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ORIGINAL RESEARCH PAPER

# Development and validation of RP-HPLC method with UV-DAD detection for simultaneous determination of acesulfame K, sodium saccharin and aspartame in beverages

Mirjana S. Jankulovska*, Teodora Josimovska and Lenche Velkoska-Markovska

Ss. Cyril and Methodius University in Skopje, Faculty of Agricultural Sciences and Food – Skopje, North Macedonia

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**ABSTRACT**

Artificial sweeteners are low-calorie substances used as food additives with aim to impart a sweet taste to beverages without adding significant calories. Due to the regulatory compliance regarding the type and the amount of artificial sweetener, and due to the large consumption of beverages and the effects of artificial sweeteners on human health, their identification and quantification is of a great importance. In this research simultaneous determination of acesulfame K (ACE-K), sodium saccharin (Na-SAC) and aspartame (ASP) as the most commonly used sweeteners in beverages was performed with a reversed – phase high performance liquid chromatography (RP – HPLC) with diode array detection (DAD). The best separation of the analytes was achieved on a Poroshell 120 EC-C18 (3.0 × 50 mm, 2.7 µm) column and isocratic elution with a mobile phase consisted of acetonitrile and diluted phosphoric acid (pH = 3.8) with 7/93 volume ratio (*V*/*V*), and flow rate of 1 mL $min^{-1}$. The chromatographic process was followed at 195, 220 and 230 nm, under constant column temperature (25 °C). Under these chromatographic conditions, the total time of analysis was less than 5 min. The developed method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). The LOD under established chromatographic conditions was 0.03, 0.07 and 0.17 mg $L^{-1}$ for Na-SAC, ACE-K and ASP, respectively. The amount of artificial sweeteners in analyzed samples ranged from 30.32 to 148.37 mg $L^{-1}$ for ACE-K, from 16.10 to 93.05 for Na-SAC, and from 6.06 to 512.72 for ASP. The validated method was successfully applied for determination of analytes in different commercially available beverages.

**KEYWORDS**

acesulfame K, sodium saccharin, aspartame, beverages, RP-HPLC

## 1. INTRODUCTION

Food additives are widely used by the modern food technology production, fulfilling consumer demands. They are substances added to food to maintain or improve its quality, freshness, taste, texture or appearance [1]. In addition to other compounds, food additives also include artificial sweeteners, which are the most commonly used in the last decade. Artificial sweeteners are expected to provide sweetness without causing unpleasant taste, be low-calorie, economical to produce, thermally stable and should not be cancerogenic or mutagenic [2]. In the past, sugar was mainly used to sweeten food, and it was causing problems like obesity, which could be reason for heart disease and other lifestyle illnesses like diabetes [3]. Possible solution of this situation is replacement of sugars with low-calorie artificial sweeteners. In a lower amount they could achieve the same sweetness of sugars, in other words they have very low amount of energy and high intensity [4].

*Corresponding author.
Tel.: +38970858045.
E-mail: jankulovska@fznh.ukim.edu.mk

A number of artificial sweeteners are available by chemical synthesis, but the most commonly used are acesulfame K (ACE-K), sodium saccharin (Na-SAC) and aspartame (ASP). ACE-K (potassium salt of acesulfame) is a calorie-free artificial sweetener. It is around 200 times sweeter than sucrose, making it a popular choice in food products and beverages where calorie reduction is desired. ACE-K has very good water solubility, it is known for its stability under a wide range of conditions such as temperature, pH (from 2.5 to 9), and usual storage conditions of food products and beverages. ACE-K is associated with slight bitter aftertaste when it is used alone and at higher concentrations. Na-SAC (sodium saccharin or only saccharine) is the oldest sweetener, it is a white, crystalline powder that is approximately 300–400 times sweeter than sucrose. Due to its high sweetness potency, only small amounts are needed to achieve the desired sweetness in beverages. The stability of SAC and its salts is not affected by pH of the environment and temperature in solid state, while in solution SAC salts have excellent hydrolytic, thermal and photostability [5]. ASP (N-L-α-aspartyl-L-phenylalanine methyl ester) is composed of two amino acids, aspartic acid and phenylalanine, linked together. It is a low-calorie artificial sweetener, approximately 200 times sweeter than sucrose and has a sugar-like taste. ASP is found in the form of white crystalline powder, which becomes colorless and odorless when dissolved, but with an intense sweet taste [6]. In aqueous solutions the stability of ASP is reduced and it is most stable at a pH value of 3–5 with an optimum of 4.2. Despite the fact that a healthy individual can safely use ASP, it is known that disproportionate intake of phenylalanine, can cause a hazard to people affected from phenylketonuria, an inherited metabolic disorder. Due to this reason, all food products containing aspartame must indicate the presence of phenylalanine on the label. In beverages could be used single low-calorie sweetener or a mix of them called blend. Nowadays, the common trend for practical reason is usage of sweetener blends, because some of the sweeteners have aftertastes that limit their application. For instance, ACE-K and ASP are commonly used as a binary blend in order to achieve a more sugar-like taste and to mask any potential aftertastes [7].

The use of artificial sweeteners is beneficial when compared with high-calorie sweeteners such as sucrose, because they are not metabolized through digestion [2]. However, although artificial sweeteners are legally used in beverages, they could be harmful especially with prolonged usage. In the literature can be found ongoing studies regarding their long-term health effects [8, 9]. Various studies have suggested potential links between artificial sweeteners and adverse health effects due to prolonged and high consumption [9–11]. Furthermore, some of them highlighted the increased cancer risk with prolonged consummation of artificial sweeteners, especially ASP and ACE-K [12]. Nowadays, the number of people who need to control their sugar intake increased, and interest for artificial sweeteners increased, too. On the other hand, the low calorie intake in the diet is very popular due to obesity issue, and children could be exposed to artificial sweeteners more than adults, because they prefer sweet food.

Artificial sweeteners are recognized as safe for consumption by regulatory agencies when used within recommended limits. Therefore, national and international regulatory agencies have established strict regulations where the maximum permitted level of artificial sweeteners in food products is defined. The Republic of North Macedonia has developed national food safety legislation, based on the Regulation (EC) No. 1333/2008 on food additives [13]. According to these regulations, artificial sweeteners are marked with capital letters (E) and a number that is different for each sweetener. The artificial sweeteners ACE-K, Na-SAC and ASP are marked with E950, E954 and E951, respectively. Their maximum permitted level in beverages (350 mg $L^{-1}$ for ACE-K, 80 mg $L^{-1}$ for Na-SAC and 600 mg $L^{-1}$ for ASP) in North Macedonia are in accordance with European regulation for food additives [13]. The Acceptable Daily Intake (ADI) levels expressed as mg $kg^{-1}$ body weight per day are 0–9 for ACE-K, 0–5 for Na-SAC 0–40 for ASP [14].

Determination of sweeteners in beverages is a crucial aspect of safety control of food products and regulatory compliance although they are considered generally safe (GRAS) substances [2]. Literature data reveled a number of analytical methods based on different principles and used for determination of artificial sweeteners in beverages. It is important to choose a method that is appropriate for the specific sweeteners present in the beverage and meets the requirements for sensitivity, accuracy, precision and regulatory compliance. Some commonly used chromatographic methods that are easy to perform include thin-layer chromatography, high-performance liquid chromatography, gas chromatography, ion chromatography, and so on. Spectroscopic techniques are proposed for a faster analytical method such as UV/Vis Spectroscopy, Fourier-Transform Infrared Spectroscopy (FTIR) and Fourier-Transform Raman spectrometry. However, UV/Vis spectroscopy is favored technique among spectroscopic methods. Other techniques that are also available for determination of artificial sweeteners are capillary electrophoresis, electroanalytical methods, colorimetric methods and others [15–18].

Lately for determination of artificial sweeteners in combination with other additives, more advanced techniques like Liquid Chromatography-tandem Mass Spectrometry (LC MS/MS) were used [19–21]. Comparing two detectors (MS and DAD), MS is more sensitive, but more expensive, too. Moreover, DAD could exhibit acceptable sensitivity for the concentrations of artificial sweeteners used in beverages. When there is a need for simultaneous determination of ACE-K, Na-SAC and ASP in food samples, more simple methodology is preferable especially for practical reasons such as efficiency, cost-effectiveness, and ease of implementation. Literature data revels that High-Performance Liquid Chromatography (HPLC) combined with different detectors is the most commonly employed technique for simultaneous determination of artificial sweeteners in food products. Due to its availability in laboratories and

the broad range of available analytical columns makes it universal technique [22–30].

Keeping in view all this, the present research deals with development and validation of selective and sensitive RP-HPLC method for identification and simultaneous determination of ACE-K, Na-SAC and ASP in various beverages available on the local Macedonian markets. The reason for defining the objectives of this research is the fact that usage of analyzed sweeteners in beverages is the most popular, while the choice of RP-HPLC as analytical method is because it is a rapid, economic and simple, and available method for almost any laboratory, as well. Depending on single-sweetener or multi-sweetener analysis in some cases, simple analytical method is more reasonable to apply compared to advance.

## 2. MATERIALS AND METHODS

### 2.1. Instrumentation

The chromatographic separation was carried out on a liquid chromatograph Agilent 1260 Infinity series equipped with vacuum degasser (G1322A), binary pump (G1312B), auto sampler (G1329B), a thermostatted column compartment (G1316A), UV/Vis diode array detector (G1316B), Chem-Station software and Poroshell 120 EC – C18 (3.0 × 50 mm, 2.7 μm) column. The pH of the solutions was measured with pH meter METTLER TOLEDO (Seven Direct SD20, Switzerland).

The mass of the analytical standards was measured with digital analytical scale with an accuracy of ± 0.1 mg (Mettler, Zurich, Switzerland). An ultrasonic bath Elma was used for sonication of the solutions. The preparation of the buffer solutions was done on a Magnetic mixer Rotamix 550 MMH Technica. Electronic pipette (HandyStep® touch S Multi Dispenser, BrandTech® Scientific, INC, USA) and micro-pipettes with different volume were used to measure the volume when preparing the standard solutions. Filtration of the phosphate buffer and water was performed on a Vacuum filtration system (SUPELCO Analytical, USA) with nitro-cellulose membrane filters (0.45 μm pore size).

### 2.2. Analytical standards and reagents

Acetonitrile (ACN) (≥99.9 %), methanol (MeOH) (>99.8%), $KH_2PO_4$ (99%), phosphoric acid (85.5%) and ultra-pure water were obtained from Fisher Chemical, USA; analytical standards of ACE-K (99.4%), Na-SAC (99.6%) and ASP (99.0%) were produced from CPAchem, Bulgaria. All chemicals were of high purity or HPLC grade.

### 2.3. Chromatographic conditions

Separation and quantification of analyzed sweeteners was performed on a Poroshell 120 EC-C18 (3.0 × 50 mm, 2.7 μm) column, at constant column temperature (25 °C). The DAD acquisition wavelength was set to scan at 195, 220 and 230 nm. The mobile phase used in this study was consisted of two solvents, acetonitrile (solvent A) and different types of compounds as solvent B (water, phosphate buffer (pH value from 3.5 to 5.0), and diluted phosphoric acid (pH value 3.8). The isocratic and gradient mode of elution was tested. The gradient program with the mobile phase ACN and water was as follows: 0–0.7 min 4% A, 0.7–2.1 min 50% A, 2.1–3.5 min 4% A, while the gradient with the mobile phase ACN and phosphate buffer (pH = 5) was 0–0.9 min 8% A, 0.9–2.6 min 50% A, 2.6–3.5 min 8%. The flow rate was 1.0 mL $min^{-1}$, and the injection volume was 2.5 μL.

### 2.4. Preparation of standard and sample solutions

The stock solutions were prepared by dissolving 0.0707 g ACE-K, 0.0203 g Na-SAC and 0.0615 g ASP in methanol/water 50/50 (*V*/*V*) in 10 mL volumetric flasks. For better dissolution, the stock solutions were sonicated in an ultrasonic bath for 15 min. Before use, the stock solutions were stored in a refrigerator at 4 °C. The test solutions of ACE-K (14.05–632.43 mg $L^{-1}$), Na-SAC (4.04–181.89 mg $L^{-1}$) and ASP (0.60–913.2 mg $L^{-1}$) were daily prepared by dilution from the stock solutions in methanol/water 50/50 (*V*/*V*) in 10 mL volumetric flasks.

In this study, sixteen different beverages were analyzed. The sample preparation was very simple. Only the carbonated beverages were sonicated in an ultrasound bath at room temperature, for 15 min. The aim was to be sure that all kind of bubbles present in the beverages, were removed. All the samples prior analyses were filtered through 0.45 μm syringe filter and 2.5 μL of each sample were injected into the HPLC system. Each analysis was repeated in triplicate, and the average values were calculated.

### 2.5. Phosphate buffer preparation

The phosphate buffer was freshly prepared by dissolving 0.1 g of potassium dihydrogen phosphate ($KH_2PO_4$) in 100 mL ultrapure HPLC grade water. For better dissolution of the salt, the solution was mixed on a Rotamix 550 electric mixer and the required pH value was adjusted using diluted phosphoric acid ($H_3PO_4$). Finally, before use the buffer was filtered using the vacuum filtration system and 0.45 μm membrane filters.

### 2.6. Identification and quantification of analytes

Identification of analytes in chromatograms involved comparison of the retention times, and the UV spectra of analytical standards of ACE-K, Na-SAC and ASP with those in the samples. By comparing, the retention times of peaks in the sample chromatogram with those of the standards, identification of analytes was made. Furthermore, by comparing the UV spectra of peaks in the unknown sample chromatogram with those of standards, the identity of analytes based on spectral similarity was confirmed. The degree of matching of the spectra was determined by the values of the match factor. Quantification of analyzed artificial sweeteners was performed using the calibration curve

method, which shows the dependence of the peak area on concentration of the analytes. Using the calibration equation, the concentration of the analytes was calculated.

## 3. RESULTS AND DISCUSSION

Beverages are one of the main products in which, in addition to other additives, artificial sweeteners are commonly used. As consumption of beverages in las decade increased, intake of artificial sweeteners increased as well. Their concentration may vary, but it must not exceed the maximum permitted level. Because of that, their identification and quantification, as well as the control of the possibility of their misuse, is of great importance. In this research, the determination of the artificial sweeteners ACE-K, Na-SAC and ASP in beverages was performed by RP-HPLC method combined with UV-DAD detection.

### 3.1. Optimization of RP-HPLC conditions – method development

Optimization of chromatographic conditions was made in order to obtain a satisfactory separation between ACE-K, Na-SAC and ASP and matrix interferences in beverages. Development of the analytical method generally involve optimization of column temperature, mobile phase composition (choice of appropriate solvents), pH of the mobile phase, elution mode (isocratic and gradient) and flow rate. These parameters are crucial for achieving efficient separation and optimal detection of the analyzed sweeteners. In order to obtain more information about the analytes and improve detection specificity ACE-K, Na-SAC and ASP and were detected using DAD. UV-DAD detection relies on the absorption of light by analytes at specific wavelengths. Therefore, selecting appropriate detection wavelengths based on the UV absorption spectra of the analyzed artificial sweeteners was essential. In this research, the chromatographic process was monitored at 195, 220 and 230 nm.

Literature data reveled that as a stationary phase for determination of artificial sweeteners or combination of artificial sweeteners and other food additives, non-polar analytical columns C18 (150 mm × 4.6 mm, 5 µm) or C18 (250 mm × 4.6 mm, 5 µm) were most frequently used [26, 27, 30–34]. In our research, chromatographic separation of the analyzed artificial sweeteners was achieved on a Poroshell EC – C18 (50 mm × 3.0, 2.7 µm) column. This column was an excellent choice, because it could be used in a wide range of pH (from 2 to 9) making it suitable for analyses of beverages with different acidity [35]. During the separation, the working temperature of a column was constant (25 °C).

The choice of the mobile phase composition was made based on the experiments with different solvents such as: ACN, water, phosphate buffer with different pH values and diluted phosphoric acid. ACN was selected as organic solvent because it has lower backpressure and shorter cutoff wavelength (190 nm). Hence, it was more suitable for determination of ACE-K and Na-SAC whose maximum UV absorption is in 210–230 nm region [23]. The influence of the different volume ratio of the solvents in the mobile phase was tested, as well, while the flow rate of a mobile phase in all experiments was 1 mL $min^{-1}$. Initially, the mobile phase consisted of ACN/water with a volume ratio from 10/90 to 1/99 (*V*/*V*) was tested. In 10/90 (*V*/*V*) volume ratio of ACN/water, ACE-K and Na-SAC were not separated, while increasing the volume of water in the mobile phase (1/99 (*V*/*V*)), their better separation was achieved. However, with increasing the amount of water, the retention time of ASP increased significantly (9.249 min), and the peaks of all analytes were not symmetrical. In order to shorten retention time of ASP the a few linear gradient elution modes were tested, among which with the following gradient: 0–0.7 min 4% A, 0.7–2.1 min 50% A, 2.1–3.5 min 4% A, gave a satisfactory separation. However, in this gradient mode ACE-K and Na-SAC were separated, but the shape of the peaks was worse compared to the isocratic elution.

In further experiments, the water in the mobile phase was replaced with phosphate buffer. According to the literature, pH of the mobile phase influence of separation of the analytes [33]. In order to see how the change in the pH of the buffer will affect the separation process of the analytes, the pH was varied from 3.2 to 5.0 during isocratic elution. When the pH of the phosphate buffer was 3.5 isocratic elution with the ratio of the mobile phase components ACN/phosphate buffer from 10/90 to 4/96 (*V*/*V*), and following gradient: 0–0.9 min 8% A, 0.9–2.6 min 50% A, 2.6–3.5 min 8% was tested. However, the best separation of analyzed sweeteners was observed when pH of the phosphate buffer was 3.8. Finally, the phosphate buffer in the mobile phase was replaced with diluted phosphoric acid (pH = 3.8). Herein volume ratios of ACN/diluted phosphoric acid (pH = 3.8) from 12/88 to 5/95 (*V*/*V*) were tested. The results showed that the best separation of the analytes with symmetrical and sharp peaks provided the mobile phase consisted of ACN/diluted phosphoric acid with volume ratio 7/93 (*V*/*V*). According to literature, successful separation of synthetic sweeteners was achieved with the mobile phase composed of phosphate buffer (pH = 3.5) in combination with organic solvent [22]. Petrova et al. (2020) for determination of ACE-K in a mobile phase used phosphate buffer with pH = 3.5 [36]. Furthermore, the artificial sweeteners in combination with other additives were successfully separated with mobile phase consisted of ACN and phosphate buffer with pH = 3.3, and pH = 3, achieved with acetate buffer [29, 30].

Taking into consideration the results from all experiments, the best chromatographic conditions for separation of ACE-K, Na-SAC and ASP were isocratic elution with a mobile phase consisted of ACN and diluted phosphoric acid (pH = 3.8) with a volume ratio of 7/93 (*V*/*V*), and a flow rate of 1.0 mL $min^{-1}$. Figures 1 and 2 shows the chromatograms of a standard mixture solution of ACE-K (632.43 mg $L^{-1}$), Na-SAC (181.89 mg $L^{-1}$) and ASP (913.2 mg $L^{-1}$), at 195 and 220 nm.

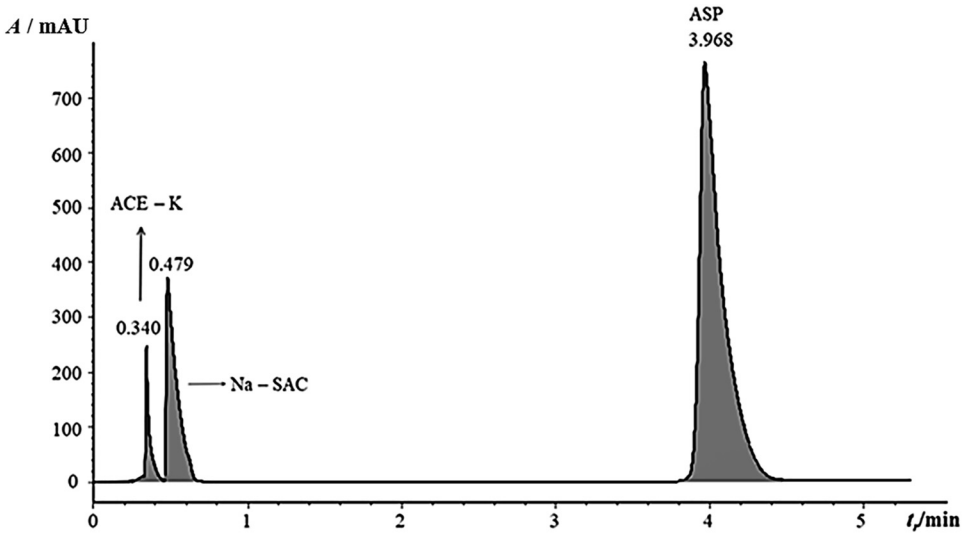


*Fig. 1.* Chromatogram of a standard mixture of ACE-K, Na-SAC and ASP, mobile phase ACN/phosphoric acid (pH = 3.8) with a volume ratio of 7/93 (*V*/*V*), 195 nm

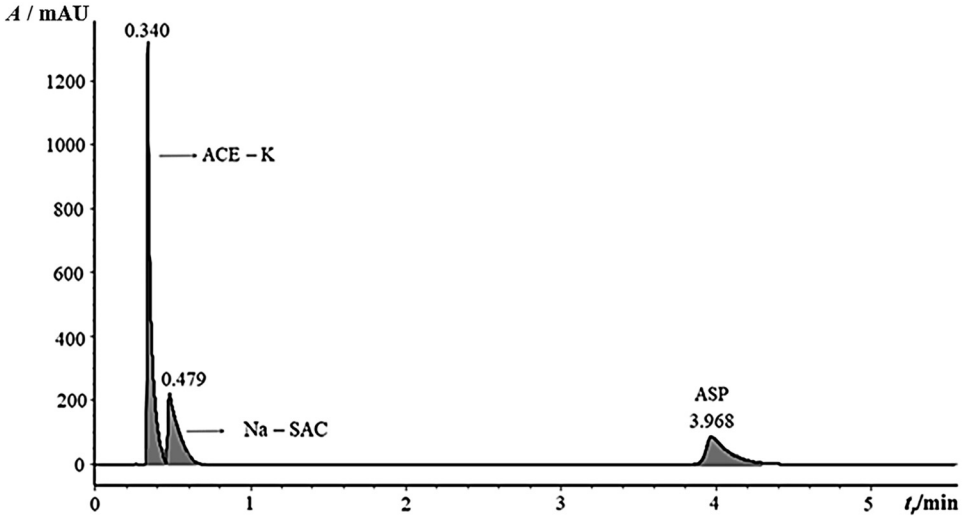


*Fig. 2.* Chromatogram of a standard mixture of ACE-K, Na-SAC and ASP, mobile phase ACN/phosphoric acid (pH = 3.8) with a volume ratio of 7/93 (*V*/*V*), 220 nm

Analyzed sweeteners have distinct retention times, allowing their identification and quantification. Under defined chromatographic conditions (Figs 1 and 2) the analytes were eluted in the following order: 0.340 min for ACE-K, 0.479 min for Na-SAC and 3.968 min for ASP. The calculated values of the retention factor (*k'*) were 1.07 for ACE-K, 1.92 for Na-SAC and 23.19 for ASP. The separation factor ($\alpha$) between the adjacent peaks of ACE-K and Na-SAC was 3.0823, suggesting a significant difference in retention between ACE-K and Na-SAC i.e. their complete separation. This was additionally confirmed with the resolution (Rs) value that was 3.43 [37, 38]. Under these chromatographic conditions, the analysis was done in less than 5 min. Hence, the proposed RP-HPLC method was faster, compared to literature data for previous reported methods, which allowed reducing the cost of analyzing the samples [24, 29–30].

## 3.2. Validation of RP-HPLC method

Validation of an RP-HPLC method was crucial to ensure its reliability, accuracy, and reproducibility. The developed RP-HPLC method for simultaneous determination of ACE-K, Na-SAC and ASP was validated according to the recommendation by the International Conference on Harmonization (ICH) Guideline Q2 (R1) [39]. Linearity, precision (intra-day and inter-day repeatability), accuracy (recovery tests), LOD and LOQ were tested as validation parameters.

***3.2.1. Linearity.*** Linearity for each of the analyzed sweeteners was tested in two concentration regions (see Table 1). Regression lines were constructed in five point by plotting the peak area against concentration of the analyte (mg $L^{-1}$). Linear relationship between peak area and concentration of analytes was observed. Calibration curve was made using the average data of three replications. The determination

*Table 1.* Validation parameters: linearity (linearity range, calibration equations and determination coefficients ($R^2$)), LOD and LOQ of ACE-K, Na-SAC and ASP (*$n$ = 3)

| Analyte | Linearity range (mg $L^{-1}$) | Calibration equations | $R^2$ | LOD (mg $L^{-1}$) | LOQ (mg $L^{-1}$) |
|---|---|---|---|---|---|
| ACE-K | 14.05–70.27 | y = 7.0126x + 6.9384 | 0.9959 | 0.07 | 0.24 |
| | 70.27–632.43 | y = 6.7573x + 8.5248 | 0.9989 | | |
| Na-SAC | 4.04–20.21 | y = 9.1327x – 0.8385 | 0.9993 | 0.03 | 0.10 |
| | 20.21–181.89 | y = 8.9609x + 2.2045 | 0.9981 | | |
| ASP | 0.60–460.00 | y = 2.5924x – 20.901 | 0.9980 | 0.17 | 0.56 |
| | 304.30–913.20 | y = 1.3811x – 13.1 | 0.9915 | | |

*n is number of replications.

coefficients were used for validation of linearity. Resulting calibration equations and determination coefficients ($R^2$) for analyzed sweeteners are given in Table 1.

The values of determination coefficients showed excellent linearity between peak area and concentration of each analyte in the tested concentration region.

***3.2.2. Sensitivity (LOD and LOQ).*** Table 1 displays the obtained LOD and LOQ values for analyzed sweeteners that were used to find sensitivity of the method towards each analyte. Evaluation of LOD and LOQ was based on signal-to-noise ratio (S/N). The LOD was estimated by gradually reducing the concentration of analytes until the peaks disappeared. The concentration that produced signal three times greater than the noise signal was considered LOD i.e. concentrations giving a S/N ratio of 3/1. The LOQ was calculated as three times the LOD i.e. concentrations giving a S/N ratio 10/1 [40]. According to the results presented in Table 1, using the proposed method minimal concentration that could be detected in samples was 0.07 mg $L^{-1}$ ACE-K, 0.03 mg $L^{-1}$ of Na-SAC and 0.17 mg $L^{-1}$ of ASP. These values were lower compared to those presented in literature for determination of various artificial sweeteners or artificial sweeteners in combination with other additives in different beverages using HPLC method. For instance, according to Gomaa et al. (2015) LOD for ACE-K was 7.17 µg $mL^{-1}$ for Na-SAC 6.84 µg $mL^{-1}$, and for ASP 2.55 µg $mL^{-1}$, while Diviš et al. (2020) found the LOD for ACE-K, Na-SAC and ASP of 0.9, 0.6 and 1.6 mg $kg^{-1}$, respectively. LOD of ACE-K, Na-SAC and ASP in research conducted by Imanulkhan et al. (2020) was 3.00, 1.16 and 2.98 mg $kg^{-1}$. Furthermore, de Querioz Pane et al. (2015) found slightly higher value for LOD of ACE-K and Na-SAC compared to our results, while for LOD of ASP they found a little lower value (0.142 µg $mL^{-1}$). Higher values compared with ours for LOD of ACE-K (1.73 mg $L^{-1}$), Na-SAC (1.58 mg $L^{-1}$) and ASP (1.60 mg $L^{-1}$) were estimated for the HPLC-DAD method applied for determination of artificial sweeteners with other additives [26, 27, 30, 32, 41]. The sensitivity of the developed method was satisfactory since LOQ values for ACE-K (0.2376 mg $L^{-1}$), Na-SAC (0.0994 mg $L^{-1}$) and ASP (0.5630 mg $L^{-1}$) were lower than the lowest concentration of the calibration curve. It is important to highlight that the obtained low LOD and LOQ values facilitate a reliable detection and quantification of analyzed sweeteners in low concentration in beverages.

***3.2.3. Precision (repeatability).*** Precision of the developed method was verified by repeatability test i.e. a mixture of analytical standards with the concentration of 351.35, 101.05 and 608.8 mg $L^{-1}$ for ACE-K, Na-SAC and ASP, respectively was repeatedly injected into the column. Eight consecutive injections (2.5 µL) from the standard mixture within a single day (intra-day precision) or over two consecutive days (inter-day precision) were made. The obtained results (Table 2) from this experiment were express as relative standard deviation (RSD).

As it could be seen from Table 2, relative standard deviations (RSD) of the retention time, peak height and peak area were used to express the precision. The obtained results demonstrate excellent intra-day precision of the used method with RSD < 1% for all tested parameters (retention time, peak area and peak height), while for the inter-day precision RSD < 2% for peak area and retention time, while for peak height RSD was greater than 2%. Taking into consideration that the beverages were analyzed without clean-up procedure, the RSD values were acceptable.

***3.2.4. Accuracy (recovery).*** The accuracy of the developed method was evaluated by recovery test i.e. using the method of standard additions. Recoveries represents a measure that indicates the degree of closeness of the obtained results with

*Table 2.* Repeatability of retention time (min), peak area and peak height ($n$ = 8)

| Parameter | ACE-K | | Na-SAC | | ASP | |
|---|---|---|---|---|---|---|
| | *RSD intra-day | RSD inter-day | RSD intra-day | RSD inter-day | RSD intra-day | RSD inter-day |
| Peak area | 0.0786 | 0.1739 | 0.1085 | 0.3456 | 0.1257 | 0.2328 |
| Peak height | 0.6198 | 6.7149 | 0.6030 | 3.3909 | 0.4907 | 13.7747 |
| Retention time | 0.1327 | 0.4425 | 0.1777 | 1.3065 | 0.2107 | 2.1310 |

*RSD – relative standard deviation (%).

the actual concentration of sweeteners in samples. In our research recoveries were evaluated at three different concentration levels ($n$ = 3) of ACE-K (70.27, 140.55 and 210.82 mg $L^{-1}$), Na-SAC (20.21, 40.42 and 60.63 mg $L^{-1}$) and ASP (121.76, 243.52 and 365.28 mg $L^{-1}$). For this reason, three different volumes of the test solution were spiked into sample S9 (refreshing, carbonated beverage), which already contained ACE-K. The concentration of the total analyte found in the sample after spiking and recoveries calculated as percentage (%) are presented in Table 3.

The lowest recovery was observed for ASP, (84.48%), while the highest recovery was noted for Na-SAC (99.83%). However, the obtained results reveled that applying this analytical method excellent recovery (in most cases near 100%) was achieved. All values varied between 84.48 and 99.83%. Considering that the samples were analyzed with minimal sample preparation, these results confirmed excellent recovery. Additional clean-up could result with better recovery values for some samples. The obtained results showed that the developed method was characterized with accuracy that will give satisfactory results in quantification of ACE-K, Na-SAC and ASP in beverages, in accordance with the acceptable values established by ICH and AOAC International [39, 42].

### 3.3. Artificial sweeteners in beverages

In the present study, applying the proposed RP-HPLC method with DAD detection the artificial sweeteners ACE-K, Na-SAC and ASP were successfully determined in different beverages (Table 4). The presence of artificial sweeteners in beverages was declared through their E numbers on the labels of the beverages, but their concentration was not declared, while in various samples, the presence of sugar was declared. Taking into consideration the adverse health effect on humans, determination of sweeteners in beverages is very important in order to verify whether their amount in the beverages is in agreement with the values indicated on the labels, and if it is in accordance with the effective legislation. This method was based on measuring the concentration (mg $L^{-1}$) of ACE-K in a concentration range between 14.05 and 632.00 mg $L^{-1}$, Na-SAC from 4.04 to 181.89 mg $L^{-1}$, and ASP from 0.60 to 913.20 mg $L^{-1}$.

*Table 3.* Recovery (%) of ACE-K, Na-SAC and ASP spiked in sample S9 ($n$ = 3)

| Analyte | Mass of analyte (μg) | Pure analyte added (μg) | Total analyte found (μg) | Recovery (%) |
|---|---|---|---|---|
| ACE-K | 0.3697 | 0.1756 | 0.5352 | 94.20 |
| | 0.3697 | 0.3513 | 0.7098 | 96.79 |
| | 0.3697 | 0.5270 | 0.8530 | 91.70 |
| Na-SAC | 0 | 0.0505 | 0.0493 | 97.60 |
| | 0 | 0.1010 | 0.1008 | 99.83 |
| | 0 | 0.1515 | 0.1509 | 99.59 |
| ASP | 0 | 0.3044 | 0.2571 | 84.48 |
| | 0 | 0.6088 | 0.5472 | 89.88 |
| | 0 | 0.9132 | 0.9079 | 99.42 |

Before quantification, the identification of analytes in the chromatograms was performed. For that purpose, the retention time of analytes in the standard mixture was compared to the retention time of each analyte in the sample chromatograms. At the same time, in order to assess the similarity and to confirm identification between the chromatograms of the standards and the samples the UV spectra of peaks in the sample chromatogram with those of the standards were compared. The obtained match factor values of ACE-K (999.924), Na-SAC (999.673) and ASP (999.202) indicated an excellent match between the two chromatograms.

Table 4 summarize the concentration (mg $L^{-1}$) for the artificial sweeteners in all analyzed samples. Artificial sweeteners were found in 13 out of 16 analyzed samples (81.25%). The obtained results correspond to the type of labeled referent value and maximum permitted level [13]. The most frequently used artificial sweeteners in beverages are ACE-K and ASP, while Na-SAC was determined only in five samples. Namely, only in three analyzed samples (Fig. 3) all analyzed artificial sweeteners were found together (S8, S15 and S16). In general, the combination of ASE-K, Na-SAC and ASP is rare in beverages available on the market. The most common combination of artificial sweeteners in beverages was a blend of ACE-K and ASP (S1, S3, S11, S13, S14, S18) (Fig. 4), whereas, combination of Na-SAC with ACE-K (no one sample) or ASP (only S17) is not common in beverages. Individually ACE-K was determined in one sample (S9), Na-SAC in two samples (S2 and S5), while ASP was found only in combination with ACE-K and Na-SAC. In a few of analyzed samples declared with sugar (S4, S6, S7 and S12) the analyzed artificial sweeteners were not detected. It can be summarized that ACE-K and ASP showed higher detection rate in analyzed samples in comparison with the detection rate for Na-SAC. Probably, Na-SAC was less used in beverages, because of its bitterness aftertaste. Furthermore, Na-SAC is more suitable sweetener for cakes due to its heat stability [34]. In their work de Queiroz Pane et al. (2015) and Imanulkhan et al. (2020) found that among others, ACE-K and ASP are the most frequently used sweeteners in diet/light/zero drinks [26, 32].

The obtained results correspond to the content of the analyzed sweeteners described on the beverages labels. In all the samples where the analytes were declared were also found, while the presence of analytes were not detected in the samples where they were not declared. The concentration of ACE-K in analyzed beverages ranged between 30.32 ± 3.83 and 148.37 ± 1.78, the concentration of Na-SAC was found to be from 16.10 ± 0.12 to 93.05 ± 7.64, while for ASP the determined concentration range was from 6.06 ± 1.29 to 512.72 ± 2.07. The concentration of ACE-K and ASP in all of the analyzed samples was within the maximum permitted level, while the concentration of Na-SAC in the two of the analyzed beverages (S8 and S15) exceeded it (85.43 and 93.06 mg $L^{-1}$). However, the possibility to exceed ADI drinking beverages with artificial sweeteners is almost impossible. The highest concentration of ACE-K (148.37 mg $L^{-1}$) and ASP (512.72 mg $L^{-1}$) was determined in S13, while 93.05 mg $L^{-1}$ for Na-SAC was determined as

*Table 4.* Concentration (mg $L^{-1}$) of analyzed sweeteners (ACE-K, Na-SAC and ASP) in the analyzed beverages

| Samples | Labeled values | ACE-K mg $L^{-1}$ ± SD | Na-SAC mg $L^{-1}$± SD | ASP mg $L^{-1}$ ± SD |
|---|---|---|---|---|
| S1 (carbon tablets with vitamin C with lemon flavour), pH = 4.45 | E950 and E951 | 36.42 ± 0.54 | nd* | 351.08 ± 3.56 |
| S2 (isotonic, non-carbonated, sports beverage with red food flavour), pH = 3.78 | E954 | nd | 16.10 ± 0.12 | nd |
| S3 (isotonic, refreshing, low-energy, non-carbonated, sports beverage), pH = 3.43 | E950 and E951 | 74.22 ± 0.69 | nd | 133.66 ± 0.81 |
| S4 (refreshing, non-carbonated beverage), pH = 3.22 | Declared with sugar | nd | nd | nd |
| S5 (non-carbonated, multivitamin flavored sports beverage), pH = 3.86 | E954 | nd | 49.95 ± 0.39 | nd |
| S6 (non-carbonated, refreshing beverage), pH = 3.48 | Declared with sugar | nd | nd | nd |
| S7 (non-carbonated, refreshing beverage with herbal extracts and black tea), pH = 3.46 | Declared with sugar | nd | nd | nd |
| S8 (refreshing, carbonated beverage without energy value), pH = 3.47 | E950, E954 and E951 | 30.32 ± 0.38 | 85.43 ± 0.44 | 46.16 ± 0.55 |
| S9 (refreshing, carbonated beverage without energy value) pH = 3.06 | E950 | 147.92 ± 1.02 | nd | nd |
| S10 (refreshing, carbonated beverage), pH = 2.82 | Declared with sugar | nd | nd | nd |
| S11 (refreshing, carbonated beverage made from plant extracts), pH = 3.07 | E950 and E951 | 36.74 ± 0.75 | nd | 6.06 ± 1.99 |
| S12 (refreshing, carbonated beverage) pH = 2.94 | Declared with sugar | nd | nd | nd |
| S13 (refreshing, carbonated beverage without energy value), pH = 2.94 | E950 and E951 | 148.37 ± 1.76 | nd | 512.72 ± 2.07 |
| S14 (refreshing, carbonated beverage without energy value), pH = 2.96 | E950 and E951 | 146.58 ± 1.21 | nd | 448.18 ± 1.60 |
| S15 (refreshing, carbonated beverage with sweeteners), pH = 3.54 | E950, E954 and E951 | 32.63 ± 0.61 | 93.05 ± 7.6 | 324.30 ± 6.06 |
| S16 (non-carbonated, energy beverage, with sweeteners and high caffeine content) | E950, E954 and E951 | 133.25 ± 9.51 | 79.29 ± 1.05 | 14.02 ± 0.32 |
| S17 (refreshing, carbonated beverage with sugar and sweeteners) | E954 and E951 | nd* | 72.46 ± 2.02 | 14.56 ± 0.16 |
| S18 (refreshing, carbonated beverage with herbal extracts, without energy value) | E950 and E951 | 35.82 ± 2.20 | nd | 11.98 ± 1.68 |

*nd – not detected.

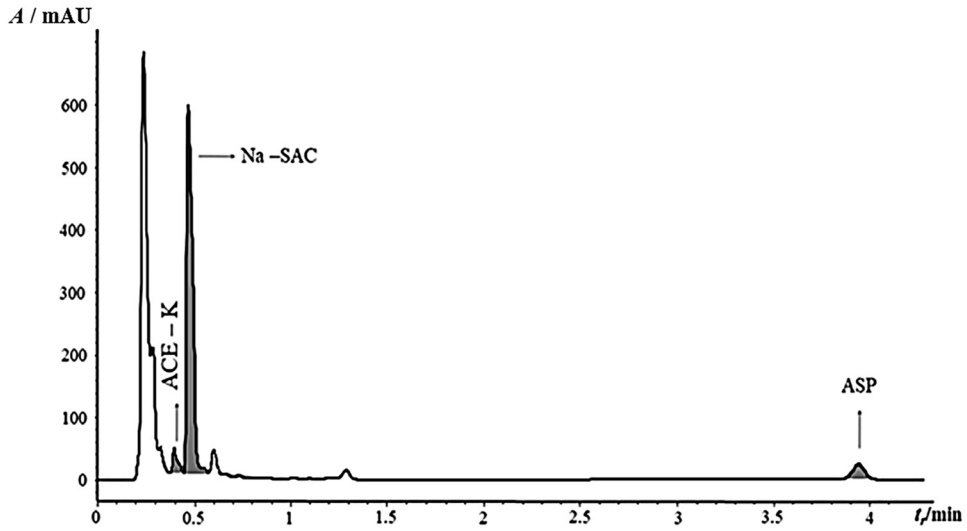


*Fig. 3.* Chromatogram of a sample S8 under optimized conditions at 220 nm

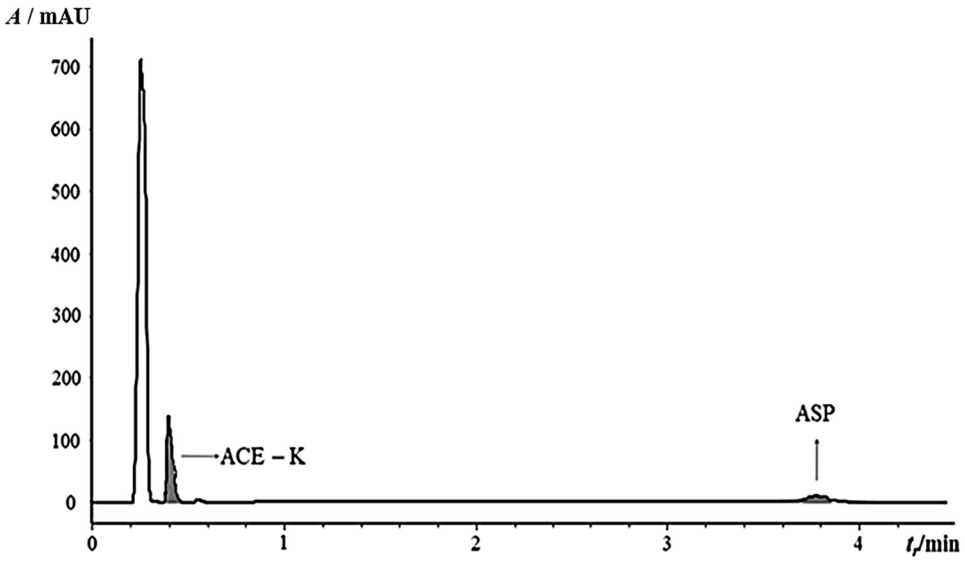


*Fig. 4.* Chromatogram of a sample S1 under optimized conditions at 220 nm

highest concentration in S15. Consumption of 250 mL of S13 in a day for average individual of 60 kg gives 4.12% of ADI for ACE-K and 5.34% for ASP, while with consumption of 250 mL of S15 gives 7.75% of ADI for Na-SAC.

## 4. CONCLUSION

In the presented research, a simple, sensitive and rapid RP-HPLC method with UV-DAD detection for simultaneous determination of the artificial sweeteners ACE-K, Na-SAC and ASP in beverages was developed and validated. The analyses were performed on an Agilent 1290 Infinity LC with Poroshell 120 EC – C18 (3.0 × 50 mm, 2.7 µm) column as a stationary phase. A mobile phase consisted of ACN and diluted phosphoric acid (pH = 3.8) in volume ratio 7/93 (*V*/*V*) was used. Successful separation was achieved in less than 5 min, with isocratic elution and 1 mL $min^{-1}$ flow rate. The developed method was validated by testing the linearity, precision, accuracy, LOD and LOQ. The obtained results revealed that among analyzed beverages, ASP and ACE-K were the most used sweeteners followed by Na-SAC (six samples). From analyzed sweeteners only Na-SAC exceed the maximum permitted level in two samples (S8 and S15). It is worth noting that the proposed method is appropriate for routine analysis because is easy to perform, it is less time consuming and less labor intensive, and it is comparable in terms of accuracy, precision and LOD with some of the published advanced analytical methods.

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