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SIMULTANEOUS DETERMINATION OF SWEETENERS AND PRESERVATIVES IN BEVERAGES BY HPLC-DAD-ELSD

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ABSTRACT
People suffering from diabetes or being overweight must severely reduce their sugar use, often seeking food with sweeteners. Often, sugar is replaced by non-nutritious sweeteners in beverages, which also contain several other substances like vitamins, caffeine, amino acids, phenolic compounds and thus increasing the shelf life of the beverages is additionally treated with the addition of preservatives. As the concentration of additives in food (including beverages) is determined by the legislation in force, it is necessary to have an appropriate analytical method for food control. Since artificial sweeteners and preservatives are very different substances, they are determined separately using different HPLC methods. In this work HPLC method combining the advantages of specific (diode array detector, DAD) and universal (evaporative light scattering detector, ELSD) detector was validated and used for simultaneous determination of benzoic acid, sorbic acid, aspartame, acesulfame K, saccharin, sucralose and steviol glycosides in sugar-free beverages. The proposed analytical method showed good linearity, precision, and accuracy. Measured limits of detection (0.6 – 11.8 mg.dm^{-3} depending on the analyte) were sufficient to analyze 5-times diluted beverage samples. The validated method has been successfully used for the simultaneous analysis of artificial sweeteners and preservatives in beverage samples (energy drinks, ice teas, carbonated drinks). Except for steviol glycosides, the concentration of monitored substances in beverages did not exceed the maximum permitted concentrations given in the valid legislation.

Keywords: sweeteners; preservatives; beverage; chromatography; DAD ELSD detectors

INTRODUCTION
Carbohydrates are the most important and quickest source of energy, accounting for more than half the energy value of our food. In addition to the natural carbohydrate content, foods are further sweetened with sugars and various sweeteners that give the products a pleasant sweet taste. Sweeteners are divided into intensive (non-nutritious) and bulk (nutritional) sweeteners **(Basoli and Merlini, 2003)**. Intensive sweeteners include both synthetic and natural sweeteners. The most commonly used are saccharin, sucralose, acesulfame K, stevioside, and rebaudioside A. The most popular bulk sweeteners are erythritol, sorbitol, xylitol, maltitol, isomalt, lactitol, and mannitol **(Mortensen, 2006)**. An increase in the number of autoimmune diseases, an ageing population, and above all, an unhealthy lifestyle is increasing the proportion of people suffering from diabetes. People with diabetes cannot use their blood glucose. This leads to a rise in blood sugar (hyperglycaemia) and other serious consequences **(Bartnik, Norhammar and Rydén, 2007)**. In addition to diabetes, excessive intake of refined sugars also poses a problem in terms of obesity and tooth decay **(Kamal, O´Toole and Bernabé, 2019)**. The use of sugar substitutes and intense sweeteners makes it possible to produce sweet foods for people suffering from diabetes while reducing the caloric value of the food at the same time it reduces the risk of obesity. The use of sweeteners in food products is governed by applicable national legislation. The list of permitted sweeteners in the Czech Republic is given in the Decree No. 122/2011.

Preservatives ensure the quality and safety of the product and prevent the adverse reactions that are responsible for food spoilage. At the same time, they inhibit the growth of undesirable microorganisms (bacteria, fungi, yeasts) and thereby prolong the shelf life of food during distribution and storage. Preservatives can be divided into natural, synthetic, and antibiotic **(Silva and Lidon, 2016)**. Preservatives used in the food industry must meet certain criteria. Preservatives and their metabolites must not be toxic or harmful. They should be readily soluble in water and have sufficient stability, even at higher temperatures. It must have antimicrobial properties within the pH range of a particular foodstuff. Preservatives should not affect the sensory properties of products and react with other food ingredients. The most common synthetic preservatives used in the food industry are benzoic acid, sorbic acid, or salts thereof.

Since the concentration of sweeteners and preservatives used in food production is limited by the laws in force it is necessary to monitor these substances in food and to have

the necessary analytical methods for this purpose. The most commonly used method for analysis of sweeteners and preservatives is high-performance liquid chromatography with UV or DAD detector **(Sik, 2012; Ha et al., 2013; Javanmardi et al., 2015; de Queiroz Pane et al., 2015)**. Since some substances absorb a small amount of radiation in the 200 – 700 nm range, universal detectors such as MS **(Yang and Chen, 2009; Di Donna et al., 2017)** or ELSD **(Wasik, McCourt and Buchgraber, 2007)** are also used. Due to the different nature of the substances, sweeteners and preservatives are usually determined using different HPLC methods separately. In this work HPLC method combining the advantages of specific and universal detectors was validated and used for simultaneous determination of benzoic acid, sorbic acid, aspartame, acesulfame K, saccharin, sucralose, and steviol glycosides in beverages.

Scientific hypothesis

By combining two detectors (DAD and ELSD) and by using HPLC it is possible to determine selected sweeteners together with preservatives in beverages using one method and one injection.

The concentration of sweeteners and preservatives used in sugar-free drinks complies with the limits set out in the applicable legislation.

MATERIAL AND METHODOLOGY

The individual standards of sweeteners and preservatives as well as formic acid, ammonium acetate, and triethylamine were purchased from Sigma-Aldrich (Germany). The purity of all standards and chemicals except stevioside and rebaudioside A was at least 99 %. The purity of stevioside was >95 % and purity of rebaudioside A was >96 %. Methanol, acetonitrile, and acetone (HPLC grade) were purchased from VWR (France). Ultrapure water with resistivity > 18 MΩcm was obtained from ELGA Purelab Classic UV (Veolia, France).

An Agilent 1260 liquid chromatograph with Poroshell 120 EC-C18 (4.6 x 150 mm, 2.7 µm) column, equipped with 1260 Infinity diode array detector (DAD) and 1260 Infinity evaporative light scattering detector (ELSD) was used in this study. The injection volume was 5 µL. The flow of the mobile phase was 0.5 $mL.min^{-1}$. The temperature in the column thermostat was 30 °C. The signal from the DAD detector was monitored at 210 nm. For ELSD detector nitrogen flow of 2 $dm^{-3}.min^{-1}$, nebulization temperature of 90 °C and evaporating temperature of 95 °C has been set.

Samples of sugar-free beverages were purchased from the supermarket located in Brno, Czech Republic. Four samples of energy drinks (S1 – S4), 2 samples of carbonated drinks (S5 – S6), and 2 samples of iced teas (S7 – S8) were purchased.

Statistical analysis

For each sample data analysis and statistical evaluation were performed in Microsoft Excel (Microsoft, USA) and XL-stat (Addinsoft, France, version 2014.5.03). Before the main data analysis, results were tested for outliners using the Grubbs test at significance level α=0.05).

RESULTS AND DISCUSSION

First, a suitable column was selected for the HPLC method to be tested. Non-polar C18 columns are most commonly used in the literature for the type of analysis required **(Grembecka et al., 2014; de Queiroz Pane et al., 2015; Sik, 2012)**. Thus, the end-capped Poroshell 120 EC-C18 column, which is packed with solid-core surface-porous microparticles and a porous silica gel outer layer, to which a non-polar dimethyl-n-octadecyl silane monolayer is bound, was chosen for the method being investigated.

The next step was to select the appropriate mobile phase. In the literature, mobile phases containing phosphate buffers are often mentioned for the analysis of sweeteners and preservatives **(Dossi et al., 2006; de Queiroz Pane et al., 2015; Zygler, Wasik and Namieśnik, 2009)**, however, the phosphate buffer is not compatible with the ELSD detector, and therefore a mobile phase with a different composition had to be chosen. Thus, the use of a mobile phase containing methanol, acetonitrile and 0.01 $mol.dm^{-3}$ ammonium acetate (mobile phase 1) and a mobile phase containing methanol (A), acetone (B) and a mixture of 0.02 $mol.dm^{-3}$ formic acid and 0.02 $mol.dm^{-3}$ triethylamine (C) (mobile phase 2) was investigated. Using Mobile Phase 1, separation of all analytes was not possible even by gradient adjustment. By using mobile phase 2, on the contrary, by optimizing the gradient, optimal separation of all analytes (except stevioside and rebaudioside A) was achieved. Stevioside and rebaudioside were mixed to one standard and quantified together as steviol glycosides (Figure 1 and Figure 2). The final gradient setting was: time 0 – 4 min 85% v/v C, 10% v/v A, 5% v/v B; time 4 – 10min 70% v/v C, 25% v/v A, 5% v/v B; time 10 – 15 min 60% v/v C, 35% v/v A, 5% v/v B; time 15 – 30 min 35% v/v C, 60% v/v A, 5% v/v B; time 30 – 40 min 85% v/v C, 10% v/v A, 5% v/v B.

In the following step, the linearity was verified. Calibration plots were constructed using mixed standards of 10, 25, 50, 100, 250, and 500 $mg.dm^{-3}$ (the 10 $mg.dm^{-3}$ standards were omitted for sucralose and steviol glycosides). Because the response function of the ELSD detector is known to be nonlinear, a logarithmic conversion for both concentration and peak area was performed. For all constructed calibration curves coefficients r^2 were >0.99 showing very good linearity in the concentration range tested.

The precision of the investigated method was verified by repeatability test during which a mixed standard of 10 $mg.dm^{-3}$ of the analytes of interest was repeatedly injected onto the column (steviol glycosides and sucralose concentration was 25 $mg.dm^{-3}$). Results from this test are presented in Table 1. The RSD values of the retention time were found to be <1%, the RSD of area and height of each analyte peak was found to be <2%.

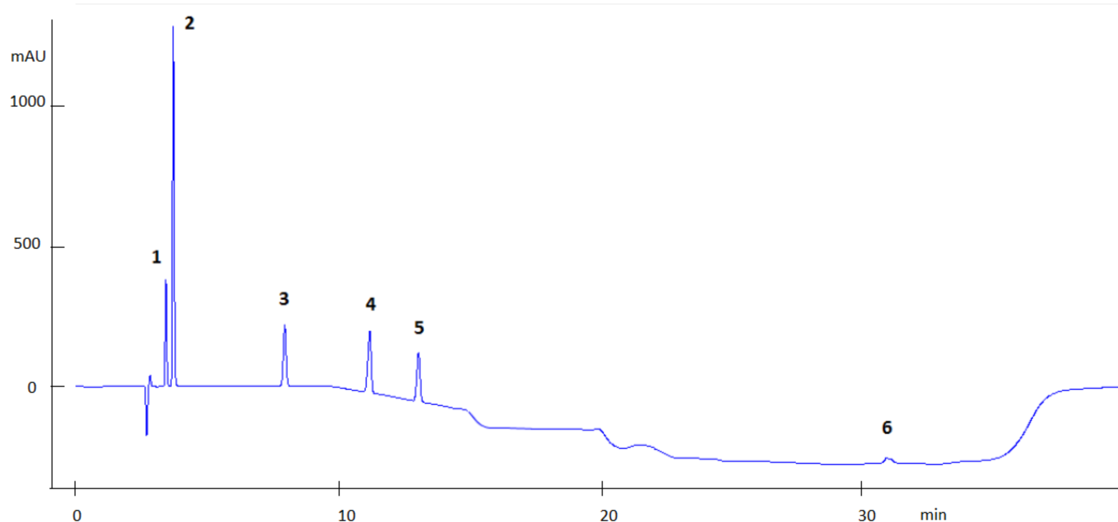

Figure 1 Chromatogram of standard (50 mg.dm^{-3}), DAD 210 nm.
Note: 1 = acesulfam K, 2 = saccharin, 3 = aspartam, 4 = benzoic acid, 5 = sorbic acid, 6 = steviol glycosides.

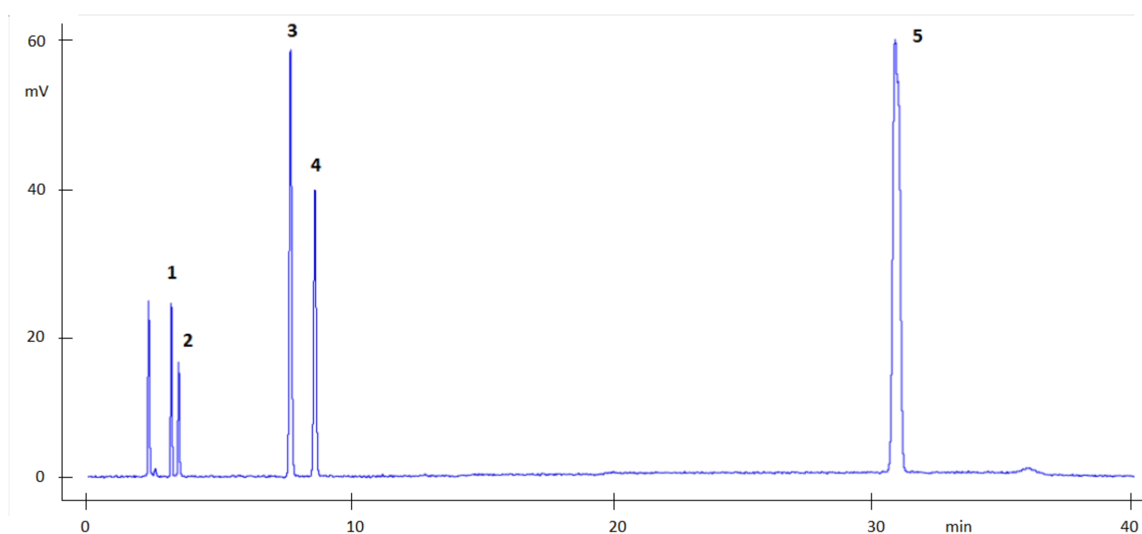

Figure 2 Chromatogram of standard (50 mg.dm^{-3}), ELSD.
Note: 1 = acesulfam K, 2 = saccharin, 3 = aspartame, 4 = sucralose, 5 = steviol glycosides.

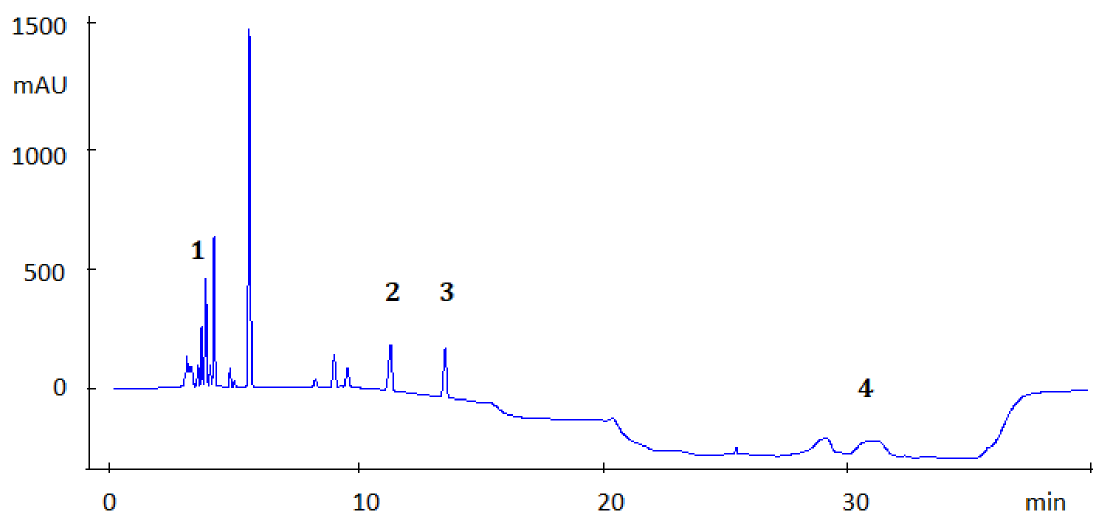

Figure 3 Chromatogram from the analysis of the real sample (S6), DAD 210 nm.
Note: 1 = acesulfame K, 2 = benzoic acid, 3 = sorbic acid, 4 = steviol glycosides.

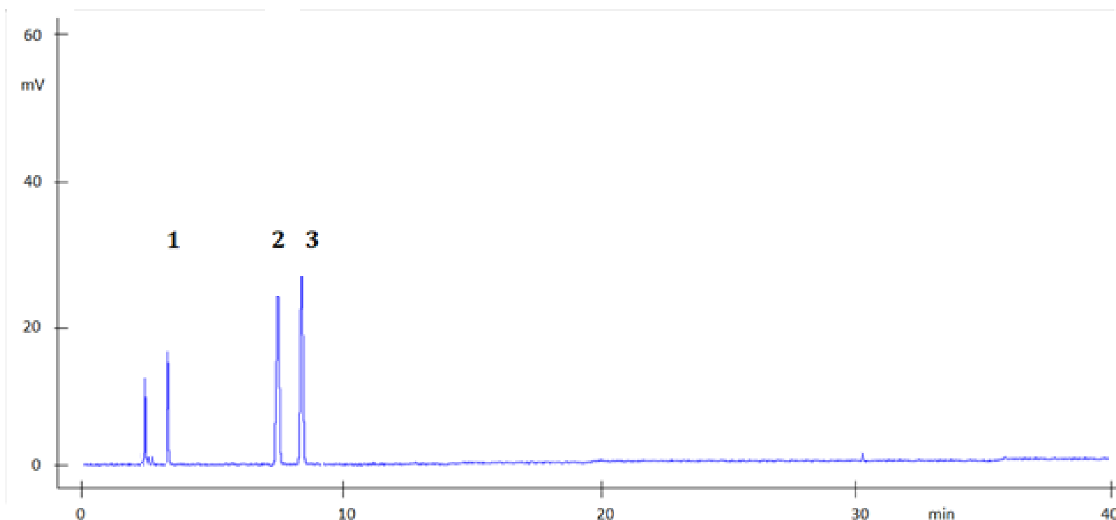

Figure 4 Chromatogram from the analysis of the real sample (S7), ELSD.
Note: 1 = acesulfame K, 2 = aspartame, 3 = sucralose.

Table 1 Repeatability of retention time (min), peak hight and peak area, (n = 6).

	Mean[a]	RSD[a]	Mean[b]	RSD[b]	Mean[c]	RSD[c]
ACS	3.46	0.37	119	0.75	457	0.86
SAC	3.70	0.47	352	0.36	1384	0.33
ASP	7.37	0.55	50	1.50	336	0.81
SUC	8.69	0.68	1.27	1.77	7.9	1.72
SG	31.20	0.17	9.8	1.70	57	1.72
BAC	11.73	0.53	57	1.42	471	0.93
SAC	12.82	0.53	52	1.06	431	1.26

Note: ACS = acesulfame K, SAC = saccharin, ASP = aspartame, SUC = sucralose, SG = steviol glycosides, BAC = benzoic acid, SAC = sorbic acid, RSD = relative standard deviation (%), [a] = Repeatability of retention time, [b] = Repeatability of peak hight, [c] = Repeatability of peak area.

Table 2 Concentration of sweeteners in analysed beverages.

Sample	**sweeteners**				
	ACS (mg.dm^{-3} ±SD)	**SAC (mg.dm^{-3} ±SD)**	**ASP (mg.dm^{-3} ±SD)**	**SUC (mg.dm^{-3} ±SD)**	**SG (mg.dm^{-3}± SD)**
S1	248 ±20	<0.6	122 ±7	<10	<12
S2	190 ±11	<0.6	120 ±4	<10	<12
S3	211 ±9	<0.6	<1.6	270 ±15	<12
S4	207 ±8	<0.6	159 ±7	<10	<12
S5	<0.9	<0.6	<1.6	<10	231 ±18
S6	278 ±12	<0.6	<1.6	75 ±6	222 ±14
S7	186 ±9	<0.6	120 ±10	173 ±13	<12
S8	191 ±10	94 ±5	103 ±8	<10	<12

Note: ACS = acesulfame K, SAC = saccharin, ASP = aspartame, SUC = sucralose, SG = steviol glycosides, S1 – S4 energy drinks, S5 – S6 carbonated drinks, S7 – S8 iced teas.

Table 3 Concentration of preservatives in analysed beverages.

Sample	**preservatives**	
	Benzoic ac. (mg.dm^{-3} ±SD)	**Sorbic ac. (mg.dm^{-3} ±SD)**
S1	<5.5	<4.4
S2	117 ±5	214 ±11
S3	120 ±9	223 ±15
S4	<5.5	<4.4
S5	159 ±8	180 ±7
S6	147 ±12	135 ±5
S7	<5.5	<4.4
S8	<5.5	104 ±9

Note: S1 – S4 energy drinks, S5 – S6 carbonated drinks, S7 – S8 iced teas.

Limits of detection and quantification were determined from calibration lines after repeated injection of a mixed standard of 10 $mg.dm^{-3}$ of the analytes of interest (steviol glycosides and sucralose concentration was 25 $mg.dm^{-3}$) according to the method described by **Shirivastava and Gupta (2011)**. The limit of detection was determined to be 0.9 $mg.dm^{-3}$ for acesulfame K, 0.6 $mg.dm^{-3}$ for saccharin, 1.6 $mg.dm^{-3}$ for aspartame, 10.4 $mg.dm^{-3}$ for sucralose, 5.5 $mg.dm^{-3}$ for benzoic acid, 4.4 $mg.dm^{-3}$ for sorbic acid and 11.8 $mg.dm^{-3}$ for steviol glycosides. Limit of quantification was determined to be 2.7 $mg.dm^{-3}$ for acesulfame K, 1.9 $mg.dm^{-3}$ for saccharin, 4.9 $mg.dm^{-3}$ for aspartame, 31.4 $mg.dm^{-3}$ for sucralose, 16.5 $mg.dm^{-3}$ for benzoic acid, 13.3 $mg.dm^{-3}$ for sorbic acid and 35.4 $mg.dm^{-3}$ for steviol glycosides.

The accuracy of an analytical method was determined by performing a recovery test. The background concentration of analytes of interest in the sample used for the recovery test was 278 $mg.dm^{-3}$ (acesulfame K), 75 $mg.dm^{-3}$ (sucralose),
222 $mg.dm^{-3}$ (steviol glycosides), 147 $mg.dm^{-3}$ (benzoic acid) and 125 $mg.dm^{-3}$ (sorbic acid). The sample was further spiked with all analytes at a concentration of 50 $mg.dm^{-3}$ and then analysed again. The concentration of analytes in the sample after spiking was 319 $mg.dm^{-3}$ (acesulfame K), 45 $mg.dm^{-3}$ (saccharin), 48 $mg.dm^{-3}$ (aspartame), 115 $mg.dm^{-3}$ (sucralose), 278 $mg.dm^{-3}$ (steviol glycosides), 245 $mg.dm^{-3}$ (benzoic acid) and 171 $mg.dm^{-3}$ (sorbic acid) which corresponds to recovery between 90 and 98%. Based on the measured results, it can be stated that the proposed method has very good accuracy.

After validation of the HPLC-DAD-ELSD method, this method was applied to the analysis of real samples. Results from the analysis are presented in Table 2 and Table 3 and the chromatogram obtained from the analysis of a real sample is shown in Figure 3 and Figure 4. Saccharin was detected only in one sample at the concentration of 94 ±5 $mg.dm^{-3}$. The most common sweetener in the beverages analyzed was acesulfame K, whose concentration ranged from 186 to 278 $mg.dm^{-3}$. The use of other sweeteners varied depending on the type of sample analyzed and their concentration in beverages was around 200 $mg.dm^{-3}$. Measured results are consistent with data published by other authors. **Sik (2012)** analyzed 56 soft drinks and only in 10 samples he detected the use of saccharin (27 – 78 $mg.dm^{-3}$). The concentration of acesulfame K in soft beverages is given in the literature in the range of 3 – 258 $mg.dm^{-3}$, the concentration of aspartame in the range of 27 – 559 $mg.dm^{-3}$, sucralose in the range of 13 – 152 $mg.dm^{-3}$ and steviol glycosides in the range of 3 – 83 $mg.dm^{-3}$. **(Sik, 2012; Ha et al., 2013; Grembecka et al., 2014; de Queiroz Pane et al., 2015; Yongsun Lee et al. 2017; Di Donna et al., 2017)**. Not all samples contained preservatives. The measured concentration of benzoic acid in beverages was about 150 $mg.dm^{-3}$. Sorbic acid was found at a higher concentration. The concentration ranged from 104 to 223 $mg.dm^{-3}$. The measured concentrations are comparable with those published by other authors **(Grembecka et al., 2014)**, however, in some cases extremely high concentrations of monitored preservatives in beverages can be found **(Javanmardi et al., 2015)**. The sweeteners and preservatives identified in all samples examined corresponded to the composition on the product packaging. Except for steviol glycosides, the concentration of monitored substances in beverages did not exceed the maximum permitted concentrations given in the valid legislation. The maximum permitted concentration of steviol glycosides in beverages is 80 $mg.dm^{-3}$. This limit was exceeded by about three times in two samples.

CONCLUSION

The scientific hypothesis that by the combination of two detectors (DAD and ELSD) with HPLC it will be possible to determine sweeteners and preservatives in beverages using one method and one sample injection was confirmed. Using non-polar C18 stationary phase, mobile phase containing methanol, acetone, and a mixture of 0.02 $mol.dm^{-3}$ formic acid and 0.02 $mol.dm^{-3}$ trimethylamine were found as the most suitable. The proposed analytical method showed good linearity, precision, and accuracy. Measured limits of detection were sufficient to analyze 5-times diluted beverage samples. The concentration of monitored additives in beverages was following valid legislation. Only the amount of steviol glycosides in two samples was exceeded by about three times the maximum allowed content in beverages.

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