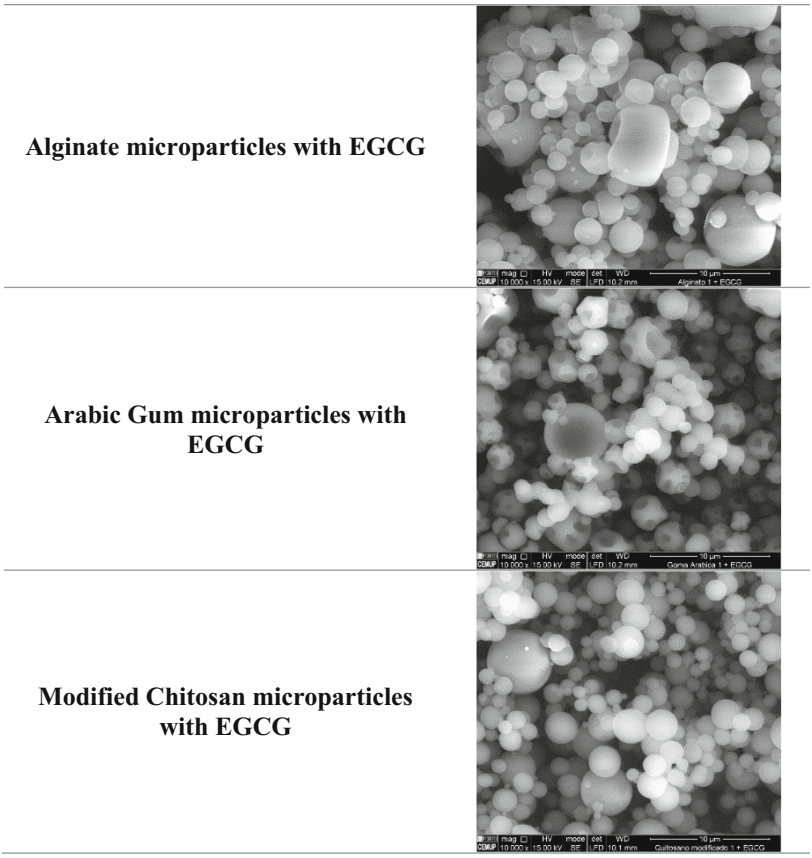

Fig. 3. SEM images of EGCG microparticles prepared with 3 different biopolymers (alginate, arabic gum and modified chitosan). SEM specifications: magnification of 10 000 ×, beam intensity (HV) of 15.00 kV, distance between the sample and the lens (WD) around 10 mm.

In terms of size, it is possible to verify in all the samples a heterogeneous size distribution, characteristic of the spray drying technique, existing some small microparticles (smaller than 1 μm) located on the middle of the microparticles with big dimensions.

In preliminary results, it was also verified that the presence or not of bioactive compound does not affect the morphology of the microparticles. Similar results were obtained by Estevinho and co-authors [1, 7, 11], when encapsulated other bioactive compounds by spray-drying method, with some of these biopolymers.

In Fig. 5, SEM images of polyphenol microparticles (curcumin and resveratrol), prepared with a combination of methods emulsification + spray drying, are presented. Spherical and irregular polyphenol microparticles with a very rough surface were produced.

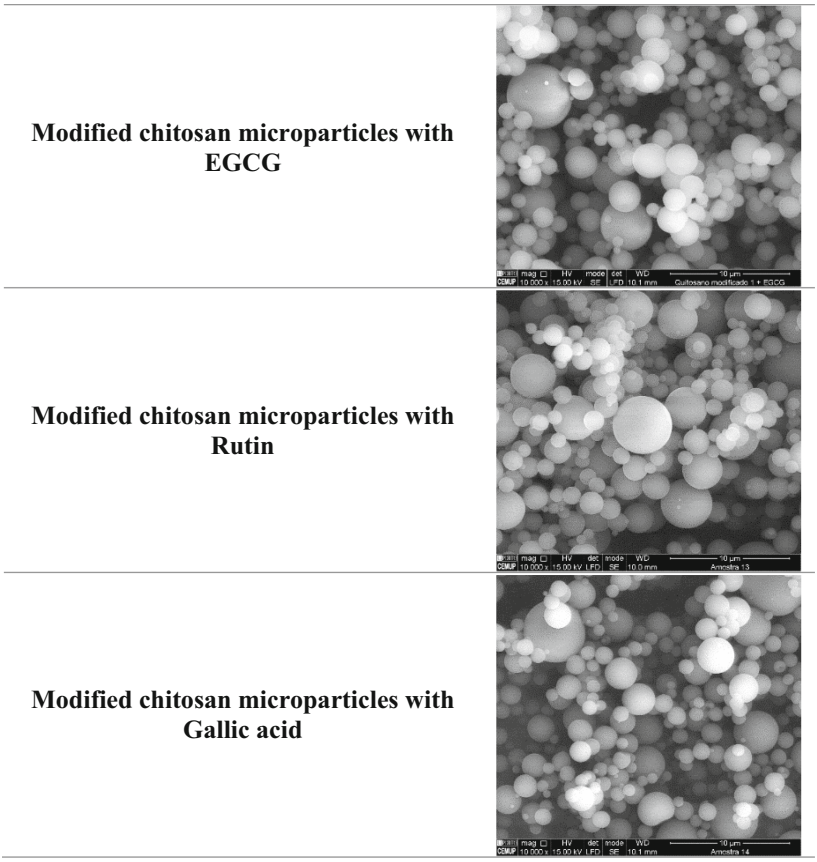

Fig. 4. SEM images of polyphenol (EGCG, rutin and gallic acid) microparticles prepared with modified chitosan. SEM specifications: magnification of 10 000×, beam intensity (HV) of 15.00 kV, distance between the sample and the lens (WD) around 10 mm.

3.3 Particle Size Analysis

In the development of new food products there are many additional factors that need to be considered for the acceptance and commercialization. Some of them are directly dependent on the final particle size and shape of the microparticle system. Therefore, the size homogeneity of the microparticles is very important to guarantee stability and similarity of the final products, replicability and also to predict the microparticles behavior regarding their incorporation in food products and release profiles [1].

Fig. 5. SEM images of polyphenol (curcumin and resveratrol) microparticles prepared with arabic gum (10%) by emulsification+spray drying. SEM specifications: magnification of 10 000 ×, beam intensity (HV) of 15.00 kV, distance between the sample and the lens (WD) around 10 mm.

The size of the particles is normally controlled by the experimental conditions used in the experimental procedure, namely in the spray dryer equipment. In this work, all the polyphenol microparticles prepared were analyzed considering a differential volume size distribution (Table 2).

Analyzing the particles by laser granulometry it is possible to verify that the microparticles prepared with different polyphenols (EGCG, curcumin and resveratrol) with different encapsulation approaches but with arabic gum, presented a mean size very similar. On the other hand, there was a big variation in the mean size of the microparticles prepared with modified chitosan (EGCG, rutin and gallic acid).

Table 2. Mean size of the microparticles prepared with the different polyphenols, different biopolymers as encapsulating agents and using different microencapsulation methodologies.

Polyphenol encapsulated	Encapsulating agent	Mean size (μm)
EGCG	Alginate	5.1
EGCG	Arabic Gum	7.2
EGCG	Modified chitosan	36.1
Rutin	Modified chitosan	5.8
Gallic acid	Modified chitosan	10.7
Curcumin	Arabic gum	7.2
Resveratrol	Arabic gum	8.2

3.4 Controlled Release Studies and Kinetic Models

The release profiles were obtained for the polyphenol microparticles produced with a EGCG, rutin, and gallic acid, in deionized water, at room temperature (Fig. 6 and 7).

The deionized water (pH 5.6) was used in these assays, as release medium, because it represents the most common solvent used in food industry and in the preparation of food products. Figure 6 has the representation of the release profiles obtained for the EGCG with different encapsulating agents (gum Arabic < 5 min, modified chitosan < 10 min and sodium alginate < 17 min). The release was total and the release times varied with the encapsulating agents used (Table 3).

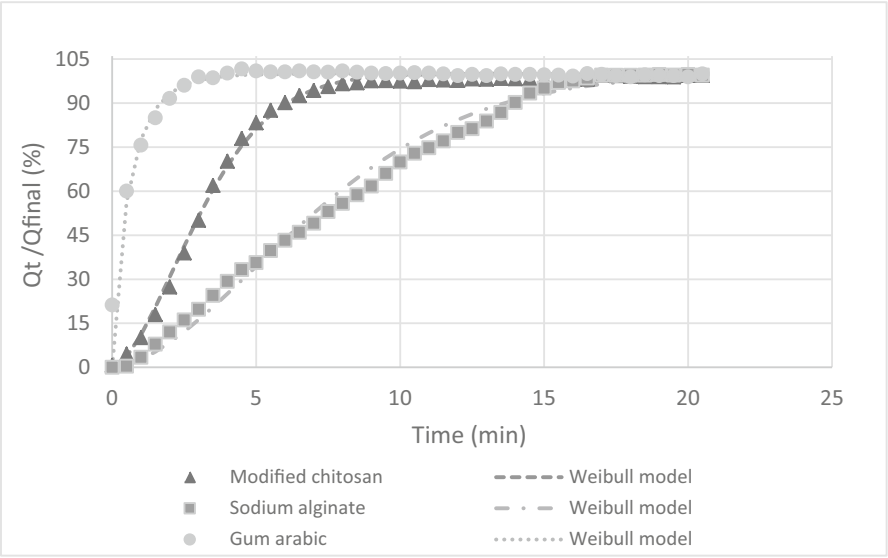

Fig. 6. Comparison between the 3 different encapsulating agents, same core material (EGCG).

In Fig. 7, the release profiles obtained for different polyphenols microencapsulated with the same encapsulating agent - modified chitosan, are presented. The release profiles are very similar, with a total release achieved in 9 min.

The experimental release profiles were adjusted to the Weibull model (Eq. 1) with a high correlation coefficient (>0.96) (Table 3). The parameter τd obtained directly from the experimental release profiles (for 63.2% of the release) is very similar to the parameter obtained from the model.

An additional information that it is obtained analysing the release profiles is the microencapsulation efficiency. The efficiency of encapsulation was close to 100% with the exception of the microparticles prepared with arabic gum and containing EGCG (79%) (Table 3).

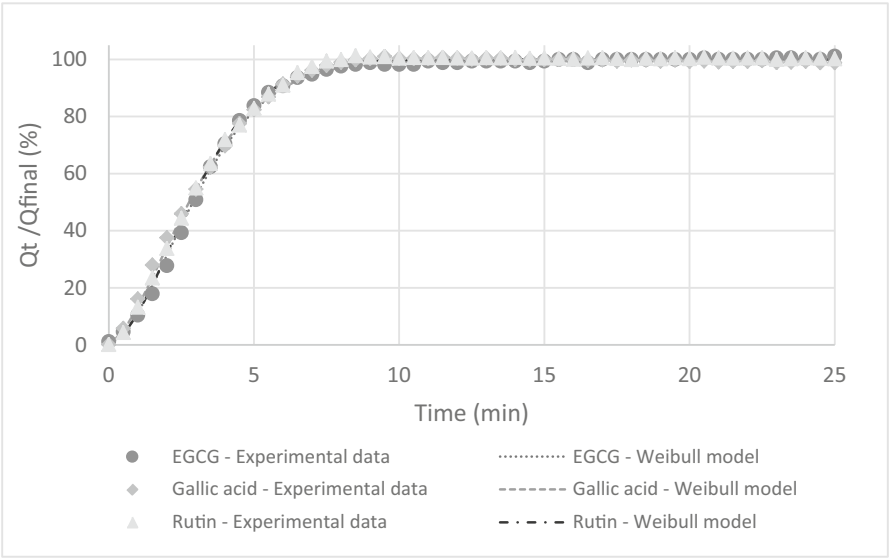

Fig. 7. Comparison between the 3 different polyphenols, same encapsulating agent (modified chitosan).

The controlled release studies of resveratrol and curcumin microparticles were performed in coconut oil, from microparticles prepared with arabic gum (10% (w/V)). These microparticles were prepared by a combination of microencapsulation methodologies, emulsification plus spray drying. The release profiles obtained are presented in Fig. 8. The coconut oil was selected as a medium for the release test of the curcumin and resveratrol microparticles, because presents a good biocompatibility with the Human body, can be used in several applications in food, pharmaceutical and cosmetic industry and can also simulate a lipophilic phase of the body.

Table 3. Encapsulation efficiency, time parameters of the release and parameters and correlation coefficients of the Weibull model applied to the experimental results (EGCG, Rutin and gallic acid microparticles).

Microparticles	Total release (min)	$\tau d_{experimental\ values}$ (min)	$\tau d_{calculated}$ (min)	β	R^2	Encapsulation efficiency (%)
EGCG/Alginate	17	9.5	8.3	1.7	0.97	100
EGCG/Arabic gum	4	1.0	0.6	0.9	0.96	79
EGCG/Modified chitosan	9	4.0	3.6	1.7	0.99	99
Rutin/Modified chitosan	9	3.5	3.3	1.6	0.99	100
Gallic acid/Modified chitosan	9	3.5	3.3	1.7	0.99	100

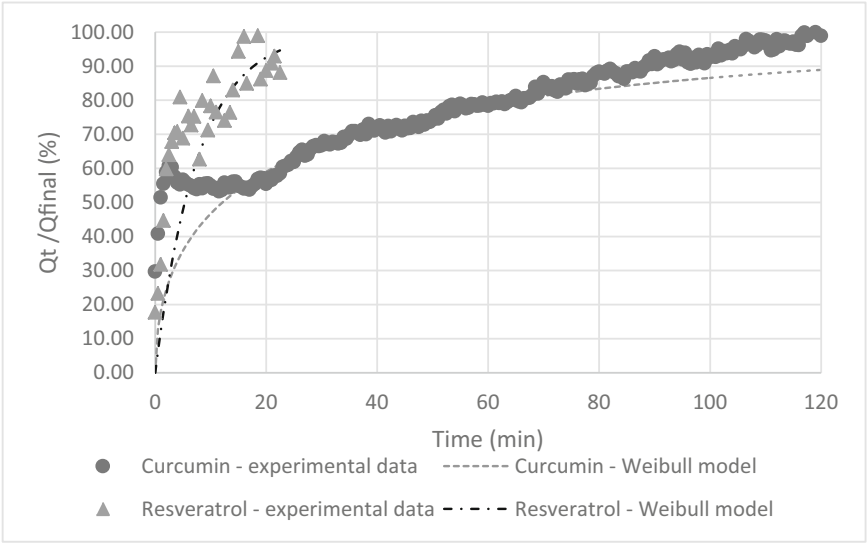

Fig. 8. Comparison between the 2 different polyphenols (curcumin and resveratrol), same coating polymer (Gum arabic).

The total release was obtained after 23 min (resveratrol) or 110 min (curcumin) and the encapsulation efficiency ranged between 70 and 82% (Table 4). The release profiles were also studied and adjusted to the Weibull model (Eq. 1). Fitting the experimental results to a linearized equation of the Weibull model it was possible to obtain acceptable R^2 coefficients, indicating that this model adjusts to the experimental data obtained.

Table 4. Encapsulation efficiency, time parameters of the release and parameters and correlation coefficients of the Weibull model applied to the experimental results (curcumin and reveratrol microparticles).

Microparticles	Total release (min)	$\tau d_{experimental\ values}$ (min)	$\tau d_{calculated}$ (min)	β	R^2	Encapsulation efficiency (%)
Curcumin/ arabic gum	110	25.5	25.1	0.50	0.97	70
Resveratrol/ arabic gum	23	8	3.8	0.49	0.84	82

4 Conclusions

In this study, two different microencapsulation approaches were studied: the most soluble polyphenols (rutin, gallic acid, and epigallocatechin gallate (EGCG)) were

microencapsulated directly by a spray drying technique; curcumin and resveratrol were microencapsulated considering a two steps approach: emulsification followed by spray drying.

Different morphologies were obtained depending on the biopolymer used (rough, smooth, regular and irregular) and on the microencapsulation procedure. Considering the results of different polyphenols (rutin, gallic acid and EGCG) microencapsulated with the same encapsulated agent, it appears that the bioactive compound (polyphenol) does not influence the morphology of the microparticles.

High efficiencies of encapsulation were obtained, in some cases equal to 100%.

Microparticles made of gum arabic released faster the polyphenol in deionized water, than modified chitosan or alginate microparticles. In comparison, modified chitosan and sodium alginate were better wall materials for this process, and sodium alginate allowed the slowest release of the compound in the aqueous media.

Microparticles prepared with resveratrol and curcumin had their release profiles simulated in coconut oil. These releases are slower and more unstable. The total release was obtained after 23 min (resveratrol) or 110 min (curcumin) and the encapsulation efficiency ranged between 70 and 82%.

All the experimental results were studied considering the Weibull model.

Thus, the polyphenol microparticles prepared with biopolymers by a spray drying process have demonstrated a great potential for the encapsulation and protection of photosensitive bioactives for food-related applications. Further research in this area should focus on the stability of encapsulated micro-particles in certain food systems, due to the complexity and possible impact of food ingredients on release rates and stability.

These microparticles can be easily incorporated in commercial instantaneous powder food products, which can be fortified with bioactive compounds.

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Trichloroacetic and Nitric Acid Extraction of Ca, Mg, Fe, Zn, Na and K from Whey Samples

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Abstract. Whey is a liquid by-product in cheese production. Although whey is a by-product it contains soluble compounds and it can be nutritionally valuable. Metals are among many different compounds that exist in whey. Nowadays there are many different methods for metal determination. First step in analysis of metals is sample preparation. For the sample preparation of whey, concentrated nitric acid is often used. This method uses corrosive strong acid, and it has a rather long heating under reflux. Because of this fact about nitric acid digestion, arises the aim of this research: is it possible to extract metals from whey samples with trichloroacetic acid (TCA) which is less toxic and has much easier procedure? In this research contents of iron, zinc, calcium and magnesium were measured by atomic absorption spectroscopy with flame atomization, FAAS. Concentrations of sodium and potassium were measured by flame photometry. Whey samples had different origin: from cow's, sheep's and goat's milk. Results show differences in metal content in different whey samples. Extraction of metals by concentrated nitric acid and TCA show statistically significant difference for iron, zinc, calcium and magnesium. Results gained from two different extraction of sodium and potassium are not statistically different, and TCA extraction could be used as an alternative in sample preparation for sodium and potassium determination in whey samples.

Keywords: Whey · Extraction · TCA · Metals · Determination

1 Introduction

Whey appears in cheese and casein production as a by-product [1]. Whey contains valuable ingredients and can be used or processed in a variety of ways [2]. Industrial processing of whey as a by-product in the production of cheese or casein, began in Bosnia and Herzegovina in 1960, although its nutritional and therapeutic properties have long been known. During curdling, almost all soluble salts and microelements of milk, as well as salts added in cheese production, pass into whey. Whey is rich in calcium, potassium, magnesium, phosphates, chlorides. It also contains sodium, iron, copper, zinc, cobalt and manganese [3]. Macronutrients such as sodium, potassium, magnesium and calcium are a significant group of nutrients from milk that the human body needs in amounts greater

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than 100 mg per day for optimal functioning. Sodium is a basic element in extracellular fluid and is known to be involved in the functioning of nerves and muscles. It also regulates plasma volume and acid-base balance. Potassium is the basic cation in the intracellular fluid, and participates in the regulation of osmotic pressure, blood pressure and acid-base balance. Calcium is very important in building and maintaining strong bones and teeth. It is involved in the work of the nervous system, muscle contraction and blood clotting. Magnesium is primarily an intracellular element that plays various roles in DNA metabolism and synthesis and muscle relaxation [4]. Since whey is well known for its health benefits and it' mineral content, it is very important to adequately measure metal content. The well-known digestion of whey samples is in concentrated nitric acid under reflux. This method uses corrosive acid which develops toxic fumes during the process [5]. The chemical process of nitric acid decomposition is shown in the Eq. 1.1.

$$4HNO_3 \rightarrow 4NO_2 + O_2 + 2H_2O \tag{1.1}$$

One of the products of nitric acid decomposition is NO_2 gas. NO_2 - Nitrogen dioxide is toxic to humans when inhaled. The compound is acrid and easily detectable by smell at low concentrations. However, low concentrations (4 mg/kg) will anesthetize the nose, thus creating a potential for overexposure. Symptoms of poisoning tend to appear several hours after inhalation of a low but potentially fatal dose. Since this method is dangerous for human health and it is time consuming (lasts for over two hours). The question that arises is: is it possible to extract metals from whey samples by less toxic and less time-consuming method? The method for extraction of metals with TCA was at the beginning introduced for milk [6]. In this research we investigated TCA extractability of six metals with different behaviour: two metals are alkali metals: sodium and potassium, two are alkali earth metals: magnesium and calcium, and two are heavy metals: iron and zinc.

2 Materials and Methods

2.1 Materials

As a material for performing the experimental part of the work, three types of whey were used, as follows:

1. cow's milk whey,
2. sheep's milk whey,
3. goat's milk whey.

Whey samples are obtained in the household, according to the following recipe: the cheese is made from fresh cow's, goat's or sheep's milk, which is previously strained and cooled. The cooled milk is poured into a stainless-steel metal container and heated in an oven until it reaches a temperature of about 35 °C. After that, pure rennet is added to the milk (two tablespoons are added to 15 L of milk). The milk is mixed gently until a lump formed. The formed pear is cut into cubes to release the whey. The curd is then strained through gauze, separating the whey from the curd. Then the pear moulds are formed, after which they are salted and stored in tubs for storage. Whey samples

were stored in adequate conditions, in plastic packaging, at refrigerator temperature until the beginning of laboratory analyses before which the samples were prepared in an appropriate manner.

2.2 Methods

In the samples, we initially measured the values of the basic quality parameters: pH, electrical conductivity and redox potential. Second part of investigation was extraction and analysis of metals from whey samples and their results comparison.

pH Value Determination. The pH value in whey samples was measured using a Mettler Toledo MP 230 pH meter. When measuring the pH, a buffer with a pH of 7.00 and 4.00 was used. Procedure: As the samples were in liquid form, no additional sample preparation was required. The samples were poured into plastic cups that were placed on a pH meter stand into which the electrode for pH determination was immersed, after each measurement the electrode was rinsed with distilled water. Three independent measurements were performed for each sample and the mean value was calculated.

Electrical Conductivity Measurement. The electrical conductivity in whey samples was measured using a Mettler Toledo MC 126 conductometer. The conductometer must be calibrated with standard solutions before use. Procedure: The samples were poured into plastic cups into which an electrode for measuring electrical conductivity was then immersed, after each measurement the electrode was rinsed with distilled water. After the sound signal, the measured values were recorded from the conductometer display. Three independent measurements were performed for each sample and the mean value was calculated based on them.

Reduction – Oxidation Potential Measurement. Redox potential in whey samples was measured using an ORP-meter. Procedure - As the samples are in liquid form, no additional sample preparation was required. The samples were poured into plastic cups into which the electrode was then immersed. Three independent measurements were performed for each sample and the mean value was calculated based on them.

Determination of Metal Content

Sample Preparation

Two extraction methods were used to prepare whey samples:

1. method of wet digestion under reflux in concentrated nitric acid and
2. method of extraction in 24% trichloroacetic acid (TCA)

Method of Wet Digestion in Concentrated Nitric Acid. Procedure: 1 g of the sample is placed into a 250 ml flask and 5 ml of concentrated nitric acid (HNO_3) was added. The flask was placed on the heating plate and digested in the digester for 1 h and 30 min, under a reflux. After digestion, 2 ml of demineralized water and 3 ml of 30% H_2O_2 were added to the flask and filtered through the slow filtering filter paper into a 50 ml volumetric flask. The volume was made up with distilled water, and pour into storage tubes until the determination.

Method of Extraction with 24% TCA. Procedure: To obtain 24% TCA 120 g TCA was placed into 500 mL glass beaker then 380 ml of distilled water was added. The extraction mixture was made by pipetting 5 ml of sample and 50 ml of dissolved acid into plastic bottles. After that, the bottles were shaken for 30 min. After every 5 min there is a break in shaking for 1 min. The samples were then poured into plastic tubes and carried to centrifugation (six places) at 4000 rpm for 5 min. After centrifugation, the samples were filtered through filter paper and stored in test tubes and until determination.

Metal Content Determination. After preparation of the samples, the metal content was measured by the atomic absorption spectrophotometry (FAAS) with flame atomization, and by the method of flame photometry (FP). The content of iron, zinc, calcium and magnesium were measured by FAAS. Contents of sodium and potassium were measured by FP.

Atomic Absorption Spectrophotometry. Measurements were conducted on atomic absorption spectrophotometer, Shimadzu AA-7000. For measurement of all metal different concentrations of standards were used. Addition of lanthanum solution (La) was necessary for measurement of calcium and magnesium. Procedure: After performing metal extraction using the above methods, i.e., performing sample preparation, we determined the absorption of standards for each element and started measuring each sample on an Atomic Absorption Spectrophotometer.

Flame Photometry. The concentration of sodium and potassium were measured by flame photometer FP902, PG instruments. The procedure with standards was similar to procedure with atomic absorption spectrophotometry.

Statistical Evaluation. The results of the research were processed using the statistical program SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) through the following analyses:

- descriptive statistics (arithmetic mean or average).
- factor and two-factor ANOVAs: When the p-value was less than 0.05 (because the error of the first type is 5%), the difference between the compared groups was considered statistically significant. Otherwise, the differences between the compared groups were not considered statistically significant.
- Tukey test: The differences between the mean values are separated by the Tukey test. When the p-value was less than 0.05 (because the error of the first type is 5%), the difference between the compared groups was considered statistically significant.
- Standard deviation (SD) and coefficient of variance (CV) for estimating the precision of the methods for metal analysis.

3 Results and Discussion

In this section, the results are presented with tables or graphics. The section is divided into two mayor parts: 1. Basic quality parameters and 2. Metal's content. After results, the discussion is presented.

3.1 Results of the Basic Quality Parameters

The average results of three measurements of basic quality parameters are shown in the Table 1.

Table 1. Basic quality parameters of whey

Sample	pH	EC (mS/cm)	ORP (mV)
Cow's whey	6.54	6.69	86
Goat's whey	6.33	5.98	72
Sheep's whey	6.13	6.31	82

The average pH value of the tested whey samples was 6.34. The highest pH value was measured in cow's milk whey samples, with a mean value of 6.54. The other two samples had a slightly lower pH value, i.e., goat's milk whey 6.33 and sheep's milk whey 6.13. All samples were slightly acidic, which is the case in other studies [7].

The average value of electrical conductivity in the tested whey samples was 6.33 mS/cm. The highest electrical conductivity was measured in cow's milk whey samples, with a mean value of 6.69 mS/cm. The lowest values were measured for goat's milk whey, with a mean value of 5.98 mS/cm, while for sheep's milk whey it was 6.13 mS/cm. The electrical conductivity of investigated whey is higher than found in [8]. The high electrical conductivity suggests higher amount of ions presence in the sample. The highest electrical conductivity was measured in cow's whey – the cow's whey contains more ions (metals) when compared to other samples.

The average value of redox potential in the tested whey samples was 80. The highest redox potential was measured in whey samples from cow's milk, with a mean value of 86. The lowest value was measured in goat's milk whey, with a mean value of 72, while in whey of sheep's milk was 82. The positive (+ sign) results of the redox potential measurements tell us that the whey samples are oxidative medium. The redox potential partially controls bacterial development [9].

3.2 Results of Metal Content with Two Methods of Sample Preparation

Metal Determination with Digestion in Concentrated Nitric Acid. Results gained after the digestion in concentrated nitric acid are shown in Table 2.

Table 2. Results of metal content after extraction with concentrated nitric acid

Metal (mg/kg)	Cow's whey	Goats' whey	Sheep's whey
Ca	36.4	32.4	28.0
Mg	6.8	6.8	9.6
Zn	5.6	3.7	7.3

(*continued*)

Table 2. (*continued*)

Metal (mg/kg)	Cow's whey	Goats' whey	Sheep's whey
Fe	2.0	1.9	4.8
Na	465.0	274.8	414.2
K	1615.5	1205.2	1301.4

Metal Determination with Digestion in TCA. Results gained after the digestion in TCA are shown in Table 3.

Table 3. Results of metal content after extraction with TCA

Metal (mg/kg)	Cow's whey	Goats' whey	Sheep's whey
Ca	23.8	26.8	22.3
Mg	3.4	4.4	5.6
Zn	0.4	0.3	0.5
Fe	0.8	0.8	2.0
Na	484.7	368.3	508.3
K	1353.3	1220.0	1273.3

Table 2 shows the amounts of, calcium, magnesium, zinc, iron, sodium and potassium after extraction with concentrated nitric acid. The highest value of calcium was in cow's milk whey (36.4 ppm), and the lowest in sheep's milk whey (28.0 ppm). Analysis of variance in calcium showed that there are statistically significant differences between whey samples, which was confirmed by the Tukey test. The highest values of magnesium were in sheep's whey (9.6 ppm), while the other two samples had approximately equal values, cow whey (6.8 ppm) and goat whey (6.8 ppm). For magnesium, analysis of variance showed that there was a statistically significant difference between the samples. The Tukey test showed that there is a difference between sheep whey compared to cow's and goat's milk whey. Sheep's milk whey had the most zinc (7.3 ppm) and goat's milk whey the least (3.7 ppm). The results obtained after extraction of iron by concentrated nitric acid had a statistically significant difference. The highest value of iron was in whey from sheep's milk (4.8 ppm), and the lowest in whey from cow's milk (2.0 ppm). For sodium, whey from cow's milk (465.0 ppm) had the highest value, and whey from goat's milk (274.8 ppm) had the lowest. The highest value of potassium was in cow's milk whey (1615.5 ppm), the lowest in goat's milk whey (1205.2 ppm). Analysis of variance (ANOVA) showed that there were no statistically significant differences between the tested samples ($p < 0.05$) for potassium, which was confirmed by the Tukey test. The results of this research are in correspondence with almost all measured metals researched in [10].

Table 3 shows the concentrations of investigated metals after extraction with TCA. Extraction of calcium showed the highest value in goat's whey (26.8 ppm). Highest amount of magnesium was found in sheep's whey (5.6 ppm). The highest value of zinc was in sheep's whey (0.5 ppm), but the difference was not large, as shown by the analysis of variance, which showed that there was no statistically significant difference between the samples. Analysis of extracts with TCA showed the highest iron value was in sheep's milk whey (2.0 ppm), while cow's and goat's whey had equal values (0.8 ppm). Analysis of variance (ANOVA) for iron showed that there was no statistically significant difference between the tested samples ($p < 0.05$), which was confirmed by the Tukey test. When it comes to sodium, the highest value was found in sheep whey (508.3 ppm), and the lowest in goat whey (368.3 ppm). Highest potassium value was found in cow's whey (1353.3 ppm).

Comparison of Metal Content to Recommended Daily Intake. The average value of iron from all measured values was 2.06 mg/kg. The recommended amount of iron per day is 14 mg. 14 mg of iron is contained in around 7 L of whey. The average value of magnesium was 4.47 mg/kg. The recommended daily intake of magnesium is 320–420 mg, and it is contained in around 71–93 L of whey. The average value for calcium from all measured was 28.27 mg/kg. The recommended daily intake is 1000–1300 mg, and it is contained in around 35 to 45 L of whey. The average value for zinc in all measured samples was 2.96 mg/kg. The recommended daily intake is 8.6–10 mg, and it is contained in amount of 3–3.4 L of whey. The average value of potassium from all measured samples was 1328,12 mg/kg. The recommended value is 4700 mg, and it is contained in around 3.5 L of analysed whey. The recommended value for sodium daily intake is 1500 mg, and it is contained in approximately 3.5 L of analysed whey.

Comparison of the Methods for Sample Preparation. Comparison of the results gained by two different methods of sample preparation are shown in Figs. 1, 2, 3, 4, 5 and 6.

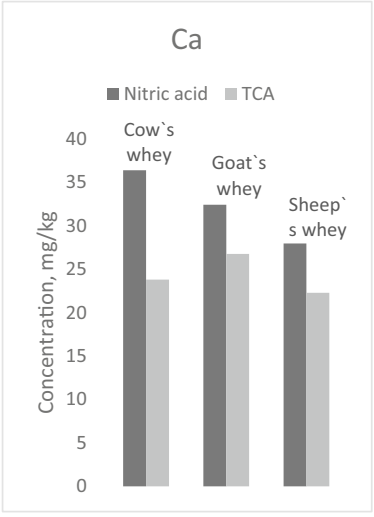

Fig. 1. Comparison of Ca analysis

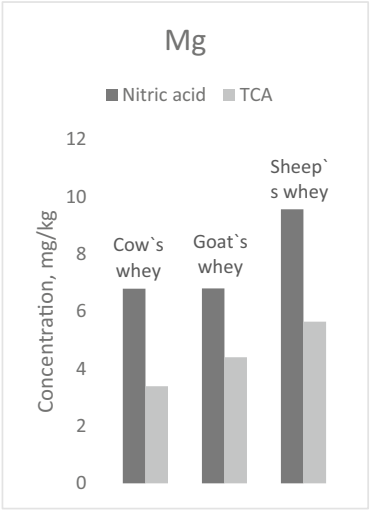

Fig. 2. Comparison of Mg analysis

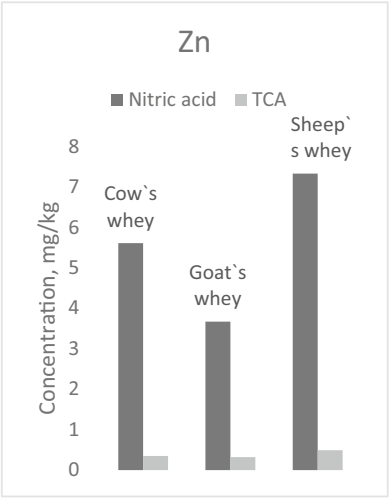

Fig. 3. Comparison of Zn analysis

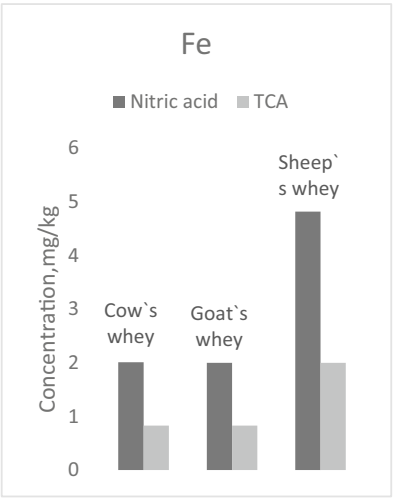

Fig. 4. Comparison of Fe analysis

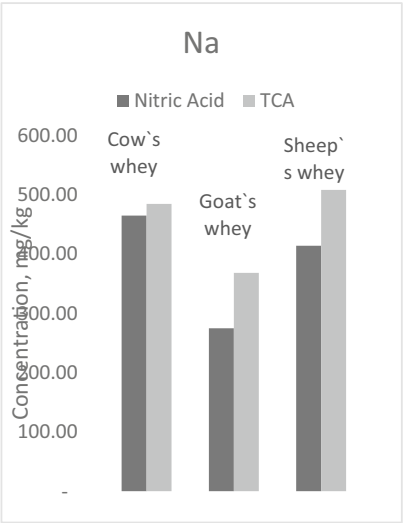

Fig. 5. Comparison of Na analysis

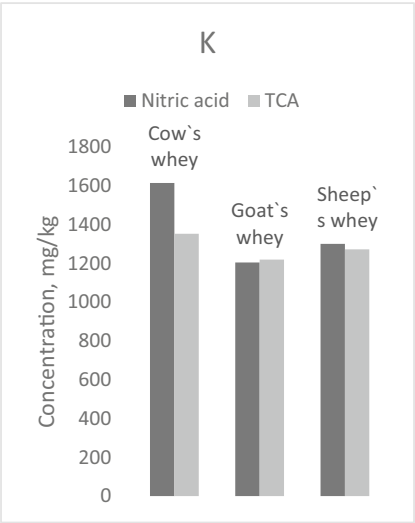

Fig. 6. Comparison of K analysis

The different extraction procedures resulted in a different amount of metals. In almost every case the concentrated nitric acid extraction resulted in higher metal concentration. The differences in extractability of metals could be the result of metal bonding to proteins of whey. One of the most important proteins of whey is α-lactalbumin to which calcium and iron are directly incorporated [11, 12]. Analysis of variance for iron, calcium magnesium and zinc, showed statistically significant differences between these methods. The highest difference in concentration of metals gained by two different methods of

extraction was found in case of zinc. The possible reason for that could be in strong interactions of zinc with whey proteins [13]. Analysis of variance (ANOVA) showed that there were no statistically significant differences ($p < 0.05$) between these two methods in case of potassium and sodium. TCA method that is simpler and less toxic than the method of digestion in concentrated nitric acid, could be used for extraction of sodium and potassium from whey samples. Factors affecting the binding of a metal to proteins include the metal properties, such as the valence state, ionic radius, charge-accepting ability, and free metal concentration in the respective biological compartment [14]. Because potassium and sodium have low complexation ability, which is derived by the fact that they are monovalent ions, and they have low charge density, they are not bonded to whey proteins, thus can be easily extracted by TCA.

3.3 Precision of the Applied Methods for Metal Determination

The precession of applied methods of metal extraction was determined by means od standard deviations between three replicates and coefficients of variance, which is presented in Table 4.

Table 4. Standard deviations and coefficients of variance of applied extraction methods

Metal	Whey type	STD of Nitric acid extract measurements (mg/kg)	CV	STD of TCA extract measurements (mg/kg)	CV
Ca	Cow's whey	1.78	0.05	0.31	0.01
	Goat's whey	0.95	0.03	0.33	0.01
	Sheep's whey	3.33	0.12	0.25	0.01
Mg	Cow's whey	0.62	0.09	0.13	0.04
	Goat's whey	0.65	0.09	0.27	0.06
	Sheep's whey	1.50	0.16	0.80	0.14
Na	Cow's whey	94.42	0.02	9.02	0.20
	Goat's whey	6.15	0.02	7.21	0.02
	Sheep's whey	65.49	0.01	7.21	0.16
K	Cow's whey	428.91	0.26	15.27	0.11
	Goat's whey	21.26	0.02	26.45	0.021
	Sheep's whey	175.42	0.13	15.27	0.01
Fe	Cow's whey	1.88	0.93	0.92	1.10
	Goat's whey	1.23	0.62	0.41	0.50
	Sheep's whey	1.33	0.27	1.70	0.85
Zn	Cow's whey	4.19	0.74	0.06	0.18
	Goat's whey	0.79	0.21	0.04	1.25
	Sheep's whey	3.99	0.54	0.25	0.51

Based on the standard deviation from the mean value, we can say that method for extraction of metals in concentrated nitric acid gives higher standard deviations, in almost every case. The results gained with flame photometry gave the higher standard deviations, the reason for this lies probably in the wavelengths selection made by filters, which give a wide range of wavelengths (precision is low). The method of extraction in TCA is more precise. Coefficient of variance which is standard deviation divided by mean value is below one[1] for every sample.

4 Conclusions

- Extraction of metals with concentrated nitric acid gives higher amounts of metals.
- It is not possible to extract all metals content with TCA. Similar results with different extraction procedures were found in case of natrium and potassium.
- Although the TCA method is more precise, we suggest usage of concentrated nitric acid for metals extraction, because of higher extractability.
- It is possible to extract alkali metals with TCA.
- From the above results we conclude that the tested metals are present in all whey samples, but that their concentration cannot meet the daily needs.
- Because of high standard deviations between three replicates in case of flame photometry method, we suggest usage of atomic emission spectroscopy (AES). AES method has much more precise wavelength selection.

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[1] Coefficient of variance below one is considered low variance, while coefficient of variance above one is considered high variance.

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The Effect of Plant Variety and Addition of Plant Distillation Products on Biopolymer Film Properties

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Abstract. Natural polymers (proteins, lipids and polysaccharides) are processed as biopackaging materials regarding concerns on environmental waste problems that are mainly caused by non-biodegradable materials, such as petrochemical-based polymer packaging materials as well as the customer's requests for environmentally-safe materials and high quality foodstuff. However, biopolymer based film has not sufficient adequate mechanical properties and barrier properties as well. In this regard, this paper aimed to describe how various plant distillation products (essential oils and hydrolats) affect biopolymer film properties. In addition, two plant varieties were examined: *Mentha mohito* and *Mentha spicata.* Gelatin was chosen as biopolymer matrix carrier for active components (essential oils and hydrolats) as it makes colorless, tasteless and odorless biopolymer films. Gelatin films were prepared as 10% solutions with addition of glycerol as plasticizer. Afterwards two essential oils were added *Mentha mohito* and *Mentha spicata*) in a percent of 0,1% and 0,5%. The other samples were prepared in a way that water was replaced with hydrolats (which lag behind after distillation of *Mentha mohito* and *Mentha spicata)* in a percent of 10% and 50%. Mechanical (thickness, tensile strength and elongation at break), physico-chemical (moisture content, swelling and solubility), structural (FTIR), and antioxidative properties (DPPH) of the synthetized active biopolymer films were examined. Obtained results showed dose-dependent effect of added active components on biopolymer films properties, which was most evident related to the antioxidative properties. Addition of active components improved examined mechanical and physico-chemical properties. The difference between the applied varieties of plant distillation products was also reported. Results shown present introductory research in demonstrating the potential use of active biopolymer films as packaging material useful in the food industry.

Keywords: Gelatin · Essential oils · Hydrolats · *Mentha mohito* · *Mentha spicata* · Properties

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1 Introduction

Polymer packaging materials, synthesized from unrenewable energy sources, are significantly damaging the environment (Lazić and Popović 2015). Biodegradable polymers are becoming increasingly significant because they are functionally equal but environmentally better than synthetic packaging materials. Biodegradable materials of natural origin (biopolymers) have strong barriers to different environmental conditions, which qualifies them for packing the food products.

Biodegradable biopolymers, such as polysaccharides, proteins and lipids are extensively used to produce biopolymer edible films, in order to bring nonrenewable energy consumption down, as well as environmental and ecological pressure. It is possible to produce composite bio-based films (Popović 2013, Hromiš et al. 2019) using food industry byproducts in order to maximize the usage od byproducts and to reduce packaging waste. Among the biopolymers that have been considered for preparing bio-based films, polysaccharides (Hasan et al. 2020) and proteins (Wang et al. 2019) must be highlighted. Polysaccharide films showed good mechanical properties, good barrier properties to gases and aromas and ability to carry active compounds (Mikkonen et al. 2007). Protein biofilms have been shown to be a good barrier to oxygen (Popović et al. 2018) and to be appropriate for the biopolymer materials synthesis, whose properties can be improved by different types of modification (physical, chemical and enzymatically), in order to obtain bio-material with adequate properties.

There are different sources of proteins to produce biofilms, but gelatine based edible films have been widely used to develope biodegradable materials due to their good film-forming ability (Wang et al. 2015). However, the use of gelatine based films are limited, because of lower mechanical properties then commercially used polymers as well as poor water vapour barrier characteristics.

The use of active compounds in different edible films preparation, such as plant essential oils with hydrophobic properties, can improve water vapor barrier properties and mechanical properties as well (Espino-Manzano et al. 2020). In order to produce active gelatin films, many different types of essential oils have been added (palme, clove, oregano, orange and ginger) (Da Silva et al. 2021; Espino-Manzano et al. 2020; Zhang et al. 2017).

The essential oils usage is widely known, but limited by their strong flavour. However, blending them into a polymer film matrix represents an alternative in order to reduce their sensory impact (Ruiz-Navajas et al. 2013). However, the non-polar nature of the essential oils requires the different techniques of homogenization intending to incorporate them into the aqueous film forming dispersions of hydrocolloids, such as gelatin or starch. Moreover, there is possibility to enhance functional film's properties, because of the lipid molecules and their potential interactions with the polymer chains in film's matrix (Chiralt et al. 2015).

In the essential oils production there are byproducts that left behind. Hydrolates (hydrosols or aromatic waters) are obtained by steam distillation from the raw material. They consist of the water found in the raw plant material and contain great amount of biologically active volatile compounds. Aćimović et al. (2020) reported that hydrolates have a much softer scent and lower biological activity than the correspondent essential oils, but they still can be used to develop biopolymer films.

In this context, the head aim of this study was to analyze the effect of two plants *(Mentha mohito* and *Mentha spicata*) distillation products (essential oils and hydrolats) on the functional properties of gelatin films, in terms of the mechanical, physico-chemical, structural properties, as well as in terms of their antoxidative activity.

2 Materials and Methods

2.1 Material

Bovine gelatin type B was obtained from Sigma-Aldrich (Darmstadt, Germany). *Mentha spicata* and *Mentha mohito* essential oils, as well as *Mentha spicata* and *Mentha Mohito* hydrolats were acquired from Institute of field and vegetable crops (Novi Sad, Serbia), and Tween 80 ($C_{64}H_{124}O_{26}$) and glycerol ($C_3H_8O_3$) were provided by Sigma Aldrich (Darmstadt, Germany).

2.2 Preparation and Casting of Active Films

Active biopolymer films based on gelatine were prepared using the casting method. The film-forming suspension of gelatine from bovine skin, Type B (10 %, w/w) in deionized water was produced with 30% glycerol addition of (w/w, per weight of gelatine). *Mentha spicata* and *Mentha mohito* oils were previously emulsified with Tween 80 at 25% (w/w, based on essential oil) and essential oils were incorporated into the film-forming suspension (at concentrations of 0.1% (v/v) and 0.5% (v/v) respectively) byusing an ultra-turrax homogenizer for 2 min at 10 000 rpm. *Mentha spicata* and *Mentha mohito* hydrolats were added instead of deionized water in appropriate percentages (10% and 50%), respectively. The emulsions were casted onto Teflon-coated Petri dishes and thosefilms were dried for 48 h, under room conditions (23 ± 2 °C, 50 ± 5% RH). The films obtained were labeled in such a way:

- gelatine film with 0.1% (v/v) of *Mentha spicata* and *Mentha mohito* essential oil (MS 0.1%; MM 0.1%),
- gelatine film with 0.5% (v/v) *of Mentha spicata* and *Mentha mohito* essential oil (MS 0.5%; MM 0.5%),
- gelatine film with 10%*Mentha spicata* and *Mentha mohito* hydrolat (v/v) (MS 10%; MM 10%),
- gelatine film with 50% *Mentha spicata* and *Mentha mohito* hydrolat (v/v) (MS 50%; MM 50%),
- control gelatine film without essential oils or hydrolats.

2.3 Characterisation of Films

Mechanical Properties

Micrometer Digico 1 was used to measure film thickness with 0.001 mm sensitivity (Tesa, Renens, Switzerland). Eight thickness measurements were carried out. The results were

obtained by calculating the arithmetic mean of eight random measurements from the surface of each sample.

In accordance with ASTM standard method D882-10 tensile strength (TS) and elongation to break (EB) were examined on the Instron Universal Testing Instrument Model No 4301 (Instron Engineering, Canton, Massachusetts, USA). The initial grip separation was set at 50 mm, while the cross-head speed was set at 50 mm/min. For each sample there were eight times repeated examinations. All the film samples were cut in same dimensions (15 × 90 mm).

The tensile strength (TS) in MPa and elongation at break (%EB) were calculated using Eqs. (1) and (2), respectively.

$$TS = Fm/A \tag{1}$$

where TS: tensile strength (MPa); Fm: maximum force the rupture of the film is occured (N); A: cross-sectional area of the film examined (m^2).

$$EB = E_a/d_{initial} \times 100 \tag{2}$$

where: EB: elongation at break (%); Ea: sample extension (mm); dinitial: initial claw separation distance (50 mm).

Physico-Chemical Properties

Before water (moisture) content determination, film samples (2 × 2 cm) were conditioned in different starting atmospheres during 48 h (room and refrigerated conditions). Film samples were weighed (w_1), then dried at 105 °C ± 2 °C for 24 h and weighted (w_2) again. Based on Rhim et al. (1998) water content (WC) was determined as the percentage of initial film weight lost during drying and reported on a wet basis Eq. (3). For each storage condition three measurements of water content were conducted.

$$W_C = 100 \times \frac{w_1 - w_2}{w_1}[\%] \tag{3}$$

where: *w1* and *w2* are the weights of the wet and air-dried samples.

For swelling ability determination pieces of films (1 × 2 cm) were cut, weighed in air-dried conditions (w_d) and then immersed in deionized water (25 °C) for 2 min. Wet samples were wiped with filter paper to remove excess liquid and weighted (w_w). Swelling was determined by the modified method described by Bigi et al. (2001). The amount of adsorbed water was calculated according to Eq. (4) and expressed as percentage:

$$\text{Swelling ability} = 100 \times \frac{W_w - W_d}{W_d}[\%] \tag{4}$$

where: *ww* and *wd* are the weights of the wet and air-dried samples.

As explained by the method in the study of Kaewprachu et al. (2017) the film solubility was measured. For total soluble matter determination, small pieces of film (2 × 2 cm) were dried in the oven at 105 °C ± 2 °C for 24 h to obtain the initial dry mass of the film. After drying, films were placed into test tubes containing 20 ml of deionized water, after what test tubes were covered, gently shaken and left at room temperature

for 24 h. The remaining pieces of film were dried in the oven at 105 °C ± 2 °C for 24 h to obtain final dry mass of the film. The percentage of total soluble matter (% TSM) of the films was calculated using the Eq. (5).

$$\mathrm{TS}M\,[\%] = 100 \times \frac{W_1 - W_2}{W_1}[\%] \tag{5}$$

where: *w1* and *w2* are initial dry mass before the test and final dry mass after the test.

Antioxidative Activity

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the active films was evaluated according to the procedure reported by Morales and Jimenez-Perez (2001), with several modifications. Briefly, an aliquot of 200 µl of the sample was added to 1 ml of a daily prepared DPPH solution (74 mg/l) in ethanol. Film pieces (10 × 10 mm) were mixed with 2 ml of the DPPH ethanolic solution and left on a shaker for 24 h. The absorbance values were measured at 520 nm (T80 UV–Vis Spectrophotometer; PG Instruments, Lutterworth, UK) and the DPPH radical scavenging activity was calculated by the following equation Eq. (6):

$$\text{DPPH radi. scav. acti.} = \frac{\text{Ablank} - \text{Asample}}{\text{Ablank}} \times 100 \tag{6}$$

where A blank is the absorbance value at 520 nm of the ethanolic solution of DPPH without the added sample and A sample is the absorbance value at 520 nm of the ethanolic solution of DPPH with the added sample.

Structural Properties

Fourier Transform Spectroscopy

FTIR analysis of the film samples was carried out in the wave number range 4000 to 400 cm^{-1}, at a resolution of 4 cm^{-1}, using the IR spectrophotometer, Nicolet IS10, Thermo Scientific (Massachusetts, USA) and attenuation total reflection (ATR) extension. Before measurement, background shot was taken before the analysis of each sample, following each sample was scanned 32 times. IR spectrophotometer is controlled via software Omnic 8.1. (Thermo Fisher Scientific, MA, USA), which was also used to operate the FTIR spectrometer, collect and present all the datas given.

Statistical Analysis

Descriptive statistical analyses for calculating the means and the standard error were completed using MicroSoft Excel software (MicroSoft Office 2010). All obtained results were expressed as the mean value ± standard deviation (SD).

Significance of the effect and interaction of individual factors, for every response was determinated by analysis of variance (ANOVA) and application of post-hoc Tukey HSD test. For ANOVA analysis, StatSoft Statistica ver.12.0 software package was used.

3 Results and Disccusion

3.1 Mechanical Properties

The visual appearance of the product and packaging directly influences consumer purchasing. Related to that, gelatine films are visually appealing due to its transparency, above all. In the Fig. 1. gelatine films with *Mentha mohito* and *Mentha spicata* oils (0.1%; 0.5%) in the first row and gelatine films with *Mentha mohito* and *Mentha spicata* hydrolats (10%; 50%) in the second row are shown.

Referring to visual examination obtained films were homogeneous, transparent, fragile, but easy to examine and handle. Films were not greasy on the surface and sticky as well. Films to which both essential oils were added had a mild odor of oil added, while those with hydrolats were almost neutral. Higher concentrations of oil (0.5%) and hydrolats (50%) contributed to slightly more intensive film color and odor for both plant species (*Mentha mohito* and *Mentha spicata).*

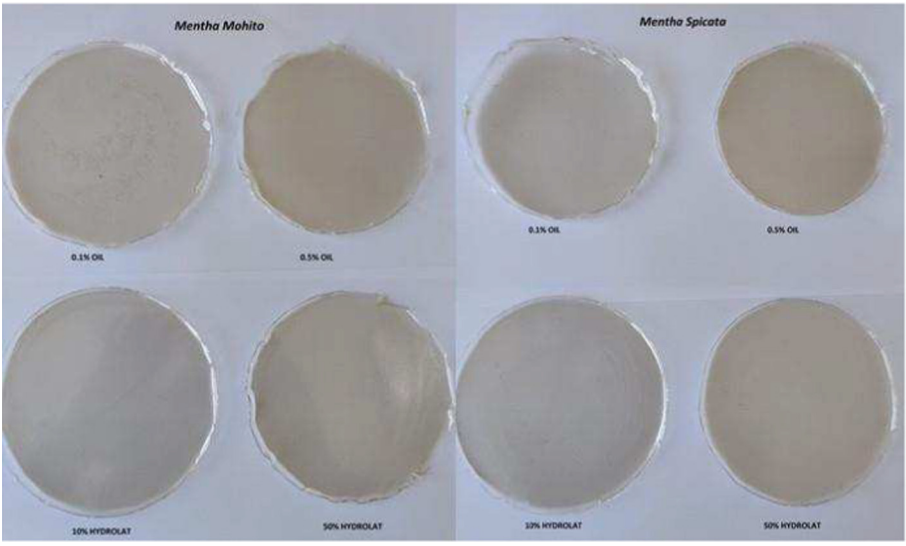

Fig. 1. Gelatine films with *Mentha mohito* and *Mentha spicata* oil (0.1%; 0.5%), in the first row, respectively; Gelatine films with *Mentha mohito* and *Mentha spicata* hydrolat (10%; 50%), in the second row, respectively

The film thickness is an important parameter which can influence other film properties (mechanical, optical and water vapour permeability) and its application area. Film thickness was quiet uniform, in the range from 99.50 to 106.54 μm (Table 1). Very small values of standard deviation confirmed film uniformity. The thickness of the films containing essential oils was greater than the control film and the films with hydrolates, for both plant species (*Mentha mohito* and *Mentha spicata* oil), but there is no statistically significant differences among values.

The films with 0.5% essential oils had the greatest thickness, followed by the films with 50% corresponding hydrolats. This small grow trend in thickness is probably related to different surface stresses and the size of the oil droplets incorporated in film matrix (Tongnuanchan and Benjakul, 2014).

Table 1. Gelatine based active films mechanical properties with different amount of added essential oil/hydrolat (mean ± SD)

	Thickness (μm)	Tensile Strength (MPa)	Elongation at break (%)
Control	99.91 ± 3.08^{a}	43.02 ± 4.78^{a}	10.31 ± 0.23^{a}
MM 0.1%	102.21 ± 3.90^{a}	32.33 ± 1.58^{b,c}	14.05 ± 2.41^{a,b}
MM 0.5%	106.54 ± 4.75^{a}	29.17 ± 0.33^{b}	18.87 ± 0.47^{b}
MM 10%	96.59 ± 3.18^{a}	42.25 ± 5.49^{a}	10.37 ± 3.09^{a}
MM 50%	101.09 ± 3.78^{a}	38.88 ± 4.04^{a-c}	12.79 ± 2.62^{a,b}
MS 0.1%	99.50 ± 3.53^{a}	30.31 ± 0.36^{b,c}	12.35 ± 4.77^{a}
MS 0.5%	106.5 ± 3.09^{a}	19.59 ± 3.54^{d}	16.36 ± 0.53^{a,b}
MS 10%	100.5 ± 3.50^{a}	42.73 ± 3.12^{a}	11.98 ± 0.65^{a}
MS 50%	104.5 ± 3.54^{a}	36.51 ± 2.33^{a-c}	12.84 ± 0.27^{a,b}

$^{a–d}$Different letters in superscript of the same table column indicate on statistically significant difference between values, at level of significance of $p < 0.05$ (based on post-hoc Tukey HSD test)

Table 1 shows that the addition of both essential oils and both hydrolats decreases the tensile strength (TS) value, which indicates a loss of macromolecular mobility which is consistent with the research of Souza et al. (2013). Decrease in value was correlated with concentration of added oil/hydrolat. Tensile strength amounting 43.02% for control gelatine film remain almost the same in films with 10% hydrolats for both the species. More remarkable decreasement in TS values is visible in samples with 0.1% oil (32.33% and 30.31%) and 0.5% oil (29.17% and 19.59%) with more significant difference in sample with the lowest TS value for film with *Mentha spicata* essential oil (19.59%).

Elongation at break values (Table 1) of the tested films increased, which is consistent with the other authors findings (Da Silva et al. 2021; Zhang et al. 2017), demonstrating that incorporation of higher oil concentration strengthen the film matrix, making it more elastic.

Same as tensile strength, increase in value was correlated with concentration of added oil/hydrolat. Higher concentration of oil (0.5%) contributed to higher values for elongation at break (16.36–18.87%) in comparison with TS value for control sample (10.31%). However, there was not observed significant difference related to the used plant species, except for samples where 0.5% *Mentha spicata* oil was added. There is more remarkable increasement in tensile strength values in comparison with samples where 0.5% *Mentha mohito* oil was added.

To maintain packaging integrity and to retain the characteristics of the food inside of packaging, packaging material must be resistant to the pressure during the transport, handling and application. Souza et al. (2013) reported that values for tensile strength and elongation at break of hydrocolloid films are low in comparison with synthetic polymers, but good enough to find an application for food packaging.

3.2 Physico-Chemical Properties

Some of the film properties (especially mechanical) are affected by the moisture content. Film flexibility can be decreased due to lower moisture content, because water is acting like a plasticizer. The moisture content of gelatine-based films was 9.28–16.09% (Table 2), depending on the film formulation. The moisture content of gelatine-based films was affected by the presence of higher oil percentage (0.5%) for both plant species (9.28% ad 11.20%) when compared with control sample (16.09%). The addition of 50% hydrolats led to a decrease in moisture content of gelatine film with *Mentha mohito* hydrolat (10.28%) more then *Mentha spicata* hydrolat, where the moisture content value remained almost the same as for control sample (14.45%).

Table 2. Gelatine based edible films water content (%), solubility (%) and swelling (%) with different amount of added essential oil/hydrolat (mean ± SD)

	Moisture content (%)	Solubilty (%)	Swelling (%)
Control	16.09 ± 2.22^{d}	62.60 ± 4.26^{c}	139.89 ± 9.59^{b}
MM 0.1%	$10.81 \pm 1.89^{a-c}$	$51.09 \pm 2.19^{a,b}$	$117.56 \pm 8.90^{a,b}$
MM 0.5%	9.28 ± 1.97^{a}	48.71 ± 1.69^{a}	114.59 ± 8.95^{a}
MM 10%	$11.93 \pm 1.75^{a-d}$	55.65 ± 2.31^{b}	$136.87 \pm 7.85^{a,b}$
MM 50%	$10.28 \pm 1.90^{a,b}$	$52.09 \pm 1.98^{a,b}$	$129.12 \pm 7.58^{a,b}$
MS 0.1%	$11.92 \pm 1.68^{a-d}$	$54.20 \pm 2.01^{a,b}$	$120.15 \pm 8.66^{a,b}$
MS 0.5%	$11.20 \pm 1.74^{a-c}$	$50.04 \pm 1.89^{a,b}$	$118.78 \pm 9.18^{a,b}$
MS 10%	$15.23 \pm 0.39^{c,d}$	$53.82 \pm 1.79^{a,b}$	$136.79 \pm 4.15^{a,b}$
MS 50%	$14.45 \pm 0.89^{b-d}$	$52.30 \pm 1.69^{a,b}$	$130.91 \pm 6.97^{a,b}$

a-d Different letters in superscript of the same table column indicate on statistically significant difference between values, at level of significance of $p < 0.05$ (based on post-hoc Tukey HSD test)

The water solubility of prepared gelatine films with essential oils/hydrolats is presented in Table 2. The presence of hydrophilic compounds should increase water solubility of films, whereas hydrophobic compounds decrease it, as claimed by Kavoosi et al. (2013). Water solubility of control film was the highest, so the decreasement in water solubility is result of the essential oil addition. The highest measured value of solubility was 62.6% for the control film sample and the lowest (48.71% and 50.04%) in samples with 0.5% of *Mentha mohito* and *Mentha spicata* oils, respectively. There is

no statistically significant differences between species used for samples with 0.1% oil and samples with 50% hydrolate. Among samples where 0.5% oil and 10% hydrolate were added there are statistically significant differences among plant species, which is not in accordance with the values presented, probably due to method sensitivity. Present study results showed that the film solubility followed the equal trend as moisture content which is in accordance with other studies (Da Silva et al., 2021; Akhter et al., 2019).

It is of great importance to predict stability and eventual quality changes of biopolymer-based films. The main reason for this is their low resistance to water, so it is preferably to add lipid phase to the biopolymer film matrix, because it can resulted in swelling degree changes. Swelling of the films decreased with the addition of higher amount of essential oils, whereas the addition of hydrolats did not affect the swelling ability (Table 2). It is obvious that there is no significant difference regarding the species added for samples with 0.1% of oil added. However samples with 0.5% oil are different slightly more, where samples with 0.5% *Mentha mohito* oil showed greater ability to reduce swelling. Film samples with hydrolats are not crucially different from control film. Similarly, there is no statistically significant differences among swelling values for both plant species where hydrolates were added.

3.3 Antioxidative Activity

There are different methods for determining the antioxidant activity of biopolymer films. DPPH free radical scavenging is generally used method, because it is simple, quick and economical mean to evaluate the antioxidant activity. This test shows that the analyzed gelatine films can "scavenge" free radicals that act as free hydrogen atoms or as electron donors. Gelatine film without oil and hydrolat added has not shown any antioxidative activity, as it is expected.

From the Fig. 2. it can be seen that the presence of oil compared to hydrolates contributed to significantly higher values of antioxidant activity. Between the two plant species used, slightly higher activity is observed in *Mentha spicata* oil and its hydrolate. Also from the figure it can be seen that the values obtained for 0.1% of the essential oil applied corresponded to the values obtained for 50% of the applied hydrolate for *Mentha spicata* species, whereas values for 0.1% *Mentha mohito* essential oil applied corresponded to the values obtained for 10% its hydrolat.

It has been proven that the addition of essential oils increased scavenging activity of the control film with no antioxidant activity. The antioxidant values are directly proportional to the added amount of essential oils (dose-dependent), that is in accordance with the findings of other groups of authors (Da Silva et al. 2021; Espino-Manzano et al. 2020).

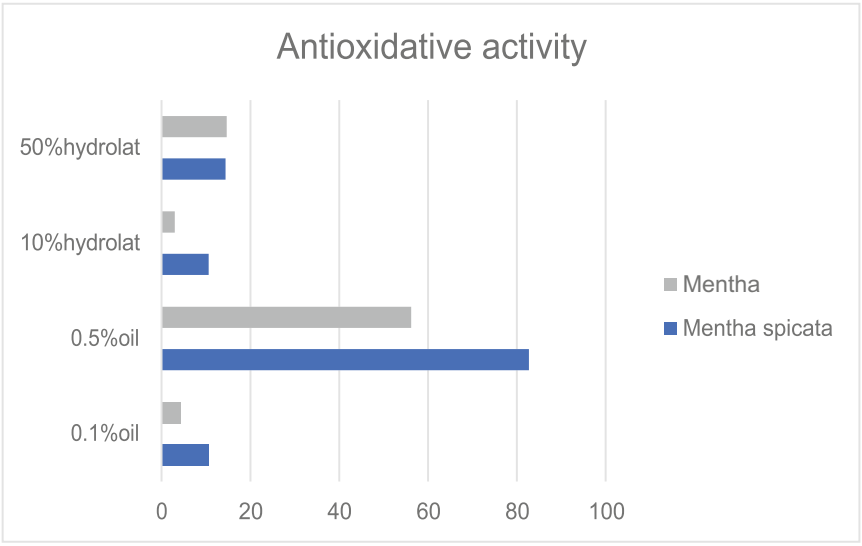

Fig. 2. Antioxidative activity of gelatine films with *Mentha mohito* and *Mentha spicata* essential oils and their hydrolats

3.4 Structural Properties

The final structure of biopolymer films containing lipid phase (essential oils) can be affected by the structural characteristics and stability of the film-forming emulsion. Film surface may contain irregularities, promoted by essential oils addition, because of non-miscible lipid compounds, that are incorporated in aqueous biopolymer film matrix. According to authors (Aewsiri et al. 2009; Jongjareonrak et al. 2008) characteristic bands of gelatine films are at approximately 3299, 1658, 1544, and 1243 cm^{-1}. Those wavelengths are corresponding to amides A, amide-I, amide-II and amide-III, respectively. Based on the recorded spectra of all samples, no significant differences between individual spectra were found. Characteristic peaks are present in the control sample as well as in all other samples with added oils/hydrolates (Fig. 3).

Despite there are no obvious differences in spectras shown, for the purpose of further studies areas with minor variations at certain wavelengths should be mentioned. The most obvious deviations, concerning the value of absorbance, are identified in the area of wavelengths 3000–3500 cm^{-1} (Fig. 4).

It was noticed that the absorbance value of the control film (without added active plant components) is highest at each wavelength, which means that the realized bonds are weaker in the samples in which hydrophobic plant compounds were added. The bonds are weak due to the presence of oil/ hydrolat in the system, regardless of the plant species.

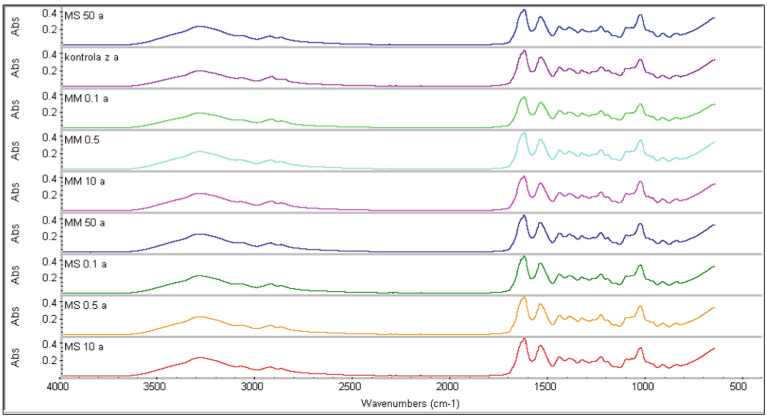

Fig. 3. FTIR spectra of control film, gelatine films with *Mentha mohito* (MM) and *Mentha spicata* (MS) oils (MM 0.1a; MM 0.5 and MS 0.1a; MS 0.5a) and gelatine films with *Mentha mohito* (MM) and *Mentha spicata* (MS) hydrolats (MM 10a; MM 50a and MS 10a; MS 50a)

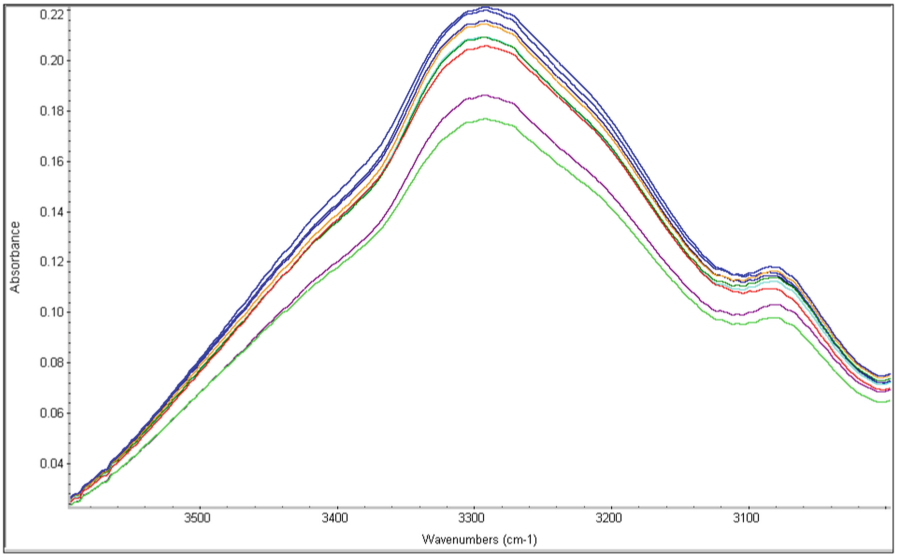

Fig. 4. FTIR spectras of previously mentioned samples, shown on common scale in the area of 3000–3500 cm^{-1} wavelength.

4 Conclusion

Although further investigation into the performance of gelatine edible films has to be conducted, as well as studies into the optimization of essential oils and hydrolats concentrations, improvements in examined mechanical, physico-chemical properties and antioxidative properties were found. The difference between the applied plant species was not significant. Addition of 0.5% for both *Mentha mohito* and *Mentha spicata* oils

resulted in improved functional properties of the films unlike the films with lower oil concentration (0.1%) and corresponding hydrolats, as a byproducts that left behind in essential oil distillation.

Taking all facts into consideration, results shown present introductory research in demonstrating the potential approach in use of active gelatin-based films as a great source for commercial packaging material production.

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Author Index

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