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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR DETERMINATION OF PRESERVATIVES IN BEVERAGES

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Abstract
Benzoic and sorbic acids, and their sodium, potassium and calcium salts are widely used as preservatives in acidic foods and beverages. They inhibit the growth of molds and yeasts, and are also effective against wide range of bacteria which explains the benefit of their usage. The health effects have led to limitation on the concentrations that can be used in food and beverages. Because of that, the analytical determination of these preservatives is important for consumer interest and protection. Therefore, a new reversed-phased HPLC method for a fast, simple, accurate and precise determination of potassium sorbate and sodium benzoate in different beverages is developed. In this purpose, a HPLC system equipped with UV diode array detection is employed. Separation and determination of investigated preservatives are performed using Purospher® STAR RP-18 (30 mm x 4 mm; 3 μm) analytical column. Methanol/phosphate buffer (pH = 3.70) (20/80, *V/V*) is used as a mobile phase, with flow rate of 1 mL/min, constant column temperature of 25°C and UV detection at 225 nm and 255 nm. Successful separation conditions are obtained using an isocratic elution within 6 min. Accuracy, precision, limit of detection, limit of quantification, and linearity range are evaluated. The separation factor value of 1.128 showed that this method can be successfully used for simultaneous determination of benzoates and sorbates. The obtained data indicated that the concentration of investigated preservatives found in the analyzed beverages available on the local Macedonian markets is in accordance with Macedonian maximum levels for food safety.
Key words: benzoic acid, benzoates, sorbic acid, sorbates, beverages, determination, HPLC.

Introduction

Preservatives are natural or synthetic chemicals that are added to different products such as food, pharmaceuticals, cosmetics and biological samples to prevent spoilage from microbial growth and undesirable chemical reaction (Ranken *et al*., 2005). Chemical preservation has become an increasingly important practice in modern food technology with the increase in production of processed and convenience foods. In order to enhance preservative effectiveness combination of two or more preservatives often is used (Russell *et al*., 2003).

Beverages are rich in vitamins and minerals and as a result of that they are an attractive environment for microbes. Furthermore, the water activity is also important factor that has significant influence of the microbial activity. However, the usually low pH of beverages due to carbonation, the sugar content in some of them, and the addition of preservatives help in inhibition of the growth of microbes. The type of chemical preservative that can be used in beverages depends on the chemical and physical properties of both, the preservative and the beverage. In addition, the pH of the beverages, the presence of vitamins, the type of packaging and the storage conditions will determine whether preservatives are necessary and what type should be used to prevent microbial growth (Tfouni *et al*., 2002).

The main preservatives that can be used and are allowed in beverages are sorbic acid (E200) and benzoic acid (E210), as well as their corresponding salts. Sodium benzoate (E211) is the sodium salt of benzoic acid and is widely used in acidic media to inhibit yeasts, molds, and bacterial growth. Potassium sorbate (E202) is the potassium salt of sorbic acid and generally is used to inhibit mold growth in juices. The salts are used in carbonated, non-alcoholic and juice beverages because they work best between pH levels of 2

and 4 (Ferrand *et al*., 2000). Although these compounds are generally recognized as safe due to their low risk of acute and chronic toxicity, they have limitations of usage in protecting human health (Pylypiw *et al*., 2000; Mamur *et al*., 2010).

Safety for the human consumption of these products is regulated in each country by national regulations based on codes and standards derived by the Codex Alimentarius Commission. European Union (EU) member states use European legislation for microbiological criteria, food additives and general hygiene requirements for the production, storage and trade of food products, as well as specific requirements for quality and safety of beverages (Directive No 95/2/EC, 2005). The Republic of Macedonia has developed national legislation for food safety, primarily based on European legislation and Codex standards. According to these regulations maximum permitted concentrations are 150 mg/L for sodium benzoate and 300 mg/L for potassium sorbate if they are used separately, and 150 mg/L for sodium benzoate and 250 mg/L for potassium sorbate, if they are used in combination. These maximum levels are expressed as benzoic and sorbic acid, not the salts.

The analytical determination of these preservatives is not only important for quality assurance purposes, but also for consumer interest and protection. In the literature there are several analytical methods that can be applied for determination of preservatives. The most common analytical method for the determination of benzoic acid (BA), sorbic acid (SA), sodium benzoate (SB) and potassium sorbate (PS) are UV spectrophotometry, gas-liquid chromatography (GLC), thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) with different detection (Wood *et al*., 2004; Dong *et al*., 2006; Bahremand *et al*., 2013). Many of the reported methods use complicated pre-treatment procedures such as solvent extraction method, while HPLC offers simple sample preparation limited to filtration, degasification and dilution. Furthermore, this method is widely used because of the possibility to analyse preservatives with high precision, and with low limit of detection. This shows that HPLC is a powerful technique in the area of preservative analysis (Isabel *et al*., 2001; Küçükçetin *et al*., 2008).

The present work deals with the development and characterization of rapid, economic and simple RP-HPLC method for simultaneous determination of potassium sorbate and sodium benzoate in beverages. The developed method is applied to the analysis of these preservatives in seven samples available on the local Macedonian markets.

Materials and methods

Instrumentation

The chromatographic analyze is carried out with Agilent 1260 Infinity Rapid Resolution Liquid Chromatography (RRLC) system equipped with: vacuum degasser (G1322A), binary pump (G1312B), auto sampler (G1329B), a thermostatted column compartment (G1316A), UV/Vis diode array detector (G1316B) and ChemStation software. For optimization of the method following three analytical columns are used: Hypersil ODS (25 cm x 0.46 cm; 5 μm), LiChrospher 60RP-select B (12.5 cm x 0.4 cm; 5 μm) and HS (High Speed) type LiChroCART® 30-4 HPLC-Cartridge Purospher® STAR RP-18 (30 mm x 4 mm; 3 μm). The pH measurements are performed with a Radiometer pH-meter (Copenhagen, DK). An ultrasonic bath "Elma" is used for degasification of solutions.

Chemicals

Sodium benzoate (99.9%) and potassium sorbates (99.9%) are produced from Supelco, acetonitrile (ACN) and methanol (MeOH) used for the preparation of the mobile phase (Sigma Aldrich-Germany) are HPLC grade. Ultra pure water used for chromatography processing is obtained from Water Purification System TKA Smart 2 Pure 12 UV/UF. The components of phosphate buffer (H_3PO_4 (85.5 %) and K_2HPO_4 (99.0 %)) are from Sigma Aldrich, Germany. Phosphate buffer is prepared dissolving 0.5 g K_2HPO_4 in 500 mL ultra pure water and adjusted to the pH 3.70 with H_3PO_4.

Chromatographic conditions

The mobile phase composed of methanol and phosphate buffer (pH 3.70) (20:80, *V*/*V*) is used. The optimal wavelength for detection is set at 225 nm and 255 nm for sodium benzoate and potassium sorbate, respectively. The chromatographic separation is achieved with isocratic elution (2.5 μL of sample are injected into chromatographic system) at a flow rate of

1.0 mL/min and column temperature of 25 ºC. The peaks of the determined preservatives are identified by their UV spectrum and by comparing the retention time with that of the standard.

Preparation of standard and sample solutions

The stock solutions are prepared by dissolving 0.0365 g sodium benzoate and 0.0774 g potassium sorbate in methanol in 10 mL volumetric flasks. Then they are degassed in an ultrasonic bath for 15 minutes. All prepared stock solutions are stored in a refrigerator at 4 ºC. The stock solutions are used to prepare sample solutions with different concentration of SB (30 mg/L, 60 mg/L, 90 mg/L, 120 mg/L and 180 mg/L) and PS (62.6 mg/L, 125 mg/L, 187.1 mg/L, 250 mg/L and 374 mg/L) dissolved in methanol/water 50/50 (*V*/*V*) in 10 mL volumetric flasks. In order to obtain calibration curves, 2.5 µL of each working solution are injected in the chromatograph three times. The obtained chromatograms are analyzed considering area and height of the peaks. The developed method was used for determination of benzoate and sorbate contents in seven different samples taken from Macedonian market. They are prepared by filtering through a 0.45 µm membrane filter (T-Spartan) and after that they are injected into the system without clean-up procedures prior to determination by HPLC (Veerabhadrarao 1987).

Results and discussion

Determination of food preservatives sodium benzoate and potassium sorbate in beverages is performed using a rapid, economic and simple RP HPLC method. The UV-Vis spectra using photodiode array detector of sodium benzoate and potassium sorbate in 50/50 (*V*/*V*) methanol/water solution show that sodium benzoate has an absorption maximum at 225 nm, while potassium sorbate has an absorption maximum at 225 nm. Therefore, the detection of sodium benzoate and potassium sorbate is carried out at these wavelengths, respectively.

In order to separate preservatives an attempt is made using the Hypersil ODS (25 cm x 0.46 cm; 5 µm) column with a mobile phase consisted of acetonitrile/water 80/20 (*V*/*V*), flow-rate of 1 mL/min, and column temperature of 25 ºC. Unfortunately, the peaks of benzoic acid (I) and potassium sorbate (II) are not separated. There is only one peak with fronting and retention time of 2.5 min (Figure 1). The changes in the composition of the mobile phase (till 55 % ACN and 45 % water) did not produce satisfactory separation.

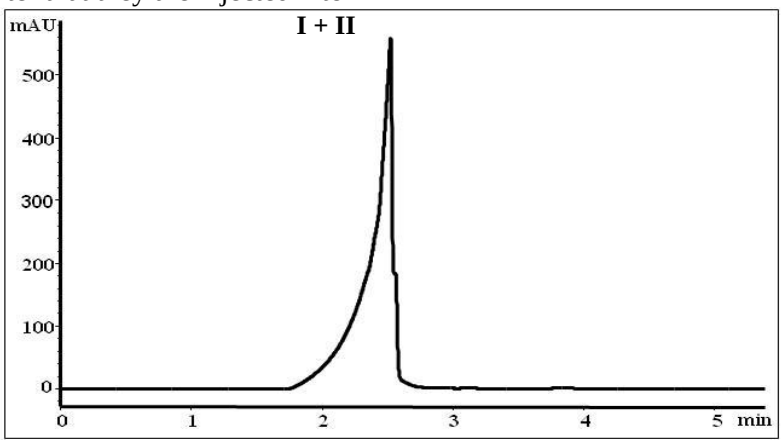

Figure 1. Chromatogram of benzoic acid (I) and potassium sorbate (II) obtained on Hypersil ODS (25 cm x 0.46 cm; 5 µm) column; mobile phase acetonitrile/water 80/20 (*V*/*V*), flow rate 1 mL/min, column temperature 25 ºC and UV detection at 220 nm.

Similarly, when LiChrospher 60 RP-select B (12.5 cm x 0.4 cm; 5 µm) column is used a good separation is not obtained with the mobile phase consisted of acetonitrile/water 50/50 (*V*/*V*), flow-rate of 1mL/min and column temperature of 25 ºC. The peak shapes of benzoic acid (I) and potassium sorbate (II) are not acceptable for quantitative determination of these two compounds (Figure 2).

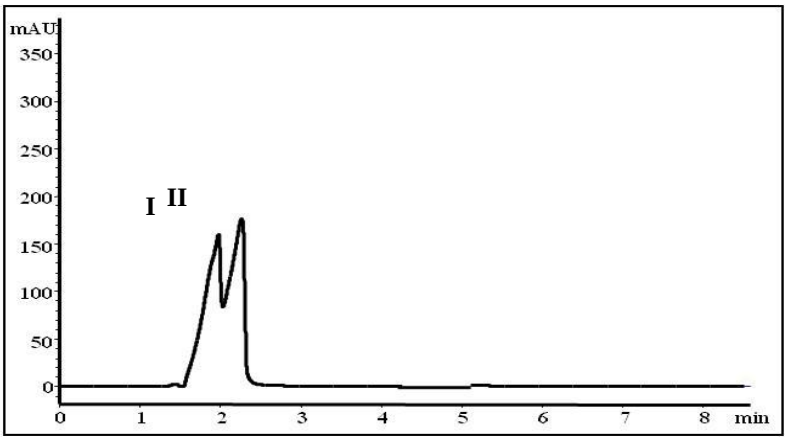

Figure 2. Chromatogram of benzoic acid (I) and potassium sorbate (II) obtained on LiChrospher 60 RP-select B (12.5 cm x 0.4 cm; 5 μm) column; mobile phase acetonitrile/water 50/50 (*V*/*V*), flow rate 1 mL/min, column temperature 25 ºC and UV detection at 220 nm.

The previous results indicated that the changes in the stationary phase are necessary to be done. Hence, in the further investigation the HS (High Speed) type LiChroCART® 30-4 HPLC-Cartridge Purospher® STAR RP-18 (30 mm x 4 mm; 3 μm) is employed. In order to obtain better separation the changes in the mobile phase are also made. The change of the solvent is a potentially useful variable for optimizing the separation. ACN is a stronger solvent than MeOH and can typically elute analytes faster in RP HPLC at similar concentration. On the other hand, MeOH may be preferred compared to ACN for separation of some ionic samples. Furthermore, the buffers have the greater solubility in methanol-water mixtures compared to mobile phases that contain ACN. Taking into consideration these facts, methanol is used as a best choice of organic solvent in the mobile phase.

The investigated preservatives can exist either into the nondissociated and ionized form, depending on the mode of separation. Hence, the chromatography process is important to be performed at a well defined pH for separation of ionizable compounds. For this purpose buffers are suitable choice because they can control the pH of the mobile phase and neutralize the charge on the silica surface of the stationary phase. Additionally, the buffers can act as ion pairing agents which can neutralize analyte charge. Inorganic buffers are usually relatively stable and because of that they are often used. In this investigation the phosphate buffer (pH 3.70) is used because its ionization occurs over pH range defined by p*K*a ± 1.5 (p*K*a = 4.19 for benzoic acid and p*K*a = 4.76 for sorbic acid). The pH dependence of the retention values can serve as a guide to select the optimum pH value for the isocratic separation of a particular mixture. As expected, retention time decreases when dissociation of the solutes increases, probably as a result of interactions with the stationary phase. If the sample is consisted only of acidic compounds the pH of the solution should be low to suppress dissociation and to obtain stronger retardation and better separation (Snyder *et al*., 1997; Meyer *et al*., 2010; Dong *et al*., 2006; Davidson *et al*., 2005).

In order to obtain satisfactory separation mobile phase consisted of methanol/phosphate buffer (pH 3.70) with different volume ratio are tested. The obtained chromatograms showed that the best separation with sharp and symmetrical peaks for sodium benzoate (I) and potassium sorbate (II) is achieved with the mobile phase composed of methanol/phosphate buffer (pH 3.70) 20/80 (*V*/*V*), flow-rate of 1 mL/min, injection volume of 2.5 μL, constant column temperature of 25 ºC and UV detection at 225.4 nm and 255.4 nm (Figure 3). The retention time under these chromatographic conditions is 4.823 min for benzoic acid and 5.407 min for potassium sorbate. The values of the retention factor (*k*) are 17.915 and 20.203 for sodium benzoate and potassium sorbate, respectively. The separation factor (α) between the adjacent peaks of sodium benzoate and potassium sorbate is 1.128.

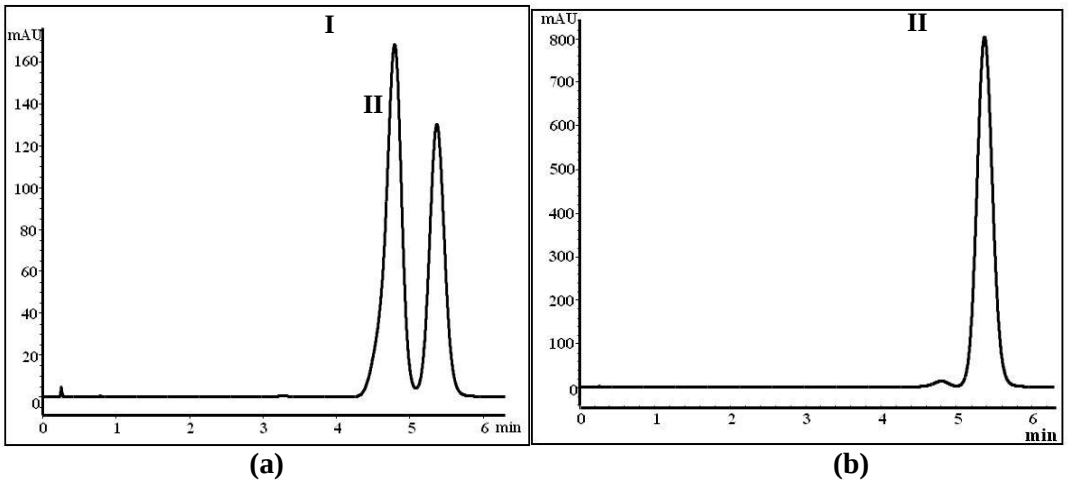

Figure 3. Chromatogram of benzoic acid (I) and potassium sorbate (II) obtained on HS (High Speed) type LiChroCART® 30-4 HPLC-Cartridge Purospher® STAR RP-18 (30 mm x 4 mm; 3 µm) column; mobile phase methanol/phosphate buffer (pH 3.70) 20/80 (*V*/*V*), flow rate 1 mL/min, column temperature 25 ºC and UV detection at 225.4 nm (a) and 255.4 nm (b).

The calibration curve for sodium benzoate and potassium sorbate are plotted on the basis of peak areas of chromatograms obtained for different concentrations of sample solutions. The curves followed Lambert-Beer's law in the concentration range of 30 mg/L, 60 mg/L, 90 mg/L, 120 mg/L and 180 mg/L for sodium benzoate, and 62.6 mg/L, 125 mg/L, 187.1 mg/L, 250 mg/L and 374 mg/L for potassium sorbate. Peak area/peak heights versus concentration are used to construct calibration curves. The correlation coefficients (R^2) indicated that the peak area is a preferable variable for further accuracy testing (Table 1). The limit of detection (LOD) is determined as signal to noise ratio 3:1, while the limit of quantification (LOQ) is determined as signal to noise ratio 10:1 (Saad *et al*., 2005). LOQ is referred to the lowest level of the compound which can be determined with an acceptable degree of confidence. The obtained values for LOD and LOQ are given in table 1. According to these results, the developed method allows to identify and quantify the preservatives in the obtained concentration range.

Table 1. Statistical evaluation of calibration curves for sodium benzoate and potassium sorbate

compound	linearity range (mg/L)	regression equation	R^2	LOD (mg/L)	LOQ (mg/L)
SB (225.4 nm)	30-180	y = 8466.90x + 234.04	0.9906	0.0038	0.0128
		y = 343.40x + 62.21	0.8893		
PS (225.4 nm)	62.6-374	y = 17619.46x + 1407.11	0.9869	0.0030	0.0099
		y = 1105.81x + 125.91	0.9885		

Precision of the method is calculated from the values of retention time, area and height of the peaks present in the chromatograms of the standard solutions containing both sodium benzoate and potassium sorbate in one day and three consecutive days (Lough *et al*., 1996). The inter-day (n = 3) and intraday (n = 8) repeatability is evaluated, by eight successive injection of the mixture containing 120 mg/L sodium benzoate and 250 mg/L potassium sorbate (Meyer *et al*., 1994).

Table 2. Relative standard deviation values (RSD, %) of the inter-day and intraday precision

parameter	sodium benzoate		potassium sorbate	
	RSD % inter-day (n = 3)	RSD % intraday (n = 8)	RSD % inter-day (n = 3)	RSD % intraday (n = 8)
peak area	0.17	0.22	0.18	0.22
peak height	0.08	1.78	0.06	1.28
retention time	0.09	1.06	0.16	1.36

Presented data of RSD % calculated using the retention time, peak area and peak height values demonstrate excellent inter-day precision of the used method (RSD < 1 %), while the intraday precision is satisfactory when the data of the peak area are used.

In order to verify accuracy of the analytical procedure, the recovery studies are carried out. The accuracy of the procedure is tested using method of standard additions and expressed as the deviation between calculated mean value of analyte amounts obtained by the analysis and the true (known) value of the analyte amounts spiked to a selected sample of beverage which contained both analyzed preservatives (Snyder *et al*., 1997). Recoveries are evaluated at three different levels of concentration for SB (18.75 mg/L, 37.50 mg/L and 75.00 mg/L) and PS (31.30 mg/L, 62.60 mg/L and 125.00 mg/L), corresponding to the minimum, the maximum and an intermediate value of the linearity range (Table 3). In cases where preservatives in food are analyzed recoveries in the range 80 - 110 % are acceptable (AOAC, 2011).

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

compound	mass of analyte (μg)	pure analyte added (μg)	total analyte found (μg)	recovery (%)	RSD (%)
SB (225.4 nm)	0.1258	0.0469	0.1715	99.32	0.09
	0.1258	0.0938	0.1987	90.50	0.06
	0.1258	0.1875	0.2892	92.31	0.15
PS (255.4 nm)	0.0272	0.0783	0.0960	91.00	1.75
	0.0272	0.1565	0.1764	96.05	0.09
	0.0272	0.3125	0.2938	86.46	0.16

The presented data suggested that the proposed method is characterized with high repeatability and it can be used for determination of preservatives in beverages.

The developed RP-HPLC method is applied to determine the sodium benzoate and potassium sorbate in real samples under the defined experimental conditions. Seven different samples marked as: A, B, C, D, E, F, G and H are purchased randomly from Macedonian market. Samples are prepared simply by filtering through 0.45 μm filters (T-Spartan) and injected (2.5 μL) into the HPLC system without any pre-treatment steps. Each analysis is repeated in triplicate. The identity of SB and PS is confirmed using the values of the match factor obtained by comparison of the peaks of SB (999.929) and PS (999.974) of pure analytical standards with those of the sample. The results for determination of sodium benzoate (benzoic acid) and potassium sorbate (sorbic acid) in seven different samples of beverages are presented in Table 3.

Table 4. Na-benzoate (mg/L) and K-sorbate (mg/L) in different beverages

Sample	Preservative content			Referent values labeled on product [mg/L]
	SB [mg/L]	PS [mg/L]	Total [mg/L]	
A	53.64	26.48	80.12	SB and PS are present*
B	50.32	10.88	61.2	SB and PS are present*
C	45.64	**ND	45.64	SB max 120 mg/L
D	44.88	52.72	97.6	SB and PS max 250 mg/L
E	59.44	103.48	162.92	SB and PS max 400 mg/L
F	57.16	89.32	146.48	SB and PS max 400 mg/L
G	50.60	12.88	63.48	SB (0.12 g/L) and PS (0.036 g/L)

*the correct content of SB and PS is not labeled; **ND-not detected

From the results presented in the table 3 it can be seen that the mean concentration of SB and PS found in the analyzed beverages is below the maximum permitted level.

Conclusion

The developed RP HPLC method is adequate for the determination of sodium benzoate and

potassium sorbate in different beverages with good analytical performances under the optimized experimental parameters. It is simple, rapid, reliable and allows good recoveries of benzoates and sorbates in the concentration range usually present in the beverages. The obtained results showed that the concentration of SB (or BA) and PS (or SA) varied between different kinds of beverages samples, with levels lower than the maximum values established by national and international legislation.

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