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# Development and validation of an RP-HPLC method for the simultaneous determination of artificial sweeteners, preservatives and caffeine in non-alcoholic beverages

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**Development and validation of an RP-HPLC method for the simultaneous determination of artificial sweeteners, preservatives and caffeine in non-alcoholic beverages**

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**ABSTRACT**

Consumption of a wide variety of beverages has increased considerably in modern diets. These products frequently contain additives such as artificial sweeteners, preservatives, and caffeine to enhance flavor, stability, and functional properties. However, due to the potential adverse health effects associated with excessive intake of these compounds, the development of reliable analytical methods for their determination is of considerable importance. Therefore, the aim of this study was to develop and validate a rapid reversed-phase high performance liquid chromatography (RP-HPLC) method coupled with ultraviolet diode-array detection (UV-DAD) for the simultaneous determination of acesulfame K, sodium saccharin, caffeine, aspartame, sodium benzoate, and potassium sorbate in non-alcoholic beverages.

Optimal chromatographic separation was achieved on a Poroshell 120 EC-C18 column using isocratic elution with a mobile phase consisting of methanol and phosphoric acid solution (pH = 3.8) in a volume ratio of 20:80, at a flow rate of 1.0 mL/min and a constant column temperature of 25 °C. Detection was performed at 195, 230, 260, and 275 nm. Under the optimized chromatographic conditions, the run time was less than 4.5 minutes. The validated method demonstrated excellent linearity ($R^2 > 0.999$), satisfactory intra-day and inter-day precision (RSD = 0.02 – 2.94 %), and good accuracy, with recoveries ranging from 91.11 to 108.99 %. The developed method was successfully applied for the simultaneous determination of the target analytes in commercially available non-alcoholic beverages. Compared with previously reported methods, the proposed method provides shorter analysis time and simple sample preparation, making it suitable for routine quality control analysis.

**Keywords:** artificial sweeteners, caffeine, preservatives, non-alchoholic beverages, RP-HPLC method

## 1. INTRODUCTION

The use of additives in food and beverages dates back to the earliest civilizations, where natural substances such as salt, honey, vinegar, and spices were commonly used to extend shelf life and enhance flavor [1]. Today, with the advancement of the modern food industry, synthetic additives are increasingly used. They are chemical substances that are added to food products and help to extend the shelf life, as well as to improve or maintain the microbial and chemical stability, nutritional quality, sensory characteristics, and the quality and safety of food products [2].

Non-alcoholic beverages represent a widely consumed group of products, including carbonated, fruit-based, functional, and energy drinks. Food additives play a crucial role in ensuring microbiological stability, safety, and acceptable sensory quality of these beverages. Their use is strictly regulated and based on scientifically established risk and safety assessments to protect consumer health.

Among the most commonly used additives in non-alcoholic beverages are preservatives and artificial sweeteners.

Preservatives are added to beverages to improve stability and extend shelf life. They effectively inhibit microbial growth while maintaining the organoleptic characteristics of the product. Their use not only ensures improved beverage safety, but also mitigates quality deterioration resulting from microbiological, enzymatic, and physicochemical processes [3].

Among others, sodium benzoate (SB) and potassium sorbate (PS) are some of the most frequently used preservatives in beverages (Fig. 1).

SB (E211) is a sodium salt of benzoic acid and is commonly used antimicrobial preservative in acidic beverages, including carbonated soft drinks and fruit-based beverages. Its antimicrobial efficacy is strongly pH-dependent, as the undissociated form of benzoic acid predominates at pH values below 4.5 and inhibits the growth of yeasts, molds, and some bacteria by disrupting intracellular metabolic activity [4]. Due to its high water solubility, stability, and effectiveness at low concentrations, sodium benzoate is widely applied within regulatory limits established by international food safety authorities [5].

PS (E202) is the potassium salt of sorbic acid and, like SB, is widely used as an antimicrobial preservative in beverages to inhibit the growth of yeasts, molds, and certain bacteria, thereby extending product shelf-life and maintaining microbiological safety. Owing to its high water solubility and sensory neutrality, it is well suited for liquid formulations such as fruit juices, soft drinks, and flavored beverages. The antimicrobial efficacy of PS derives from the dissociation to sorbic acid in aqueous environments. The undissociated sorbic acid can penetrate microbial cell membranes, disrupting metabolic processes and inhibiting cell growth. Its activity is pH-dependent, with optimal effectiveness in acidic conditions typically found in beverages (pH ~ 3.0 – 6.5). This makes it well-suited for fruit juices and soft drinks, where pH is maintained below ~ 4.5 for stability and safety [6, 7].

Although SB and PS are classified as “Generally Recognized as Safe” (GRAS), several studies have raised concerns regarding their potential adverse health effects, particularly with long-term exposure [8].

For example, combining ascorbic acid with SB in beverages could lead to the formation of benzene, especially when the drinks are stored at higher temperatures for longer periods of time. Benzene is a chemical classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1) [5, 9]. SB has also been associated with urticaria, asthma, dermatitis, life-threatening reactions, abdominal pain, vomiting, eye irritations, hyperactivity in children, and other behavioral disorders [10, 11].

Research studies have demonstrated that potassium sorbate may induce oxidative stress, chromosomal damage, and genotoxic effects [12], as well as reduced lifespan, impaired fertility, and increased cellular apoptosis [13]. Furthermore, alterations in liver inflammatory responses and changes in gut microbiota composition have been reported [14]. In addition, potassium sorbate has been associated with hypersensitivity reactions, including oral, ocular, and cutaneous itching, nasal congestion, rhinorrhea, and gastrointestinal discomfort [11].

The preference for sweetness is biologically innate; however, high intake of added sugars has been linked to increased energy consumption and poor dietary quality, contributing to obesity, type 2 diabetes, and cardiovascular disease. Artificial sweeteners, also referred to as intense sweeteners or synthetic sugar substitutes, provide sweetness with little or no caloric contribution and are widely used in beverage formulations to reduce sugar content while maintaining sensory acceptability. Their use supports public health strategies aimed at lowering the consumption of high-calorie foods and reducing the risk of obesity, type 2 diabetes, and dental caries [15, 16].

Among the most commonly used artificial sweeteners in beverages are acesulfame potassium (acesulfame K, ACK), sodium saccharin (SS), and aspartame (ASP), (Fig. 1).

ACK (E950) is a high-intensity sweetener, approximately 180 – 200 times sweeter than sucrose. It is characterized by a clean sweet taste and a slight sweet aftertaste, along with excellent thermal and pH stability, which makes it suitable for use in carbonated and shelf-stable beverages. ACK is frequently blended with other sweeteners, such as saccharin and aspartame, to mask bitterness and achieve synergistic enhancement of sweetness

[17, 18]. However, the use of ACK should be approached with caution, as some studies have suggested potential adverse health effects under certain conditions or at high exposure levels. Reported effects include neurological symptoms, gastrointestinal disturbances, and indications of hepatic and renal stress [15].

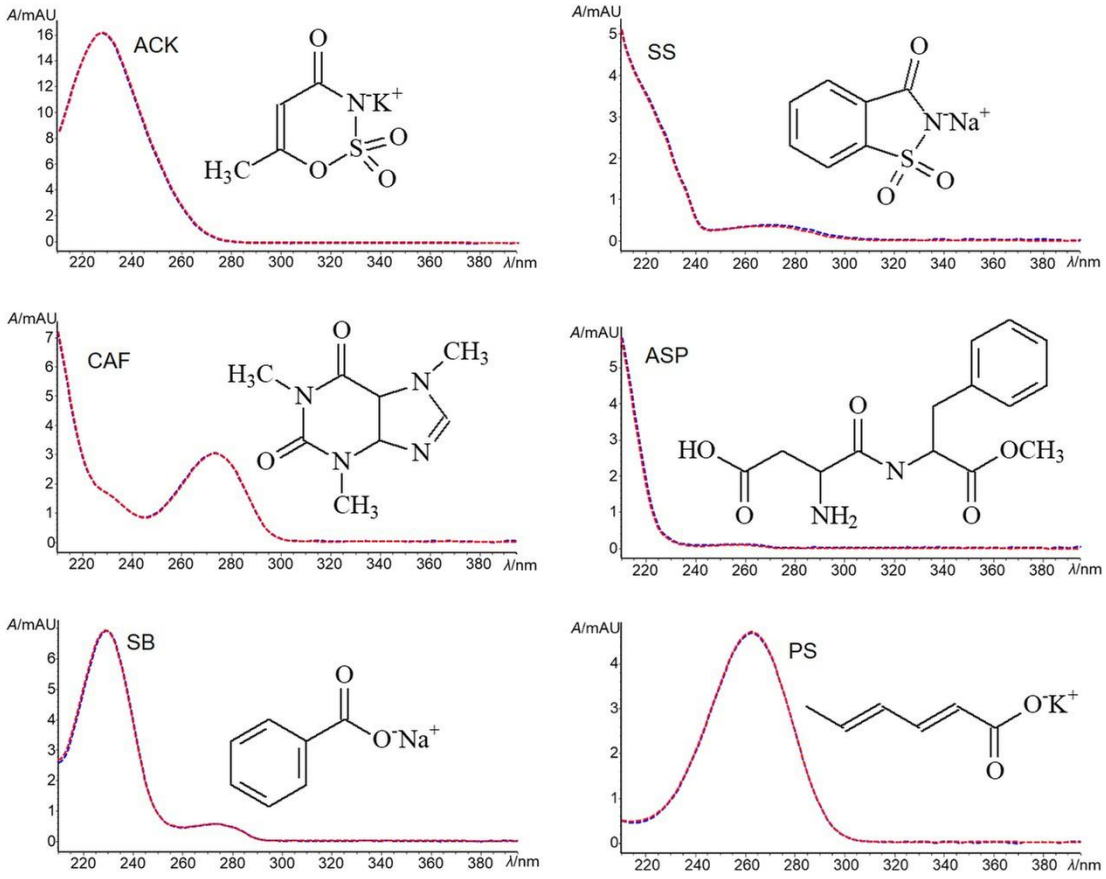



**Fig. 1** Structural formulas and overlayed UV spectra of acesulfame K (ACK), sodium saccharin (SS), caffeine (CAF), aspartame (ASP), sodium benzoate (SB), and potassium sorbate (PS) in the standard solution and the sample, with the mobile phase methanol and phosphoric acid (pH = 3.8), (20/80, *V/V*)

SS (E954) is one of the earliest artificial sweeteners, exhibiting high sweetening potency (approximately 300 – 500 times sweeter than sucrose), high water solubility, and excellent stability across a wide pH range and during heat treatment. Its characteristic bitter or metallic aftertaste can be mitigated by blending it with other sweeteners and therefore, it is commonly used in combination with ACK and ASP in food products [18]. As a low-calorie sugar substitute, SS is widely applied in sugar-free formulations. However, concerns have been raised regarding its potential effects on glucose metabolism and gut microbiota, particularly with regular consumption [19].

ASP (E951) is approximately 200 times sweeter than sucrose and provides a clean sweet taste with a prolonged aftertaste. It is frequently used in combination with other sweeteners, such as SS and ACK. ASP is readily soluble in water and exhibits optimal stability within a pH range of 3.0 – 4.3. However, its limited stability at elevated temperatures and during prolonged storage restricts its application primarily to non-heat-treated beverages [18]. ASP is a low-calorie artificial sweetener widely used due to its favorable sensory profile and absence of a bitter aftertaste. In July 2023, a joint evaluation by the World Health Organization (WHO), the International Agency for Research on Cancer (IARC), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) classified aspartame as possibly carcinogenic to humans (Group 2B), while reaffirming that intake within the established acceptable daily intake (ADI) remains safe [20]. This classification has attracted significant attention from both consumers and beverage manufacturers, particularly given the widespread use of aspartame in artificially sweetened beverages, which constitute a substantial segment of the global beverage market [21].

In addition to artificial sweeteners and preservatives, caffeine is a common additive in an increasing number of beverages and energy drinks.

Caffeine is a naturally occurring methylxanthine alkaloid and one of the most widely consumed psychoactive substances worldwide. It is commonly found in coffee, cocoa, soft drinks, and energy drinks and acts

as a stimulant of the central nervous system [22]. Caffeine intake has been associated with increased arousal and alertness, improved cognitive performance, enhanced perceived energy, reduced perception of fatigue, and positive effects on mood [23]. However, excessive caffeine intake, particularly from beverages such as energy drinks, has been associated with adverse effects including tachycardia, nausea and vomiting, cardiac arrhythmias, seizures, and, in rare cases, fatal outcomes. Additional reported effects include increases in blood pressure, anxiety and other psychiatric symptoms, and an increased risk of dependence in susceptible individuals [24]. The physiological effects of caffeine are strongly influenced by dose, age, sex, duration of exposure, and individual sensitivity. Caffeine and energy drinks are generally not recommended for children and adolescents [25].

The use of food additives in non-alcoholic beverages is regulated to ensure consumer safety, despite many of these substances being classified as Generally Recognized as Safe (GRAS) by regulatory authorities. Within the European Union, Regulation (EC) No 1333/2008 [26] establishes harmonized rules for the authorization and conditions of use of food additives, based on scientific risk assessments conducted by the European Food Safety Authority (EFSA). Although preservatives, artificial sweeteners, and caffeine are widely used and technologically justified, scientific evidence has demonstrated that excessive or long-term exposure to some of these compounds may be associated with adverse health effects [5, 7, 22, 27 – 29]. Therefore, both health-based guidance values, such as the Acceptable Daily Intake (ADI), and product-specific Maximum Permitted Levels (MPLs) are essential regulatory tools to limit consumer exposure and ensure that the cumulative intake of these additives remains within safe margins. Together, ADI and MPL values provide a scientifically grounded framework for protecting human health while allowing the controlled use of food additives in beverage formulations [26, 30, 31].

Accordingly, Table 1 presents the Acceptable Daily Intake (ADI) values established by EFSA, FDA, and the Codex Alimentarius Commission, together with the corresponding Maximum Permitted Levels (MPLs) for the investigated additives in non-alcoholic beverages, as regulated under Regulation (EC) No 1333/2008 [5, 7, 22, 26 – 32].

Table 1. Acceptable Daily Intake (ADI) values and Maximum Permitted Levels (MPLs) for selected additives in non-alcoholic beverages

| **Compound** | **ADI (mg/kg bw/day)** | **MPL in non-alcoholic beverages** | **References** |
|---|---|---|---|
| Sodium benzoate | 0 – 5 (group ADI for benzoates) | 150 mg/L (alone or with sorbates) | [5, 26, 30] |
| Potassium sorbate | 3 (group ADI for sorbates) | 300 mg/L (alone);<br>250 mg/L (with benzoate) | [7, 26, 30] |
| Acesulfame potassium | 15 | 350 mg/L | [26, 29, 30] |
| Aspartame | 40 (EFSA, EU);<br>50 (FDA, USA) | 600 mg/L | [26, 27, 30, 31] |
| Sodium saccharin | 9 | 80 mg/L | [26, 28, 30] |
| Caffeine | No ADI (intake guidance: ≤ 400 mg/day (adults)) | No MPL established; typically, ≤ 320 mg/L in energy drinks;<br>mandatory labeling required above 150 mg/L | [22, 32] |

Given the widespread use of preservatives, artificial sweeteners, and caffeine in non-alcoholic beverages, together with established ADI and MPLs values, the availability of reliable analytical methods is essential to ensure regulatory compliance and consumer safety. Numerous analytical approaches for the determination of these compounds have been reported in the literature, including electrophoretic, electroanalytical, colorimetric, and spectroscopic techniques, as well as chromatographic methods [33, 34]. Among the latter, gas chromatography coupled with mass spectrometry (GC–MS) [35], liquid chromatography–mass spectrometry (LC–MS) [36], and, most commonly, high-performance liquid chromatography (HPLC) combined with UV–Vis or diode array detection (UV-DAD) have been extensively applied [37 – 42].

However, despite the large number of available analytical methods, the simultaneous determination of these compounds in complex beverage matrices remains analytically challenging due to their belonging to different chemical classes, resulting in diverse physicochemical and spectral characteristics as well as a wide range of concentrations. Furthermore, routine quality control and regulatory monitoring demand robust, accurate, and time-efficient methods capable of analyzing multiple additives within a single analytical run. Therefore, the aim of the present study was to develop and validate a reversed-phase high-performance liquid chromatography (RP-HPLC) method with UV-DAD detection for the simultaneous determination of sodium benzoate, potassium sorbate, acesulfame potassium, aspartame, sodium saccharin, and caffeine in non-alcoholic beverages. Furthermore, the developed method was successfully applied to the simultaneous determination of the investigated analytes in commercially available non-alcoholic beverages from the local market.

## 2. MATERIALS AND METHODS

### 2.1. Instrumentation

The chromatographic analyses were performed using Agilent 1260 Infinity Rapid Resolution Liquid Chromatography (RRLC) system equipped with vacuum degasser (G1322A), binary pump (G1312B), autosampler (G1329B), a thermostatted column compartment (G1316A), ultraviolet diode array detector (UV-DAD) (G1316B), and ChemStation software (version A.01.01). Two analytical columns with identical stationary phase characteristics but different internal diameters were evaluated, namely Poroshell 120 EC – C18 (50 × 3.0 mm, 2.7 μm) and Poroshell 120 EC – C18 (50 × 4.6 mm, 2.7 μm), manufactured by Agilent Technologies (USA).

A digital analytical balance (ME 204, Mettler Toledo, Switzerland) with an accuracy of ± 0.0001 g was used to weighing the analytical standards. The pH of the solutions was measured using a pH meter (Seven Direct SD 20, Mettler Toledo, Switzerland) equipped with a magnetic stirrer (EasyMix, Mettler Toledo, Switzerland). Precise volumes of standard solutions, working solutions, and samples were measured using an electronic pipette (HandyStep touch S, BRAND, Germany) with interchangeable piston tips (0.01 – 5 mL). An ultrasonic bath (Elmasonic Select 100, Elma, Germany) was used to facilitate the dissolution of analytical standards and for degassing (ultrasonication) of the samples prior to analysis. Phosphate buffer solutions were filtered using a vacuum filtration system (Supelco Analytical, USA) with nitrocellulose membrane filters (0.22 μm pore size).

### 2.2. Analytical standards and reagents

Pure analytical standards of acesulfame K (99.4 %), sodium saccharin (99.6 %), caffeine (99.9 %), aspartame (99.0 %), sodium benzoate (99.9 %), and potassium sorbate (99.9 %) were purchased from CPAchem Ltd. (Bulgaria). HPLC-grade acetonitrile, methanol, and water (Fisher Chemical, USA) were used for the preparation of the mobile phase.

Potassium dihydrogen phosphate (> 99 %), manufactured by Fisher Chemical (USA), and phosphoric acid (85.35 %), manufactured by Fisher Chemical (UK), were used for the preparation of phosphate buffer solutions. For pH meter calibration, standard buffer solutions with pH values of 4.0 and 7.0 (Fisher Chemical, Belgium) were used.

The study included 16 commercially available non-alcoholic beverages of different brands and compositions, selected to cover a wide range of products containing artificial sweeteners, preservatives, and caffeine. The analyzed products comprised carbonated and non-carbonated beverages, fruit juices, iced teas, energy and sports drinks, which were randomly sampled from local markets.

### 2.3. Preparation of of standard solutions

For the preparation of stock solutions, the following amounts of analytical standards were accurately weighed (± 0.0001 g): 0.0694 g of acesulfame K, 0.0273 g of sodium saccharin, 0.0512 g of caffeine, 0.1201 g of aspartame, 0.0324 g of sodium benzoate, and 0.0610 g of potassium sorbate. Each weighed standard was quantitatively transferred into a 10 mL volumetric flask, dissolved, and diluted to volume with HPLC-grade water. To ensure complete dissolution, the solutions were sonicated in an ultrasonic bath for 15 min at a temperature not exceeding 25 °C. The stock solutions were stored in a refrigerator at 4 °C until analyses.

Six mixed working standard solutions with different mass concentrations were prepared by transferring appropriate aliquots (50, 100, 300, 500, 700, and 900 μL) of each individual stock solution into the same 10 mL volumetric flask, followed by dilution to volume with water. The working standard solutions were freshly prepared on the day of analysis. Each working standard solution was injected in triplicate into the HPLC system using an injection volume of 2.5 μL.

To determine the accuracy of the method, known volumes of each stock solution (400, 500, and 600 μL), corresponding to three concentration levels of each analyte, were added to a 10 mL volumetric flask containing a blank matrix, and the volume was adjusted to the mark with the blank sample. An energy drink sample was used as the blank matrix in this study. For each concentration level, three replicate solutions were prepared, and each solution was injected five times into the chromatographic system using an injection volume of 2.5 μL. Prior to injection, all solutions were filtered through nylon syringe filters with a pore size of 0.22 μm.

### 2.4. Preparation of sample solutions

Sample preparation in the present study was simple and required minimal pretreatment. To remove dissolved gases, carbonated beverages were degassed in an ultrasonic bath for approximately 15 min at a

temperature not exceeding 25 °C. Prior to HPLC analysis, all samples were filtered through a nylon syringe filter with a pore size of 0.22 μm. Each sample was analyzed in triplicate using an injection volume of 2.5 μL.

### 2.5. Preparation the phosphate buffer solutions

Phosphate buffer solutions at different pH values were freshly prepared by dissolving 0.1 g of potassium dihydrogen phosphate ($KH_2PO_4$) in 100 mL of HPLC-grade water. Each solution was stirred on a magnetic stirrer to ensure complete dissolution, and the desired pH was adjusted using dilute phosphoric acid ($H_3PO_4$). Prior to use, each buffer solution was filtered through a 0.22 μm nitrocellulose membrane using a vacuum filtration system.

### 2.6. Preparation of phosphoric acid solution

HPLC-grade water was placed in a beaker, and diluted phosphoric acid ($H_3PO_4$) was added dropwise under stirring until a pH of 3.8 was achieved. Before use, the mobile phase was filtered through a 0.22 μm nitrocellulose membrane filter.

### 2.7. Chromatographic Conditions

Chromatographic separation was performed on a Poroshell 120 EC-C18 analytical column (50 × 3.0 mm, 2.7 μm). The mobile phase consisted of methanol and phosphoric acid solution (pH = 3.8) in a volume ratio of 20:80 (*V*/*V*). The flow rate was set at 1.0 mL/min, and isocratic elution was applied throughout the analysis. The column oven temperature was maintained at 25 °C.

UV–DAD detection was carried out at wavelengths of 195, 230, 260, and 275 nm. Identification of the analytes was based on a comparison of their retention times and UV spectra with those of the corresponding analytical standards. Quantification was performed using external calibration curves at 230 nm for acesulfame K and sodium benzoate, 195 nm for sodium saccharin and aspartame, 260 nm for potassium sorbate, and 275 nm for caffeine.

### 2.8. Method validation

The developed RP-HPLC method was validated in accordance with internationally recognized guidelines for analytical method validation, including specificity, selectivity, linearity, limits of detection and quantification, precision, accuracy, and robustness [43, 44]. Method validation was performed using retention time, peak area, and peak height data obtained at a detection wavelength of 230 nm for acesulfame K and sodium benzoate, 195 nm for sodium saccharin and aspartame, 260 nm for potassium sorbate, and 275 nm for caffeine.

**Specificity and selectivity**. Specificity and selectivity were evaluated by comparing chromatograms of standard solutions and spiked beverage samples to verify the absence of interfering matrix components at the retention times of the investigated analytes.

**Linearity**. Linearity was evaluated by analyzing six working standard solutions containing all analytes at different concentration levels within the selected calibration range. Calibration curves were constructed by plotting peak areas against the corresponding mass concentrations of each analyte. Linear regression analysis was performed, and the linearity of the method was assessed based on the correlation coefficients ($R^2$).

**Limits of detection and quantification**. The limits of detection (LOD) and quantification (LOQ) for each analyte were determined based on signal-to-noise ratios of approximately 3:1 and 10:1, respectively. LOD and LOQ values were calculated using diluted standard solutions injected under the optimized chromatographic conditions.

**Precision**. The precision of the method was evaluated in terms of intra-day (repeatability) and inter-day (intermediate precision). Intra-day precision was assessed by analyzing standard mixtures at selected concentration levels ten times within the same day, whereas inter-day precision was evaluated by repeating the analysis on three days under the same experimental conditions. Precision was expressed as the relative standard deviation (RSD, %) of peak areas, peak height and retention times for each analyte.

**Accuracy**. The accuracy of the method was evaluated by recovery studies using the standard addition method. Known amounts of each analyte at three concentration levels were added to a blank matrix, and the samples were analyzed under the optimized chromatographic conditions. Accuracy was expressed as percentage recovery (R, %), calculated by comparing the measured concentration with the added concentration.

**Robustness**. The robustness of the developed RP-HPLC method was evaluated by applying the optimized chromatographic conditions to an alternative analytical column with identical stationary phase characteristics but a different internal diameter (Poroshell EC-C18, 50 × 4.6 mm, 2.7 μm). The influence of the column dimension on retention times, peak shapes, and resolution was examined.

The validated method was subsequently applied to the simultaneous determination of sodium benzoate, potassium sorbate, acesulfame K, aspartame, sodium saccharin, and caffeine in commercially available non-alcoholic beverages.

## 3. RESULTS AND DISCUSSION

Considering the high consumption of non-alcoholic beverages and the presence of multiple food additives with different physicochemical properties, the chromatographic conditions were developed and optimized to enable reliable simultaneous determination of the investigated analytes. Therefore, several parameters affecting chromatographic separation were evaluated in order to achieve satisfactory resolution, good peak shape, reproducible retention, and reliable detection of all target compounds within a single analytical run.

### 3.1. Development of RP-HPLC method

**Selection of the stationary phase**. The selection of an appropriate stationary phase represents a crucial step in chromatographic method development, particularly when the simultaneous determination of analytes with diverse physicochemical properties is required. Reversed-phase C18 columns are widely applied for the separation of food additives due to their ability to provide adequate retention and resolution for compounds with varying polarity and chemical characteristics. Numerous studies have demonstrated the successful application of RP-HPLC using C18 stationary phases for the determination of artificial sweeteners, preservatives, and caffeine in beverage matrices, either individually or simultaneously [37, 39, 40]. In recent years, core–shell C18 columns have gained increasing attention, as they offer higher separation efficiency, improved peak shape, and reduced analysis time compared to fully porous particles, while maintaining moderate backpressure [45, 46]. Additionally, building on our previous research on the successful determination of artificial sweeteners [41], as well as preservatives and caffeine [42] in various soft drinks, the present study aimed to investigate whether the use of a Poroshell 120 EC-C18 column would enable efficient separation of all target analytes within a single analytical run. This column provides chemical stability in a wide pH range (2 - 9), as well as resistance to dehydration and degradation when working with aqueous mobile phases or organic solvents (such as methanol and acetonitrile). The column shows excellent reproducibility, symmetrical peaks and high selectivity in complex matrices [47]. Accordingly, a Poroshell 120 EC-C18 column was selected to achieve reliable and efficient simultaneous separation of the investigated compounds within a short analysis time.

In addition to the selection of the stationary phase, the influence of the column internal diameter on chromatographic performance was evaluated as part of the method optimization. Two Poroshell 120 EC-C18 columns with identical stationary phase characteristics but different internal diameters (50 × 3.0 mm and 50 × 4.6 mm, 2.7 μm) were evaluated during the optimization process. The comparison was focused on key performance parameters, including retention behavior, peak shape, resolution, analysis time, solvent consumption, and method sensitivity.

Although both columns provided good chromatographic performance, expressed as symmetrical and narrow chromatographic peaks for all investigated analytes, the use of the 3.0 mm column resulted in lower retention times and consequently shorter run times and reduced solvent consumption. Based on these observations, the Poroshell 120 EC-C18 column (50 × 3.0 mm, 2.7 μm) was selected for further optimization and full validation of the developed RP-HPLC method. The column with a larger internal diameter (4.6 mm) was subsequently used as part of the robustness testing to assess the performance of the method under modified chromatographic conditions.

In spite of the fact that both gradient [37, 39, 48] and isocratic [40 – 42] elution modes have been widely reported in the literature for the determination of artificial sweeteners, preservatives, and caffeine in beverages, the present study aimed to develop a simple and robust RP-HPLC method suitable for routine analysis. Therefore, chromatographic separation was performed under isocratic elution conditions, using a constant mobile phase composition throughout the analysis. Isocratic elution is often preferred for routine quality control due to its simplicity, improved method robustness, easier reproducibility, and reduced method development time, particularly when adequate separation of all analytes can be achieved within a single run [49].

A constant flow rate of 1.0 mL/min was applied, as commonly recommended for short C18 columns to ensure a balance between analysis time, chromatographic efficiency, and system backpressure [46]. In addition, the column oven temperature was maintained at 25 °C to ensure stable retention times and reproducible chromatographic behavior, since temperature fluctuations can significantly influence analyte retention and peak shape in reversed-phase liquid chromatography [49].

The choice of the wavelength at which the chromatographic analysis was performed was made based on the UV spectra of the components of interest recorded in a mobile phase composed of methanol and phosphoric acid solution (pH = 3.8) with a volume ratio of 20/80 (Fig. 1). As can be seen from Fig. 1, acesulfame K and sodium benzoate show maximum absorption at a wavelength of approximately 230 nm, while saccharin sodium

and aspartame have a high absorption at around 195 nm. Caffeine has a maximum absorption at around 275 nm, and potassium sorbate at around 260 nm. Hence, the chromatographic process was monitored at 195, 230, 260 and 275 nm.

**Mobile phase composition and pH optimization**. Furthermore, the composition and pH of the mobile phase were systematically investigated, as these parameters have a pronounced effect on the retention, resolution, and peak shape of the investigated analytes.

During method development, various mobile phase compositions were systematically evaluated in order to achieve adequate separation, acceptable peak shape, and stable retention of all target analytes within a single chromatographic run. The tested mobile phases consisted of different combinations of phosphate buffer, phosphoric acid solution, and methanol and acetonitrile, as organic modifiers. The investigated analytes include weak acids and ionic compounds, whose ionization state strongly depends on the pH of the mobile phase. According to chromatographic theory and previous studies, operating under mildly acidic conditions suppresses the dissociation of acidic analytes, leading to improved retention, reduced peak tailing, and better peak symmetry on C18 stationary phases [49]. In agreement with these findings, it was observed that mobile phases adjusted to acidic pH values provided improved chromatographic performance compared to neutral conditions. Therefore, the pH of the aqueous phase was adjusted within the range of 3.5 – 4.3, which is commonly reported in the literature as suitable for the separation of preservatives, artificial sweeteners, and caffeine using reversed-phase HPLC [40–42].

The investigation of the optimal mobile phase composition was initiated using acetonitrile/phosphate buffer (pH = 3.5) in a ratio of 8:92 (*V*/*V*). These conditions previously enabled satisfactory separation of benzoates and sorbates on another C18 column [40]. However, when applied to the present column, sodium benzoate and potassium sorbate eluted almost simultaneously and could not be separated.

To improve retention and separation, the proportion of acetonitrile was reduced (3 – 8 %). Nevertheless, decreasing the content of the stronger eluent did not lead to separation of the analytes in their mixture. Moreover, injection of individual analytes under these conditions resulted in asymmetric peaks with double and triple peak formation. The occurrence of multiple peaks for a single compound may arise from inappropriate chromatographic parameters, improper sample–mobile phase compatibility, or heterogeneities in the stationary phase [50]. Therefore, further optimization focused on adjustment of the mobile phase pH.

The same mobile phase composition was examined at pH = 3.7, 3.8 and 4.3. At pH = 3.7, using acetonitrile/buffer ratios of 10:90 and 15:85 (*V*/*V*), certain analytes showed improved peak symmetry; however, acesulfame K and sodium saccharin were not baseline-separated, while aspartame and sodium benzoate produced broad and diffuse peaks. Such peak broadening and distortion may result from solvent-strength mismatch and viscous-drag effects [51].

Further experiments were conducted at pH = 3.8. A mobile phase of 15:85 (*V*/*V*) acetonitrile/buffer, previously reported for the separation of caffeine, sodium benzoate and potassium sorbate in beverages [42], did not provide satisfactory separation in this case: acesulfame K and sodium saccharin remained partially overlapped, and sodium benzoate exhibited a broadened peak of poor shape.

To enhance resolution, the acetonitrile fraction was further decreased to 10 %, 8 % and 7 %. At 10:90 (*V*/*V*), both the acesulfame K/sodium saccharin and sodium benzoate/potassium sorbate pairs were not baseline-resolved and exhibited peak broadening. A composition of 7:93 (*V*/*V*), previously successful for the separation of acesulfame K, sodium saccharin and aspartame [41], still resulted in broad and poorly shaped peaks for sodium benzoate and potassium sorbate.

Although partial separation of the analytes was observed using acetonitrile/buffer (pH = 3.8) in a ratio of 10:90 (*V*/*V*), the separation was not sufficient to ensure reliable quantification. This was confirmed by peak purity values below 900, indicating spectral interference between co-eluting compounds and insufficient chromatographic resolution.

In order to improve selectivity, the buffer pH was increased to 4.3 while maintaining acetonitrile as the organic modifier. However, with acetonitrile/buffer (10:90, pH = 4.3), no effective separation was achieved. The chromatogram showed unstable baseline and broadened, irregular peaks, confirming that acetonitrile did not provide adequate selectivity for simultaneous determination of the investigated compounds on this column.

Therefore, acetonitrile was replaced with methanol. Although acetonitrile generally provides lower viscosity and shorter analysis time, methanol often offers different selectivity in reversed-phase chromatography and improved resolution for structurally related polar additives, particularly artificial sweeteners, while maintaining stable baseline at low UV wavelengths. Similar behavior has been reported for determination of food additives in beverages [52].

Mobile phases consisting of methanol and phosphate buffer were investigated at 10:90 and 20:80 (*V*/*V*) ratios and at pH = 3.7 and 3.8. Using methanol/buffer (10:90, pH = 3.8), sodium benzoate and potassium sorbate produced broadened peaks of poor shape. Increasing the methanol fraction to 20 % significantly improved resolution. At pH = 3.7, baseline separation of all analytes was obtained; however, a pronounced baseline disturbance (“hump”) appeared before the sodium benzoate peak, interfering with quantification.

To eliminate this effect, the buffer pH was slightly increased to 3.8. Even a small pH adjustment in reversed-phase chromatography can significantly influence analyte ionization and selectivity, as a change of only 0.1 pH units may markedly alter relative retention [53]. After this adjustment, the baseline disturbance was minimized and clean separation with symmetrical peaks was achieved.

From the investigations performed so far, the best separation of the components was obtained using a mobile phase composed of methanol and phosphoric buffer (pH = 3.8) in a ratio of 20/80 (*V/V*), producing sharp, symmetrical chromatographic peaks of regular shape.

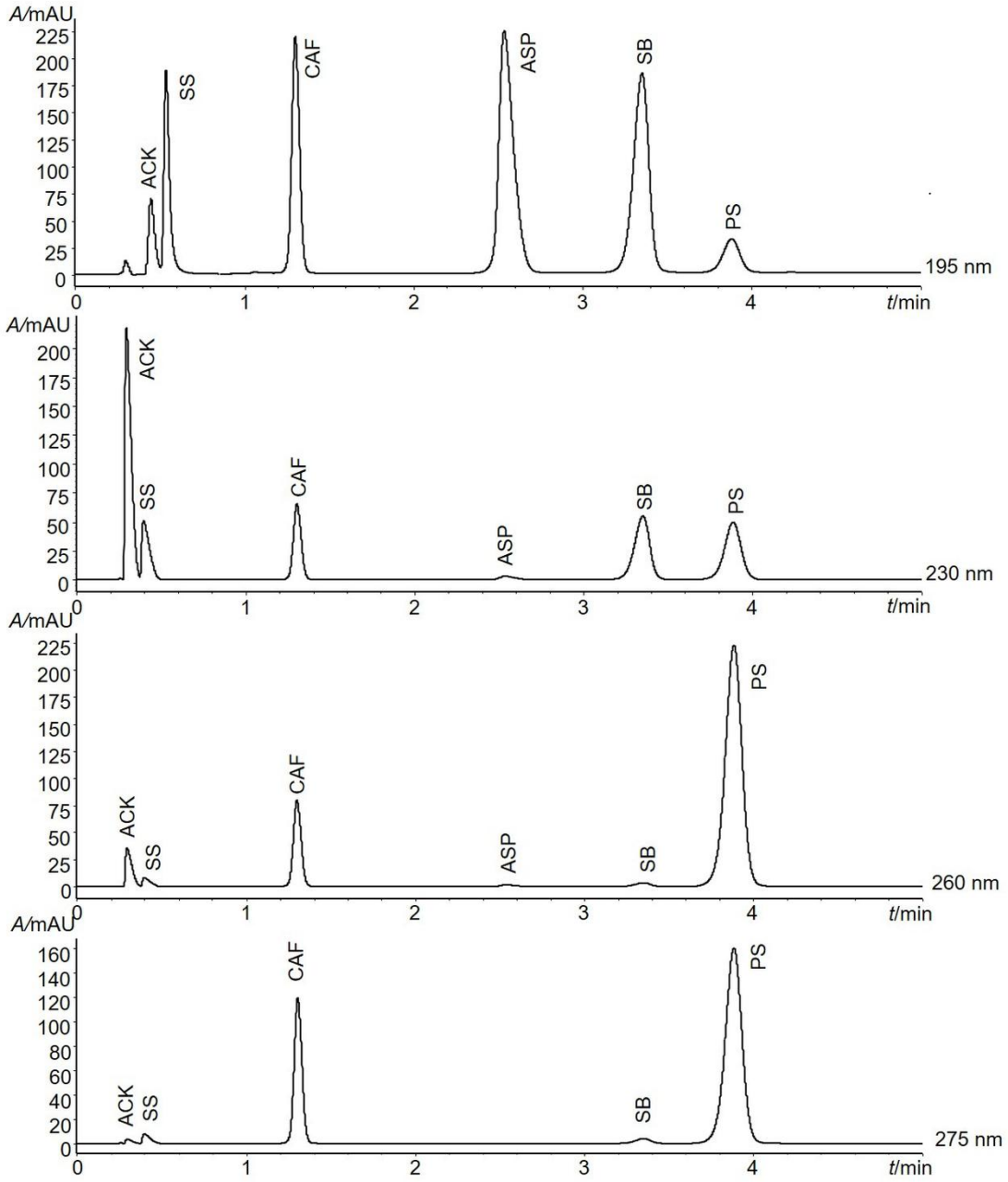



**Fig. 2** Chromatograms obtained from standard mixture of acesulfame K (ACK), sodium saccharin (SS), caffeine (CAF), aspartame (ASP), sodium benzoate (SB), and potassium sorbate (PS) on a Poroshell 120 EC–C18 column (50 × 3.0 mm, 2.7 µm), with methanol/phosphoric acid solution (pH = 3.8), (20/80, *V/V*) as mobile phase

However, phosphate buffers have limited solubility in mobile phases containing organic modifiers and may precipitate within the chromatographic system. Precipitation of buffer salts inside the column, capillaries and detector flow cell can distort the UV signal and lead to increased baseline noise, instability and split peaks, thereby reducing method robustness [54]. For this reason, the phosphate buffer was replaced with a phosphoric acid solution. No significant differences were observed in terms of analyte separation, while improved baseline stability

and method reliability were achieved. Based on the obtained results, the optimal chromatographic conditions for the developed RP-HPLC method for the simultaneous determination of acesulfame K, sodium saccharin, caffeine, aspartame, sodium benzoate and potassium sorbate in beverages were established as follows: an isocratic mobile phase composed of methanol and phosphoric acid solution (pH = 3.8) in a volume ratio of 20:80, flow rate of 1 mL/min, column temperature of 25 °C, UV detection at 195, 230, 260 and 275 nm, and an injection volume of 2.5 μL (Fig. 2 ). Under these conditions, a stable baseline with negligible noise was obtained, and the chromatographic peaks were sharp, symmetrical and sufficiently separated, enabling reliable qualitative identification and accurate quantitative determination of all analytes.

The chromatographic performance of the developed method was evaluated using retention factor ($k'$), separation factor ($\alpha$) and resolution ($R_s$), which represent the principal parameters describing separation efficiency. The obtained values for the dead time ($t_0$), retention times ($t_r$), retention factors ($k'$), separation factors ($\alpha$) and resolution ($R_s$) are presented in Table 2.

Table 2. Retention times ($t_R$), retention factors ($k$'), separation factors ($\alpha$) and resolution ($Rs$) of the investigated analytes

| **Analyte** | $t_r$ / **min** | ***k'*** | ***α*** | ***Rs*** |
|---|---|---|---|---|
| Acesulfame K | 0.276 | 1.00 | 1.36 | 1.73 |
| Sodium saccharin | 0.326 | 1.36 | 6.15 | 23.35 |
| Caffeine | 1.294 | 8.38 | 2.00 | 14.64 |
| Aspartame | 2.448 | 16.74 | 1.38 | 8.05 |
| Sodium benzoate | 3.319 | 23.05 | 1.16 | 4.36 |
| Potassium sorbate | 3.828 | 26.74 | - | - |
| Dead time | 0.138 | - | - | - |

As can be seen from the Table 2, the values for the retention factors ranged from 1 to 26.74. According to chromatographic theory, $k'$ values between approximately 1 and 20 are generally considered optimal for liquid chromatographic separations, ensuring sufficient interaction of analytes with the stationary phase without excessive band broadening or unnecessarily long analysis times [49]. Although the two last-eluting compounds exhibited a slightly higher k′ value (> 20), the total chromatographic run time was approximately 4.5 min, indicating that the retention did not negatively affect the practical efficiency of the method.

The selectivity of the separation was confirmed by separation factors in the range of 1.1 – 6.15. Values of $\alpha \geq 1.1$ are considered sufficient for effective separation between adjacent peaks [49], indicating adequate differences in analyte–stationary phase interactions.

The quality of separation was further evaluated through resolution values ranging from 1.73 to 23.35. A resolution value of 1.5 corresponds to baseline separation, while higher values indicate complete peak independence [49]. Since all peak pairs showed $R_s$ values above 1.5, baseline separation was achieved for all investigated analytes, ensuring reliable identification and accurate quantification without mutual interference.

Overall, the combination of acceptable retention behavior, sufficient selectivity and high resolution confirms that the developed method provides efficient chromatographic separation within a short analysis time. The results demonstrate that the selected chromatographic conditions allow simultaneous determination of artificial sweeteners, preservatives and caffeine in beverage samples with both high separation efficiency and high sample throughput.

In comparison with previously reported RP-HPLC methods for similar analytes, which commonly require 9 – 20 min per chromatographic run [37, 39, 55], the developed method provides significantly shorter analysis time (≈ 4.5 min) while maintaining baseline separation. Such reduction in run time considerably increases sample throughput and decreases solvent consumption without compromising chromatographic performance, which represents an important advantage in routine quality control analysis.

After achieving satisfactory separation of the analytes on this column, we aimed to evaluate whether the optimized chromatographic conditions could also be applied to another column with the same stationary phase but a different internal diameter. Therefore, the method was tested on a Poroshell 120 EC–C18 column (50 × 4.6 mm, 2.7 μm).

From the chromatogram obtained (Fig. 3), it can be observed that the method provided efficient separation of all analytes using the alternative column. The analytes were completely separated, exhibiting symmetrical peaks with appropriate peak shape. However, longer retention times were observed, namely 0.689 min for acesulfame K, 0.931 min for saccharin sodium, 2.820 min for caffeine, 5.465 min for aspartame, 7.863 min for sodium benzoate, and 8.793 min for potassium sorbate. Consequently, the total chromatographic run time was approximately 9.5 minutes, which is more than twice as long as that obtained with the column of smaller internal diameter. This also implies higher consumption of organic solvent. For these reasons, method validation was performed using the column with the smaller internal diameter.

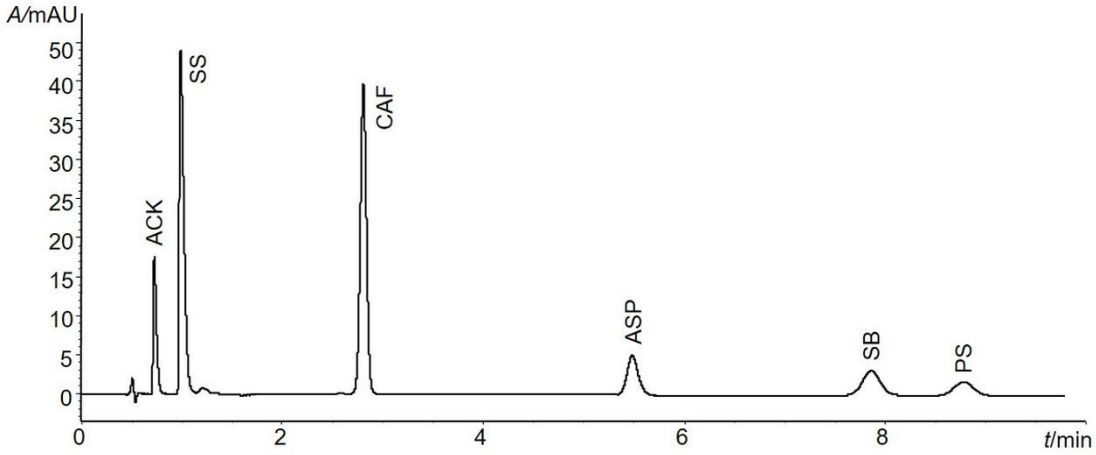



**Fig. 3** Chromatogram obtained from standard mixture of acesulfame K (ACK), sodium saccharin (SS), caffeine (CAF), aspartame (ASP), sodium benzoate (SB), and potassium sorbate (PS) on a Poroshell 120 EC–C18 column (50 × 4.6 mm, 2.7 µm), with methanol/phosphoric acid solution (pH = 3.8), (20/80, *V*/*V*) as mobile phase, at 195 nm

### 3.2. Method validation

The developed RP-HPLC method was validated in accordance with internationally accepted guidelines for analytical method validation [43, 44] in order to confirm its suitability for the simultaneous determination of artificial sweeteners, preservatives, and caffeine in beverage samples. The validation was performed with respect to specificity, selectivity, linearity, limits of detection and quantification, precision, accuracy, and robustness.

**Specificity and selectivity.** The specificity and selectivity of the developed RP-HPLC method were evaluated to ensure reliable identification and quantification of the target analytes in the presence of other components commonly found in beverage matrices. Specificity was assessed by comparing chromatograms obtained from standard solutions and spiked beverage samples, since a true blank matrix was not available. The absence of interfering peaks at the retention times of the investigated analytes was verified using the standard addition approach.

Selectivity of the method was further confirmed by evaluation of peak purity and spectral matching. The identity of each analyte was verified by overlapping the UV-Vis spectrum of the pure analytical standard with the corresponding absorption spectrum obtained from the tested beverage sample. The match factor values obtained from spectral overlap were 999.928 for acesulfame K; 999.936 for sodium saccharin; 999.976 for caffeine; 999.989 for aspartame; 999.916 for sodium benzoate and 999.976 for potassium sorbate, indicating excellent spectral agreement (Fig. 1).

These results demonstrate that the components of the sample matrix do not interfere with the target analytes, confirming the high specificity and selectivity of the proposed method for the simultaneous determination of acesulfame K, sodium saccharin, caffeine, aspartame, sodium benzoate, and potassium sorbate in beverage samples.

**Linearity**. The linearity of the developed RP-HPLC method was evaluated by analyzing a series of 6 standard mixtures containing the investigated analytes at different concentration levels. Calibration curves were constructed by plotting peak area or peak height against the corresponding mass concentration for each analyte. The method exhibited good linearity within the tested concentration range given in the Table 3. A linear regression model was applied for all analytes, and the resulting calibration curves demonstrated excellent linear relationships. The linearity, limits of detection (LOD), and limits of quantification (LOQ) of the developed RP-HPLC method are summarized in Table 3.

As can be seen from Table 3, for all analytes, coefficients of determination ($R^2$) higher than 0.9990 (when the peak area was taken as the dependent variable) and higher than 0.9936 (when the peak height was taken as the dependent variable) were obtained, indicating a strong correlation between the detector response and analyte concentration within the tested range and confirming the suitability of the method for quantitative analysis of artificial sweeteners, preservatives, and caffeine in beverage samples. Compared to the data published in the literature, the linearity of the method described in this study is better than the presented RP-HPLC methods for the determination of artificial sweeteners [41], preservatives and caffeine [42], as well as for the determination of the investigated additives [55] in beverages, which obtained values for the coefficients of determination higher than 0.99 in relation to the peak area. In the studies of Székelyhidi et al. [39] and Imanulkhan et al. [37] the obtained values for $R^2$ in relation to the peak area are higher than 0.9995 and 0.9991, respectively, and are comparable to those obtained in this study. Although calibration curves based on both peak height and peak area showed

acceptable linearity, peak area provided superior linear correlation ($R^2 \geq 0.9990$). Consequently, peak area was selected as the preferred response for quantification of all analytes in subsequent analyses.

As can be seen from the Table 3, the developed RP-HPLC method provided low LOD and LOQ values, enabling reliable detection and quantification of the investigated additives at low concentrations in beverage samples. The obtained results demonstrated adequate sensitivity for the simultaneous determination of artificial sweeteners, preservatives and caffeine in non-alcoholic beverages. Furthermore, the achieved sensitivity was better than that reported in several previously published HPLC methods for similar analytes [37, 39, 48].

Table 3. Linearity, limits of detection (LOD) and limits of quantification (LOQ) of the developed method

| Analyte | Linearity range (mg/L) | Regression equation | $R^2$ | LOD (mg/L) | LOQ (mg/L) |
|---|---|---|---|---|---|
| Acesulfame K | 34.91 – 628.38 | y* = 8.9495x - 26.488<br>y** = 5.3158x - 125.42 | 0.9990<br>0.9965 | 0.035 | 0.115 |
| Sodium saccharin | 13.70 – 246.69 | y* = 29.387x - 269.4<br>y** = 10.911x - 80.367 | 0.9992<br>0.9943 | 0.008 | 0.027 |
| Caffeine | 25.62 – 461.25 | y* = 9.7968x - 225.92<br>y** = 2.9314x - 34.292 | 0.9995<br>0.9992 | 0.016 | 0.052 |
| Aspartame | 60.66 – 1091.79 | y* = 13.094x - 61.345<br>y** = 1.5113x + 62.847 | 0.9990<br>0.9945 | 0.061 | 0.201 |
| Sodium benzoate | 16.21 – 291.87 | y* = 1.7546x + 288.96<br>y** = 0.2503x + 43.645 | 0.9993<br>0.9939 | 0.09 | 0.297 |
| Potassium sorbate | 30.53 – 549.54 | y* = 16.525x + 367.77<br>y** = 1.4275x + 136.25 | 0.9995<br>0.9936 | 0.043 | 0.143 |

y* – peak area; y** – peak height; x – concentration (mg/L) of the analyte

**Precision.** As part of method validation, intra-day and inter-day precision were evaluated. Intra-day precision was assessed by performing ten consecutive injections (2.5 µL) of a standard mixture containing all investigated analytes at a concentration of 349.1, 137.05, 256.25, 606.55, 162.15 and 305.3 mg/L for acesulfame K, sodium saccharin, caffeine, aspartame, sodium benzoate, and potassium sorbate, respectively, within a single day. For each analyte, the mean value ($\bar{x}$), standard deviation (SD), and relative standard deviation (RSD, %) were calculated based on retention time ($t_R$), peak area (A), and peak height (H). Inter-day precision was determined by repeating the same analytical procedure on three consecutive days. The obtained RSD values for all evaluated parameters over the three-day period are summarized in Table 4. As can be seen from Table 4, for intra-day precision the values obtained were in the range of 0.02 – 1.58 % for retention time, 0.07 – 2.14 % for peak area and 0.10 – 2.53 % for peak height, while the RSD values for inter-day precision ranged from 0.28 – 1.84 % for retention time, 0.88 – 2.05 % for peak area and 1.54 – 2.94 % for peak height. It can be concluded that the lowest precision (the highest RSD values) was obtained for peak height, both for intra-day and inter-day precision, but despite this these values are less than 3 %. The obtained RSD values demonstrating good precision of the developed RP-HPLC method in compliance with ICH [43] and AOAC [44] acceptance criteria, especially for retention time and peak area, which values range up to 2.14 %. Literature data show that the results obtained from this study are better than those obtained by Dossi [55], which for peak area are 1.87 – 4.34 %, and are comparable and slightly better than Imanukken [37], which for peak area range up to 2.53 %.

### 3.2. Accuracy

The accuracy of the developed RP-HPLC method was evaluated by recovery studies using the standard addition method. Known amounts of each analytical standard were added to an energy drink sample and the resulting solutions were analyzed under the optimized chromatographic conditions. The selected energy drink was used as a blank matrix for spiking experiments, although it contained all investigated analytes.

Three concentration levels were tested for each analyte (corresponding to 400, 500, and 600 µL of the respective stock solutions, Table 5), and five replicate solutions were prepared at each level. Accuracy was expressed as percentage recovery (R, %), calculated by comparing the measured concentration with the actual concentration. The mean recovery values (R, %) and relative standard deviations (RSD, %) are summarized in Table 5.

As can be seen from Table 5, the obtained recoveries for all investigated analytes ranged from 91.11 to 108.99 %, and RSD values associated with recovery measurements were ranged from 0.18 to 2.39 %. The recovery and RSD values obtained for all analytes were within the acceptable limits according to ICH [43] and AOAC [44] guidelines, which range from 80 % to 120 %, demonstrating the good accuracy of the proposed method. The RSD values associated with recovery measurements were low (≤ 2.39 %), indicating good repeatability of the recovery

experiments. These results confirm that the developed RP-HPLC method provides accurate quantification of artificial sweeteners, preservatives, and caffeine in complex beverage matrices.

Table 4. Intra-day and inter-day precision of the developed RP-HPLC method

| | | **Intra-day precision ($n$ = 10)** | | | | | | **Inter-day precision ($n$ = 3 days)** | |
|---|---|---|---|---|---|---|---|---|---|
| | | **I day** | | **II day** | | **III day** | | | |
| **Analyte** | | **$\bar{x}$ ± SD** | **RSD (%)** | **$\bar{x}$ ± SD** | **RSD (%)** | **$\bar{x}$ ± SD** | **RSD (%)** | **$\bar{x}$ ± SD** | **RSD (%)** |
| Acesulfame K | $t_r$ (min) | 0.276 ± 0.0003 | 0.11 | 0.276 ± 0.0011 | 0.42 | 0.276 ± 0.0006 | 0.23 | 0.276 ± 0.0008 | 0.28 |
| | $A$ (mAU*s) | 3187.89 ± 11.18 | 0.35 | 3104.92 ± 6.03 | 0.19 | 3072.08 ± 30.26 | 0.98 | 3121.63 ± 52.83 | 1.69 |
| | $H$ (mAU) | 1709.34 ± 6.72 | 0.39 | 1763.48 ± 19.50 | 1.10 | 1737.03 ± 38.41 | 2.21 | 1736.62 ± 33.10 | 1.90 |
| Sodium saccharin | $t_r$ (min) | 0.325 ± 0.0012 | 0.37 | 0.328 ± 0.0024 | 0.73 | 0.326 ± 0.0003 | 0.10 | 0.326 ± 0.0021 | 0.63 |
| | $A$ (mAU*s) | 3813.99 ± 67.90 | 1.78 | 3755.79 ± 37.82 | 1.01 | 3682.50 ± 55.73 | 1.51 | 3750.76 ± 76.37 | 2.03 |
| | $H$ (mAU) | 1568.28 ± 28.96 | 1.85 | 1589.64 ± 33.53 | 2.11 | 1572.49 ± 39.83 | 2.53 | 1576.80 ± 34.49 | 2.19 |
| Caffeine | $t_r$ (min) | 1.297 ± 0.0034 | 0.26 | 1.285 ± 0.0028 | 0.22 | 1.299 ± 0.0010 | 0.07 | 1.294 ± 0.0068 | 0.53 |
| | $A$ (mAU*s) | 2261.35 ± 1.62 | 0.07 | 2270.05 ± 4.16 | 0.18 | 2254.64 ± 33.70 | 1.49 | 2262.01 ± 20.10 | 0.88 |
| | $H$ (mAU) | 706.53 ± 1.05 | 0.15 | 706.81 ± 12.95 | 1.83 | 715.38 ± 12.56 | 1.76 | 709.57 ±10.90 | 1.54 |
| Aspartame | $t_r$ (min) | 2.464 ± 0.0094 | 0.38 | 2.424 ± 0.028 | 1.14 | 2.456 ± 0.0076 | 0.31 | 2.448 ± 0.0242 | 0.99 |
| | $A$ (mAU*s) | 8093.47 ± 83.31 | 1.03 | 8042.64 ± 121.09 | 1.50 | 7994.07 ± 94.43 | 1.31 | 8043.39 ± 405.71 | 1.31 |
| | $H$ (mAU) | 1033.33 ± 24.52 | 2.37 | 989.66 ± 23.91 | 2.42 | 1018.26 ± 24.56 | 2.41 | 1013.75 ± 29.84 | 2.94 |
| Sodium benzoate | $t_r$ (min) | 3.237 ± 0.0028 | 0.08 | 3.353 ± 0.029 | 0.86 | 3.365 ± 0.0008 | 0.02 | 3.319 ± 0.061 | 1.84 |
| | $A$ (mAU*s) | 587.58 ± 11.54 | 1.96 | 585.12 ± 11.37 | 1.94 | 593.79 ± 12.70 | 2.14 | 588.83 ± 12.05 | 2.05 |
| | $H$ (mAU) | 81.58 ± 0.77 | 0.95 | 81.00 ± 1.95 | 2.40 | 78.42 ± 1.48 | 1.89 | 80.33 ± 2.00 | 2.48 |
| Potassium sorbate | $t_r$ (min) | 3.825 ± 0.0054 | 0.14 | 3.770 ± 0.060 | 1.58 | 3.890 ± 0.0010 | 0.02 | 3.828 ± 0.060 | 1.57 |
| | $A$ (mAU*s) | 5812.06 ± 104.30 | 1.80 | 5845.84 ± 115.22 | 1.97 | 5822.20 ± 67.13 | 1.15 | 5826.70 ± 95.40 | 1.64 |
| | $H$ (mAU) | 723.95 ± 15.86 | 2.19 | 701.81 ± 16.26 | 2.32 | 712.34 ± 16.11 | 2.26 | 712.70 ± 18.03 | 2.53 |

Table 5. Accuracy (recovery) of the developed RP-HPLC method ($n$ = 5)

| Analyte | Concentration of analytes in blank sample (mg/L) | Pure analyte added (mg/L) | Total analyte found (mg/L) ± SD | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| Acesulfame K | 182.47 | 279.28 | 486.85 ± 0.64 | 108.99 | 0.21 |
| | | 349.1 | 527.78 ± 0.78 | 98.91 | 0.23 |
| | | 418.92 | 567.04 ± 0.69 | 91.80 | 0.18 |
| Saccharin sodium | 77.78 | 109.64 | 183.06 ± 1.90 | 96.03 | 1.81 |
| | | 137.05 | 203.51 ± 3.01 | 91.74 | 2.39 |
| | | 164.46 | 227.62 ± 1.85 | 91.11 | 1.24 |
| Caffeine | 290.73 | 205.00 | 492.10 ± 4.29 | 98.23 | 2.13 |
| | | 256.25 | 540.47 ± 2.56 | 97.46 | 1.02 |
| | | 307.5 | 594.93 ± 7.18 | 98.92 | 2.36 |
| Aspartame | 104.96 | 485.24 | 547.17 ± 3.08 | 91.13 | 0.70 |
| | | 606.55 | 662.50 ± 6.44 | 91.92 | 1.15 |
| | | 727.86 | 781.59 ± 8.20 | 92.96 | 1.21 |
| Sodium benzoate | 168.00 | 129.72 | 292.18 ± 1.68 | 95.74 | 1.35 |
| | | 162.15 | 323.02 ± 2.39 | 95.61 | 1.54 |
| | | 194.58 | 357.08 ± 3.52 | 97.18 | 1.86 |
| Potassium sorbate | 199.80 | 244.24 | 447.92 ± 3.46 | 101.59 | 1.40 |
| | | 305.3 | 491.91 ± 4.14 | 95.68 | 1.42 |
| | | 366.36 | 543.83 ± 5.00 | 93.90 | 1.45 |

According to the literature data, Székelyhidi et al. [39] in their work on the determination of the tested additives and caffeine in sugar-free beverages obtained recoveries in the range of 95.5 – 113 % and RSD < 2.49 %. Imanulkhan et al. [37] obtained recoveries in the range of 95.30 – 101.41 % for the determination of the tested additives and caffeine in powdered drinks. Furthermore, by applying the RP-HPLC method developed by Jankulovska et al. [41], for the determination of artificial sweeteners in soft drinks they obtained values of 84.48 – 99.83 %, and when applying another RP-HPLC method for preservatives and caffeine [42] they obtained values in the range of 95.99 – 101.5 %. Comparing the results obtained using the presented developed method and those in the literature, comparable or slightly better results were obtained.

**Robustness.** The robustness of the developed RP-HPLC method was evaluated by examining the influence of small, deliberate variations in chromatographic conditions on the analytical performance of the method. Robustness testing was performed in order to assess the reliability of the method under normal laboratory conditions and its suitability for routine analysis.

The robustness of the method was investigated by applying the optimized chromatographic conditions on two Poroshell 120 EC-C18 columns with identical stationary phase characteristics but different internal diameters (3.0 mm and 4.6 mm). The effect of column internal diameter on retention time, peak shape, and detector response was evaluated. Despite differences in retention times, satisfactory separation and acceptable peak symmetry were achieved for all investigated analytes on both columns.

The results obtained show that the developed RP-HPLC method was robust, as small variations in the chromatographic conditions was not significantly affect the separation and quantification of the analytes. This confirms the reliability and applicability of the method for the routine determination of artificial sweeteners, preservatives and caffeine in non-alcoholic drinks.

## 4. Application of the validated RP–HPLC method in beverages

The applicability of the developed RP-HPLC method was evaluated by analyzing a variety of commercially available non-alcoholic beverages obtained from the local market. The selected samples represented different beverage categories, including carbonated and non-carbonated soft drinks, fruit juices, iced teas, energy drinks, and sports drinks, in order to demonstrate the versatility of the method for different beverage matrices.

All samples were prepared according to the described sample preparation procedures and analyzed under the optimized chromatographic conditions. The investigated analytes (acesulfame K, sodium saccharin, aspartame, caffeine, sodium benzoate, and potassium sorbate) were successfully identified and quantified in the analyzed samples based on their retention times and UV spectra, in comparison with the corresponding analytical standards. The obtained values for the match factor of the overlapped spectra of the examined components in the analyzed samples and the standard mixture were above 999, which indicates a high similarity between the spectra and confirms that the obtained chromatographic peaks certainly originate from the components of interest.

The obtained results for the determined analytes in the analyzed beverage samples are presented in Table 6, and the chromatogram of the analytes detected in sample 15 is presented in Figure 4.

The results of the analysis obtained were compared with the maximum permitted concentrations according to the Regulation (EC) No. 1333/2008 [26] and with the Regulation (EU) 1169/2011 on the provision of food information to consumers [32]. Since the adverse effects of the additives tested on human health are known, it is very important to confirm whether their quantity in beverages is in accordance with the values specified in the Regulation [26]. Therefore, the existence of a simple and rapid, and at the same time accurate and precise analytical method for the identification and quantitative determination of the additives tested is of particular importance. The research showed that all of the selected beverages listed the additives present by name or E-number, but their content was not stated. Only for caffeine, in the example with "high caffeine content", the content was stated, while in the cola drinks its content was not stated. In the sample of iced tea, where caffeine is naturally found, it was not stated there. Also, in accordance with Regulation (EU) 1169/2011 [32], aspartame was correctly indicated on the label of the beverage ("contains a source of phenylalanine").

The most detected sweeteners in selected samples were the blend of acesulfame K and aspartame, which were determined in four samples, mainly in sports and energy drink samples (S10, S13, S15, S16), and in two of them, declared as zero sugar energy drinks (S15 and S16), they are, also, in combination with sodium saccharine. The determined concentrations were range of 39.05 - 178.72 mg/L for ACK, 30.22 – 101.74 mg/L for ASP, and 74.28 mg/L (S15) and 79.63 mg/L (S16) for SS. Imanulkan et al. [37] also found that ACK and ASP were the most commonly used sweeteners, but at much higher concentrations. Their obtained values were in the wider concentration range of 70.68 – 491.17 mg/L for ACK and 156.75 – 495.24 mg/L for ASP, while saccharin was found in only one sample, at a concentration of 117,97 mg/L. Székelyhidi et al. [39], also obtained a wider concentration range of ACK (16 – 238 mg/L), aspartame (42.2 – 490 mg/L) and SS (12.3 – 86.0) in sugar-free beverages. Jankulovska et al. [41] detected a range of acesulfame K concentrations from 30.32 to 148.37 mg/L, which values are similar to this study, and also the wider range of concentrations for ASP (6.06 – 512.72 mg/L) and SS (16.10 – 93.05 mg/L) in beverages.

Table 6. Concentration (mg/L) of the analytes in the analyzed non-alcoholic beverage samples

| Samples | Declared additives | Acesulfame K (mg/L) | Sodium saccharin (mg/L) | Caffeine (mg/L) | Aspartame (mg/L) | Sodium benzoate (mg/L) | Potassium sorbate (mg/L) |
|---|---|---|---|---|---|---|---|
| S1 (refreshing carbonated cola drink) | Caffeine | ND* | ND | 94.97 | ND | ND | ND |
| S2 (refreshing carbonated cola drink) | Caffeine | ND | ND | 92.96 | ND | ND | ND |
| S3 (refreshing carbonated pear flavor drink) | E211, E202 | ND | ND | ND | ND | 44.63 | 33.08 |
| S4 (refreshing carbonated orange juice) | Potassium sorbate | ND | ND | ND | ND | ND | 207.62 |
| S5 (refreshing carbonated lemon juice) | Potassium sorbate | ND | ND | ND | ND | ND | 213.87 |
| S6 (refreshing carbonated tonic drink) | / | ND | ND | ND | ND | ND | ND |
| S7 (iced peach tea with black tea extract) | / | ND | ND | 37.37 | ND | ND | ND |
| S8 (peach and apple fruit nectar) | / | ND | ND | ND | ND | ND | ND |
| S9 (100 % orange juice) | / | ND | ND | ND | ND | ND | ND |
| S10 (refreshing non-carbonated, low-energy isotonic sports drink with lemon, grapefruit and orange flavor) | Acesulfame K, Aspartame | 46.11 | ND | ND | 30.22 | ND | 216.55 |
| S11 (refreshing non-carbonated isotonic sports drink with grapefruit and lemon flavor) | Sodium benzoate, Potassium sorbate | ND | ND | ND | ND | 123.12 | 71.60 |
| S12 (natural mineral sparkling water) | / | ND | ND | ND | ND | ND | ND |
| S13 (energy drink with coffee, taurine and vitamins, contains a source of phenylalanine, carbonated) | Acesulfame K, Caffeine (max 40 mg/100 mL), Aspartame | 139.23 | ND | 369.17 | 64.05 | ND | ND |
| S14 (energy drink with high caffeine content, carbonated) | Caffeine (max 32 mg/100 mL), Sodium benzoate, Potassium sorbate | ND | ND | 252.89 | ND | 124.97 | 180.11 |
| S15 (energy drink with bubble gum flavor, high caffeine content, contains a source of phenylalanine, zero sugar) | E950, E954, E211, E202, Caffeine (max 32 mg/100 mL), Aspartame | 178.72 | 74.28 | 283.47 | 88.03 | 97.55 | 120.63 |
| S16 (refreshing energy drink with sweeteners, high caffeine content, contains a source of phenylalanine, zero sugar) | Acesulfame K, Sodium saccharin, Caffeine (max 32 mg/100 mL), Sodium benzoate, Aspartame | 39.05 | 79.63 | 279.83 | 101.74 | 121.38 | ND |
| ND* – not detected | | | | | | | |

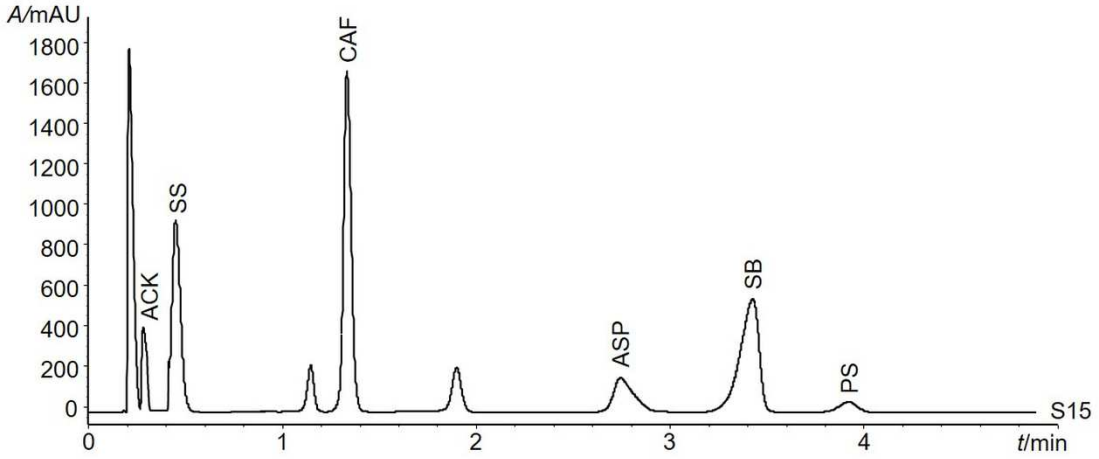



**Fig. 4** Chromatogram of sample 15 under optimized chromatographic conditions at 195 nm

Lino et al. [56] detected acesulfame K with an average concentration of 72, 48 and 128 mg/L in soft drinks, mineral water-based soft drinks, and nectar samples, respectively, while the content of ASP was ranged of 73 – 89 mg/L in soft drink samples. The obtained results for SS were similar to those obtained by Dossi et al. [55] in diet orange-flavored juice sample at a concentration of 79.1 mg/L.

Caffeine was detected in cola drinks (S1, S2) with similar values and average concentration of 93.96 mg/L, and also, in all analyzed energy drinks (S13 - S16) which contained significantly higher concentrations (252.89 mg/L – 369.17 mg/L). The caffeine concentrations were in accordance to declared data, which is in line with the requirements for labeling of products with high caffeine content according to Regulation (EU) No 1169/2011 [32]. Also, although caffeine is not declared, caffeine content was detected and determined in a sample of iced tea containing black tea extract and it was found to be 37.37 mg/L. Its presence is due to the fact that caffeine is naturally present in black tea [57]. Given that this amount of caffeine is low, its inclusion in the declaration is not mandatory. Aşçı et al. [58] determined a caffeine concentration of 142.20 mg/L in an energy drink, and on the other hand, Mazdeh et al. [59] obtained significantly higher caffeine content in sports drinks ranged 293.40 – 607.32 mg/L, while no caffeine was detected in sport drink samples in this study. The values for caffeine in energy drinks detected by Jankulovska et al. [42] (269.8 – 323.1 mg/L) are comparable to obtained results in this study.

The most common additives in soft drinks are preservatives, which were detected in 50 % of soft drinks, including drinks with not added additives. PS was found in 7 and SB in 5 samples, of which in 4 drinks these preservatives were present together. The determined concentrations ranged from 44.63 to 124.97 mg/L for sodium benzoate and from 33.08 mg/L to 216.55 mg/L for potassium sorbate, which is in accordance with the MPL [26]. However, despite not exceeding the MPL value for PS in one sport drink sample (S10) the concentration of PS was determined at 216.5 mg/L, but the presence of PS was not indicated on the declaration, which indicates an inadequate labelling or insufficient transparency in the declaration process, which is a violation of the standard of consumer information [32]. For comparison, Székelyhidi et al. [39] determined concentrations of sodium benzoate in the range of 22.2 to 150 mg/L and 49.7 – 278 mg/L for potassium sorbate in sugar-free beverages. Additionally, Jankulovska et al. [42], obtained concentrations of 116.91 – 172.92 mg/L for sodium benzoate and 73.12 - 219.01 mg/L for potassium sorbate in sports drinks.

The obtained results revealed that the determined concentrations of all analytes were in accordance with the MPL [26], but in one sample (S10) non-compliance with the declared data on the label was observed as specified in Regulation (EU) 1169/2011 [32], where the presence of sorbate was not indicated.

The obtained results confirmed that the developed RP-HPLC method is suitable for the simultaneous determination of artificial sweeteners, preservatives, and caffeine in complex beverage matrices. The method demonstrated good applicability, selectivity, repeatability and accuracy providing reliable quantification of the investigated analytes in commercially available non-alcoholic beverages in about 4.5 min.

## 5. CONCLUSIONS

This study describes the development and validation of a rapid and reliable RP-HPLC method for the simultaneous determination of acesulfame K, sodium saccharin, caffeine, aspartame, sodium benzoate, and potassium sorbate in non-alcoholic beverages. Optimal chromatographic separation was achieved using a Poroshell 120 EC-C18 column (50 × 3.0 mm, 2.7 μm) and isocratic elution with a methanol/phosphoric acid solution (pH = 3.8) mobile phase (20:80, *V/V*). Under the optimized chromatographic conditions, all analytes were successfully separated within 4.5 min.

Method validation confirmed satisfactory selectivity, linearity, accuracy, precision, robustness, as well as low LOD and LOQ values, demonstrating that the proposed RP-HPLC method is suitable for rapid and simultaneous analysis of the selected additives and caffeine in beverages. The applicability of the method was verified through the analysis of 16 commercially available non-alcoholic beverages, including carbonated and non-carbonated drinks, fruit juices, iced teas, energy drinks, and sports drinks.

The obtained results indicate that the developed method is appropriate for routine quality control and monitoring of additives and caffeine in beverage samples. Future studies may evaluate the applicability of the method to more complex food matrices, which would require additional validation.

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**COMPETING INTERESTS**

The authors have no competing interests to declare that are relevant to the content of this article.

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**REFERENCE**

1. Asif S, Bule M, Khan F, Niaz K (2020) In: Nabavi SM, Nabavi SF, Loizzo MR, Tundis R, Devi KP, Silva AS (ed) Food Additives and Human Health, Bentham Science, Singapore. https://doi.org/10.2174/9789811446139120010004
2. Tenuta MC, Leporini M (2020) In: Nabavi SM, Nabavi SF, Loizzo MR, Tundis R, Devi KP, Silva AS (ed) Food Additives and Human Health, Bentham Science, Singapore. https://doi.org/10.2174/9789811446139120010005
3. Quansah JK, Saalia FK (2024) In: Lagouri VS (ed) Food Additives - From Chemistry to Safety, IntechOpen, London. https://doi.org/10.5772/intechopen.114573
4. Shahmohammadi M, Javadi M, Nassiri-Asl M (2016) An Overview on the Effects of Sodium Benzoate as a Preservative in Food Products. Biotech Health Sci. https://doi.org/10.17795/bhs-35084
5. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) (2016) Scientific Opinion on the re-evaluation of benzoic acid (E 210), sodium benzoate (E 211), potassium benzoate (E 212) and calcium benzoate (E 213) as food additives. EFSA J. https://doi.org/10.2903/j.efsa.2016.4433
6. Stopforth J, Kudron T (2020) In Davidson PM, Taylor TM, David JRD (ed) Antimicrobials in food, CRC Press, Boca Raton. https://doi.org/10.1201/9780429058196
7. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) (2015) Scientific Opinion on the re-evaluation of sorbic acid (E 200), potassium sorbate (E 202) and calcium sorbate (E 203) as food additives. EFSA J. https://doi.org/10.2903/j.efsa.2015.4144
8. Yazdanfar N, Manafi L, Ebrahiminejad B, Mazaheri Y, Sadighara P, Basaran B, Mohamadi S (2023) Evaluation of sodium benzoate and potassium sorbate preservative concentrations in different sauce samples in Urmia, Iran. J. Food Prot. https://doi.org/10.1016/j.jfp.2023.100118
9. Azuma SL, Quartey NK-A, Ofosu IW (2020) Sodium benzoate in non-alcoholic carbonated (soft) drinks: Exposure and health risks. Sci. Afr. https://doi.org/10.1016/j.sciaf.2020.e00611
10. Mirza N, Mubarak H, Haider S, Farrukh S, Khan QU, Yasin S, Husain A, Qayyum MA, Lodhi MI, Ismail QM, Shah MA, Noor T (2024) In Lagouri VS (ed) Food Additives - From Chemistry to Safety, IntechOpen, London. https://doi.org/10.5772/intechopen.114357
11. Shaikh S, Kalshetti MS, Patil RY (2018) HPLC method development for simultaneous estimation of sodium benzoate and potassium sorbate in food products. Asian j. appl. sci. technol. https://api.semanticscholar.org/CorpusID:201606293
12. Pongsavee M, Mishra R (2021) Potassium sorbate induces oxidative stress and genotoxicity in human lymphocytes. Indian J Med Forensic Med Toxicol. https://doi.org/10.37506/ijfmt.v15i2.14795

13. Zhang X, Zhang Q, Song X, Yang W, Cheng A, Zhang J, Dong W (2024) Toxicity evaluation of potassium sorbate in vivo with Drosophila Melanogaster. Insects. https://doi.org/10.3390/insects15090703
14. Xiao N, Ruan S, Mo Q, Zhao M, Liu T, Feng F (2024) Effects of potassium sorbate on systemic inflammation and gut microbiota in normal mice: A comparison of continuous intake and washout period. Food Chem Toxicol. https://doi.org/10.1016/j.fct.2024.114443
15. Krishnasamy K In: Himmerich H (ed) Weight management, IntechOpen, London. https://dx.doi.org/10.5772/intechopen.93199
16. Aiello F (2020) In Nabavi SM, Nabavi SF, Loizzo MR, Tundis R, Devi KP, Silva AS (ed) Food Additives and Human Health, Bentham Science, Singapore. https://doi.org/10.2174/9789811446139120010013
17. Bhattacharya S (2023) In Bhattacharya S (ed) Snack Foods, Academic Press, London. https://doi.org/10.1016/B978-0-12-819759-2.00003-3
18. Garba AI, Unar NB, Kelechi AJ (2024) In Lagouri VS (ed) Food Additives - From Chemistry to Safety, IntechOpen, London. https://www.intechopen.com/chapters/89364
19. Roth K, Lück E (2011) Die Saccharin-Saga: Ein Molekülschicksal. Chem. Unserer Zeit. https://doi.org/10.1002/ciuz.201100574
20. WHO (2023) Aspartame hazard and risk assessment results released. https://www.who.int/news/item/14-07-2023-aspartame-hazard-and-risk-assessment-results-released. Accessed 20 January 2026.
21. Miller C, Ettridge K, Wakefield M, Pettigrew S, Coveney J, Roder D (2020) Consumption of sugar-sweetened beverages, juice, artificially-sweetened soda and bottled water: An Australian population study. Nutrients. https://doi.org/10.3390/nu12030817
22. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) (2015b) Scientific Opinion on the safety of caffeine. EFSA J. https://doi.org/10.2903/j.efsa.2015.4102
23. Temple JL, Bernard C, Lipshultz SE, Czachor JD, Westphal JA, Mestre MA (2017) The safety of ingested caffeine: A comprehensive review. Front. Psychiatry. https://doi.org/10.3389/fpsyt.2017.00080
24. Kaur A, Yousuf H, Ramgobin-Marshall D, Jain R (2022) Energy drink consumption: A rising public health issue. Rev Cardiovasc Med. https://doi.org/10.31083/j.rcm2303083
25. Soós R, Gyebrovszki Á, Tóth Á, Jeges S, Wilhelm M (2021) Effects of caffeine and caffeinated beverages in children, adolescents and young adults: Short review. Int J Environ Res Public Health. https://doi.org/10.3390/ijerph182312389
26. European Commission (2008) Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December on food additives. OJEU. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32008R1333. Accessed 14 January 2026
27. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) (2013) Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive. EFSA J. https://doi.org/10.2903/j.efsa.2013.3496
28. EFSA Panel on Food Additives and Flavourings (FAF) (2024) Re-evaluation of saccharin and its sodium, potassium and calcium salts (E 954) as food additives. EFSA J. https://doi.org/10.2903/j.efsa.2024.9044
29. EFSA Panel on Food Additives and Flavourings (FAF) (2025) Re-evaluation of acesulfame K (E 950) as food additive. EFSA J. https://doi.org/10.2903/j.efsa.2025.9317
30. Codex Alimentarius Commission. General Standard for Food Additives (GSFA). FAO/WHO (2019) https://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/gsfa/en. Accessed 14 January 2026
31. U.S. Food and Drug Administration (FDA) (2023) Aspartame and other sweeteners in food. https://www.fda.gov/food/food-additives-petitions/aspartame-and-other-sweeteners-food. Accessed 14 January 2026
32. European Commission (2011) Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, OJEU. https://eurlex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32011R1169. Accessed 14 January 2026
33. Salwan S, Passi I, Kumar B (2022) Various Analytical Methods for Estimation of Potassium Sorbate in Food Products: A Review. Curr. Anal. Chem. https://doi.org/10.2174/1573411018666220820122912
34. Tallam AK, Sahithi A, Nuli M, (2023) A review on different kinds of analytical methods are performed for the analysis of food preservatives in food. J. Innov. Appl. Pharm. Sci. https://doi.org/10.37022/jiaps.v8i1.414
35. Al-Bratty M, Alhazmi HA, ur Rehman Z, Javed SA, Ahsan W, Najmi A, Khuwaja G, Makeen HA, Khalid A (2020) Determination of caffeine content in commercial energy beverages available in saudi arabian market by gas chromatography-mass spectrometric analysis. J. Spectrosc. https://doi.org/10.1155/2020/3716343
36. Park J, Lee MH, Lee JE, Cho YS, Choi JY (2026) Green and validated ultra-performance liquid chromatography-tandem mass spectrometry methods for multiclass food additive determination in beverages and candies. J. Food Compos. Anal. https://doi.org/10.1016/j.jfca.2026.109001
37. Imanulkhan I, Setyaningsih W, Rohman A, Palma M (2020) Development and validation of HPLC-DAD method for simultaneous determination of seven food additives and caffeine in powdered drinks. Foods. https://doi.org/10.3390/foods9081119

38. Sezgin B, Arli G, Can NÖ (2021) Simultaneous HPLC-DAD determination of seven intense sweeteners in foodstuffs and pharmaceuticals using a core-shell particle column. J. Food Compos. Anal. https://doi.org/10.1016/j.jfca.2020.103768
39. Székelyhidi R, Ajtony Z, Lakatos E, Hegyi O, Sik B (2023) Optimization and validation of HPLC–DAD method for simultaneous analysis of sweeteners, preservatives, and caffeine in sugar-free beverages. Eur. Food Res. Technol. https://doi.org/10.1007/s00217-023-04328-4
40. Petanovska-Ilievska B, Velkoska-Markovska L, Jankulovska MS (2017) Development of reverse-phase high-performance liquid chromatography method for simultaneous determination of sodium benzoate and potassium sorbate in beverages. Acta Chromatogr. https://doi.org/10.1556/1326.2017.29.3.05
41. Jankulovska MS, Josimovska T, Velkoska-Markovska L (2025) Development and validation of RP-HPLC method with UV-DAD detection for simultaneous determination of acesulfame K, sodium saccharin and aspartame in beverages. Acta Chromatogr. https://doi.org/10.1556/1326.2024.01239
42. Jankulovska MS, Stojchevska S, Velkoska-Markovska L (2025) Development and validation of an RP-HPLC method for the determination of caffeine and preservatives (sodium benzoate and potassium sorbate) in sports and energy drinks. J. Food Meas. Charact. https://doi.org/10.1007/s11694-025-03319-1
43. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (2005) ICH Harmonised Tripartite Guideline, Validation of analytical procedures: Text and methodology Q2(R1). https://database.ich.org/sites/default/files/Q2(R1)%20Guideline.pdf
44. AOAC Official Methods of Analysis (2016) Appendix F: Guidelines for standard method performance requirements, AOAC International: Rockville, MA, USA. https://www.aoac.org/wp-content/uploads/2019/08/app_f.pdf. Accessed 20 December 2025
45. Kirkland JJ, Schuster SA, Johnson WL, Boyes BE (2013) Fused-core particle technology in high-performance liquid chromatography: An overview. J Pharm Anal. https://doi.org/10.1016/j.jpha.2013.02.005
46. Gritti F, Guiochon G J (2012) Mass transfer kinetics, band broadening and column efficiency in core–shell particle columns. J. Chromatogr A. https://doi.org/10.1016/j.chroma.2011.04.058
47. Agilent Technologies (2009) Agilent Poroshell 120 EC-C18 Threaded Column.
48. Diviš P, Jurečková Z, Vespalcová M, Pořízka J, Punčochářová L (2020) Simultaneous determination of sweeteners and preservatives in beverages by HPLC-DAD-ELSD. Potravinarstvo. https://doi.org/10.5219/1339
49. Snyder LR, Kirkland J.J, Dolan JW (2010) Introduction to modern liquid chromatography (3rd ed.). John Wiley & Sons, Hoboken, New Jersey. https://doi.org/10.1002/9780470508183
50. Cichocki A (2024) Peak splitting in HPLC: Causes and solutions. Separation Science. https://www.sepscience.com/peak-splitting-in-hplc-causes-and-solutions-9379. Accessed 20 January 2026
51. Zhang Y, Lucy CA (2014) Effect of injection matrix concentration on peak shape and separation efficiency in ion chromatography. J. Chromatogr. A. http://dx.doi.org/10.1016/j.chroma.2014.10.056
52. Jankulovska MS, Velkoska-Markovska L, Petanovska-Ilievska B, Trpkovska S (2016) High-performance liquid chromatography method for determination of preservatives in beverages. JAFES. https://journals.ukim.mk/index.php/jafes/article/view/1081.
53. Chromacademy. The Theory of HPLC: Chromatographic Parameters. Crawford Scientific. https://www.chromacademy.com. Accessed 20 January, 2026
54. Dong MW, Boyes BE (2018) Modern Trends and Best Practices in Mobile-Phase Selection in Reversed-Phase Chromatography. LCGC North Am. https://www.chromatographyonline.com/authors/barry-e-boyes. Accessed 20 January, 2026
55. Dossi N, Toniolo R, Susmel S, Bontempelli G (2006) A simple and sensitive HPLC-UV method for the simultaneous determination of sweeteners in beverages. Chromatographia. https://doi.org/10.1365/s10337-006-0793-y
56. Lino CM, Costa I M, Pena A, Ferreira R, Cardoso SM (2008) Estimated intake of the sweeteners, acesulfame-K and aspartame, from soft drinks, soft drinks based on mineral waters and nectars for a group of Portuguese teenage students. Food Addit. Contam. Part A. https://doi.org/10.1080/02652030802195309
57. Attar A, Altikatoglu YM (2022) The analysis of methylxanthine fractions obtained from Camellia sinensis cultivated in Turkey and effects on the in vitro inhibition of CYP2D6 enzyme. Biotechnol Appl Biochem. https://doi.org/10.1002/bab.2325
58. Aşçı B, Dinç Zor, Ş, Aksu Dönmez Ö (2016) Development and validation of HPLC method for the simultaneous determination of five food additives and caffeine in soft drinks. Int. J. Anal. Chem. https://doi.org/10.1155/2016/2879406
59. Mazdeh FZ, Moradi Z, Moghaddam G, Moradi-Khatoonabadi Z, Aftabdari FE, Badaei P, Hajimahmoodi M (2016) Determination of synthetic food colors, caffeine, sodium benzoate and potassium sorbate in sports drinks. Trop. J. Pharm. Res.. https://doi.org/10.4314/tjpr.v15i1.25