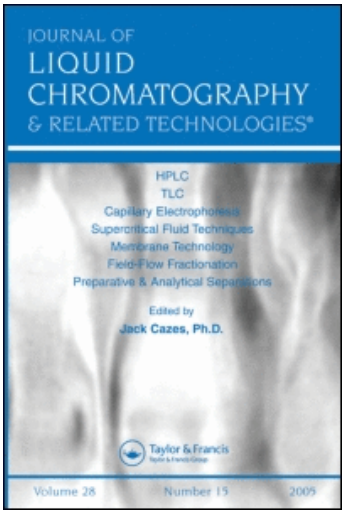

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SOLID-PHASE EXTRACTION AS A CLEAN-UP PROCEDURE FOR THE LIQUID CHROMATOGRAPHIC DETERMINATION OF BENZOIC AND SORBIC ACIDS IN FRUIT-DERIVED PRODUCTS

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ABSTRACT

A liquid-chromatographic procedure is proposed for the simultaneous determination of benzoic and sorbic acids in fruit-derived products. Prior to the chromatographic separation, a clean-up pre-treatment of the sample is carried out off-line, using successively an anionic resin and a C_{18} stationary phase as sorbent beds. After elution from the cartridges, the acids are separated and quantitated by LC with UV-absorbance detection. The validation tests show that the technique can be applied to different types of fruit-derived products (i.e. jams, dried fruits and almond paste).

INTRODUCTION

Benzoic and sorbic acids can be used as preservatives and/or fungistatics in fruit-derived products, but are subject to EC regulations, the authorized limits being dependent on the type of products and the additive. The restrictions are particularly strict for benzoic acid. Its addition in combination with sorbic acid is often not allowed. A method is needed to detect unauthorized additions.

In a previous paper,[1] we proposed a sensitive thin-layer chromatographic method for the determination of benzoic and sorbic acids in fruit beverages. It allows the simultaneous determination of these acids without interference by most of the commonly used preservatives. However, column liquid chromatography is by far the most applied technique, because of its selectivity, flexibility and the possibility of automation. It has been used for the determination of both benzoic and sorbic acids in citrus fruits,[2] orange juice,[3] foodstuffs,[4] soft drinks,[5,6] meat,[7] fish product,[8] milk, and dairy products.[6,9,10] In these procedures, filtration, centrifugation and/or liquid-liquid extraction were used prior to the chromatographic separation. However these clean-up steps are inadequate for achieving a sensitive determination for most fruit-derived products.

Solid-phase extraction was also used in the determination of preservatives in orange juice,[11] jams[12] and foodstuffs such as cheese, cakes, yogurts and other samples which may form emulsions during the extraction step.[13] We applied[12] the clean-up on C_{18} to the determination of benzoic and sorbic acids in apricot and raspberry jams, which allowed 5 and 3 mg/kg of benzoic and sorbic acids to be respectively detected. However, this extraction procedure is still inadequate for a sensitive determination of these acids in orange jams, because of the presence of interfering peaks in the chromatogram, mainly at the retention time of benzoic acid.

In this paper a more efficient clean-up procedure, which uses two different sorbent beds in succession, is proposed for fruit-derived products.

First of all, benzoic and sorbic acids are retained in their ionized forms at pH 7.5 on a cartridge packed with an anionic resin modified with a trimethylaminopropylsilane. Potential interfering substances such as flavors, carotens, and compounds giving high viscosity (pectins,sugars ...) pass through the sorbent bed and are removed. Subsequently, the fixed acids are eluted at pH 3 with a phosphate buffer solution. The eluate is collected on a second cartridge packed with an octadecyl silica conditioned at pH 2. At this pH value, both acids are retained on the sorbent bed in their molecular forms.

while the residual polar compounds are removed. The preservatives are finally eluted from the C_{18} cartridge with methanol and the resulting extract is chromatographed. The chromatographic separation is carried out on a C_{18} stationary phase with a mobile phase consisting of phosphate buffer (pH 6.5) and acetonitrile (94 + 6, v/v). The analytes are detected and quantitated at 234 nm, which is close to the wavelength of maximum absorbance of benzoic acid. This allows a sensitive determination of this latter.

EXPERIMENTAL

Apparatus

Extractor for solid phase extraction VAC ELUT SPS 24 (Analytichem International).

SAX BOND ELUT cartridges, 2.8 mL, packed with anionic exchange resin, 40 µm (Analytichem International). C_{18} BOND ELUT cartridges 3 mL, 40 µm (Analytichem International).

Centrifuge capable of operating at a rotational frequency of 4000 rpm.

Liquid chromatograph Hewlett Packard 1050 fitted with a Rheodyne injection valve, a 10 µL injection loop, a variable wavelength detector and an integrator.

Operating Conditions

Column: Lichrospher RP_{18} 5 µm, 250 x 4 mm (Merck) connected to a guard-column Lichrosorb RP_{18} 4 x 4 mm (Merck). Mobile phase : 0.03 M phosphate buffer pH 6.5 - acetonitrile (94 + 6, v/v) filtered through a 0.45 µm Millipore filter. Flow rate : 1 mL/min. Temperature : 22 ± 1°C. Detector : set at λ = 234 nm.

Reagents

Acetonitrile : HPLC grade. Methanol and other chemicals : analytical grade. 0.05M and 0.3M sodium hydroxide aqueous solutions. 1M phosphate buffer solution pH 3 obtained by dissolving 27.2 g KH_2PO_4 in 200 mL distilled water then adjusting at pH 3 ± 0.1 by addition of a 50 % (v/v) phosphoric acid

solution. 0.1M hydrochloric acid solution. 0.03M phosphate buffer pH 6.5 obtained by dissolving respectively 0.816 g of KH_2PO_4 in 200 mL distilled water (sol'n 1) and 2.556 g of Na_2HPO_4 in 600 mL distilled water (sol'n 2); the pH of solution 1 was adjusted to pH 6.5 ± 0.1 with solution 2.

Standard Solutions

Stock solution of sodium benzoate 1.18 g/L (corresponding to 1 g/L benzoic acid) prepared by dissolving 59 mg of sodium benzoate in 50 mL distilled water. Stock solution of potassium sorbate 1.34 g/L (corresponding to 1 g/L sorbic acid) prepared by dissolving 67 mg of potassium sorbate in 50 mL distilled water.

Mixed standard solutions prepared by suitable dilution of each stock solution with methanol to match the respective concentrations of the different test solutions.

Sample Preparation

The samples were homogeneized for 3 min in a laboratory mixer at 7500 rpm. For dried stone fruits (prunes) pits were removed and the material was blended until a homogeneous sample was obtained.

Test portion : 10 g of jam or jelly or 5 g of dried fruits or almond paste were weighed up to 0.01 g in duplicate. For dried stone fruits, a test portion of pulp corresponding to 5 g of the whole fruit was weighed. The samples were mixed thoroughly in a mortar with a few mL of tepid water (<40°C), then adjusted at pH 7.5 ± 0.1 with 0.3M sodium hydroxide solution and quantitatively transferred in a volumetric flask (20, 25, 50, or 100 mL); the solutions were made up to volume with washing water, then transferred into a centrifuge tube. The solutions were centrifuged at 4000 rpm for 10 min. except for almond paste sample (20 min). The supernatant was decanted off and filtered through a cellulose filter. The filtrate was used for the solid phase extraction-step.

Extraction and Clean-up

Conditioning of the anionic resin : 6 mL of distilled water, then 2 mL of methanol and then 6 mL of distilled water (previously adjusted at pH 7.5 ± 0.1 by addition of a 0.05 M sodium hydroxide solution) were successively passed

through the SAX cartridge. The sorbent bed should not be allowed to dry out during the conditioning step. One mL aliquot of filtrate was applied on the top of the cartridge (flow rate regulated at ca. 0.5 mL/min). The cartridge was washed with 3 x 2 mL of distilled water (adjusted at pH 7.5 ± 0.1). The eluents were discarded and the cartridge was dried by suction with vacuum.

Conditioning of the C_{18} cartridge

Two mL of methanol and 2 mL of 0.1M HCl were passed through the C_{18} cartridge successively; then 2 mL of 0.1M HCl were added without aspiration. The cartridge should not be allowed to dry during this step. The C_{18} cartridge was connected to the SAX cartridge via an adaptor.

The analytes were eluted from the SAX cartridge by passing successively 2 x 2 then 1 mL of 1M phosphate solution (pH 3). The eluates were collected on the C_{18} cartridge. The SAX cartridge was dried by suction with vacuum.

The SAX cartridge and the adaptor were discarded and the C_{18} cartridge was washed with 3 x 2 mL of 0.1M HCl. The cartridge was air dried under vacuum. The analytes were eluted by 2 x 2 mL of methanol. Eluates were collected, made up to 4 mL with methanol and filtered through a 0.45 µm Millipore filter before injection. The extracts can be kept for at least 12 h, except prune extracts which should be injected within 6 h following their preparation.

Chromatography

The solutions were injected in duplicate onto the chromatograph, bracketting standard and test solutions.

Peak height or area measurements were used to calculate the concentration of the analytes in the samples analyzed.

RESULTS AND DISCUSSION

A representative chromatogram of a mixed standard solution is shown in Fig. 1. Typical retention times were 4.98 min.(k'=1.43) and 6.02 min. (k'=1.93) for benzoic and sorbic acid respectively. The plate number was about 2200 for each acid and the resolution was ca 2 between the two compounds. The ratio of peak intensity (sorbic/benzoic acid = 2.4) is in agreement with the respective molar absorptivities of these acids at 234 nm (about 13000 and 5900)

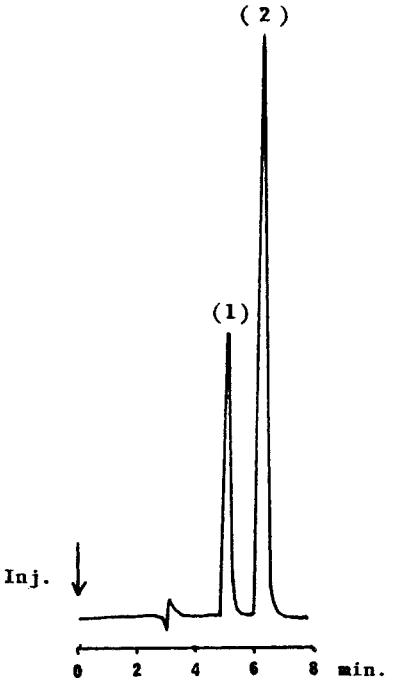


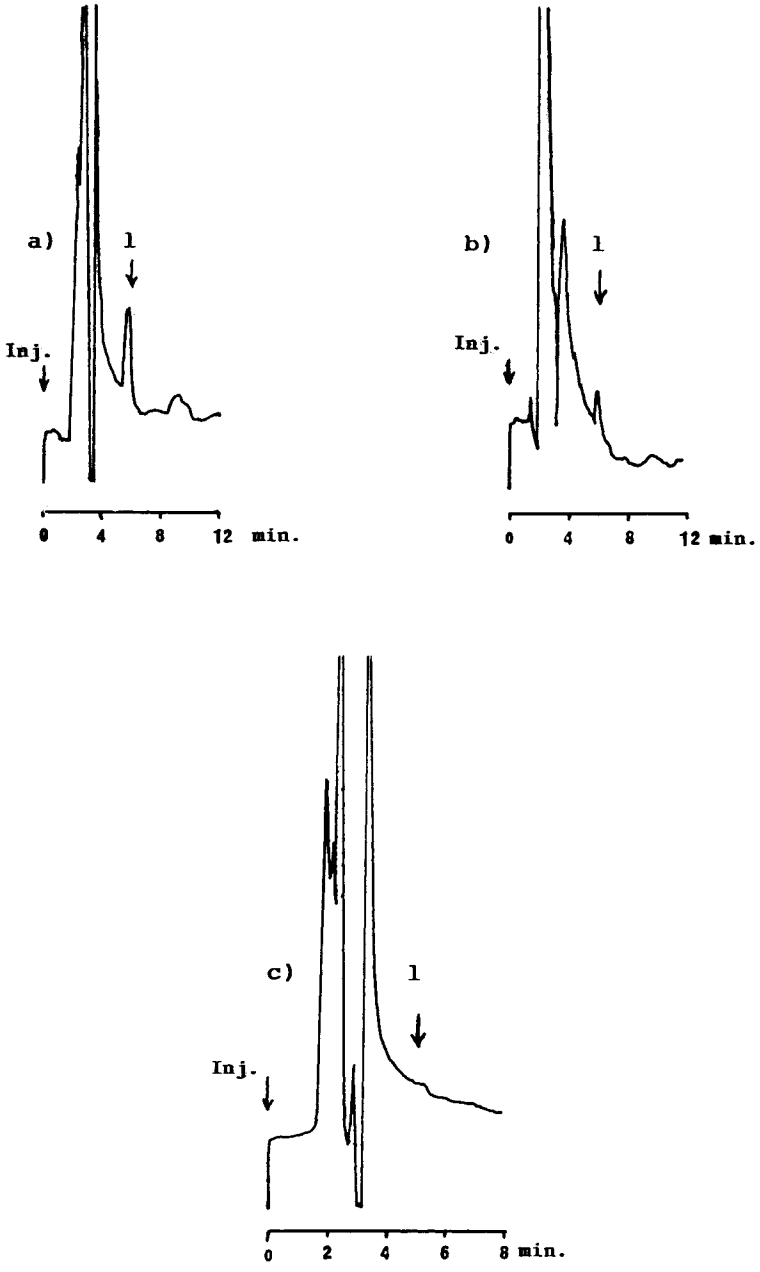
Figure 1. Chromatogram of a mixed standard solution : (1) Benzoic acid 1.25 mg/L, (2) Sorbic acid 1.25 mg/L.

Validation Tests

Linearity of the calibration graph

The linearity of the variation of the peak height (or peak area) as a function of the concentration was assessed from duplicate injections of mixed standard solutions (n=6) in a concentration range of 0.5-20 mg/L for each additive. The correlation coefficient was better than 0.999 and the intercept was not significantly different from zero (P=0.05). This allows only one standard solution to be used for the determinations.

Figure 2. **(right)** Chromatograms from unspiked raspberry jam extracts: a) Clean-up on C_{18} cartridge b) Clean-up on SAX cartridge c) Clean-up on two sorbent beds (SAX + C_{18}).



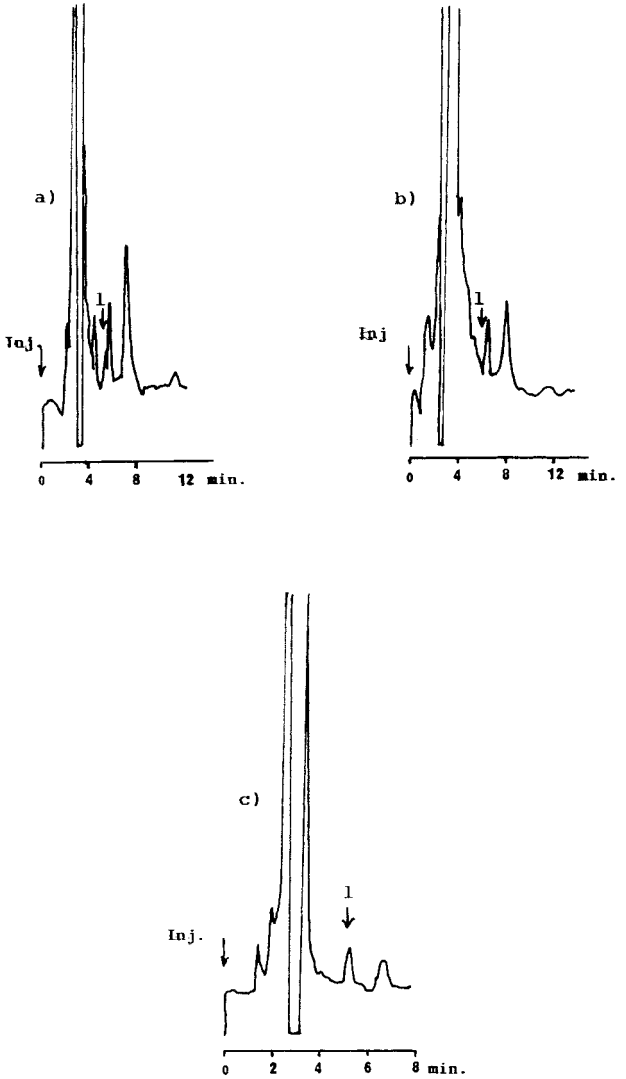


Figure 3. Chromatograms from unspiked orange jam extracts : a) Clean-up on C_{18} cartridge b) Clean-up on SAX cartridge c) Clean-up on two sorbent beds (SAX + C_{18}).

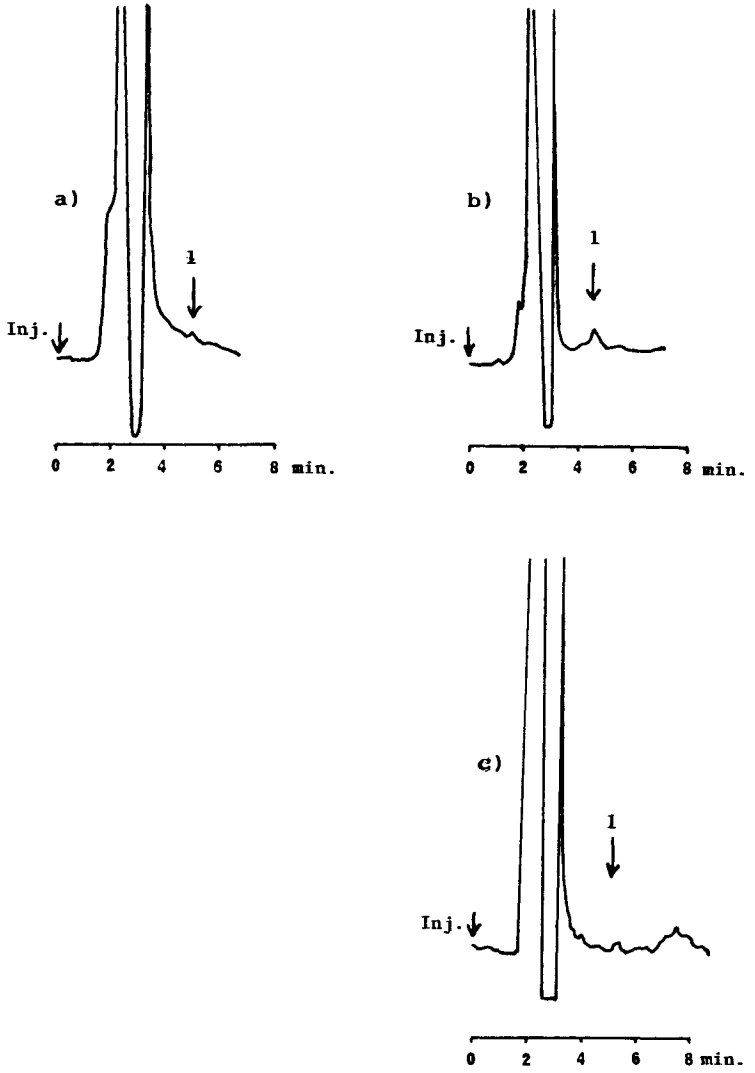


Figure 4. Chromatograms from unspiked sample extracts: a) Prune b) Almond paste c) Bilberry jam.

Table 1

Repeatability (RSD, %) of LC Analysis*

Sample	Spiked With			
	Benzoic Acid		Sorbic Acid	
	50 mg/kg	30 mg/kg	50 mg/kg	30 mg/kg
Raspberry jam	0.88	1.76	0.38	1.02
Orange jam (marmalade)	0.58	1.74	0.58	0.32
Bilberry jam	1.09	1.39	0.79	0.86
Chestnut jam	0.89	1.96	0.36	0.38
Dried apricot	3.61	5.81	1.51	3.49
Prune	1.50	3.17	1.04	1.93
Almond Paste	3.30	3.20	1.23	0.89

* n = 7 injections

Specificity

The specificity of the test procedure was assessed by injecting the blank matrix of different fruit-derived products. The efficiency of a clean-up step using two different beds in succession is illustrated in Fig. 2 and 3, which present the comparative chromatograms from unspiked raspberry and orange jams extracted on C_{18} or SAX. Chromatograms from other types of matrices treated as indicated in the procedure are given in Fig.4.

The small peak observed at the retention time of benzoic acid in some chromatograms (Fig. 3 and 4) corresponds to about 3-4 mg/kg, 1.5-2 mg/kg and 1-1.5 mg/kg of benzoic acid in marmalade, prune samples and almond paste, respectively. These concentrations are assumed to be due to natural benzoic acid at trace levels in fresh fruits.[14] This is in agreement with the natural presence of benzoate in the range of 0.1-2.0 mg/kg[15] which was recently noted in a collaborative study devoted to the determination of benzoate in orange juices. We have also mentioned in the experimental part that prune extracts should be analyzed within 6 hours following their preparation. After this delay, an artefact peak which was assumed to be a decomposition product of the matrix was observed at the retention time of sorbic acid. Its formation is minimized if the extract is kept at 4°C and in the dark.

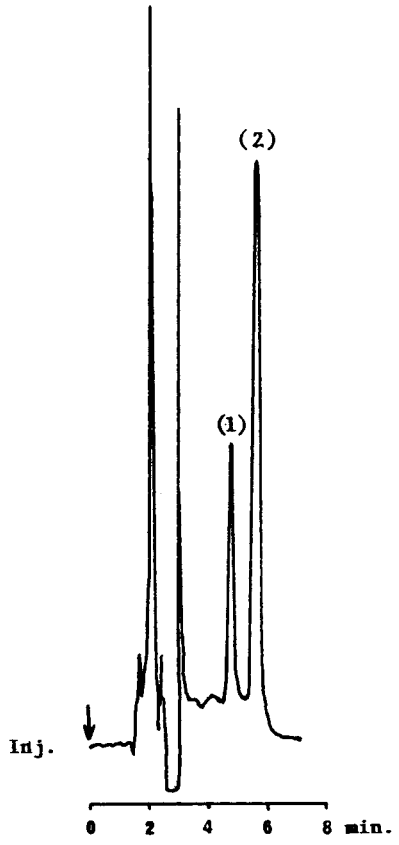


Figure 5. Chromatogram from an almond paste extract spiked with benzoic acid (1) and sorbic acid (2), 30 mg/kg each.

Repeatability and Accuracy

The repeatability of the LC analysis was assessed by injecting, seven times successively, the mixed standard solutions at two concentration levels (1mg/L and 10 mg/L). The RSD was always better than 3%. The repeatability was also tested on extracts from samples spiked (30 and 50 mg/kg) with a mixed standard solution of benzoate and sorbate (corresponding to 100 mg/L sorbic and benzoic acids). The results are listed in Table 1.

Table 2

Accuracy and Repeatability of the Test Procedure*

Sample	Analyte	Concentration Added (mg/Kg)	Concentration Added (mg/Kg)	Recovery (%)	RSD (%)
Raspberry jam	Benzoic acid	50	49.76	99.52	1.83
		30	29.65	98.83	2.15
	Sorbic acid	50	48.10	96.20	1.82
		30	29.14	97.13	2.31
Orange jam (marmalade)	Benzoic acid	50	52.07	104.13	0.98
		30	31.19	103.96	2.03
	Sorbic acid	50	49.71	99.41	0.21
		30	29.77	99.23	1.53
Bilberry jam	Benzoic acid	50	49.96	99.93	0.95
		30	29.63	98.77	0.79
	Sorbic acid	50	49.93	99.86	0.97
		30	29.58	98.59	0.77
Chestnut jam	Benzoic acid	50	49.81	99.62	0.74
		30	29.99	99.97	2.12
	Sorbic acid	50	49.99	99.98	0.34
		30	30.09	100.32	0.61
Dried apricot	Benzoic acid	50	53.88	107.77	3.54
		30	33.95	113.18	3.82
	Sorbic acid	50	50.50	101.00	5.70
		30	28.88	96.27	4.36
Prune	Benzoic acid	50	51.97	103.95	0.84
		30	31.84	106.13	1.56
	Sorbic acid	50	49.92	99.84	0.80
		30	30.31	101.05	1.25
Almond paste	Benzoic acid	50	51.54	103.08	3.06
		30	31.51	105.04	2.58
	Sorbic acid	50	49.84	99.68	2.27
		30	30.17	100.59	2.55

* n = 7 extractions

The repeatability and accuracy of the whole test procedure (extraction step and chromatography), was assessed by extracting, seven times, spiked samples at the two same concentration levels. A representative chromatogram from a spiked matrice is given in Figure 5. Recovery results in Table 2 show that in all cases the recovery is close to 100%, with a RSD lower than 4%. A slight overestimation (shown mainly on 30 mg/kg spiked samples) can be noted for benzoic acid in apricot (jam and dried fruits), marmalade, prune, and almond paste samples. It is probably due to the natural presence of benzoic acid.

Limits of Detection

The examination of the chromatograms from the different matrices allows the detection limits (S/N=3) to be evaluated to ca 0.6 mg/kg for benzoic acid and 0.25 mg/kg for sorbic acid in the samples analyzed. The limits of quantification can be evaluated to be in the range of 2-5 mg/kg for benzoic acid and 1-3 mg/kg for sorbic acid.

CONCLUSION

The validation tests which have been carried out show the efficiency of a double step extraction prior to liquid chromatographic separation for determining sorbic and benzoic acids in fruit-derived products.

Recovery studies and repeatability tests performed at 30 and 50 mg/kg show that the procedure is free from systematic and random errors. The detection limits which have been evaluated for benzoic acid allow to assume that the proposed procedure should be suited to detect its unauthorized addition in the presence of sorbic acid.

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