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ОДРЕДУВАЊЕ НА ДИАЦЕТИЛ ВО ПАВЛАКА: КОМПЛЕМЕНТАРНА СПЕКТРОФОТОМЕТРИСКА И GC-MS АНАЛИЗА

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КРАТОК ИЗВАДОК

Кај ферментираните прехранбени производи диацетилот (2,3-бутандион) е важно соединение одговорно за аромата, што е биосинтетизирано од млечно-киселинската бактерија која користи цитрат. Ефектот на диацетилот може да биде позитивен кај одредени ферментирани млечни производи како на пример кај павлаката. Заради тоа, потребен е едноставен и сигурен метод за одредување на диацетилот и останатите α-дикарбонилни соединенија во павлаките. Реакцијата помеѓу диацетил и *о*-фенилендиамин (OPDA) во водна средина дава стабилен 2,3-диметилхиноксалин, кој е попогоден за анализа со УВ спектрофотометрија. Реакциските услови беа оптимизирани и спекрофотометриската анализа ја дава вкупната концентрација на α-дикарбонилните соединенија. Методата за одредување на диацетил во павлака прикажна во овој труд, е поосетлива и поспецифична во споредба со модифицираната Вестерфелдова метода. Со комбинирање на спектрофотометриската метода со GC-MS анализа може да се идентификуваат хиноксалинските деривати од различни α-дикарбонилни соединенија. Утврдно е дека и во отсуство на гасен хроматограф, може да се користи само спектрофотометриската метода за испитивање на диацетилот, најзастапеното α-дикарбонилно соединение (>90%), одговорно за аромата во испитуваните павлаки.

Клучни зборови: диацетил, α-дикарбонилни соединенија, спектрофотометрија, гасна хроматографија-масена спектрометрија, млечни производи

ASSAYING DIACETYL IN SOUR CREAM: A COMPLEMENTARY SPECTROPHOTOMETRIC AND GC-MS ANALYSIS

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SUMMARY

Diacetyl (2,3-butanedione) is an important aroma compound, biosynthesized in fermented food by lactic acid bacteria that utilizes citrate. The effect of diacetyl can be positive in dairy products such as sour cream. Therefore, a reliable and simple method for determination of diacetyl and if

possible other α-dicarbonyl compounds in sour cream is needed. The reaction between diacetyl and *o*-phenylenediamine (OPDA) in aqueous medium yields a stable 2,3-dimethylquinoxaline derivative highly suitable for analysis via UV spectrophotometry. The reaction conditions were optimized and spectrophotometric analysis gave the total concentration of the α-dicarbonyl compounds present in sour cream. The method presented in this work for determination of diacetyl in sour cream by derivatization with *o*-phenylenediamine is more sensitive and more specific than the modified Westerfeld's method. By combining the spectrophotomeric method with GC-MS analysis, one can identify the quinoxaline derivatives from different α-dicarbonyl compounds. The major aroma compound in the examined sour creams is diacetyl (> 90 %) and in absence of gas chromatography the spectrophotometric method may be used alone.
Keywords: diacetyl; α-dicarbonyl compounds; spectrophotometry; gas-chromatography-mass spectrometry; dairy product

INTRODUCTION

Fermented foods, including milk and dairy products, have played important roles in the diet of humans worldwide for thousands of years. Milk has been known as nature's most complete food. Milk is an aqueous solution and it is important source of proteins, lactose, minerals and certain vitamins necessary for growth and development (Coultate, 2002). As a process, fermentation consists of the transformation of simple raw materials into a range of value-added products by utilizing the phenomena of the growth of microorganisms and their activities on various substrates. Numerous health and functional attributes of fermented dairy products are ascribed to the microorganisms that induce physical and/or chemical modifications of milk components. In addition to the products that consumers traditionally associate with milk, such as cheese, butter, and yoghurts, several products contain milk as a source of nutrients with important and unique properties. The development of new dairy products and the improvement to their safety are due to the developments of food technology, which can be able to reply successfully to the challenges of consumers and the industry (Tamime, 2005). Because citrate is present at a low level in milk, specific addition of this compound should be a way to increase biosynthesis of diacetyl (2,3-butanedione) in some fermented dairy products, such as sour cream, for which it is important flavour component. Certain *Lactic acid bacteria* (LAB) have significant commercial interest, either as starter cultures for industrial fermentations (e.g. for production of yoghurt, cheese, sour cream, butter and other fermented-milk drinks) (Fox and McSweney, 1998).

In general, flavour from dairy products is made up of a large number of volatile compounds such as: carbonyl compounds, primary and secondary alcohols, esters, ethers, aliphatic and aromatic hydrocarbons. The major contributor to desirable flavour and aroma in certain fermented dairy products is diacetyl. This compound is formed during the fermentation of milk and the rate of formation and the stability of diacetyl in fermented dairy products depend on the growth medium, temperature, pH and the presence or absence of oxygen (Vahcic et. al., 2000; Taylor and Linforth, 2010). As a consequence a number of methods for quantitative determination of diacetyl have been reported. In general, methods are based on spectophotometry and chromatography.

One of the spectrophotometric methods is based on the conversion of diacetyl into dimethylglyoxime and subsequent conversion into a coloured metal complex (Pack et. al, 1964). The advantage of this method is the determination of diacetyl in beer, but the dairy products are more complex and it is uncertain reaction between the formation of the metal complex and some compounds in the dairy products. The other spectrophotometric method which is more accurate for the determination of diacetyl in fermented dairy products is based

on the Westerfeld's method (Westerfeld, 1945) in which both of aroma flavour compounds diacetyl and acetoin (3-hydroxy-butane-2-one) react with creatine in the presence of 1-naphthol to form a chromogenic compound that can be quantified spectrophotometrically at 525 nm. The disadvantage of the method is that the rate of development of the colour with creatine and alkali is difficult to control and the colour is unstable. The pale pink color that developes after one hour fades away due to redox processes. Also, 1-naphthol must be freshly sublimed and kept in dark because it turns to reddish-brown color and intereferes with the analysis.

There has been constant progress in the development of analytical methods for detecting volatile compounds such as aroma compounds. The development of efficient analytical tool, such as gas chromatography (GC), high performance liquid chromatography (HPLC) and related techniques has greatly contributed to progress in this area (Parish et. al. 1990). A sensitive HPLC method was developed with derivatization of the compounds bearing certain functional groups such as: carbonyls and alcohols by introducing specific chromophores with absorption in the UV-VIS region (Hiroaki et. al., 1990). These methods have been used especially for quantifying carbonyl compounds with the reagent 2,4-dinitrophenylhydrazine (DNPH) to form the corresponding colored 2,4-dinitrophenylhydrazones. The main advantage of the method is successfully determination of diacetyl and acetoin in variety of fermented food. The disadvantages of the method are the expensive instrumentation and mobile phase such as acetonitrile.

Gas chromatograph equipped with autosampler coupled with mass spectrometer is a successful matching procedure that compares unknown mass spectra and their intensities with those of the reference library (Mariaca and Bosset, 1997; Landaud et. al., 1998).

Regarding headspace GC techniques, the sample to be analyzed is a gas phase aliquot containing volatiles released from the condensed phase. The detection of flavour compounds greatly depends on their concentration and vapour pressure, as well as on the temperature and matrix of the food product. The main advantage of the method is its direct determination of volatile compounds in the sample, but the unstable compounds may decompose thermally (Richelieu et. al., 1997; Dupire et. al., 1998). The primary problem of the method is the low concentration of flavour compounds. α-acetolactic acid is an unstable compound that chemically decarboxylates to diacetyl and acetoin during headspace gas chromatography, leading to an overestimation of diacetyl and acetoin.

Other methods worth mentioning are the fluorometric methods (Li et. al., 2009; Guerra Hernandez et.al., 1995; Mariaud, and Levillain, 1994; Voulgaropoulos et. al., 1991) which are based on quantitative preparation of fluorescent derivatives. Their general applicability is rather limited because of not easily accessible derivatizing reagents and the need for expensive and accurate spectrofluorimeters.

Most of the commonly used derivatization reagent is based on *o*-phenylenediamine (1,2-diaminobenzene), which forms quinoxaline derivatives upon reaction with α-dicarbonyl compounds (Rodrigues and Barros, 1997; Pejin et. al., 2006). The reaction between diacetyl and OPDA yields 2,3-dimethylquinoxaline, which has desirable properties for spectrophotometric and GC studies and has been applied for detection and quantification of diacetyl in alcoholic beverages (De Revel et. al., 2000; Dwyer and Fillo, 2006). The goal of this work was to investigate the derivatization procedure with OPDA for determination of diacetyl in sour cream and possibly with the aid of GC-MS to identify the main dicarbonyl compounds present.

MATERIALS AND METHOD

Reagents

Ammonium chloride (Merck), methanol (Alkaloid), *o*-phenylendiamine 99,5 % (Aldrich), diacetyl (Aldrich), dichloromethane (Alkaloid), *n*-hexane (Merck), ethyl ethanoate (Merck), sodium tungstate (Merck), 1-naphthol (Merck), sodium hydroxide (Alkaloid), sulfuric acid (Alkaloid), creatine (BDH), anhydrous sodium sulfate (Alkaloid) were used without further purification. The following sour creams were analyzed: Kiselo vrhnje- Z'bregov, Sour cream Meggle, Pavlaka-Bitolska mlekara, Pavlaka-Zdravje Radovo.

Instrumentation

Melting points were determined using Mel-Temp apparatus and were uncorrected. The IR spectrum was recorded on Varian 3100 FT-IR spectrophotometer Perkin as KBr pellets.. The gas chromatography mass spectrometric analyses were performed on a Varian 450 gas chromatograph equipped with autosampler (Varian CP 8410) coupled with Varian 300 SQ mass spectrometer. The capillary column VF-5MS was 0.25 mm in diameter and 30 m in length and with 0.25 μm film thickness. The carrier gas was helium (1 mL/min flow), the transfer line was set at 230 °C and the ion source at 200 °C. The following temperature program was used: 40 °C, 3 min, 10 °C/min to 250 °C, hold 10 min. The solvent delay was set at 2.3 minutes and the masses from 45 to 350 amu were scanned in electron impact (EI) mode. TLC was carried out using Merck pre-coated plates (60 F_{254}, 250 μm) and the plates were visualized at 254 nm using CAMAG ultraviolet lamp. For spectrophotometric measurements, all samples were analyzed on Varian Cary 50 UV-VIS spectrophotometer.

Synthesis of 2,3-dimethylquinoxaline

2,3-Dimethylquinoxaline was prepared by slight modification of the procedure described by Darbi et. al (2008). To a stirred solution of diacetyl (0.224 g, 2,6 mmol) in 5 mL methanol in 25 mL Erlenmeyer flask, ammonium chloride (50 mol%) and *o*-phenylenediamine (0.224 g, 2.0 mmol) in 5 mL methanol were added. The mixture was stirred at a room temperature for 30 minutes and the progress of the reaction was monitored by the thin layer chromatography (TLC) using a mixture of ethyl ethanoate and *n*-hexane (1:3). The R_f values of *o*-phenylendiamine and the desired product were 0.07 and 0.28, respectively. The reaction mixture was transferred into separatory funnel and diluted with 40 mL of water. The aqueous layer was extracted with dichloromethane (3 x 20 mL) and the combined organic layers were dried over anhydrous sodium sulfate. Upon removal of the solvent yellowish-brown solid was obtained. Recrystallization from 1:1 dichloromethane/hexane by slow evaporation on benchtop gave 0.224 g (71%) of 2,3-dimethylquinoxaline as small off-white crystals; mp 104-107 °C (lit. (Darbi et. al. 2008) 104-108 °C); **GC-MS:** R_t = 14.15 min; $\mathbf{R_f}$ (1:3 ethyl ethanoate and *n*-hexane)=0.28; **IR** (KBr disc): 3107, 3079, 3050, 3031, 3013, 2997, 2964, 2916, 1953, 1491, 1437, 1399, 1364, 1327, 1211, 1165, 1136, 989, 905, 821, 763, 671, 613; **EI-MS** (*m/z*, rel. intensity): 159 (M^+ + 1, 10%), 158 (M^+, 86%), 143 (M^+- CH_3, 3 %), 118 (9%), 117 (100%), 77 (20%), 76 (28%), 50 (14%).

Spectrophotometric derivatization studies of diacetyl with *o*-phenylendiamine

A 9.4 x10^{-5} M aqueous solution of 2,3-dimethylquinoxaline was prepared. The solution was placed in quartz cuvette and scanned from 200 to 400 nm. The maximum absorbance at pH 7 was at 315 nm. By addition of two drops of concentrated hydrochloric acid (pH = 1) the maximum absorbance shifted to 336 nm. Control UV experiment with *o*-phenylendiamine and subsequent acidification showed that it does not have significant absorbance at 336 nm. A 0.005 % solution (2 mL) of OPDA in 4 M HCl was placed in a quartz quvette and 5 μL of 1%

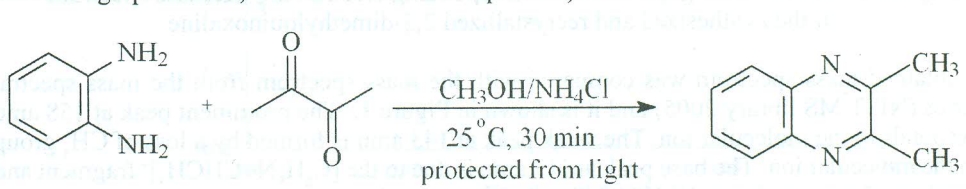
diacetyl in water was added directly into the cuvette. The reaction progress (i.e. formation of 2,3-dimethylquinoxaline) was monitored between 200 – 400 nm. The optimal reaction time was determined to be 30 minutes and the reaction must be carried out in the dark to avoid photochemical reactions of OPDA.

Spectrophotometric determination of diacetyl in sour creams via derivatization with OPDA
The sour cream (3 g) was dispersed in water (15 mL) and was gravity filtered into a clean flask. To 10 mL of the obtained clear filtrate, 0.5 mL of 0.1 % OPDA in 4 M HCl was added and the mixture was allowed to react in the dark for 30 min. Right before the analysis 2 mL of 4 M HCl was added and the absorbance is recorded at 336 nm. Appropriate calibration curve was generated using standard solutions of diacetyl in water with the following concentrations: 0.5, 1, 3,5, 8 and 10 mg/L.

Spectrophotometric determination of diacetyl in sour creams using the modified Westerfeld's method
The fermented milk product (1 g) was diluted with 1 mL of water, 1 mL of 10% sodium tungstate was added followed by 1 mL of 0.33 M sulfuric acid. The reaction mixture was gravity filtered and 0.4 mL of the filtrate was placed in a clean test tube. The filtrate is treated with 4.6 mL of water, 1 mL of 0.5% creatine in water and 1 mL of 5% 1-naphthol in 2.5 M aqueous sodium hydroxide. The test tube was closed and shaken well and was allowed to stand for 1 hour at ambient temperature. After that time, pink color developed and the spectrophotometric measurement was done at 525 nm. It is important to carry out the spectrophotometric analysis after exactly 1 hour because the color fades fast afterwards. The calibration curve was generated following the above-mentioned procedure using standard solutions of diacetyl in milk (Bitolska mlekara "Bimilk" 3.2% fat content) with the following concentrations: 0, 50, 150, 250, 350 mg/L.

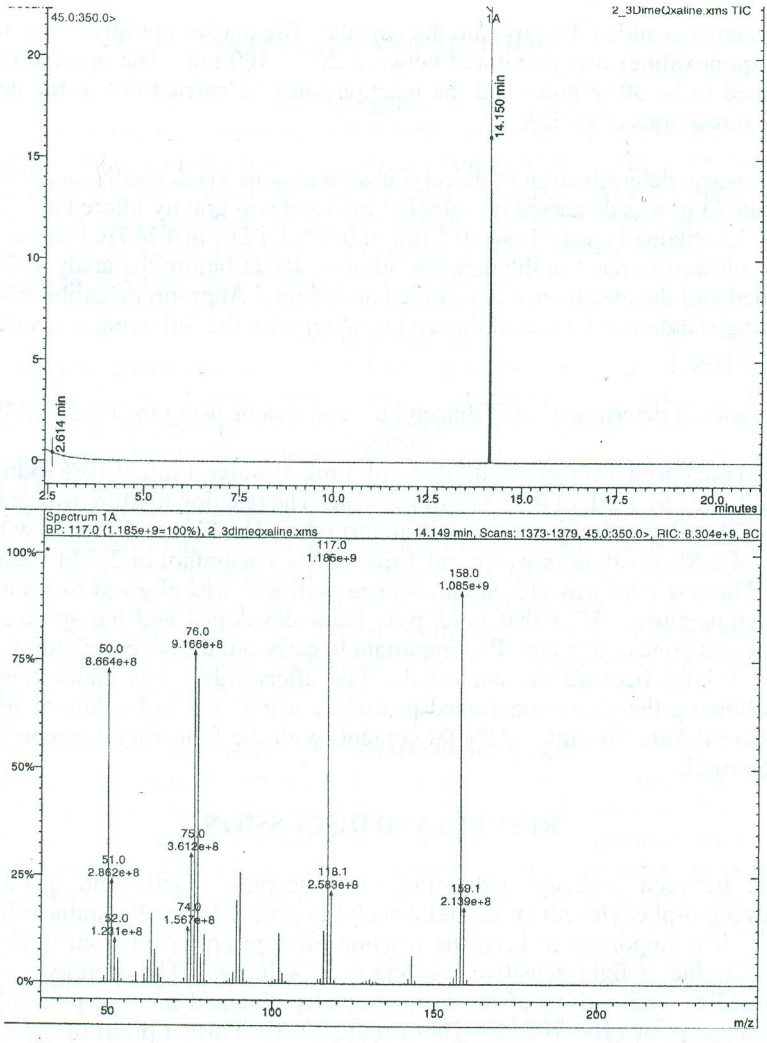
RESULTS AND DISCUSSION

The reaction between *o*-phenylenediamine and diacetyl is facile and quantitative. The reaction between *o*-phenylenediamine and diacetyl to give 2,3-dimethylquinoxaline is shown in scheme 1. It is important to keep the reaction mixture protected from light because the *o*-phenylenediamine is light sensitive, especially in solution. The identity of the product was determined by comparison of the experimentally obtained melting point (104-107 °C) with the literature value (104-108 °C) (Darbi et.al., 2008). Further proof of the identity came from spectroscopic methods (IR, UV-Vis and MS). The product after the recrystallization was of analytical purity as indicated by TLC (one spot) and GC-MS analyses (one peak, clean chromatographic trace, with no detectable impurities).



o-phenylenediamine diacetyl 2,3-dimethylquinoxaline

Шема 1. Синтеза на 2,3-диметилхиноксалин со аналитичка чистота
Scheme 1. Synthesis of analytically pure 2,3-dimethiquinoxaline



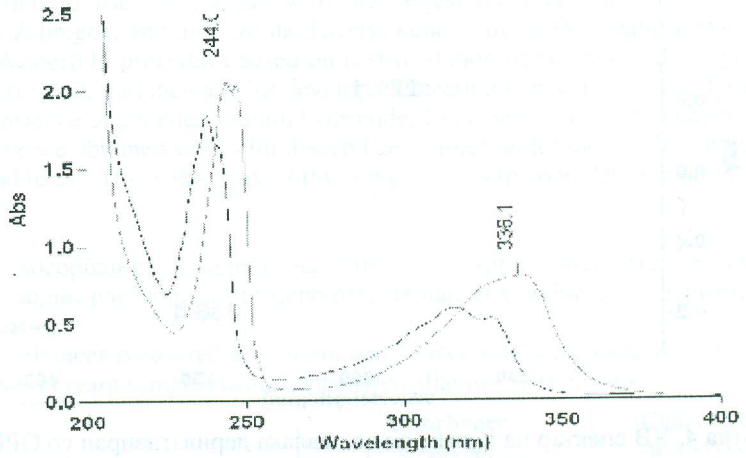
Слика 1. Гасен хроматограм и соодветниот масен спектар на синтетизираниот и прекристализиран 2,3-диметилхиноксалин

Figure 1. Gas chromatogram and the corresponding electron impact mass spectrum of the synthesized and recrystallized 2,3-dimethylquinoxaline

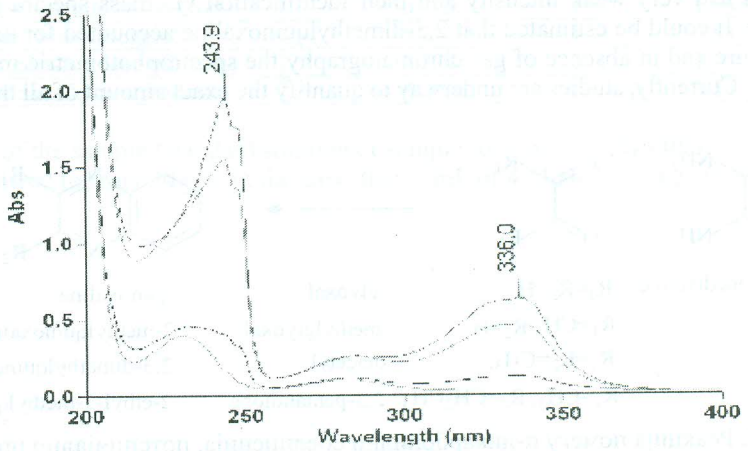
The obtained mass spectrum was compared with the mass spectrum from the mass spectral libraries (NIST MS library 2005) and it is shown in Figure 1. The prominent peak at 158 amu corresponds to the molecular ion. The weak peak at 143 amu is formed by a loss of CH_3 group from the molecular ion. The base peak at 117 amu is due to the $[C_6H_5N{=}CHCH_3]^{+\cdot}$ fragment and the peak at 77 amu is due to $[C_6H_5]^+$ fragment.

In figure 2 the UV absorption spectra of pure 2,3-dimethylquinoxaline in water at pH =7 and at pH=1 are depicted. It can be observed that upon acidification the maximum absorbance peaks shift to longer wavelength (bathochromic shift) upon acidification (from 315 nm to 336 nm). This is suitable for the spectrophotometric analysis since in acidic medium the OPDA exhibits

insignificant absorption at 336 nm. We have managed to reproduce the UV absorption spectrum of 2,3-dimethylquinoxaline by reaction of equimolar quantities of OPDA and diacetyl in water followed by acidification (Figure 3). The reaction conditions for UV studies were optimized and the most important conclusions were that the reaction should be carried out in the dark (30 min) and the reaction mixture should be acidified with 4 M HCl right before the spectrophotometric analysis.

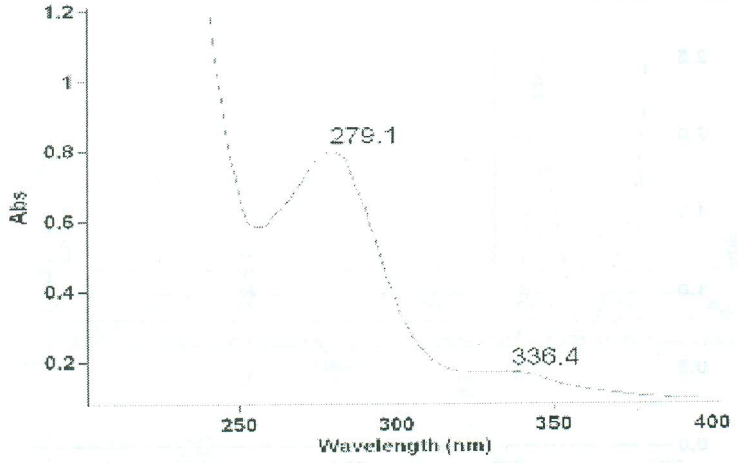


Слика 2. УВ спектар на синтетизираниот 2,3-диметилхиноксалин во вода (pH=7) и во вода закиселена со 4 M HCl (pH=1, спектарот со обележаните λ_{max} вредности)
Figure 2. UV spectra of synthesized 2,3-dimethylquinoxaline in water (pH = 7) and in water acidified with 4 M HCl (pH=1, the trace with labeled λ_{max} values)



Слика 3. УВ спектроскопско следење на реакцијата помеѓу диацетил и OPDA во вода при што се добива 2,3-диметилхиноксалин
Figure 3. Monitoring the reaction between diacetyl and OPDA in water to give 2,3-dimethylquinoxaline via UV spectroscopy

The main α-dicarbonyl compounds potentially present in the fermented food are: glyoxal, methylglyoxal, diacetyl and 2,3-pentandione. The last two are the most likely to be found, if one takes into consideration the volatility, reactivity and the metabolitic pathways. In principle, it is possible to react OPDA and the appropriate α-dicarbonyl compound to give quinoxaline derivative shown in scheme 2.



Слика 4. УВ спектар на примерок од павлака дериватизиран со OPDA и закиселен со 4 M HCl

Figure 4. UV spectrum of sour cream sample derivatized with OPDA and acidified with 4 M HCl

Preliminary GC-MS studies carried out after extraction of aqueous layer after derivatization, showed the presence of 2,3-dimethylquinoxaline, suggesting the presence of diacetyl, but the other peaks had very weak intensity and their identification via mass spectra matching was not reliable. It could be estimated that 2,3-dimethylquinoxaline accounted for more than 90% of the mixture and in absence of gas chromatography the spectrophotometric method may be used alone. Currently, studies are underway to quantify the exact amount of all the quinoxaline derivatives.

o-phenylenediamine	$R_1=R_2=H$	glyoxal	quinoxaline
	$R_1=CH_3, R_2=H,$	methylglyoxal	2-methylquinoxaline
	$R_1=R_2=CH_3,$	diacetyl	2,3-dimethylquinoxaline
	$R_1=CH_3, R_2=CH_2CH_3$	2,3-pentandione	2-ethyl-3-methylquinoxaline

Шема 2. Реакција помеѓу α-дикарбонилни соединенија, потенцијално присутни во ферментираните млечни производи, и о-фенилендиамин при што се добиваат хиноксалински деривати

Scheme 2. Reaction between α-dicarbonyl compounds potentially present in fermented dairy products and o-phenylenediamine to give quinoxaline derivative

We have decided to test the newly developed spectrophotometric method for determination of diacetyl using sour creams, because the obtained filtrate was clear and it did not need further treatment. It is important to note that the sample must be acidified prior to the spectrophotometric analysis (measurement at 336 nm) so that the effects of the matrix can be avoided (Figure 4). Using appropriate standard solutions and calibration curve we obtained values for diacetyl concentration from four commercial sour creams ranging from 0.84 mg/L to 1.57 mg/mL (Table 1).

We have decided to use the sample with the largest diacetyl concentration-Sour cream Kiselo vrhnje- Z'bregov, and analyze its diacetyl content using the established IDF method, the modified Westerfeld procedure based on first oxidation of acetoin to diacetyl by sodium tungstate/sulfuric acid, and then a color developing reaction between diacetyl, 1-naphthol and creatine in a presence of aqueous sodium hydroxide. Using appropriate standard solutions and calibration curve we obtained values for diacetyl concentration for the Kiselo vrhnje- Z'bregov of 62 mg/L (Table 2). This value was 39 times higher than the one determined via the OPDA derivatization.

Табела 1. - Абсорбанци одредени на 336 nm и концентрациите на диацетил во стандардните водни раствори и примероците на павлака добиени со дериватизација со о-фенилендиамин

Table 1. - Absorbances measured at 336 nm and concentration of diacetyl standard aqueous solutions and sour cream samples using o-phenylenediamine derivatization*

	Absorbance (336 nm) $A_{336\ nm}$	**Concentration of diacetyl (mg/L)**
Standard solution 1	0.142	0.50
Standard solution 2	0.191	1.00
Standard solution 3	0.384	3.00
Standard solution 4	0.578	5.00
Standard solution 5	0.869	8.00
Standard solution 6	1.066	10.00
Sour cream (Kiselo vrhnje- Z'bregov)	0.245	1.56
Sour cream (Sour cream-Meggle)	0.175	0.84
Sour cream (Pavlaka-Bitolska mlekara)	0.234	1.45
Sour cream (Pavlaka-Zdravje Radovo)	0.200	1.11

*To the 10 mL of the sample (standard solution or sample) 0.5 ml of 0.1% OPDA in 4 M HCl was added , allowed to react 30 min in the dark, then 2 mL of 4 M HCl was added.

Табела 2. - Абсорбанца одредена на 525 nm и концентрациите на диацетил во стандардните водни раствори и примероците на павлака добиени со модифицираната Вестерфилдова метода

Table 2. - Absorbance measured at 525 nm and concentration of diacetyl standard aqueous solutions and sour cream sample using modified Westerfeld's method*

	Absorbance (525 nm) $A_{525\ nm}$	**Concentration of diacetyl (mg/L)**
Standard solution 1 (blank unfermented milk)	0.061	0
Standard solution 2	0.163	50
Standard solution 3	0.455	150
Standard solution 4	0.691	250
Standard solution 5	0.955	350
Sour cream (Kiselo vrhnje- Z'bregov)	0.212	62

*See methods and material section for experimental details

The large difference in the obtained concentrations can be explained by the fact that using our procedure only the α-diketones (which are manily represented by diacetyl) are determined. With the modified Westerfeld's procedure the total diacetyl and acetoin is determined. Additionally, using sodium tungstate/sulfuric acid (i.e oxidizing agent/acidic medium), there is a great possibility that the remaining α-acetolactate undergoes chemical oxidative decarboxylation to give additional diacetyl. So, in fact with the modified Westerfeld's method we are measuring the total concentration of diacetyl, acetoin and α-acetolactate. The concentrations of diacetyl in commercial sour creams determined by our method (see Table 1) are in agreement with literature values for fermented milk 0.8 mg/L to 5.0 mg/L (Ott et.al., 2000).

CONCLUSION

The method presented in this work for determination of diacetyl in sour cream by derivatization with *o*-phenylenediamine is more sensitive and more specific than the established modified Westerfeld's method. By combining the spectrophotomeric method with GC-MS analysis one can identify the quinoxaline derivatives from different α-dicarbonyl compounds. The major aroma compound in the examined sour creams is diacetyl (> 90 %) and in absence of gas chromatography the spectrophotometric method may be used alone.

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