Original Research Paper

Acta Chromatographica 29(2017)3, 345–358
DOI: 10.1556/1326.2017.29.3.05

Development of Reverse-Phase High-Performance Liquid Chromatography Method for Simultaneous Determination of Sodium Benzoate and Potassium Sorbate in Beverages

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Summary. Reverse-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous determination of sodium benzoate and potassium sorbate in beverages was developed using high speed column. The simple and rapid reverse-phase method for quantitative determination of both preservatives was established on LiChroCART® Purospher STAR RP-18e (30 mm × 4 mm; 3 μm) column, mobile phase consisted of acetonitrile–phosphate buffer (pH = 3.5) in volume ratio of 8:92 (*v*/*v*), flow rate of 1 mL min^{-1}, ultraviolet (UV) detection at 195 nm for sodium benzoate and 260 nm for potassium sorbate, and constant column temperature at 25 °C. Linearity, precision, accuracy, limit of quantification (LOQ), and limit of detection (LOD) were tested for method validation. Linearity range for sodium benzoate was 6.04–200.27 mg L^{-1} (R^2 = 0.999) while, for potassium benzoate (R^2 = 0.999), 12.19–406.36 mg L^{-1}. The RSD values ≤1.03% demonstrate excellent intra-day precision. LOD for sodium benzoate and potassium sorbate was 0.004 and 0.003 mg L^{-1}, while LOQ was 0.012 and 0.009 mg L^{-1}, respectively. This method was applied for quantitative determination of investigated preservatives in beverages which were taken from Macedonian markets.

Key Words: RP-HPLC method, sodium benzoate, potassium sorbate, UV detection, beverages

Introduction

Chemical preservation has become an important practice in modern food technology with increase in production of processed and convenience products. Processed food available in the markets often contains different types of food additives, among which preservatives are playing important role. Preservatives are defined as substances that increase the food preservation time by protecting them against the damages due to microbiological, enzymatic, or chemical changes of foods [1]. Nowadays, the very popular

First published online: October 11, 2016 *ISSN 2083-5736 © The Author(s)*

and commonly used preservatives are benzoic and sorbic acid, which are mostly used in the form of the well soluble sodium, potassium, or calcium salts [2]. They are most active in food at low pH value and ineffective in food at neutral pH values. At the same time, the bacterial action of these acids increases in the presence of NaCl [3].

Sodium benzoate (E211) is sodium salt of benzoic acid, and it is used in a variety of products, such as food, cosmetics, and pharmaceuticals, but more commonly in beverages to preserve freshness [4, 5]. The undissociated form, formed from the salt dissolved in solution, is responsible for the antibacterial activity, which is optimum at a pH value between 2.5 and 4.0 (benzoic acid pK_a 4.19). Benzoic acid and its salts could react with the ascorbic acid within some drinks, forming small quantities of benzene which is cancerous [6]. Potassium sorbate (E202) is the potassium salt of sorbic acid which acts efficiently against the growth of yeasts, molds, and some bacteria. It acts at low pH, but it is also efficient over a wide pH range, including high levels (up to 6 pH value). The sorbates are physiologically harmless and less toxic compared to benzoates but may still influence the taste of the food [7].

The excess amounts of preservatives can be harmful to human health, and because of that, there are limitations of the concentration of preservatives that can be used [8]. The permitted quantities of benzoic and sorbic acids in soft drinks are regulated with Macedonian regulation for food safety according to European Union Legislation: (EU) No. 1129/2011 [9, 10]. When both the acids are used solely, their maximum level is established to 300 mg L^{-1} for sorbic acid and 150 mg L^{-1} for benzoic acid. When these acids are found in combination, the usage of sorbic acid is set at 250 mg L^{-1}. These limits are expressed as benzoic and sorbic acid, not the salts.

In order to ensure that preservatives in beverages comply with regulations, appropriate methods are required to evaluate and to determine their quantity. Frequently used method for determination of preservatives is high-performance liquid chromatography because it separates them from the sample matrix providing accurate results [11–15]. Other important thing is that aqueous beverage samples can be directly injected in the column without prior derivatization. It makes high-performance liquid chromatography (HPLC) technique a superior analytical procedure for detection and quantification of preservatives in food and beverages [16–18]. On the other hand, there is a constant need to improve this method in order to obtain better analytical results, to shorten the time required for the analysis and to reduce the cost. Therefore, the aim of this investigation was to develop a simple reverse-phase high-performance liquid chromatography (RP-HPLC)

method for simultaneous determination of sodium benzoate and potassium sorbate in beverages. Accuracy (recovery), limit of detection (LOD), limit of quantification (LOQ), precision, and linearity range were evaluated for method validation. The developed method was tested to analyze these preservatives in fifteen different beverages available in the local Macedonian markets.

Experimental

Equipment and Chemicals

The chromatographic analysis was carried out with 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–visible (UV–vis) diode array detector (G1316B), and ChemStation software. For optimization of the method, the following two high speed (HS) columns for separation of sodium benzoate and potassium sorbate were used: LiChroCART® Purospher STAR RP-18e (30 mm × 4 mm; 3 μm; Merck) and HS 3 × 3 C_{18} (3.3cm × 0.46 cm, 3 μm; Perkin Elmer). Before use, all beverages were filtered with 0.45 μm Iso-Disc PTFE (Supelco, Germany) syringe filters, while carbonated beverages were degassed in an ultrasonic bath.

The pure analytical standards of sodium benzoate (99.9%) and potassium sorbate (99.9%) were produced by Supelco, Germany. Both of them were used without further purification. The HPLC-grade chemicals employed for the preparation of the mobile phase acetonitrile, K_2HPO_4, H_3PO_4, and water were produced by Sigma-Aldrich, Germany.

Chromatographic Conditions

The mobile phase contained a mixture of acetonitrile and phosphate buffer adjusted to pH = 3.5 (8:92, *v*/*v*). The buffer solution was prepared by dissolving of 0.3 g K_2HPO_4 in 300 mL water and adjusted to pH = 3.5 with H_3PO_4. The chromatographic separation was achieved with isocratic elution at a flow rate of 1.0 mL min^{-1} and column temperature at 25 °C. 2.5 μL of sample was injected into chromatographic system. The run time of the analysis was 7.5 min. To carry out a more sensitive determination, the detection of benzoic and sorbic acids was done at the 195, 230, and 260 nm

wavelengths and quantification was performed, for each wavelength, by a calibration curve. The peaks of the determined acids were identified by comparing their UV spectra and retention times with those of the standards.

Preparation of Standard and Sample Solutions

Stock solutions of sodium benzoate and potassium sorbate were prepared by dissolving 0.0224 g and 0.0339 g of the pure analytical standards in mixture of acetonitrile–water (50:50, v/v) in a 10-mL volumetric flask. All solutions were stored in a refrigerator at 4 °C. The stock solutions were used to prepare mixed standards in a volumetric flask of 10 mL with different concentrations of sodium benzoate (6.00–200.27 mg L^{-1}) and potassium sorbate (12.16–406.36 mg L^{-1}) used to obtain the calibration curves. These solutions were prepared in seven concentration levels, from 27, 80, 179, 358, 537, 716, and 895 μL of stock solution of sodium benzoate and 36, 108, 240, 480, 720, 960, and 1200 μL of potassium sorbate in 10-mL volumetric flasks, and filled to the mark with the mixture of acetonitrile–phosphate buffer (50:50, v/v). The chromatographic measurements corresponding to the calibration curve were recorded the same day in decreasing order of concentration. Calibration curves for sodium benzoate and potassium sorbate were obtained with three injections (2.5 μL of each) of these solutions. The area and height of individual peaks and the corresponding concentration (mg L^{-1}) of sodium benzoate and potassium sorbate were used to construct the standard curves. Least squares linear regression analysis method was used to determine the slope, intercept, and the correlation coefficients of the standard plots [19].

The fifteen different beverages (ice teas, cola drinks, energy drink, flavored drinks, and fruit nectars) were used for testing efficiency of the developed method. Before injection in the chromatographic system, the samples were filtered through 0.45 μm Iso-Disc PTFE syringe filters and, from the clean filtrate, three injections were performed with 2.5 μL each. All beverages were directly analyzed without cleanup procedures prior the HPLC determination [20]. Each sample was triple analyzed and data were averaged.

The solutions for recovery experiment were prepared by adding a known amount of sodium benzoate (23.94, 47.88, and 71.83 mg L^{-1}) and potassium sorbate (50.12, 99.90, and 150.02 mg L^{-1}) in a volumetric flask of 10 mL filled to the mark with ice tea which contained both analyzed preservatives. Each of the spiked solutions was analyzed in triplicate (2.5 μL injections).

Results and Discussion

Reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed for simultaneous determination of sodium benzoate and potassium sorbate in beverages. UV diode array detection was used to confirm the peak purity and analyte peak identity [21]. The UV spectra of sodium benzoate and potassium sorbate are presented in *Fig. 1*.

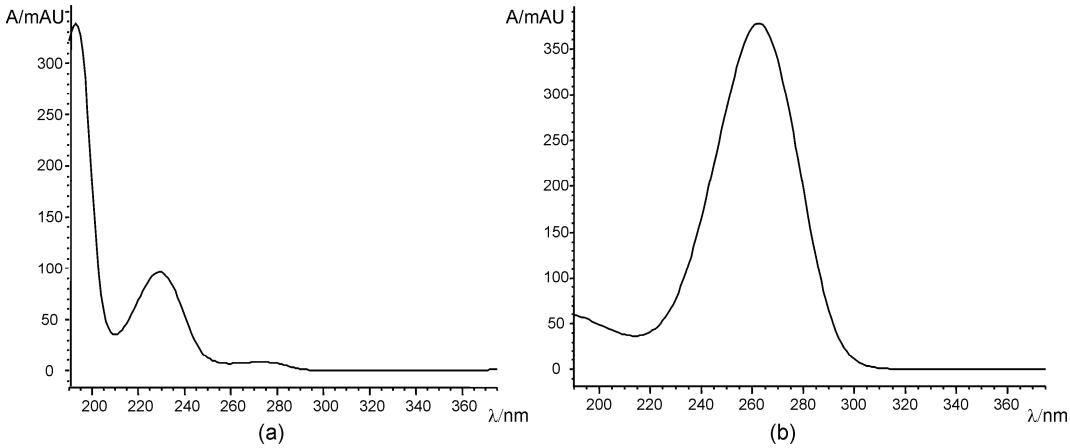

Fig. 1. UV spectra of pure analytical standards of sodium benzoate (a) and potassium sorbate (b)

From *Fig. 1*, it is evident that sodium benzoate has two UV maxima located at ca. 195 and 230 nm, while potassium sorbate has maximum near 260 nm. Therefore, the chromatograms data were recorded using these three wavelengths. If it is needful to detect both preservatives with a good sensitivity at a single wavelength, λ = 230 nm is the best compromise for sodium benzoate and potassium sorbate.

Mobile phase consisting of acetonitrile and phosphate buffer (pH = 3.5) was used to find the correct volume ratio for satisfactory separation at isocratic mode of elution. In order to separate tested analytes two HS columns: LiChroCART® Purospher STAR RP-18e (30 mm × 4 mm; 3 μm) and HS 3 × 3 C_{18} (3.3 cm × 0.46 cm, 3 μm; Perkin Elmer) were tested.

The first column was tested with mobile phase consisting of acetonitrile and phosphate buffer (pH = 3.5) in volume ratio of 20:80, 10:90, 8:92, and 7:93 (*v*/*v*). The best separation, with symmetrical peak shapes and good purity index, was achieved with mobile phase adjusted to 8:92 (*v*/*v*), flow ratio

of 1 mL min^{-1}, run time of 7.5 min, and column temperature of 25 °C. Reducing organic solvent in mobile phase increased its polarity, and because of that, interactions of preservatives with nonpolar C_{18} stationary phase are stronger. Hence, the retention time was a bit longer compared to the methods which used more organic solvent in the mobile phase [22, 23]. The mean retention times for sodium benzoate and potassium sorbate were about 6.08 min and 6.65 min, respectively. The obtained value for separation factor (α = 1.10) indicated that the analytical method proposed in this study successfully separates the analytes. The determined column dead time was 0.24 min, so the calculated retention factors (k) were 24.33 for sodium benzoate and 26.71 for potassium sorbate.

The chromatograms of a standard solution of sodium benzoate (120.16 mg L^{-1}) and potassium sorbate (243.84 mg L^{-1}) obtained with the experimental conditions described in Experimental section are presented in *Fig. 2*.

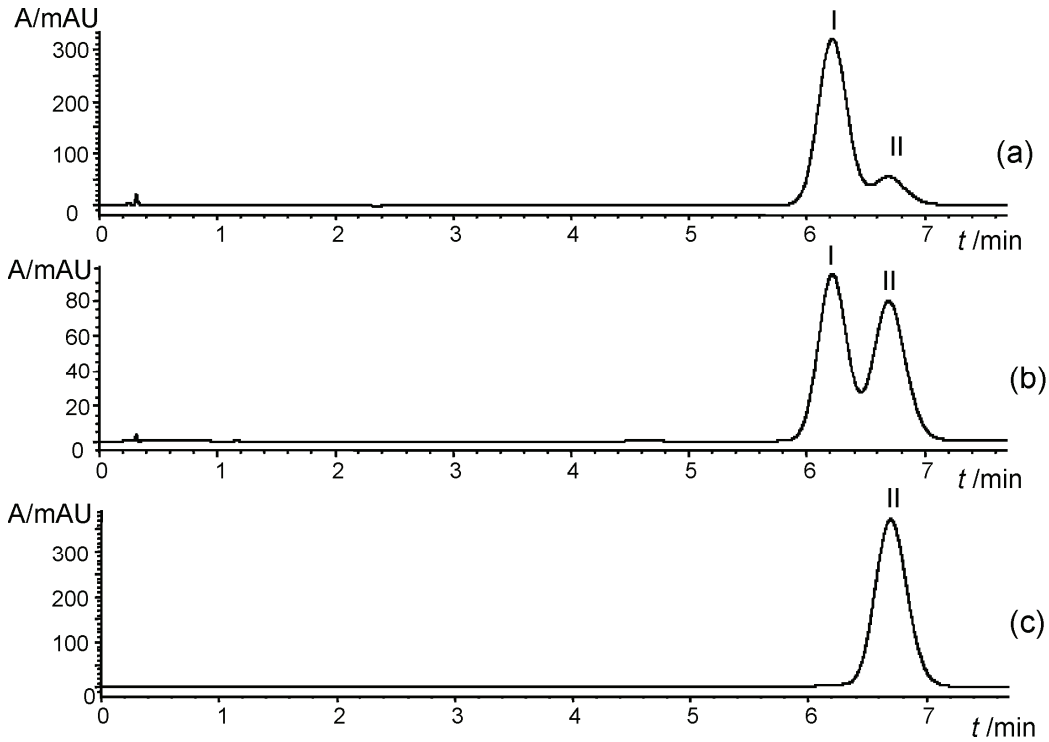

Fig. 2. Chromatograms of a standard mixture of (I) sodium benzoate (120.16 mg L^{-1}) and (II) potassium sorbate (243.84 mg L^{-1}) on the LiChroCART® Purospher STAR RP-18e (30 mm × 4 mm; 3 μm) column with acetonitrile–phosphate buffer (pH = 3.5) (8:92, v/v) as mobile phase at a) 195 nm, b) 230 nm and c) 260 nm

Further investigations were performed on a HS 3 × 3 C_{18} (3.3 cm × 0.46 cm, 3 μm; Perkin Elmer) analytical column with mobile phase consisted of acetonitrile–phosphate buffer (pH = 3.5) with volume ratio between 7:93 (*v*/*v*) and 50:50 (*v*/*v*), at flow rate of 1 mL min^{-1}. Chromatogram recorded at 8:92 (*v*/*v*) volume ratio of acetonitrile–phosphate buffer (pH = 3.5) is presented in *Fig. 3*.

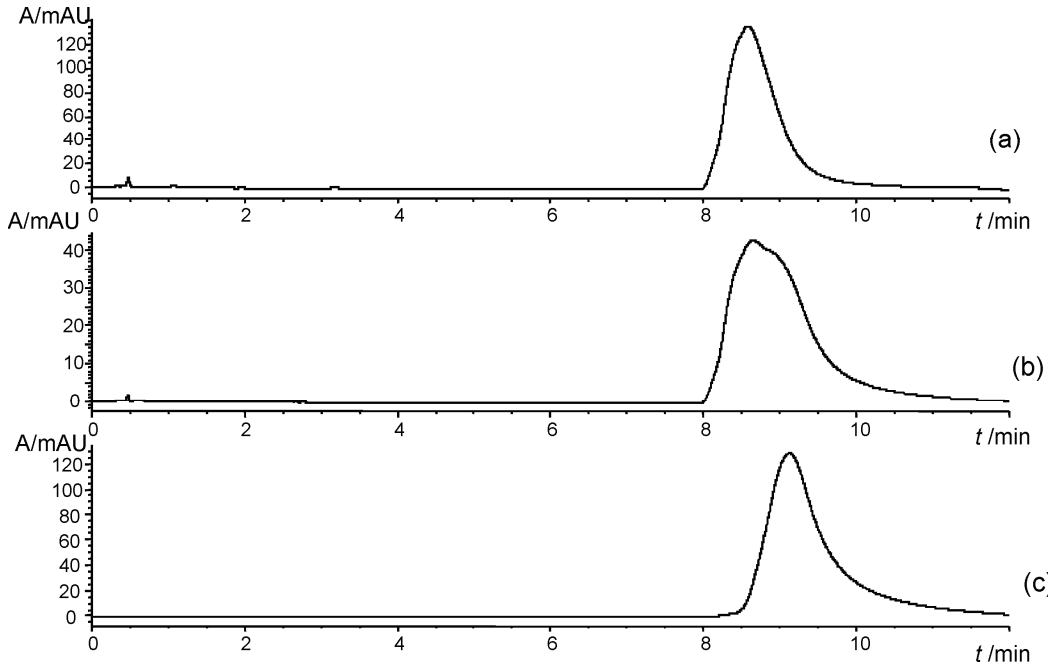

Fig. 3. Chromatograms of a standard mixture of sodium benzoate (120.16 mg L^{-1}) and potassium sorbate (243.84 mg L^{-1}) on the HS 3 × 3 C_{18} (3.3 cm × 0.46 cm, 3 μm; Perkin Elmer) column with acetonitrile–phosphate buffer (pH = 3.5) = 8:92 (*v*/*v*) as mobile phase at a) 195 nm, b) 230 nm and c) 260 nm

The obtained results show that the analyzed preservatives cannot be separated using this analytical column. Same situation was observed in increasing the percentage of organic solvent in the mobile phase.

In order to obtain calibration curves for benzoic and sorbic acid, standard solution was prepared in acetonitrile and phosphate buffer in volume ratio 1:1. Each calibration solution was analyzed in triplicate, and average value of the results was used as the representative for each point. Calibration graphs were constructed by plotting the mass of standard injected as a

function of peak area and peak height. The curves are linear over the concentration range of 6.04–200.27 mg L^{-1} for sodium benzoate and 12.19–406.36 mg L^{-1} for potassium benzoate, giving regression coefficients of 0.999 for both analytes. The mean regression equations for concentrations of sodium benzoate (195 nm) and potassium sorbate (260 nm) vs. peak area and height are given in *Table I*. The correlation coefficients (R^2 = 0.999) for both parameters (peak area and height) indicate an excellent linearity of the tested method.

Table I. Linearity range, limit of detection (LOD), limit of quantification (LOQ), regression, and correlation data of sodium benzoate (195 nm) and potassium sorbate (260 nm)

Compound	Linearity range (mg L^{-1})	Regression equation	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Sodium benzoate	6.00–200.27	$y^a = 19{,}730x - 73.89$	0.999	0.004	0.012
		$y^b = 1165x - 1.418$	0.999		
Potassium sorbate	12.16–406.36	$y^a = 12{,}544x - 76.80$	0.999	0.003	0.009
		$y^b = 684.4x - 0.94$	0.999		

[a]Area.
[b]Height.

LOD was determined as a signal to noise ratio 3:1, while LOQ value was calculated as 10 times the signal height to the baseline (S/N = 10) [24]. The determined values of LOD and LOQ for the investigated compounds are given in *Table I*. The similar results (R^2, LOD, and LOQ) were calculated from data obtained at 230 nm for both analyzed preservatives.

Precision was obtained by analyzing the standard solutions containing both sodium benzoate and potassium sorbate in 1 day and three consecutive days [25]. The inter-day (n = 3) and intra-day (n = 8) repeatability were evaluated for the retention times, peak areas, and peak heights of sodium benzoate and potassium sorbate by eight successive injections of each analytical standard with concentration of 120.16 and 243.84 mg L^{-1}, respectively *(Table II)* [26].

The RSD values (%) calculated from the data (t_r, A, and H) obtained in 1 day demonstrate excellent intra-day precision of the used method *(Table II)*. On the other hand, the inter-day precision is better with respect to the area of the chromatographic peaks, i.e., the RSD values calculated from the peak areas are lower compared to the peak heights *(Table II)*. Based on the values of RSD, further calculations were made from the area of the peaks.

Table II. Relative standard deviation (RSD %) values of the intra-day and inter-day data (t_r, *A*, and *H*)

Compound	Days	Intra-day (RSD %)			Inter-day (RSD %)		
		(t_r)/min	(*A*)	(*H*)	(t_r)/min	(*A*)	(*H*)
Sodium benzoate (195 nm)	1	0.85	1.03	0.77	5.68	0.80	4.42
	2	0.37	0.43	0.42			
	3	0.11	0.66	0.77			
Potassium sorbate (260 nm)	1	0.80	0.89	0.75	5.84	1.04	5.10
	2	0.31	0.59	0.43			
	3	0.29	0.66	0.77			

In order to verify the accuracy of the analytical procedure, recovery studies were performed by the standard addition method [27]. For that purpose, three different concentration levels of standards were spiked to a selected sample of beverages which contained both analyzed preservatives. Recoveries were calculated from the differences in total amount of either benzoate or sorbate between the spiked and unspiked samples. The results calculated from the data (area) at 195 nm for sodium benzoate and 260 nm for potassium sorbate as the mean ± standard deviation are presented in *Table III*.

The obtained values of total analyte found suggested that the proposed method can be satisfactory used for determination of preservatives in beverages. The RSD values (less than 1%, see *Table III*) indicated high repeatability of the method. Acceptance criteria for mean recovery and precision (repeatability and RSD) are in accordance with guidelines for standard method performance requirements [28].

Table III. Results from recovery studies conducted on three concentration levels (n = 3)

Compound	Mass of analyte (μg)	Pure analyte added (μg)	Total analyte found (μg) (± SD)	Recovery (%)	RSD (%)
Sodium benzoate	0.213	0.059	0.277 ± 0.0005	102.3	0.179
	0.213	0.119	0.338 ± 0.0006	102.8	0.182
	0.213	0.179	0.406 ± 0.0001	106.5	0.028
Potassium sorbate	0.255	0.125	0.378 ± 0.0001	99.2	0.025
	0.255	0.249	0.515 ± 0.0003	104.3	0.065
	0.255	0.375	0.649 ± 0.0001	107.4	0.012

The developed method was applied to the analysis of investigated preservatives in 15 different beverages randomly selected for analysis. The labels of the analyzed beverages indicated which preservative is present. In some beverages, both preservatives were present; in some, only benzoates were present. On the label of some beverages, benzoate and sorbates were declared, but the amounts were not given. Five of the analyzed beverages are declared without preservatives. All the samples were analyzed without previous preparation. Direct injection of samples allowed a rapid, cheap, and extraction-free determination with almost no analyte loss in the samples. The least-square equations were used to evaluate the concentrations of sodium benzoate and potassium sorbate in the analyzed beverages. The concentration (mg L^{-1}) of sodium benzoate and potassium sorbate found in the analyzed beverages is presented in *Table IV*.

Table IV. Concentration of sodium benzoate and potassium sorbate in beverages

Beverage	Sodium benzoate		Potassium sorbate	
	Concentration (mg L^{-1})	On label	Concentration (mg L^{-1})	On label
I (Ice tea peach)	75.55	120	NF	ND
II (Ice tea peach)	85.22	250[a]	102.18	250[a]
III (Ice tea peach)	NF	WP	NF	WP
IV (Ice tea peach)	90.49	Declared[b]	55.21	Declared[b]
V (Ice tea peach)	NF	ND	NF	ND
VI (Ice tea peach)	NF	WP	NF	WP
VII (Ice tea lemon)	NF	WP	NF	WP
VIII (Bitter lemon)	61.17	ND	19.80	ND
IX (Ice tea lemon)	72.71	120	NF	ND
X (Lemonade)	NF	WP	NF	WP
XI (Cola)	43.44	ND	26.55	ND
XII (Fresco)	73.47	ND	3.59	ND
XIII (Mojito)	52.99	120[c]	30.39	120[c]
XIV (Multired)	90.68	ND	NF	ND
XV (Strawberry)	92.75	ND	NF	ND

WP—without preservatives, ND—not declared, and NF—not found.
[a]Benzoates declared together with sorbates.
[b]Declared, but not given the amount.
[c]Declared as benzoic acid.

The data given in *Table IV* indicated that, in all cases, the mean concentration of sodium benzoate and potassium sorbate found in the analyzed beverages is below the maximum level. Representative chromatograms of beverages (a) II—Ice tea peach (declared both preservatives), (b) III—Ice tea peach (without preservatives), (c) XI—Cola (not declared both preservatives), and (d) XIV—Multired (not declared benzoates) at 230 nm are shown in *Fig. 4*.

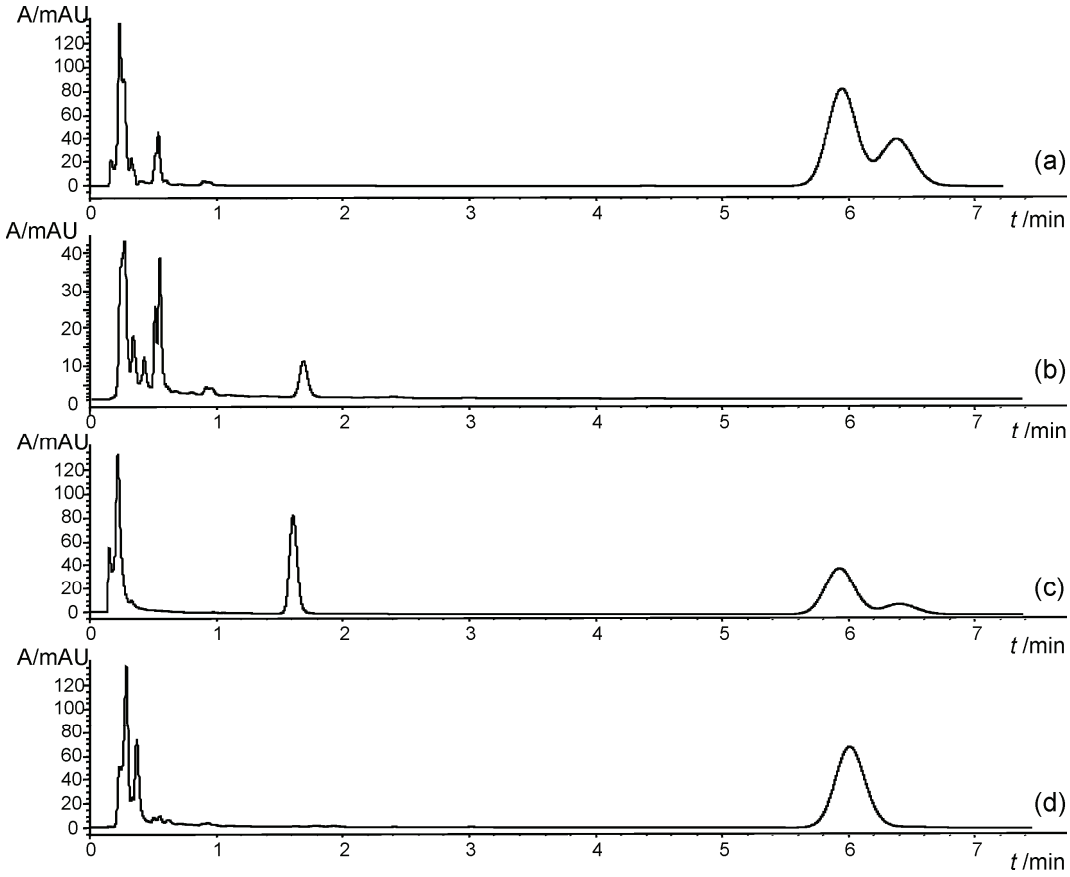

Fig. 4. HPLC chromatograms: a) II (Ice tea peach), b) III (Ice tea peach), c) XI (Cola) and d) XIV (Multired), at 230 nm

According to the obtained results (see *Table IV*), six beverages contained both sodium benzoate and potassium sorbate *(Fig. 4a)* and four contained only sodium benzoate. Five of the analyzed beverages were declared

without preservatives *(Fig. 4b)*, and the obtained results confirmed that. In beverages VIII (Bitter lemon), XI (Cola) *(Fig. 4c)*, and XII (Fresco), benzoates and sorbates were not marked on the label, but they were found. Same situation was observed for benzoates in beverages XIV (Multired) *(Fig. 4d)* and XV (Strawberry). From the declared and found amounts *(Table IV)*, it can be seen that sodium benzoate seems to be the most common preservative in beverages, despite the fact that both preservatives are permitted for use in these products.

Compared to the methods known from the literature, the presented method simplifies the analysis because pretreatment is not necessary, reduced its time and cost, and also encompasses lower level of LOD and LOQ [20, 22]. The retention time among other factors depends on composition of the mobile phase, i.e., decreasing the percentage of organic solvent, the retention time increases. Using this method, separation of the investigated preservatives was achieved in less than 7.5 min with the mobile phase consisted of 8% of organic solvent, making it more cost-effective in comparison with the methods known from the literature [29, 30].

Conclusion

Sodium benzoate and potassium sorbate are the most commonly used preservatives in beverages. A fast, simple, low-cost, selective, and reliable HPLC method for the quantitative determination of sodium benzoate and potassium sorbate in beverages was developed. This method has the advantage in utilization of high speed (HS) columns which allow short time of analysis. Moreover, the great advantage of this method was the composition of the mobile phase (acetonitrile–phosphate buffer [pH = 3.5] in volume ratio 8:92), i.e., only 8% of acetonitrile is enough for successful separation of the preservatives. Effective separation and quantification were achieved in less than 7.5 min, at flow rate of 1 mL min^{-1}. Analytical characteristics of the separation such as LOD, LOQ, accuracy, and repeatability were evaluated, and the obtained results were comparable or better compared to the results in previous studies known from literature. The results presented in this paper show that HPLC is a powerful technique in the area of preservatives determination in beverages.

Acknowledgment

The results presented here are obtained by HPLC granted within the Tempus Joint Project "Improvement and Establishment of Biotechnology in Higher Education" 145040-Tempus-2008-DE-JPCR coordinated by Prof. B. Petanovska-Ilievska.

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