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Determination of the alkyl esters of *p*-hydroxybenzoic acid in mayonnaise by high-performance liquid chromatography and fluorescence labelling

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Abstract

A reliable and sensitive high-performance liquid chromatographic method with fluorescence detection is described for the determination of methyl, ethyl and propyl *p*-hydroxybenzoates in mayonnaise with the internal standard method using *n*-butyl *p*-hydroxybenzoate as the internal standard. The parabens were extracted with acetonitrile and determined, after derivatization with 4-bromomethyl-6,7-dimethoxycoumarin, by reversed-phase liquid chromatography (C_{18} column) with fluorescence detection at $\lambda_{ex.} = 355$ nm and $\lambda_{em.} > 420$ nm using an aqueous methanolic eluent and linear gradient elution. The method was tested on a mayonnaise sample spiked with methyl, ethyl and *n*-propyl *p*-hydroxybenzoate each at different levels (100, 500 and 1000 ppm). Average recoveries ranged from 93.6 to 99.4% with R.S.D. ranging from 1.8 to 4.0%.

1. Introduction

The alkyl esters of *p*-hydroxybenzoic acid (parabens) are compounds similar to benzoic acid and are used as food additives to prevent antimicrobial food spoilage. The use of parabens is very useful as they exert antimicrobial activity at pH 7 or higher, while benzoic acid has weak antimicrobial activity at this pH. The antimicrobial activity of *p*-hydroxybenzoic acid esters increases with increasing length of the alkyl chain of the ester group, but in practice the shorter esters are commonly used because of their high solubility in water.

Many antimicrobial compounds such as sorbic acid, benzoic acid, alkyl *p*-hydroxybenzoate and sulphite are used in the food industry. Their use in foods is related to the food characteristics and to the specific laws of the different countries. In Italy the methyl, ethyl and *n*-propyl *p*-hydroxybenzoates are authorised at various concentrations in preserved fish (maximum 1000 ppm), caviar and its substitutes (maximum 1000 ppm), rennet (maximum 10 000 ppm) and mayonnaise (maximum 1000 ppm) [1].

To determine the presence of antimicrobial additives in food, samples are prepared using different procedures such as extraction with acetonitrile (MeCN) [2], extraction with a mixture of MeCN, 2-propanol, ethanol and oxalic acid [3], simple steam distillation [4,5] or the use of a Sep-Pak C_{18} cartridge [5–7] or an Extrelut column [8].

Many methods for their determination have been described. They include UV spectrophotometry [9], thin-layer chromatography (TLC)

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[10] and gas chromatography (GC). The last method is carried out by directly injecting the extract obtained from the sample [11] or by injecting the trifluoroacetyl or benzoyl derivatives [12,13].

In the last 10 years, high-performance liquid chromatography (HPLC) with UV spectrophotometric detection has been used for the determination of preservatives in different foods. In particular, methods based on reversed-phase (RP) HPLC [2–4,5–8,14–17] have been more widely applied than those based on normal-phase HPLC [18]. RP-HPLC methods include ion-pair chromatography achieved using a cationic counter ion with a mobile phase that contains a pH 3.6 buffer [11], a pH 4.4 buffer [6] or an aqueous acetic acid solution at pH 4.5 [19]. Galensa and Schäfers [13], after derivatization (benzoylation), determined esters of *p*-hydroxybenzoic acid by normal- and reversed-phase HPLC.

Recently, Burini and Damiani [20] developed an HPLC method with fluorimetric detection for the determination of sorbic acid (another preservative) using 4-bromomethyl-6,7-dimethoxycoumarin as a fluorescence derivatizing reagent. This method, partially modified, has now been extended to the determination of parabens, which are characterized by a potentially reactive phenol group. 4-Bromomethyl-6,7-dimethoxycoumarin reacts with potassium salts of the alkyl esters *p*-hydroxybenzoic acid generated *in situ* with K_2CO_3 in MeCN and in the presence of 18-crown-6 to yield the corresponding fluorescent ethers as shown in Fig. 1. The derivatized compounds are separated on a reversed-phase column with gradient elution using aqueous methanol. The derivatization reaction, which converts the parabens into their corresponding fluorophores, and the chromatographic parameters were optimized; a method for the simultaneous determination of these compounds in mayonnaise was developed.

2. Experimental

2.1. Apparatus

A Varian (Palo Alto, CA, USA) Model 5000 liquid chromatograph, a sample-injection valve (Rheodyne, Cotati, CA, USA) with a 20-μl loop and a Varian Fluorichrom fluorescence detector were used. The following settings were applied: gain and lamp, LO; attenuator, ×20; excitation filters, CS 7-60/CS 7-54 ($\lambda_{ex.} = 355$ nm); emission filters, CS 3-73/CS 4-76 ($\lambda_{em.} > 420$ nm).

Separation was performed by RP-HPLC on a 5-μm Supelcosil LC-18 column (250 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, USA). The system was interfaced with an HP 3394 computing integrator (Hewlett-Packard, Avondale, PA, USA), with attenuation ×0 and chart speed 0.2 cm/min.

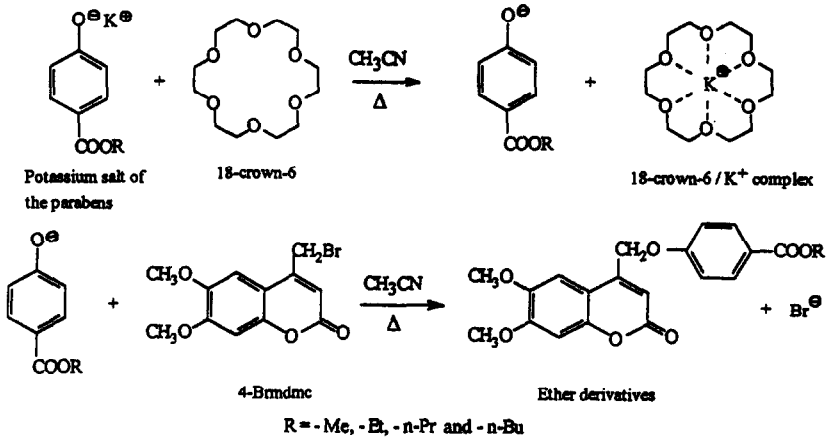

Fig. 1. Derivatization reaction for alkyl esters of *p*-hydroxybenzoic acid.

2.2. Reagents

Analytical-reagent grade methyl, ethyl, *n*-propyl and *n*-butyl *p*-hydroxybenzoate were purchased from Lancaster Synthesis (Morecambe, UK), sulphuric acid (96%) and small grains of K_2CO_3 from Carlo Erba (Milan, Italy), 4-bromomethyl-6,7-dimethoxycoumarin (4-Brmdmc) and 18-crown-6 from Sigma (St. Louis, MO, USA) and anhydrous sodium sulphate from BDH (Poole, UK). All the solvents (HPLC grade) such as acetone, water, acetonitrile and methanol were purchased from BDH.

Sulphuric acid of concentration 5 *M* was prepared by adding 14 ml of concentrated sulphuric acid to 30 ml of distilled water in a 50-ml volumetric flask. After cooling the flask, the solution was diluted to volume with distilled water.

Acetone solutions of 4-Brmdmc (1.05 mg/ml) and 18-crown-6 (1.26 mg/ml) were prepared. When refrigerated, these solution were stable for several weeks.

2.3. Preparation of standard solutions

A stock standard solution (1000 μg/ml) was prepared by dissolving 50.5 mg each of methyl, ethyl and *n*-propyl *p*-hydroxybenzoate in MeCN in a 50-ml volumetric flask. The internal standard (I.S.) solution was prepared by dissolving 51 mg of *n*-butyl *p*-hydroxybenzoate in 50 ml of MeCN. These two solutions were diluted with MeCN to obtain solutions containing 6.25 mg/ml of parabens. Aliquots of 1, 2, 3, 4, 5 and 6 ml of the solution containing methyl, ethyl and *n*-propyl *p*-hydroxybenzoate were mixed with 2 ml of the internal standard solution in separate 25-ml volumetric flasks. The solutions were then diluted to volume with MeCN. These working standard solutions, containing 0.25–1.5 μg/ml of the individual *p*-hydroxybenzoates and 0.5 μg/ml of the internal standard, were then derivatized and used to construct the calibration graph by HPLC analysis. The working standard solutions were stable for several weeks.

A solution containing 1 μg/ml of each paraben was prepared for an optimization study of the derivatization reaction.

2.4. Derivatization procedure

A 100-μl volume of the 18-crown-6 and 100 μl of the 4-Brmdmc acetone solutions were placed in a 10-ml test-tube equipped with a screw-cap and the acetone was evaporated in a gentle stream of nitrogen. Potassium carbonate (50 mg) and 0.5 ml of each working standard solution were added to the residue. The tube was then tightly closed and placed in a water-bath at 80°C for 2 min. After cooling to room temperature, 20 μl of the reaction mixture were injected into the liquid chromatograph.

Calibration graphs were obtained by plotting the peak-area ratios of methyl, ethyl and *n*-propyl *p*-hydroxybenzoate to that of the internal standard *versus* the concentration of the individual *p*-hydroxybenzoate. Fig. 2 shows a chromatogram for the derivatized standard solution containing 0.5 μg/ml of each preservative and 0.5 μg/ml of the internal standard.

2.5. Chromatographic conditions

The chromatographic conditions used were as follows: solvent A, water; solvent B, methanol; gradient elution programme, starting composition 45% of A and 55% of B, linear gradient from 55 to 100% of B in 35 min (1.29%/min), isocratic flow of 100% B for 10 min, return of the system to 55% B in 5 min; flow-rate, 1 ml/min; column pressure, initial value 105 bar, final value 46 bar; column, Supelcosil LC-18 (5 μm) (250 × 4.6 mm I.D.); fluorimetric detection.

2.6. Sample preparation

A 2-g amount of anhydrous sodium sulphate, 0.5 ml of the internal standard (1000 μg/ml), 6.5 ml of MeCN and 50 μl of 5 *M* sulphuric acid were added, in that order, to 0.5 g of sample weighed in a 10-ml test-tube equipped with

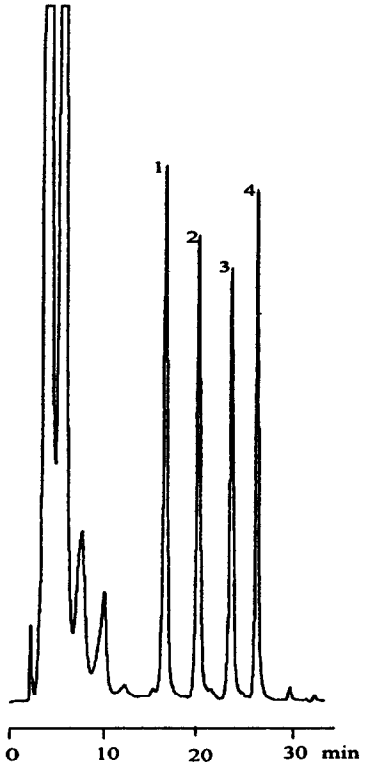

Fig. 2. Standard chromatogram of (1) methyl, (2) ethyl, (3) *n*-propyl and (4) *n*-butyl *p*-hydroxybenzoate (10 ng each).

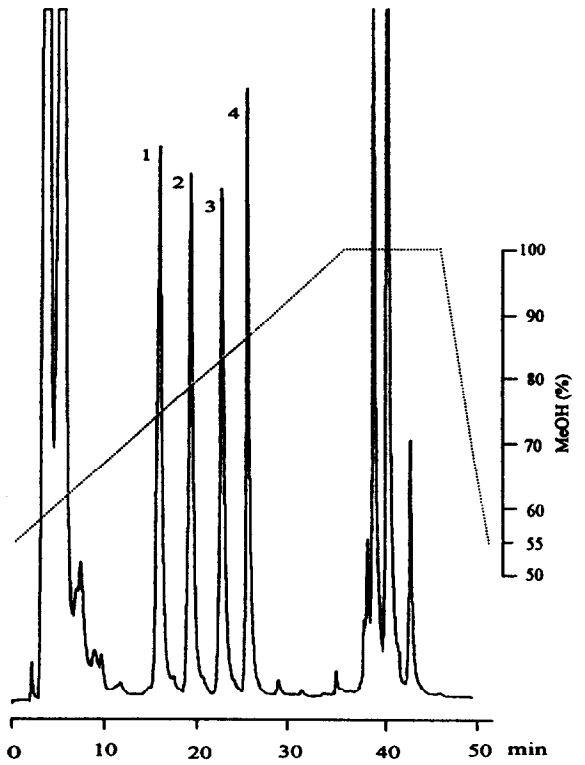

Fig. 3. Typical chromatogram of mayonnaise containing 100 μg/ml of (1) methyl, (2) ethyl, (3) *n*-propyl and (4) *n*-butyl (I.S.) paraben. Dilution factor of the extract sample = 1:2 (see Section 2.6). The linear gradient from 55 to 100% (v/v) methanol is indicated by the broken line.

screw-cap. The test-tube was closed, shaken for 20 s and centrifuged for 3 min at *ca.* 700 *g*. The supernatant was decanted into a 50-ml volumetric flask and the extraction was repeated once. Even if the solution was turbid at this stage, it was diluted to volume with MeCN (clear solution). Before derivatization and HPLC analysis this solution was diluted 1:20 with MeCN.

2.7. Determination by RP-HPLC

A 0.5-ml volume of the solution of the extract sample was derivatized as described for the working standard solutions (see section 2.4), using 200 μl of the 18-crown-6 and 200 μl of the 4-Brmdmc acetone solutions. A 20-μl volume of each solution was then injected in duplicate into the LC apparatus. The peak-area ratios of methyl, ethyl and *n*-propyl *p*-hydroxybenzoate to that of the internal standard were integrated on the calibration graphs for quantification of each paraben. The concentration of each paraben in the sample was calculated as follows:

$$\text{ppm } (\mu g/g) = \frac{c \cdot 50 \cdot 20}{m}$$

where c = concentration (μg/ml) of paraben obtained from the calibration graph, 50 = volume of the extract (ml), 20 = dilution factor of extract and m = mass of extracted sample (g). Fig. 3 shows a typical chromatogram for a sample of mayonnaise.

3. Results and discussion

The influence of temperature, time and 4-Brmdmc and 18-crown-6 concentrations on the derivatization reaction were examined; each experiment was performed as described in Section 2.4, changing one variable at a time and in each instance using a standard solution of 1 μg/ml of each paraben. Conditions of *ca.* 80°C, 2 min and a molar ratio of 30 and 40 for [4-Brmdmc]/[parabens] and [18-crown-6]/[parabens], respectively, were required for plateau formation for all the parabens. These were therefore adopted as the optimum derivatization conditions. An example of the results obtained in the optimization studies is shown in Fig. 4.

Parabens are compounds more reactive than carboxylic acids, *i.e.*, only 2 min at 80°C are required for complete derivatization.

n-Butyl *p*-hydroxybenzoate was used as the internal standard as it is similar to the sample components and further it is not allowed as an antimicrobial food additive in Italy. The internal standard (I.S.) procedure has greatest potential for precise quantitative results because the standard, which is included with each sample, eliminates the possible causes of error during extraction and derivatization of parabens. Further, changes in chromatographic conditions affect both the standard and component peaks equally.

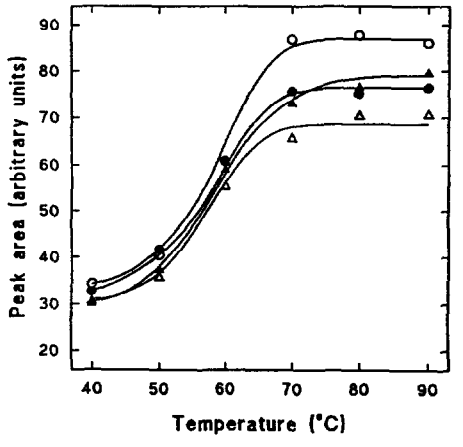

Fig. 4. Effect of temperature on the peak area for the derivatization reaction of the parabens. ○ = Methyl; ● = ethyl; △ = *n*-propyl; ▲ = *n*-butyl.

Acetonitrile was chosen as the extraction solvent of the parabens because, in addition to allowing good solubilization of the compounds of interest, it allows for their immediate derivatization without further clean-up treatment.

Calibration graphs were obtained from a plot of the peak-area ratios of methyl, ethyl and *n*-propyl *p*-hydroxybenzoate to that of the internal standard against the concentration of these preservatives. The graphs, in the concentration range 0.25–1.5 μg/ml, gave good linearity and passed through the origin. The limit of detection was 5 ng for a 20-μl injection.

For testing the method, a mayonnaise sample was selected because of the amount and frequency with which it is used with respect to preserved fish and caviar and its substitutes. The request of consumers for products without chemical additives has stimulated the food industry to put products on the market without preservatives. In fact, most of the mayonnaise on the market has no chemical additives. Because the mayonnaise samples obtained from the local stores contained no parabens, a sample spiked with known amounts of parabens was used to test the method. Parabens are most active against moulds and yeasts; the inhibitory concentrations of methyl, ethyl and *n*-propyl *p*-hydroxybenzoate range from *ca.* 80 to 1000 ppm. Based on this range of inhibitory activity and as the maximum amount of parabens authorized by Italian law is 1000 ppm, concentrations of 100, 500 and 1000 ppm were selected.

Large amounts of 4-Brmdmc and of 18-crown-6 in the derivatization of the sample solution (see Section 2.7) were verified by the presence, in the mayonnaise, of compounds that give rise other fluorophores (see peaks with high retention times in Fig. 3). The sample without addition of the parabens showed a small peak at the retention time of methyl paraben, which can yield an error of about 1% at the ppm level.

The precision of the method was assessed by submitting each sample to five runs; in addition,

Table 1
Recoveries of methyl, ethyl and *n*-propyl parabens from a mayonnaise sample

Amount added (ppm)				Amount found (ppm)				Recovery (%)		
Methyl	Ethyl	*n*-Propyl		Methyl	Ethyl	*n*-Propyl		Methyl	Ethyl	*n*-Propyl
100	100	100	Average	95.7	99.1	99.4	Average	95.7	99.1	99.4
			S.D.	1.8	3.0	3.3	S.D.	1.8	3.0	3.3
			R.S.D. (%)	1.8	3.0	3.4	R.S.D. (%)	1.8	3.0	3.4
500	500	500	Average	468.0	471.8	474.3	Average	93.6	94.4	94.9
			S.D.	17.7	13.8	12.0	S.D.	3.5	2.8	2.4
			R.S.D. (%)	3.8	2.9	2.5	R.S.D. (%)	3.8	2.9	2.5
1000[a]	1000[a]	1000[a]	Average	973.6	983.9	976.2	Average	97.4	98.4	97.6
			S.D.	32.0	39.8	35.4	S.D.	3.2	4.0	3.5
			R.S.D. (%)	3.3	4.0	3.6	R.S.D. (%)	3.3	4.0	3.6

Five replicate analyses.
[a] The maximum concentration allowed in mayonnaise in Italy.

the percentage recovery of methyl, ethyl and *n*-propyl parabens was verified by adding different amounts of each paraben to the mayonnaise sample (Table 1). The R.S.D. values in each instance ($\leq 4\%$) show the good reproducibility of the method.

The validity of the procedure is demonstrated by the recovery of parabens from samples spiked with known amounts of methyl, ethyl and *n*-propyl *p*-hydroxybenzoate (Table 1).

Benzoic acid under the conditions adopted can be partly derivatized and, if present in the sample, interfere in the determination of methyl *p*-hydroxybenzoate. The identity of each of the compounds could be specified by using the peak-area ratio at two pairs of excitation or emission wavelengths. The peak-area ratio, at two wavelength pairs, for each compound should differ at least by 5%.

Even though the derivatization step is time consuming, the HPLC determination of esters of *p*-hydroxybenzoic acid in mayonnaise as the 4-Brmdmc derivatives is of interest because fluorimetric detection is both very specific and sensitive. Further, it can be used as an alternative to the earlier HPLC–UV determinations that are complex because they require extraction and further clean-up procedures and/or eluents made with buffer solutions. In time, the salts could cause deposits in the pumps and in the capillary tubes, adversely affecting the efficiency of the chromatograph, a characteristic rarely taken into account.

4. References

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