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Optimisation of extraction procedures for analysis of benzoic and sorbic acids in foodstuffs

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Abstract

Benzoic and sorbic acids are the most commonly used preservatives in foodstuffs. They are usually analysed by RP-HPLC. However, in view of the complexity and diversity of foodstuffs composition, appropriate sample preparation procedures are required for reliable extraction of these preservatives from the matrices. Specific extraction procedures for analysis of jams, table olives, spreadable fats, sauces, fruit juices and wines were optimised. Thus, different types of food matrices were chosen, including those with high sugar content, with high fat content and beverages (with and without alcohol). A significant set of validation data was performed through recovery and precision studies. Chromatographic separation was achieved using a C18 column (S_{10} ODS_2) and acetate buffer 0.005 M (pH = 4.4)—methanol (65:35) as mobile phase, 1.4 ml/min flow rate and UV detection at 235 nm. The concentration of preservatives in the samples was calculated by external standard method. Benzoic and sorbic acids in jams, jellies and table olives were efficiently extracted with methanol after ground homogenization. Fortified samples, at 4 different concentration levels of benzoic and sorbic acids, presented average recoveries (after discarding outliers) for each preservative greater than 91% with a coefficient of variation (CV) less than 2.6%. Sorbic acid was extracted from spreadable fats and emulsified sauces with n-hexane and was back-extracted to an aqueous phase with acetate buffer 0.005 M (pH = 4.4). Recoveries were higher than 98% for two levels of concentration and CV lower than 2.9%. The preservatives extraction from fruit juices (orange, apple and pineapple) and wines required purification using a Sep-Pak C18 cartridge, and its elution with methanol. Average recoveries of benzoic and sorbic acids at two levels of concentration were greater than 94% with CV less than 4.0%. Eighty-seven commercial brands were analysed including table olives (29), jams (24), jellies (2) spreadable fats (25), sauces (3), fruit juices (10) and table wines (3). All samples conformed to the legal prescriptions.

Keywords: Benzoic acid; Sorbic acid; HPLC; Table olives; Jams; Spreadable fats; Sauces; Juices; Wines

1. Introduction

Food preservatives are added to stop or delay nutritional losses due to microbiological, enzymatic or chemical changes of foods during its shelf life. They also prevent consumer hazards due to the presence of microbial toxins or pathogenic microrganisms and economic losses due to spoilage (Davidson, 1997). The role of preservatives has become more prominent with the increase in production of processed and convenience foods.

The use of sorbic (E200) and benzoic (E210) acids, as well as their salts (E201-sodium sorbate; E202-potassium sorbate; E203-calcium sorbate; E211-sodium benzoate; E212-potassium benzoate; E213-calcium benzoate) is allowed by European legislation (Directive no. 98/72/CE) and its presence must be declared on the label.

Previous studies have reported the determination of these preservatives in orange juices, fruits, jellies and jams, sauces and other foods using HPLC (Bui & Cooper, 1987; Ferreira, Mendes, Brito, & Ferreira, 2000; Lee, 1995; Pylypiw & Grether, 2000). Different eluents were used, including phosphate buffer, methanol, tetrahydrofuran and, in other cases, acetate buffer

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and acetonitrile. However, some of these methods are not applicable to a wide range of foodstuffs or use lengthy clean up steps prior to HPLC analyses. Efficient sample clean up procedures are essential for eliminating high-molecular mass matrix interferents (e.g. proteins, fats, polysaccharides) and, also, vitamins and food additives. Thus, the aim of our research work was to optimise and validate sample preparation procedures for extraction of preservatives from different types of food matrices, including those with high sugar content, such as, jams and jellies, those with high fat content, and presenting different textures, namely, olive tables, spreadable fats and emulsified sauces. Beverages including wines and juices were also analysed.

Benzoic and sorbic acid contents in 87 food products, commercially available on the market, were evaluated after appropriate sample preparation and HPLC/UV separation in order to compare their levels with respective allowable limits.

2. Materials and methods

2.1. Apparatus

The chromatographic analysis was carried out in a high performance liquid chromatograph (Gilson Medical Electronics, Villiers le Bel, France) equipped with a 305 pump and a 7125 Rheodyne Injector with a 20 μl loop. The chromatographic separation was achieved with a C18 Spherisorb S_{10} ODS_2 chromatographic column (10 μm, 250 mm×4.6 I.D.). A Gilson 118 variable ultraviolet detector and Gilson 712 HPLC system controller software were also used.

2.2. Reagents and standards

Sodium benzoate was obtained from BDH Chemicals Co. (England), Sorbic acid, ammonium acetate, acetic acid, methanol and *n*-hexane p.a., were obtained from Merck (Darmstadt, Germany). Methanol (Lichrosolv) was obtained from Merck.

Water used for chromatography possessing a resistance greater than 15Ω was prepared by purifying demineralised water in a "Seral" system. Filtered through a membrane of 0.45 μm porosity and subsequently degassed. Methanol (Lichrosolv) was also degassed.

Standard solutions were prepared using methanol as solvent, except for spreadable fats and sauces analyses in this cases standard solutions were prepared using acetate buffer 0.005 M, pH 4.4.

2.3. Chromatographic conditions

The chromatographic separation of benzoic and sorbic acids was achieved with a reversed phase C18 column at room temperature, a flow rate of 1.4 ml/min and isocratic elution, with a mixture of acetate buffer 0.005 M (pH = 4.4) and methanol (65:35) as the mobile phase. The ultraviolet (UV) detector was set at 235 nm with a sensitivity of 0.05 AUFS.

2.4. Sampling

Eighty-seven commercial brands were assayed, which included:

- 20 table olive samples numbered from 1 to 20: 10 were black table olives (samples 1–10), nine were green table olives (samples 11–19) and one sample of turnover table olive (sample 20);
- 18 quince jam samples numbered from 21 to 38;
- 6 jams numbered from 39 to 44: two of strawberry (samples 39 and 40), other two of peach (41 and 42), one of blackberry (43) and one of apple (44);
- 2 strawberry jellies (45 and 46);
- 25 spreadable fats numbered from 47 to 71: twelve had a fat content above 60% (samples 47 to 58), while thirteen had less than 60% of fat (59–71);
- 3 emulsified sauces with less than 60% of fat (numbered from 72 to 74);
- 10 fruit juice samples numbered from 75 to 84: six of orange juice (75–80), two of apple juice (81–82) and two of pineapple juice (83–84);
- 3 table wine samples numbered from 85 to 87.

2.5. Extraction procedures

As mentioned above the matrices chose presented complex and diverse composition. Thus, appropriate extraction methods were checked for each. The precision was evaluated taking into account its repeatability. Determination of the coefficient of variation (CV%) of the extraction step was evaluated by measuring the peak area on six different extracts of the same sample. Recovery studies were performed by standard addition method and carried out in duplicate. Known sorbic or benzoic acid amounts were added to the samples. Thereafter, the respective extraction procedures were carried out and recovery percentages of sorbic and benzoic acids obtained after subtracting the previously quantified endogenous sorbic and/or benzoic acid from total contents.

For jams, jellies and table olives precipitation of fat and proteins was achieved by the addition of methanol (Ferreira et al., 2000). Approximately 2.0 g of homogenized sample was thoroughly mixed with 20 ml of methanol and the volume brought up to 25 ml with methanol. The extract was centrifuged (2500 *g*) for 5

min and the supernatant was filtered through a 0.45 μm filter membrane.

For spreadable fats and sauces a different extraction procedure was optimized owing to its physic-chemical properties. Spreadable fat or sauce (0.1 g) was first diluted with *n*-hexane (2 ml). Then a liquid-liquid extraction was performed with 10 ml of acetate buffer 0.005 M (pH = 4.4), followed by centrifugation and filtration.

Juices or wines (10 ml) were first centrifugated and extracted via a Sep-Pak® C18 disposable cartridge (Waters). The cartridge was previously activated with 2 ml of methanol and 4 ml of water. Supernatant (1 ml) was passed through the cartridge, cleaned with 4 ml of *n*-hexane and eluted with 3 ml of methanol. The extract was filtered through a 0.45 μm filter membrane.

2.6. Statistical analysis

Analysis of variance (ANOVA) was used to assess significant differences at 5% significance level. Statview™ 4.0 statistical package (Abacus concept, Berkeley, CA) was used in all statistical analyses.

3. Results and discussion

3.1. Chromatographic separation of benzoic and sorbic acids

The external standard method was used to calibrate the chromatographic system for benzoic and sorbic acids quantification. For that purpose standard solutions prepared with methanol ranging from 5 to 500 μg of benzoic acid/ml and 1 to 500 μg of sorbic acid/ml were used. Linearity between the concentration of benzoic and sorbic acids and the UV absorbance at 235 nm was obtained and correlation coefficient for each standard curve exceeded 0.9998.

Sorbic acid standard solutions prepared with acetate buffer 0.005 M pH 4.4 presented similar linearity. However, calibration curve parameters were different than those with methanol. Thus, these calibration curves were used for quantification of sorbic acid in spreadable fats and sauces.

The detection limit, calculated as the concentration corresponding to three times the background noise, was 2 μg/ml for benzoic acid and 0.5 μg/ml for sorbic acid.

The HPLC system used presented optimum separation with minimal bandwidth and short elution time (3.64 retention time for benzoic acid and 4.94 for sorbic acid). A representative chromatogram for separation of benzoic and sorbic acids in a table olive extract is shown in Fig. 1.

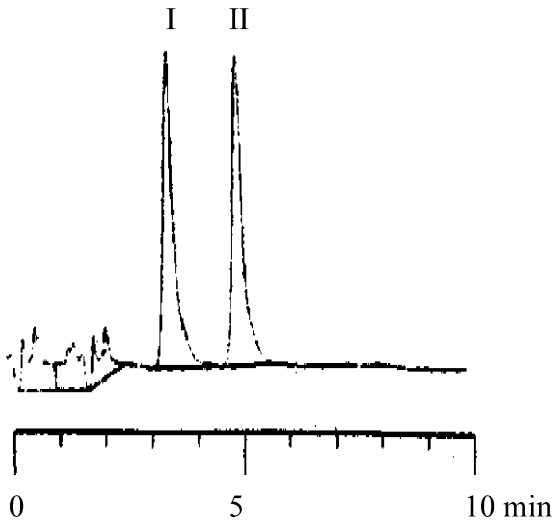

Fig. 1. HPLC chromatogram of a table olive extract (sample 9) containing: 6.9 μg/ml of benzoic acid (I) and 5.44 μg/ml of sorbic acid (II).

3.2. Extraction of benzoic and sorbic acids from different food matrices

3.2.1. Jams, jellies and table olives

Optimization of benzoic and sorbic acids analyses carried out in quince jams some years ago showed that extraction of preservatives with methanol, followed by HPLC/UV analyses was a good alternative to achieve accurate and precise results in a short period of time. Results were in good agreement with the AOAC reference methods (Ferreira et al., 2000). Similar sample preparation was used for other types of jams and table olives. Table 1 gives validation data for those matrices. Extraction recoveries for benzoic and sorbic acids, spiked at different concentrations indicate no significant effect of analyte concentration on recoveries.

The precision of the method was also evaluated in the three matrices ($n = 6$). The coefficients of variation were always lower than 2.6%

3.2.2. Spreadable fats and sauces

The extraction of sorbic acid from spreadable fats and emulsified sauces with methanol was not appropriate. It was not possible to disperse these matrices in methanol. Thus, they were diluted with *n*-hexane and thereafter, different extraction procedures were tried, including liquid–liquid extraction with different solvents (methanol, acetate buffer, water) or purification using Sep-Pak C18 cartridge. Liquid–liquid extraction with methanol was not recommended, because of the emulsion that was obtained. Liquid–liquid extraction with acetate buffer gave higher recoveries than liquid–liquid extraction with water or even with Sep-Pak C18 (Table 2). Average recoveries of 100 and 200 mg/kg of sorbic acid spiked in sample 48, which contained 466 mg of sorbic acid/kg, were 98.3 and 98.7%, respectively. The CV

Table 1
Recoveries of benzoic and sorbic acids from spiked quince jam, strawberry jam, jelly and table olives

Matrix (sample)	Benzoic acid mg/kg	CV% ($n=6$)	Sorbic acid mg/kg	CV% ($n=6$)	Added amount mg/kg	Percentage of recovery	
						Benzoic acid	Sorbic Acid
Quince jam (sample 23)	646	2.6	116	1.2	10	96.3±2.4	98.9±0.9
					50	95.4±1.2	96.2±1.6
					100	97.0±0.9	99.1±2.6
					200	98.1±1.4	95.2±0.9
Strawberry jam (sample 39)	798	2.1	–	–	10	92.4±2.2	91.4±2.1
					50	96.2±1.7	96.1±0.6
					100	98.4±0.9	93.0±2.0
					200	91.4±2.5	97.3±2.2
Strawberry jelly (sample 45)	825	1.7	–	–	10	93.2±3.2	92.4±1.1
					50	95.2±1.7	94.1±0.6
					100	98.4±0.9	91.0±3.0
					200	93.4±2.5	95.3±4.1
Table olives (sample 8)	68.0	1.9	63.7	1.2	10	96.1±0.9	92.7±2.2
					50	98.3±1.3	94.2±0.5
					100	91.3±0.8	93.6±1.2
					200	98.4±1.1	91.9±0.8

Table 2
Recoveries of sorbic acids from spiked spreadable fats and sauces

Matrix (sample)	Sorbic acid mg/kg	CV% ($n=6$)	Added amount mg/kg	Percentage of recovery		
				Liquid–liquid extraction acetate buffer	Liquid–liquid extraction water	Sep-Pak C18
Spreadable fats (sample 48)	466	3.1	100	98.6±1.1	93.0±1.2	97.0±1.1
			200	98.4±0.8	92.3±1.0	98.3±1.2
Sauce (sample 72)	820	2.8	100	98.1±1.0	94.1±1.3	97.6±1.1
			200	99.6±2.0	93.9±2.6	97.9±0.9

($n=6$) was less than 2.9%. Similar recoveries of sorbic acid (higher than 98%) were obtained for sample 72, which contained 820 mg of sorbic acid/kg and was spiked with 100 and 200 mg/kg. The CV ($n=6$) was less than 3.1%.

3.2.3. Fruit juices and wines

Purification of beverages using solid phase extraction was recommended as higher recoveries were obtained. With respect to wine analyses this sample preparation procedure eliminated interference from ethanol. The results obtained from recovery studies in fruit juices and wine samples are listed in Table 3.

Six different extractions, as described above, were made for each sample and subsequently injected. The CV ($n=6$) reported was less than 4%.

Table 3
Recoveries of benzoic and sorbic acids from spiked fruit juices and wines

Matrix (sample)	Benzoic acid mg/kg	CV% ($n=6$)	Sorbic acid mg/kg	CV% ($n=6$)	Added amount mg/kg	Percentage of recovery	
						Benzoic acid	Sorbic acid
Orange juice (sample 75)	198	3.1	39.1	2.2	25	98.3±2.1	94.0±1.5
					75	96.4±0.9	95.3±1.1
Apple juice (sample 81)	–	–	153	2.6	25	97.1±1.3	95.1±0.9
					75	97.6±2.1	96.9±0.6
Pineapple juice (sample 83)	179	4.0	52.0	1.2	25	98.9±3.8	94.1±2.9
					75	97.3±2.5	95.9±4.0
Wine (sample 85)	169	3.0	–		25	95.0±2.1	98.8±1.2
					75	96.4±3.7	94.9±3.6

3.3. Analysis of commercial samples

Benzoic and sorbic acid contents in black, green and turnover table olives were evaluated. Eight table olive samples presented benzoic acid (samples 1, 6, 8, 9, 10, 13, 15 and 20, concentrations ranged from 4.2 ± 0.2 to 199.2 ± 1.0 mg/kg of product) and 10 contained sorbic acid (samples 2, 3, 6, 7, 8, 9, 12, 16, 18 and 19, concentrations ranged from 5.3 ± 0.3 to 268.5 ± 2.1 mg/kg of product). Preservatives were not detected in five samples (4, 5, 11, 14, 17).

In all cases the levels found for benzoic and sorbic acid contents were below the maximum allowed by European legislation, 500 mg/kg for benzoic acid when used alone or 1000 mg/kg for sorbic acid. The sum of benzoic and sorbic acid contents can not exceed 1000 mg/kg (Directive no. 98/72/CE).

Sorbic and benzoic acid contents in quince jams were quite heterogenous, ranging from not detected (n.d.) to 789 ± 6 mg/kg and n.d. to 639 ± 16 mg/kg of product, respectively. The large variability among the commercial brands of quince jam, were confirmed by the high F-values ($P < 0.001$) obtained from the statistical analysis for benzoic acid ($F = 809.11$) and sorbic acid ($F = 1025.92$). European legislation established a maximum of 1500 mg/kg for total content of preservatives (sorbic and benzoic acids alone or simultaneously) (Directive no. 98/72/CE). All the samples were in accordance to that requirement. With respect to other fruit jams, and jellies they presented only sorbic acid ranging between 778 and 1103 mg/kg.

Legislation only allows the use of sorbic acid as preservative in spreadable fats, establishing a maximum of 1000 mg/kg in samples with fat content higher than 60% and 2000 mg/kg in the case of lower percentage. The results revealed that no benzoic acid or their salts were added to the samples under study. Sorbic acid was not detected in four samples (60, 61, 69 and 70). The other samples contained sorbic acid ranging from 189 to 897 mg /kg of product.

No significant differences ($P = 0.323$) were found between contents of sorbic acid in samples with a fat content above 60% and in samples with a fat content below 60%.

All fruit juices analysed presented sorbic acid and/or benzoic acid ranging from n.d. to 210 ± 5.2 and n.d. to 153 ± 1.1, respectively. All samples were under the imposed maximum concentrations of 300 mg/l for sorbic acid alone or 150 mg/l for benzoic acid alone. When used simultaneously the maximum is 250 mg/l for sorbic acid and 150 mg/l for benzoic acid. Most of the samples presented the two preservatives except four samples were sorbic acid was not found (samples 71, 72, 76, 77) and one sample (78) were benzoic acid was not detected.

In the three samples of commercial wines that were analysed only sorbic acid was detected in contents below 200 mg/l, which complied with the legislation for preservatives in wines (Directive no. 98/72/CE).

4. Conclusions

The described extraction procedures seems to fulfil the criteria of selectivity, sensitivity, reproducibility and convenience for analysing the sorbic and benzoic acid contents in the respective matrices under study. Thus, they are suitable for routine assays. Total analysis time, including extraction and HPLC analysis is lower than 20 min. A clean and interference-free baseline is typical for the sample matrices tested in this study.

The general detection of benzoic and sorbic acids, leads to the conclusion that they are commonly used as preservatives in several food products available in the retail market. All the products that declared "no preservatives" were in accordance with their label claims.

In conclusion, consumers' interests are safeguard as all samples conformed to the imposed maximum concentrations.

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