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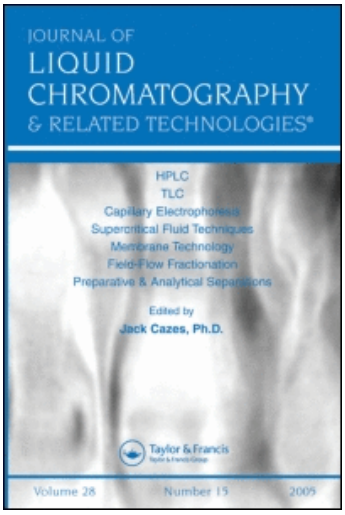

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF CHEMICAL PRESERVATIVES IN YOGURT

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ABSTRACT

Reverse phase HPLC was used to determine the preservatives methyl-, ethyl- and propyl-*p*-hydroxybenzoate and sorbic acid in aqueous solutions, using *n*-butyl-*p*-aminobenzoate as the internal standard. This method was applied to the analysis of preservatives in yogurt. The preservatives were previously identified by thin layer chromatography, and revealed at 254 nm.

INTRODUCTION

Chemical preservatives are being used in foodstuffs more and more frequently. The FAO/WHO lists preservatives under food additives. The EC definition (1) describes them as substances which, either alone or in combination, (are able to) inhibit, retard or avoid processes of fermentation, molding, putrefaction and other chemical and biological alterations in foods and drinks.

Most preservatives are organic molecules which behave as weak acids. Owing to their chemical structure, they absorb light in

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the UV region of the spectrum, a factor which makes it possible to analyze them spectrophotometrically. Beutler *et al.* (2) used an enzymatic method to determine sorbic acid in fruit preserves, and the AOAC (3) has proposed spectrophometric methods for the analysis of preservatives of cyclic structure.

Gas chromatography has also been applied in this field. The Nordic Committee on Food Analysis (4) has proposed a reference method for the determination of ascorbic and benzoid acid after extraction of the sample to obtain the trimethyl-silyl derivatives. The standards recommended for benzoic and sorbic acid were phenylacetic acid and caproic acid respectively.

Undoubtedly the most suitable quantitative method for determining preservatives in foodstuffs is high performance liquid chromatography (HPLC). Terada *et al.* (5) determined sorbic, benzoic and *p*-hydroxybenzoic acid simultaneously (in foods), as well as the methyl, ethyl and propyl esters of the latter. The extraction of these substances was performed with several different methods, including steam distillation, Sep-Pak C_{18} cartridges). These authors used reverse phase, with acetonitrile-water-0.02M phosphate buffer (7:12:1) as the eluent. A recovery rate of 93.8% was achieved in soft drinks. De la Rivera (6) determined benzoic acid in soft drinks with C_{18} column chromatography, using acetate buffer (pH 4.4)-acetonitrile (4:1).

We adapted the method of analysis used by Mase *et al.* (7) to determine esters of *p*-hydroxybenzoic acid in children's cosmetics, in order to analyze these preservatives and sorbic acid in yogurt. The preservatives were identified by thin layer chromatography with polyamide plates equipped with a fluorescence indicator (8).

MATERIALS AND METHODS

Reagents

All solvents used were of high purity grade for HPLC: methyl alcohol (Merck), benzoic acid, sorbic acid, methyl-, ethyl- and

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TABLE 1

Theoretical and Experimental Values of R_f for Preservatives Determined in Commercial Brands of Yogurt.

	R_f values	
Preservative	Theoretical	Experimental
Benzoic acid	0.48	0.40
Methyl-*p*-hydroxybenzoate	0.30	0.22
Ethyl-*p*-hydroxybenzoate	0.36	0.28
Propyl-*p*-hydroxybenzoate	0.45	0.31
Sorbic acid	0.61	0.48

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propyl-*p*-hydroxybenzoate, *n*-butyl- and *p*-amine benzoate (Sigma). 11 F_{254} polyamide plates for chromatography were from Merck.

Equipment

HPLC instrumentation consisted of a Konic model KNK-500 A chromatograph, a Konik-UV1S-200 UV absorbance detector and a Hewlett-Packard HP 3394 A computing integrator., The C_{18} columns were packed with S5 ODS 2.

Method

Thin layer chromatography was performed with ethyl alcohol solutions of the following preservatives: 0.012% benzoic acid, 0.08% methyl- ethyl- and propyl-*p*-hydroxybenzoate, 0.06% sorbic acid. Ten microliters of each solution was placed with a micropipet at a point 2 cm from the lower edge of the plate, to form bands spaced 2 cm apart. In a separation chamber, the samples were eluted with benzene-ethyl acetate-acetic acid (85:10:5). When the front had moved 10 cm, the plate was dried at room temperature. Preservatives were developed under a 254 nm UV lamp. The R_f values for each product are shown in Table 1.

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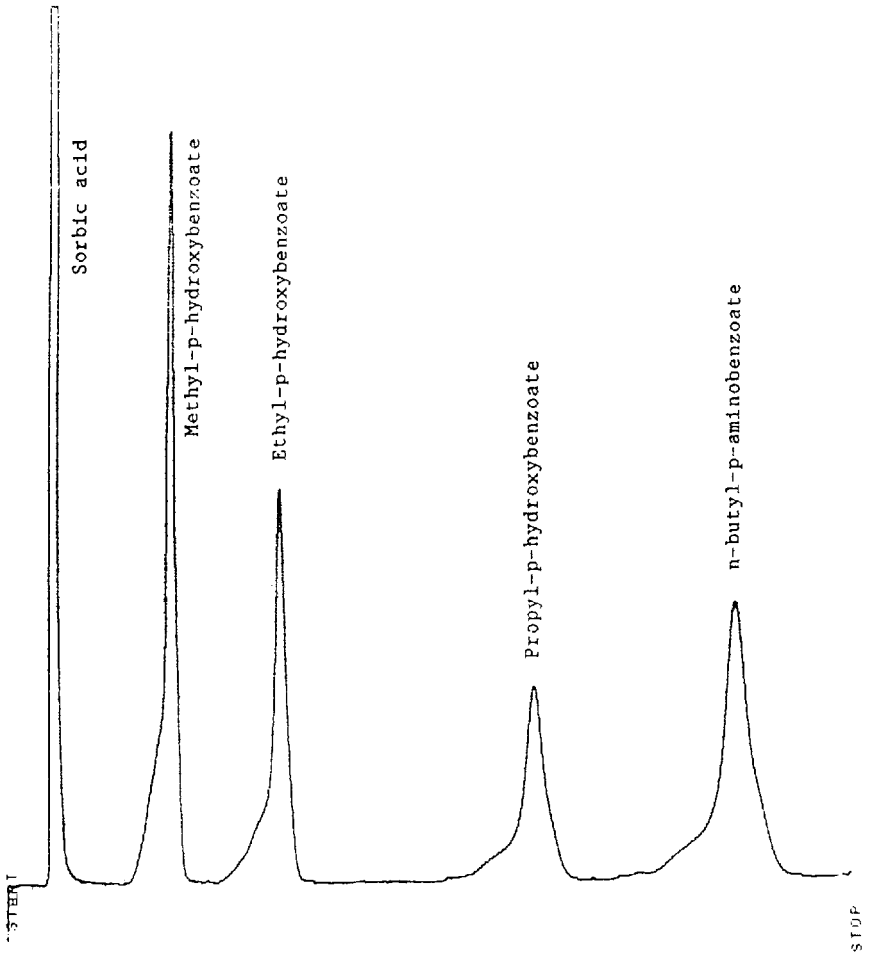

FIGURE 1. Chromatogram of a mixture of preservatives. Retention times: Sorbic acid, 1.60; Methyl-*p*-hydroxybenzoate, 4.46; Ethyl-*p*-hydroxybenzoate, 9.08; Propyl-*p*-hydroxybenzoate, 17.37; n-butyl-p-aminobenzoate, 26.30.

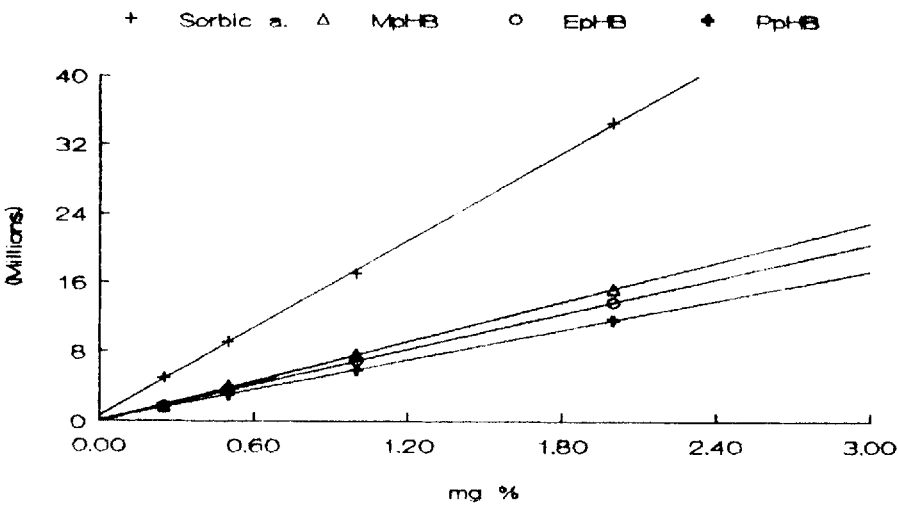

FIGURE 2. Calibration curves for linearity and sensitivity of detection. Sorbic acid, y = 16.924x + 0.558; Methyl-*p*-hydroxybenzoate, y = 7.695x + 0.063; Ethyl-*p*-hydrozybenzoate, y = 6.834x + 0.075; Propyl-*p*-hydroxybenzoate, y = 5.738x + 0.149.

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High performance liquid chromatography was carried out under the following conditions: UV detector 254 nm, eluents: methanol-water (1:1), flow rate 1 ml/min, room temperature, injection volume 20 µl, retention times: 2 mg/100 ml of each preservative in methanol. The values obtained are shown in Figure 1.

The internal standard method was used for quantitative analyses. An 0.2% solution of *n*-butyl *p*-aminobenzoate in methanol was used for the mixture of parahydroxybenzoates and sorbic acid.

Linearity and sensitivity of the detector were calculated from a series of solutions from 0.25 to 2.0 mg/100 ml. Figure 2 shows the calibration curves and the linear equations. The response factor was calculated from these data with the help of an integrating computer according the the equation $f_i = P_i/P_o.A_o/A_i$.

RESULTS

We analyzed commercial yogurts from local food stores. Preservatives were extracted as follows: to 100 g of homogenized sample we added 15 ml 10% H_2SO_4 solution and 45 ml boiling water, 10 ml 15% potassium ferrocyanide and 10 ml 30% zinc acetate. The mixture was simmered for 30 min and hot filtered with through a Buchner apparatus. If the filtrate was turbid, 2 ml potassium ferrocyanide, 2 ml zinc acetate and 20 ml saturated NaCl solution were added and the mixture was filtered again. The pH of the filtrate was adjusted to 3.0.

The preservatives were extracted three times on a 50 ml aliquot of the above mentioned mixture with 30 ml volumes of ethyl ester. The ether extracts were evaporated to dryness and the residue was redissolved in an exact volume of methanol for qualitative or quantitative analysis.

The percentage of preservative recovered was found by adding a commercial product contanining no additives to a known amount of each preservative. After extraction according to the procedure illustrated in Figure 1, quantitative analysis was carried out as follows:

The dry extract was redissolved in 5 ml methanol, and a 1 ml aliquot was dissolved in 100 ml methanol. To 1 ml of this second dilution we added 10 µl of an 0.2% solution of *n*-butyl-*p*-aminobenzoate (internal standard), and the mixture was injected into the chromatograph and run under the conditions described under Materials and Methods. The percentage recovery rate and amounts of each preservative added are shown in Table 2.

A total of 25 different commercial yogurts of 5 different brands were analyzed.

Thin layer chromatography as described above was first used for the qualitative analysis to identify the preservatives. Based on the values of R_f, the results were positive for 20 of the 25 samples analyzed. In all cases, sorbic acid was the only preservative identified (R_f=0.48).

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TABLE 2

Percentage Recovery of Preservatives after Extraction and HPLC Analysis.

Substance	ppm added	ppm found	% recovered	Standard error	Standard deviation
Sorbic acid	18	17.33	96.27	1.02	0.51
Methyl-*p*-HB	90	82.0	91.10	0.49	0.24
Ethyl-*p*-HB	90	82.3	91.44	0.53	0.35
Propyl-*p*-HB	90	85.1	94.50	0.93	0.48

HB = hydroxybenzoate

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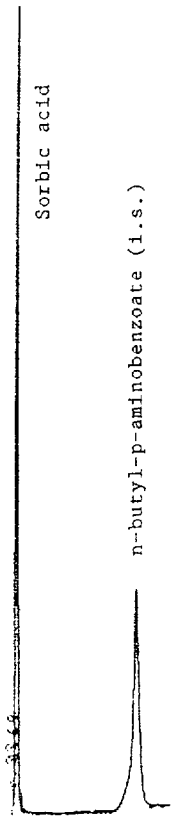

FIGURE 3. Chromatogram of sorbic acid in a sample, and internal standard.

TABLE 3

Sorbic Acid Content in Commercial Samples of Yogurt.

Sample no.	Sorbic acid	Sample no.	Sorbic acid
1	386.5	11	209.3
2	293.0	12	370.5
3	510.5	13	589.3
4	520.0	14	160.9
5	600.5	15	290.5
6	385.4	16	650.2
7	505.3	17	550.6
8	503.0	18	297.4
9	540.0	19	250.5
10	410.5	20	225.3

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The 20 positive samples were analyzed with HPLC, which yielded chromatograms like the one shown in Figure 3. The results for all samples are summarized in Table 3.

DISCUSSION

The use of *n*-butyl-*p*-aminobenzoate as the internal standard allowed us to analyze sorbic acid, methy-, ethyl- and propyl-*p*-hydroxybenzoic acid in different mixtures without resorting to the use of gradients, as the retention times used were adequate under our working conditions. The extraction process used for dairy products led to a recovery of better than 90%, which, in view of the permissible concentrations of sorbic acid in foodstuffs, makes it possible to evaluate the potential health risk of these additives. The permissible daily intake of these subtances, based on FAO/WHO recommendations, allows for a much higher content from a toxicological point of view.

The samples analyzed in this study, which was undertaken as an example of applied analytical techniques, contained amounts of sorbic acid below the 600 ppm limit permitted by international regulations.

ACKNOWLEDGEMENTS

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