SIMULTANEOUS DETERMINATION OF SORBIC AND BENZOIC ACIDS IN TOMATO SAUCE AND KETCHUP USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract
According to Directive 95/2/EC sorbic acid and its salts (E200-E203) and benzoic acid and its salts (E201-E213) belong to the preservatives allowed conditionally, and the quantities added to foods are restricted by upper limits.
As all tomato sauce and ketchup available on our market are made with sorbic and benzoic acid addition and their consumption is high, the operational parameters leading to the development of a method for the simultaneous analysis of sorbic and benzoic acid within tomato sauce and ketchup have been studied. The technique used is the high performance liquid chromatography together with diode array detection (DAD).
For analysis, the sample is extracted with water. The chromatographic separation was performed by using potassium dihydrogen orthophosphate buffer (pH 2.3) and methanol (65:35, v/v) as mobile phase, a DS Hypersil C18,5 μm column (250 mm×4,6 mm) and "diode array" detection at λ=230 nm for benzoic acid and λ=254 nm for sorbic acid. The analysis time was less than 15 min.
The method was validated in terms of sensitivity, linearity range, reproducibility, repeatability and analitycal recovery. The calibration curves showed good linearity over the concentration range of 0-50 mg/L. The method provides stable retention times and limits of detection of 2.4 and 0.4 mg/kg for benzoic and sorbic acids, respectively. The mean recovery of benzoic acid was 100.52% while for sorbic acid was 94.37%.
The presence of benzoic and sorbic acids in ketchup samples available on the Roumanian market was also determined. The concentrations were well below the limit allowed by the Directive 95/2/EC.

Keywords: benzoic acid, sorbic acid, method validation

1. INTRODUCTION

Chemical preservation has become an increasingly important practice in modern food technology with the increase in production of processed and convenience foods. These preservatives are deliberately added to stop or delay nutritional losses due to microbiological, enzymatic or chemical changes and thus increasing its shelf life. Benzoic acid and sorbic acid are generally effective to control mold and inhibit yeast growth, and against a wide range of bacterial attack [1–5].
Sorbic acid and its salts are physiologically harmless, but may still influence the taste of food. They show a strong effect on micro-organisms over a wide pH range, including high levels.
Benzoic acid and its salts may have allergic effects on susceptible persons.

The use of benzoic and sorbic acids as preservatives for various kinds of foods is permitted throughtout the European Community.
According to Directive 95/2/EC dated on 20.02.1995 [6] which, by article 1 paragraph 3 a, points that "the preservatives are substances that increase the food preservation time by protecting them against the damages caused by micro-organisms", sorbic acid (E200), potassium sorbate (E202), calcium sorbate (E203), benzoic acid (E210), sodium benzoate (E211), potassium benzoate (E212) and calcium benzoate (E213) belong to the preservatives allowed conditionally, singly or in combination.
As the directives of the European Union regulate the applicability fields and the maximum quantities for each of these preservatives, it is required that methods should be elaborated and validated for determining their quantity.
The most common analytical method for the determination of benzoic and sorbic acids has

been reversed-phase HPLC [2-5, 8-10], although other analytical methods such as TLC, capillary electrophoresis [7] and gas chromatography [10] have also been reported.
However, chromatographic reports on the simultaneous determination of benzoic and sorbic acids especially in food items are scarce [5,9]. Such a method is important as there seem to be an increasing trend in using combination of preservatives, not only in the food industry but also in pharmaceutical formulations and cosmetic products [8]. Moreover, many of the reported methods use complicated and laborintensive pre-treatment procedures such as steam distillation multiple-steps and solid-phase extractions.
Here we report on a simplified water extraction procedure followed by HPLC separation of a mixture of benzoic acid and sorbic acid. The developed method was validated to the analysis of these preservatives in tomato sauce and ketchup.

2. MATERIALS AND METHODS

Benzoic acid - certified reference material with 99.9% purity has been used, produced by Supelco (47508). All the other reagents, acetonitrile (Baker, 8257), potassium dihydrogen orthophosphate 98+% (Alfa Aesar, A12142), phosphoric acid 85% (Merk, 1805), sorbic acid 99.0% (Alfa Aesar, A16196) were of analytical purity or for chromatographic use. The water used was ultrapure, Basic TWF. The stock solution and the corresponding dilutions were made in ultra-pure water and were stored in dark places between the experiments, at low temperature (+4°C).
Mainly, the sample is extracted with water. The benzoic and sorbic acids in the sample test solution are separated by reversed phase chromatography on a 250 mm×4.6 mm i.d., 5 µm particle DS Hypersil C18 column, detected by absorbance and quantified with external calibration graphs. For the simultaneous detection of the two analytes, the detector was set at λ=230 nm for benzoic acid and λ=254 nm for sorbic acid. This setting was chosen because sorbic acid has a maximum adsorption near 254 nm.
HPLC was performed with a Surveyor Thermo Electron system comprising vacuum degasser, Surveyor Plus LCPMPP pump, Surveyor Plus ASP autosampler, diode array detector with 5 cm flow cell and Chrom Quest 4.2 software.
The determinations were made in isocratic conditions, at 40°C, using a mobile phase made of 65% phosphate solution (dissolve 6.8 g potassium dihydrogen phosphate in 900 mL water. The pH value should be adjusted to pH =2.3 with phosphoric acid and then filled to 1000 ml with water) filtered through a polyamide membrane (0.2 µm) and 35 % methanol. The volume injected was 5 µL and the flow rate of the mobile phase was 1mL/min. For preparing the sample, 2.5 g of homogenized sample are weighed, to the nearest 1 mg, into a 50 ml volumetric flask, 30 ml water are added and the flask is placed in an ultrasonic bath at 20°C for 10 minutes. The solution is then diluted to the mark with water. The test solution is filtered through a membrane filter (0.45 µm) before injection.

3. RESULTS AND DISCUSSION

Method validation. Determination of the performance parameters for the developed method

To test linearity, standard solutions of 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L and 50 mg/L were prepared and analyzed with three replicates and the results processed with Chrom Quest 4.2 software. The calibration graphs are linear, with five calibration levels. The equations of the calibration graphs, the correlation coefficients and the selected wavelengths for the analytes detection are shown in table 1.

Table 1. Equations of calibration graphs and correlation coefficients (r^2) for the two analytes

Analyte	λ, nm	Expected retention time (min)	Equation of the calibration graph	r^2
Benzoic acid	230	11.0667	y = 7.91951e-006x – 0.0223879	0.9995
Sorbic acid	254	12.0667	y = 3.66776e-006x	0.9994

The linearity range was 1–50 mg/L (y = peak area in mAU (milli absorbance units)×s; x = concentration in mg/L).
To test peak area and retention time reproducibility, Chrom Quest software allows the calculation of the relative standard deviations (RSD) for the retention time of the analytes for all levels of the calibration graph and for peak area at each calibration level.
Precision of areas must be < 2% RSD while precision of retention times must be < 0.5% RSD.
The relative standard deviations (RSD) for the retention time were 0.070% for benzoic acid and 0.066% for sorbic acid therefore, in standard solutions, the HPLC method developed for the chromatographic separation of the two analytes provides stable retention times. The calculation of peak areas led to RSD between 0.104% and 0.399% for benzoic acid and between 0.129% and 0.367% for sorbic acid. Moreover, the calculated relative standard deviations also prove stability in terms of peak height and asymmetry.
In order to establish the method traceability on real samples, a sample of ketchup available for sale was taken for analysis. The sample was analysed according to the developed method and we find 356.86 mg of benzoic acid /kg and 297.76 mg of sorbic acid /kg.
Four increasing addition levels of the two analytes were added to this sample. Thus, 0.3, 0.6, 0.9 and 1.2 mL of stock solution having a concentration of 1g/L of each analyte were added in the 50 mL volumetric flask and the solution was then diluted up to the mark with water. This might correspond to an addition of 6, 12, 18 and 24 mg/l respectively in the sample test solution. The final concentrations of the two analytes in the addition test solutions were calculated.
The four addition samples and the witness were chromatographically analyzed with three injections, according to the developed method, and for each analyte samples were treated in a way similar to a five point-calibration graph, with the calculated concentrations on the abscissa axis, as pointed above, and with the peak areas corresponding to the analytes in the addition samples on the ordinate axis. The resulted calibration graphs are linear and have five calibration levels, the first level representing the addition free test solution witness. The equations of these graphs, the correlation coefficients and the wavelengths selected for detection of the analytes are shown in table 2.

Table 2.Equations of calibration graphs and correlation coefficients (r^2) of analytes for real samples with addition

Analyte	λ, nm	Equation of the calibration graph	r^2
Benzoic acid	230	y = 7.92208e-006x – 0.814978	0.9982
Sorbic acid	254	y = 4.13970e-006x – 2.68416	0.9978

Chromatograms of one addition sample are shown in figures 1 and 2.
Note that addition samples have analytes concentrations within the linearity range of the method (0 – 50 mg/L).
Similar to the determination of the method traceability for standard solutions, we tested the reproducibility of the peak areas and retention times for the traceability on real samples, as well.
The relative standard deviations (RSD) for the retention time was of 0.215% for benzoic acid and of 0.203% for sorbic acid, therefore, on real samples, the HPLC method developed for the separation of the two analytes provides stable retention times. The calculation of peak areas led to very good RSD values below 2%, i.e. between 0.068% and 0.333% for benzoic acid and between 0.025% and 0.285% for sorbic acid. Furthermore, the relative standard deviations also prove stability in terms of peak height and asymmetry.
In order to verify the reproducibility, the standard solution of 50 mg/L was analyzed by 10 repeated injections.
For the peak areas, the relative standard deviations (RSD) were 0.105% for sorbic acid and 0.265% for benzoic acid, which shows very good reproducibility of the method developed. The data demonstrate the high reliability and precision of the Surveyor Thermo Electron system, since the criteria for

retention times and areas are fulfilled for the two compounds.

The method repeatability shows the variability noticed inside a laboratory in a short period of time, using a single operator, equipment etc.

preparing the sample and chromatographically analyzing it, according to the developed method.

The relative standard deviations for retention times, peaks areas, peaks heights and peaks asymmetries for the eight replicates are presented in table 3 and show very good

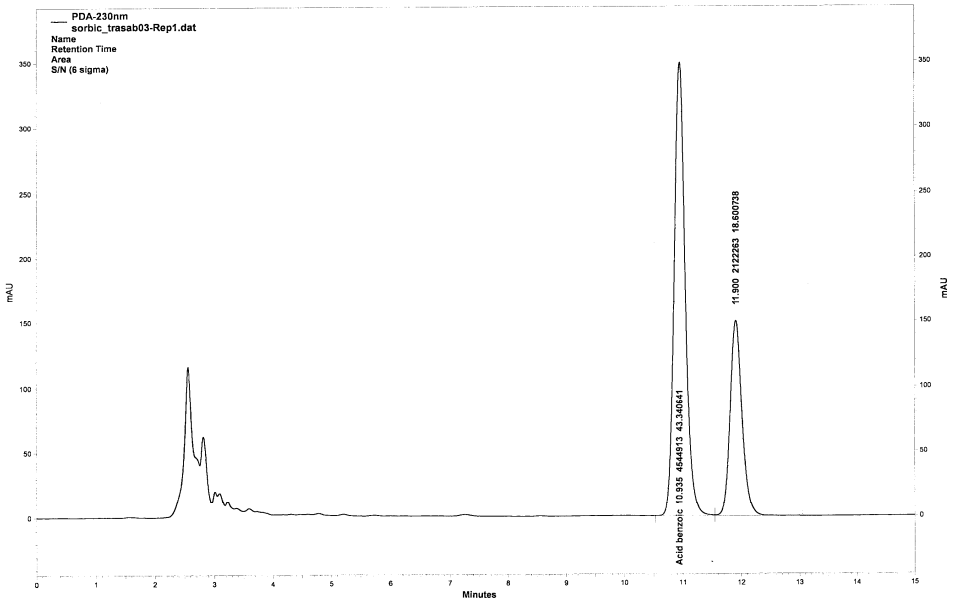

Fig.1. HPLC at λ=230 nm of a ketchup with addition (level 3).

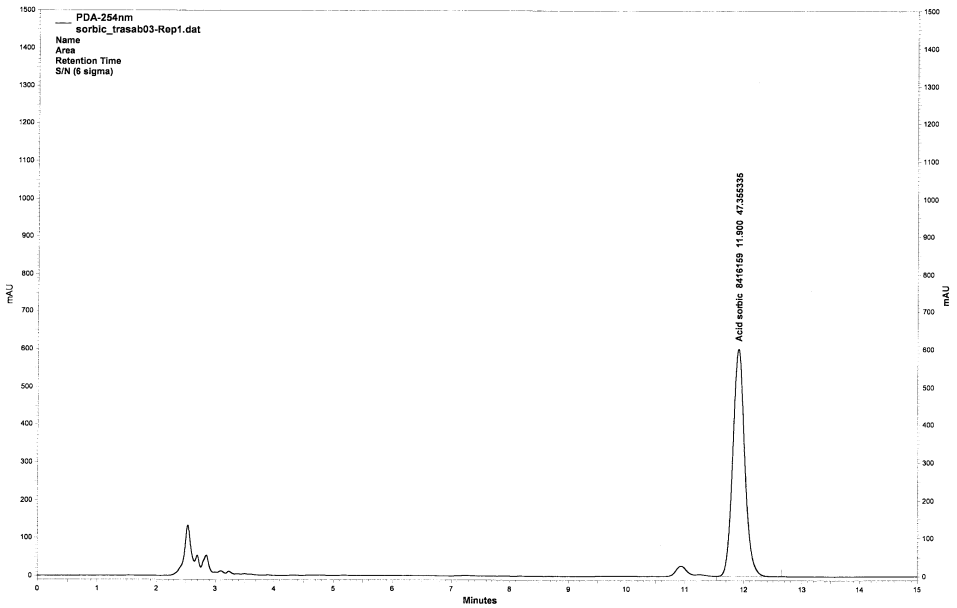

Fig.2. HPLC at λ=254 nm of a ketchup with addition (level 3).

The repeatability of the method was verified through the analysis of a ketchup sample, which was fully processed eight times, every time

repeatability of the method developed.

Table 3. The relative standard deviations for retention times, peaks areas, peaks heights and peaks asymmetries of benzoic and sorbic acids at the repeatability determinations

Analyte	λ, nm	RSD (%)			
		Retention time	Peaks areas	Peaks asymmetries	Peaks heights
Benzoic acid	230	0.215	0.333	0.214	0.353
Sorbic acid	254	0.203	0.283	0.172	0.295

The bias of an analytical method is usually determined by study of relevant reference materials or by spiking studies. A spiked recovery study was performed using an experiment similar with the one which was developed for the traceability on real samples. The mean recovery of benzoic acid was 100.52% while for sorbic acid was 94.37%.

The limit of detection (LOD) is defined as the smallest peak detected with a signal height three times that of the baseline while the limit of quantitation (LOQ) refers to the lowest level of analyte which can be determined with an acceptable degree of confidence. In our work, detection limits were estimated starting from the principle that a peak, to be detected, must have a signal-to-noise ratio > 3. The detection limit was 0.12 mg/L in the test solution for benzoic acid, which corresponds to a concentration of 2.4 mg/kg in the analyzed sample while for sorbic acid the detection limit was 0.02 mg/L, which corresponds to a concentration of 0.4 mg/kg in the analyzed sample.

The presence of benzoic and sorbic acids in ketchup samples available on the Roumanian market was also determined. Eight commercial brands of ketchup were analysed. All contained benzoic and sorbic acids. The concentration ranged from 348.06 to 472.56 mg of benzoic acid/kg and from 291.72 to 420.32 mg of sorbic acid/kg of ketchup.

4. CONCLUSIONS

An isocratic HPLC technique is described for the determination of benzoic acid and sorbic acid in industrial ketchup and tomato sauce. The chromatographic separation was achieved with a C18 column and phosphate buffer (pH=2.3) - methanol (65:35) as the mobile phase. The effluent was monitored at 230 nm for benzoic acid and at 254 nm for sorbic acid. Effective separation and quantification was achieved in less than 15 min.

The method was validated in terms of sensitivity, linearity range, reproducibility, repeatability and analytical recovery. Mean recoveries of 100.52% for benzoic acid and 94.37% for sorbic acid were obtained while detection limits of 2.4 and 0.4 mg/kg were obtained for benzoic and sorbic acids, respectively. Results were in good agreement with the reference methods. The presence of benzoic and sorbic acids in ketchup samples available on the Roumanian market was also determined. The concentrations were well below the limit allowed by the Directive 95/2/EC.

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