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Short communication

Gas chromatographic flow method for the preconcentration and simultaneous determination of antioxidant and preservative additives in fatty foods

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Abstract

A partially automated gas chromatographic method for the simultaneous determination of antioxidants (*tert*.-butyl-4-hydroxyanisole, 2,6-di-*tert*.-butyl-*p*-hydroxytoluene, *tert*.-butylhydroquinone, α-tocopherol and α-tocopheryl acetate) and preservatives (sorbic acid, benzoic acid and their esters) in fatty foods without derivatization is reported. About 95% of triglycerides from lipid material are avoided by manually extracting the samples with a mixture of solvents and the remaining by using a continuous solid-phase extraction system. By using aqueous sample solutions (in 0.1 *M* HNO_3), XAD-2 sorbent and selective elution with 200 μl of 2-propanol, only ca. 0.03% of total triglycerides present in the original sample remains in the final extract. Manual injection of the extract into a GC–flame ionization detection allows the additives to be determine at concentrations in the range 0.5–100 μg/ml with a high precision (ca. 3%). Mass spectrometry in the conventional electron impact mode, in conjunction with library search, permits the unequivocal identification of all the additives. Margarine, oil, cheese, pâté and sauce samples were analysed in this way, all with good results.

Keywords: Food analysis; Oils; Cheese; Antioxidants; Preservatives

1. Introduction

Food antioxidants are used to prevent deterioration, rancidity or discoloration through oxidation. Preservatives are also very often added to foods to slow down or inhibit microbial growth [1]. The use of these additives is subject to regulations that establish permitted compounds and their concentration limits [2]. There is a growing need for analytical control of these compounds by checking for the absence of additives banned by the European Union (e.g. *tert*.-butylhydroquinone) and determining those permitted by its regulations. Natural antioxidants are simultaneously determined by HPLC [3,4], GC [5] and capillary electrophoresis [6]; synthetic antioxidants in dehydrated soups and chewing-gum have been determined by differential-pulse voltammetry [7] and GC–MS [8], respectively. Preservatives in beverages and jams can be determined with minimal sample manipulation by TLC [9], HPLC [10] and GC–MS [11]; more recently, capillary electrophoresis was used in the presence of cyclodextrins for the separation of nine preservatives [12]. Most of these methods only allow food preservatives or antioxidants to be determined; an additional,

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simultaneous determination, however, is usually required to determine the total quality and shelf-life of the food product concerned. Simultaneous determinations of preservatives and antioxidants are usually done by HPLC [10,13], but not by GC.

Over the last ten years, solid-phase extraction (SPE) has been increasingly used for preparation purposes in the preconcentration/isolation of antioxidants and preservatives in foods [10]. The advantages and disadvantages of automatic system/manual SPE combinations were discussed [14]. Recently, our group developed on-line SPE methods for the determination of antioxidants [15] and preservatives [16] in foods by GC with flame ionization detection (FID).

In this work, we assessed the potential of continuous SPE, for reducing human intervention in the isolation of ten additives from complex matrices containing large amounts of lipids prior to manual injection into a GC–FID or GC–MS instrument. For this purpose, the influence of triglycerides on the proposed flow system was investigated. The proposed mass spectrometric method allows confirmation of all additives assayed but provides low sensitivity. Therefore, additives are quantified by FID, which is highly sensitive, inexpensive and available in many laboratories.

2. Experimental

2.1. Chemicals and standards

Antioxidants [*tert*.-butyl-4-hydroxyanisole (BHA), 2,6-di-*tert*.-butyl-*p*-hydroxytoluene (BHT), α-tocopherol (α-T), α-tocopheryl acetate (α-TA)], preservatives [benzoic acid, sorbic acid, methyl *p*-hydroxybenzoic acid (M-PHBA), ethyl *p*-hydroxybenzoic acid (E-PHBA) and propyl *p*-hydroxybenzoic acid (P-PHBA)] and 5-α-cholestane (internal standard) were all supplied by Sigma (Madrid, Spain). *tert*.-Butylhydroquinone (TBHQ) and 2-*tert*.-butyl-4-methylphenol (internal standard) were purchased from Aldrich (Madrid, Spain). Organic solvents were obtained in HPLC grade from Scharlau (Barcelona, Spain). All sample dilutions were made in ultrapure (Milli-Q) water. XAD-2 and LiChrolut-EN (polymeric styrene–divinylbenzene) sorbents, 50–100 μm, were supplied by Sigma and Merck (Darmstadt, Germany), respectively.

Additives were identified by using 10 mg/ml standard solutions in ethanol for preservatives (benzoic acid, sorbic acid, M-PHBA, E-PHBA, P-PHBA) and phenol antioxidants (BHA, BHT, TBHQ), and in *n*-hexane for tocopherols; all were stored in glass-stoppered bottles at 4°C. The optimal conditions for GC were established by using a mixture of 50 μg/ml of each additive and both internal standards in ethanol. Standard working-strength solutions of variable concentration were prepared daily by dilution of appropriate volumes of the stock solutions in 0.1 *M* nitric acid.

2.2. Equipment

Analyses were carried out on a Fisons gas chromatograph (8000 series-8035) equipped with a FID system and a fused-silica capillary column, 30 m×0.32 mm I.D. (coated with a 1 μm thick film of 5% diphenyl–95% dimethylpolysiloxane, HP-5) from Hewlett-Packard (Seville, Spain). The chromatographic conditions were as follows: an initial temperature 125°C (2 min), followed by a gradient of 10°C/min to 315°C, which was held for 2 min. The injector and flame ionization detector temperature were 250 and 310°C, respectively. Helium (6.0 grade; Air Liquide, Seville, Spain) was used as the carrier gas, at a flow-rate of 1 ml/min. In order to confirm the identity of eluted GC peaks, the gas chromatograph was coupled to a Fisons-800 mass spectrometer. The transfer line and source temperature were 250 and 200°C, respectively; the ionization energy was 70 eV. Mass spectra were recorded from m/z 50 to 500 in the electron impact mode. Samples (1 μl) were injected in the split mode (1:10 ratio).

The flow-system was constructed as described elsewhere [16]. Laboratory-made adsorption columns were constructed from PTFE capillaries of 3 mm I.D. Columns were hand-packed with different amounts of sorbent and sealed at both ends with small plugs of cotton wool to prevent material losses. Initially, the columns were conditioned with 1.5 ml 0.1 *M* nitric acid at a flow-rate of 1.5 ml/min. To avoid memory effects, the columns were flushed with acetone between successive samples.

2.3. Sample pretreatment

An amount of 0.75–2.5 g of fatty sample (margarine, oil, fresh cheese, mayonnaise or pâté) was mixed with 2 ml of saturated *n*-hexane (in acetonitrile) containing 0.625 mg of both internal standards (2-*tert*.-butyl-4-methylphenol and 5-α-cholestane). The suspension of the sample was then extracted with 10 ml of the saturated acetonitrile (in *n*-hexane)–2-propanol–ethanol (2:1:1), with gentle shaking for 5 min. The *n*-hexane phase was discarded and the other frozen at −18°C for 1 h [17] and then filtered. This procedure removed at least 95% of triglyceride in the food sample. The filtrate was placed on a flash evaporator furnished with a water bath at ≤40°C and concentrated to ca. 0.5 ml within 5 min (rapid evaporation reduced TBHQ losses). Finally the residue was dissolved in 25 ml of 0.1 *M* HNO_3 and a 5 ml aliquot was introduced into the SPE system; this afforded 3–4 replicated per sample.

2.4. Solid-phase extraction method

The manifold used for the determination of antioxidants and preservatives in foods is depicted in Fig. 1. Initially, 5 ml of standard solution or treated sample containing additives at various concentrations (0.5–100 μg/ml) plus 25 μg/ml of both internal standard was passed at 1.5 ml/min through the sorbent column (XAD-2, 50 mg) inside the loop of the injection valve (IV_1). Analytes and ca. 25% of triglyceride remaining in the sample after the pretreatment step were retained in the column and the remaining lipid fraction was sent to waste (W_1). A volume of 100 μl of *n*-hexane (from IV_2), carried by a N_2 stream, was used to flush residual sample from the column and the flow system connectors (Fig. 1A). Then, the loop of the injection valve (IV_2) was filled with eluent (2-propanol). Finally, two sequential injections of 100 μl of 2-propanol (from IV_2) were carried by the N_2 stream and passed through the minicolumn (in opposite direction to the sample) to elute adsorbed additives (Fig. 1B). The eluate (200 μl) was collected in glass vials containing anhydrous sodium sulphate, and a 1 μl aliquot was manually injected into the gas chromatograph. After each determination, the sorbent column was cleaned with 300 μl of acetone to remove residual tri-

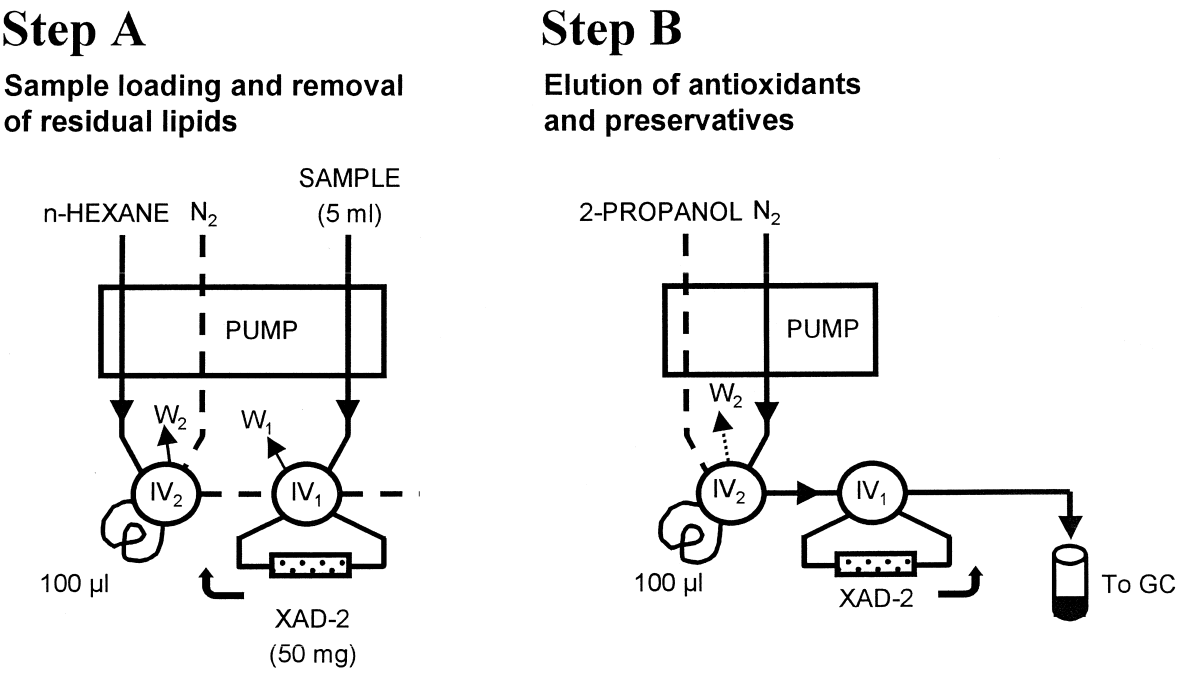

Fig. 1. Scheme of the continuous isolation/preconcentration and elution steps for the determination of antioxidants and preservatives in food samples. IV=Injection valve; W=waste; GC=gas chromatograph with FID and MS. Sample and eluent flow-rates, 1.5 ml/min.

glycerides and other adsorbed organic compounds; finally, the column was conditioned with 0.1 *M* HNO_3 at 1.5 ml/min for 1 min.

3. Results and discussion

3.1. Optimization of the preconcentration/elution process

In previous work, we developed a flow system for the separation of antioxidants in *n*-hexane medium [15] and preservatives in aqueous medium [16] in foods by GC–FID. XAD-7/XAD-2 and 2-propanol/ethyl acetate were found to be the most efficient sorbents and eluents for antioxidants/preservatives, respectively. In this work, we initially used the previously optimized system [16] for the study of the sorbent and the sample solvent. For this purpose, 5 ml of standard solutions containing 40 μg/ml of each additives dissolved in *n*-hexane, ethanol, methanol, light petroleum or distilled water (pH 5.5) were prepared. Two columns packed with 50 mg of XAD-2 and LiChrolut-EN were separately included in the flow system and the eluent was 5 ml of ethanol in all instances. On the assumption that ethanol provided quantitative elution, the following conclusions can be drawn: both sorbents were similarly efficient for preservatives, but XAD-2 was better for antioxidants; also, the highest adsorption for all analytes with both sorbents was obtained when the additive standard was dissolved in distilled water. Based on these results, and taking into account that the proposed method was intended for use with food samples (with high moisture contents), standards were dissolved in water; in addition, XAD-2 was selected as sorbent. As in previous work antioxidants were dissolved in *n*-hexane [15], in this method the sample pH was the first variable studied. As can be seen in Table 1, the optimum range was similar for all additives except natural antioxidants. Thus, the maximum additive adsorption was obtained at pH 1–5 for preservatives and synthetic antioxidants (BHA, BHT and TBHQ) and 1–3 for tocopherols. In subsequent experiments, standard solutions and samples were prepared in 0.1 *M* HNO_3. The highest adsorption was obtained above 45 mg of XAD-2, so 50 mg was selected as optimal. A sample flow-rate of 1.5 ml/min was chosen, because the signal remained constant over 0.5–0.7 ml/min, and 100 μl of *n*-hexane, carried by a N_2 stream, was used to flush and dry the column.

Table 1
Percent adsorption of preservatives and antioxidants on an XAD-2 sorbent column at a variable pH

Additive	pH				
	0.5	1–5	6	7	10
Sorbic acid	80	90	70	60	–
Benzoic acid	85	90	60	55	–
M-PHBA	60	75	65	50	25
BHA	85	90	85	75	30
BHT	45	50	40	35	–
E-PHBA	60	75	70	60	25
TBHQ	85	90	90	80	35
P-PHBA	60	75	70	65	–
α-Tocopherol	70	75[a]	–	–	–
α-Tocopheryl acetate	70	75[a]	20	–	–

[a] pH range 1–3.

Different organic solvents (methanol, ethanol, 2-propanol and ethyl acetate) were assayed for elution of preservatives and antioxidants. 2-Propanol was found to be the most effective eluent for antioxidants, (the analytical signals were 1.3-, 1.3- and 1.7-times higher than those obtained with ethanol, ethyl acetate and methanol, respectively). Similar results were obtained with ethyl acetate and 2-propanol for preservatives, so 2-propanol was selected as eluent for all additives. An injected volume of 200 μl, carried by N_2 (eluent carrier) at 1.5 ml/min was selected as optimal.

3.2. Study of triglyceride interferences

Two methods, recommended in the literature, were assayed for preliminary separation of triglycerides, using a sunflower-seed margarine as sample test. In the first method, 1 g of margarine was dissolved in 5 ml of saturated *n*-hexane in acetonitrile and extracted with 10 ml of saturated acetonitrile in *n*-hexane; the extract was concentrated to ca. 0.5 ml (≤40°C, 5 min) [18]. In the second method, following dissolution of the margarine in *n*-hexane, the extraction was done with 10 ml of 2:1:1 saturated acetonitrile–2-propanol–ethanol with homogenizing for 5 min. After freezing the extract for 1 h at −18°C, the mixture was filtered and concentrated [17]. The

residues obtained with both methods were dissolved in 25 ml of 0.1 *M* HNO_3. The triglyceride removal efficiency of both methods was determined from the initial content in the margarine sample by using the traditional transesterification to fatty acid methyl esters [19] with 5% acetyl chloride in methanol as derivatizing procedure. The derivatization reaction was manually done as described elsewhere [15]. The study revealed that ca. 65 and 5% of total triglyceride in the margarine were present in samples treated with the first [18] and second [17] method, respectively; therefore, the second method, was chosen.

The adsorption and elution efficiency for triglycerides remaining on the XAD-2 column was studied similarly to those of the additives [15]. The sorption study revealed that ca. 25% of triglyceride present in the margarine treated solution (which contained only 5% of original triglyceride) was retained on the XAD-2 column and that ca. 75% went to waste. In the elution study, triglycerides were eluted from the sorbent column by ca. 2.5% of the adsorbed fraction (viz. ca. 0.03% of triglyceride in margarine samples). In conclusion, the combination of sample pretreatment, which removes 95% of triglyceride, and SPE, which eliminates the remaining triglyceride (ca. 5%), ensures that less than 0.05% of triglyceride present in the fatty sample will remain in the final organic extract, so any interference from this type of compounds will be negligible. In order to extend its service life between samples, the sorbent column was flushed with 300 μl of acetone to ensure complete elution of residual triglyceride.

3.3. Gas chromatographic behaviour

All the additives studied exhibit good GC properties and can be determined in a direct manner (without derivatization). Only the peaks for two analytes (BHT and E-PHBA) overlapped in the chromatographic separation of the antioxidants and preservatives, which precluded their separation (Fig. 2A). While all the additives can be quantified by conventional FID, their identification must be confirmed by MS. For analyte identification purposes, the following three characteristic ions were monitored for each additive by MS (such peaks always included the strongest in bold, base peak, and that corresponding to the molecular ion, M^+ in italic): *m*/*z* values for sorbic acid, 67, **97** and *112*; 77, **105** and *122* for benzoic acid; 93, **121** and *152* for M-PHBA; 137, **165** and *180* for BHA; 57, **205** and *220* for BHT; **121**, 137 and *166* for E-PHBA; 123, **151** and *166* for TBHQ; **121**, 138 and *180* for P-PHBA; 165, 205 and ***430*** for α-T; and 166, **430** and *472* for α-TA. As can be seen from Fig. 2, corresponding to a standard solution containing all additives, the two overlapped additives (peak 5) were resolved by using a characteristic ion for each (viz. *m*/*z* **205** for BHT and *m*/*z* **121** for E-PHBA). For quantitation the strongest peak was employed except for the compounds: benzoic acid, M-PHBA, BHA and P-PHBA.

Calibration graphs were obtained by using a sample volume of 5 ml at pH 1 and the SPE system depicted in Fig. 1. The sensitivity (slope of the calibration graph), linear range, limit of detection and precision provided by the different detectors are shown in Table 2. The limit of detection (LOD) was defined as the minimum concentration providing a chromatographic signal three times higher than background noise (at the *m*/*z* value used in MS). The precision of the method (repeatability) was checked on 11 samples containing 40 μg/ml of each additive with FID and 200 μg/ml with MS detection. Several interesting conclusions can be drawn from these results. First, FID provides a higher sensitivity (ca. four times) for all additives than does MS. The linear ranges also differ; thus MS enables detection down to the 0.5 mg/ml level. Also, the limits of detections for all the analytes are lower with FID, probably as the result of its higher sensitivity. Finally, the precision, expressed as relative standard deviation, is similar with both detectors and ranges from 2.5 to 4.2%.

3.4. Determination of antioxidants and preservatives in food products

Samples were selected among those with high fat contents (viz. margarines, oils, fresh cheeses, mayonnaises and pâté); they were treated as described under Section 2.3 and the resulting aqueous phase was introduced into the SPE system. In all instances, quantitation was done by FID and confirmation of

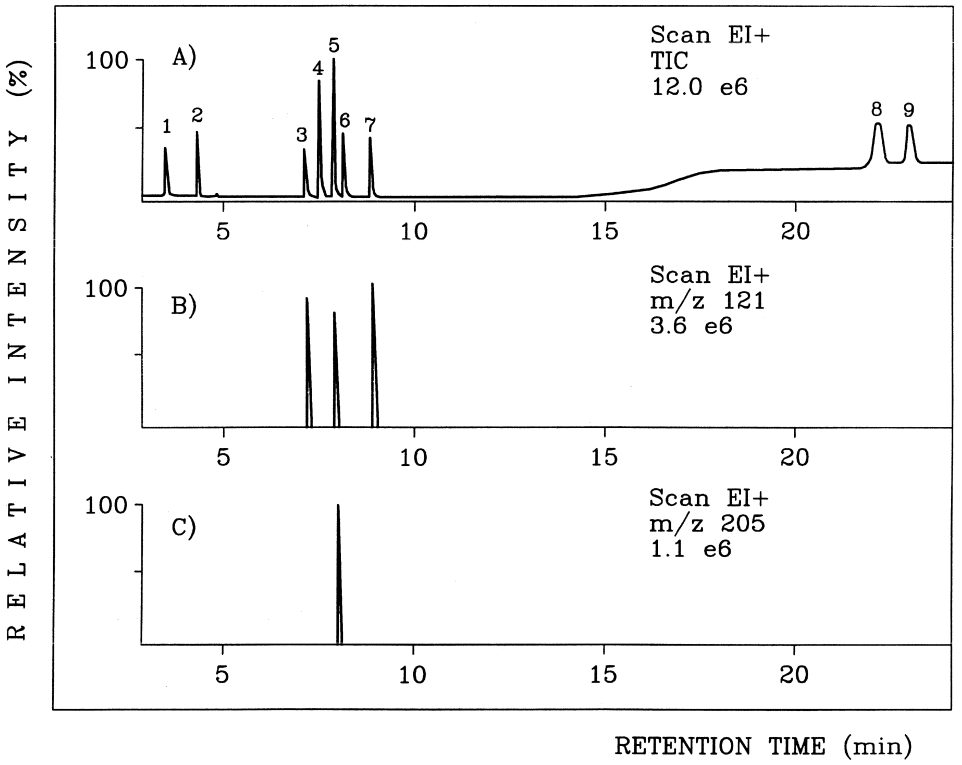

Fig. 2. Total-ion current chromatograms obtained for a standard solution containing 250 μg/ml of each additive, without specific monitoring (A) and with selected characteristic ions for E-PHBA (B) and BHT (C). 1=Sorbic acid, 2=benzoic acid, 3=M-PHBA, 4=BHA, 5=BHT+E-PHBA, 6=TBHQ, 7=P-PHBA, 8=α-tocopherol and 9=α-tocopheryl acetate.

each additive by MS. Table 3 lists the average results obtained from five individual analyses of each food sample (all determinations in the flow system were in triplicate, so $n=15$). All food products were found to contain at least one antioxidant and one preservative except for the oil sample. P-PHBA and BHT were

Table 2
Analytical figures of merit of the determination of antioxidants and preservatives

Additive	Sensitivity[a]		*m/z* (MS)	Linear range (μg/ml)		LOD (μg/ml)		RSD (%)	
	FID	MS		FID	MS	FID	MS	FID	MS
Sorbic acid	6.0×10^{-3}	1.6×10^{-3}	97	0.5–100	2–500	0.20	1.0	3.7	4.0
Benzoic acid	8.7×10^{-3}	2.2×10^{-3}	105	0.4–100	1.5–500	0.15	0.8	3.1	3.5
M-PHBA	4.6×10^{-3}	1.1×10^{-3}	152	0.5–100	2–500	0.20	1.0	2.8	3.4
BHA	10.0×10^{-3}	2.4×10^{-3}	180	0.4–100	1.5–500	0.15	0.8	3.1	3.2
BHT	3.6×10^{-3}	0.9×10^{-3}	205	0.6–100	2.5–500	0.25	1.5	2.7	3.8
E-PHBA	4.8×10^{-3}	1.2×10^{-3}	121	0.4–100	1.5–500	0.15	1.0	2.6	3.4
TBHQ	5.2×10^{-3}	1.3×10^{-3}	151	0.5–100	2–500	0.20	1.0	3.4	4.0
P-PHBA	5.1×10^{-3}	1.3×10^{-3}	138	0.5–100	2–500	0.20	1.0	2.5	3.9
α-T	0.8×10^{-3}	0.2×10^{-3}	430	1–100	4–500	0.50	2.0	3.6	4.2
α-TA	0.7×10^{-3}	0.2×10^{-3}	430	1–100	4–500	0.50	2.0	3.8	4.2

[a] Relative area (analyte/internal standard peak area ratio)/μg/ml.

Table 3
Antioxidant and preservative contents and percent RSD ($n=15$) in fatty samples as determined by the proposed SPE–GC–FID method

Food sample	Content (mg/kg fat)						
	Sorbic acid	Benzoic acid	BHA	BHT	P-PHBA	α-T	α-TA
Margarines							
Sunflower	840 (5.4)	–	170 (4.1)	–	–	150 (4.7)	–
Corn	390 (5.1)	–	–	–	–	110 (4.5)	280 (5.4)
Cheese							
Fresh	745 (4.0)	–	–	–	–	725 (4.8)	–
Light	880 (5.1)	–	–	–	–	480 (5.2)	–
Mayonnaise							
Plain	–	175 (5.7)	190 (5.3)	–	–	1080 (4.2)	–
Garlic	365 (4.1)	625 (4.8)	–	–	–	1765 (4.0)	1100 (4.5)
Light	400 (3.8)	–	–	–	–	1900 (4.7)	–
Oil							
Sunflower-seed	–	–	134 (3.7)	58 (3.5)	–	485 (4.1)	–
Pâté							
Porc	225 (5.3)	–	–	–	840 (5.4)	2100 (4.8)	–

only present in pâté and oil, respectively. M-PHBA and E-PHBA were detected in none of the samples. As expected, TBHQ was absent from all samples (in compliance with European Union regulations). All the results are below the European Union allowed levels for both individual and mixed additives [2].

Finally, in order to asses the potential of the proposed method for the determination of antioxidants and preservatives, samples were spiked with the maximum legally allowed amount of the additives in each food type [2] and analysed in quintuplicate. The recoveries obtained ranged from 92.8 to 102.5%. Fig. 3A shows the chromatogram for a plain mayonnaise sample, using mass spectrometric detection. No matrix interference was observed owing to the highly selectivity of the proposed method. By way of example, Fig. 3B illustrates the identification of one of the additives by comparing the mass spectrum for the corresponding peak with that in the library. All spectral comparisons resulted in coincidences above 85%.

4. Conclusions

Antioxidants and preservatives as additives jointly spiked to fatty food samples can be determined by GC with no prior derivatization. The problem posed by the presence of two overlapped peaks for BHT and E-PHBA (their mixture was not found in the studied samples, however) can be solved by using characteristic ions for the two in MS. However, the determination of the additives studied by MS is less sensitive than their FID quantitation.

The proposed method is not laborious but requires manual sample preparation (to remove 95% of triglycerides in fatty foods) prior to insertion into the flow system; because the system is not computer-controlled, it requires continuous intervention of the operator. However, the SPE system is highly affordable (it uses minimal amounts of solvents) and robust, and can be directly applied to foods with low fat contents.

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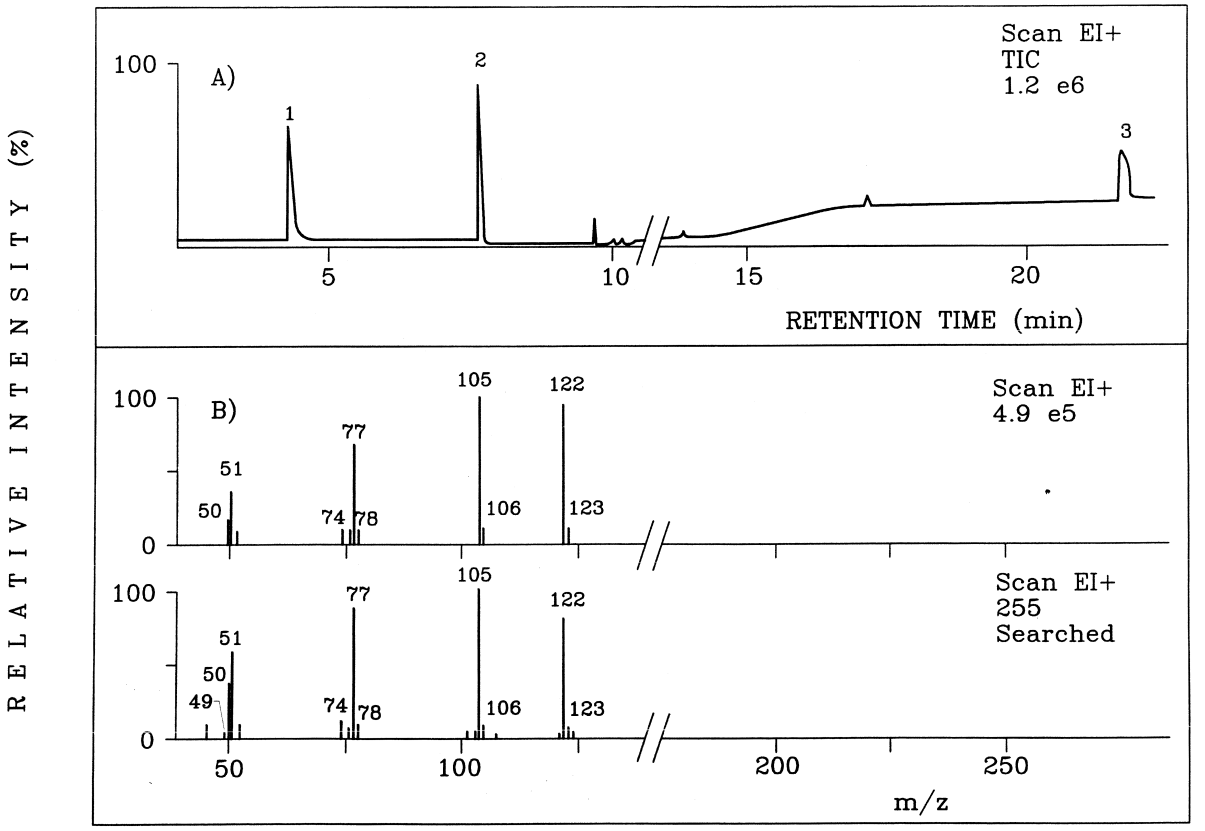

Fig. 3. Chromatogram for antioxidants and preservatives found in a plain mayonnaise sample (A). 1=Benzoic acid, 2=BHA, and 3=α-tocopherol. Electron impact full-scan mass spectra for benzoic acid in the sample and from NIST library (B).

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