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Determination of sorbic acid in margarine and butter by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A procedure is reported for the separation and determination of sorbic acid, as a derivative of 4-bromomethyl-6,7-dimethoxycoumarin, by reversed-phase high-performance liquid chromatography with fluorescence detection using enanthic acid as an internal standard. Sorbic acid, separated from samples of commercial margarine and butter by steam distillation, was evaluated using the proposed procedure and by UV absorption and visible spectrophotometric methods (AOAC). The preparation of the calibration graph and the determination of sorbic acid with the visible spectrophotometric method was improved. The sorbic acid content determined using UV and visible spectrophotometric methods was higher than that obtained with the reversed-phase high-performance liquid chromatographic method owing to the presence of interfering substances in the samples. The range of recovery and the precision of the proposed method and the reference methods are also reported.

INTRODUCTION

Sorbic acid, isolated for the first time from the berries of mountain ash (*Sorbus aucuparia* L.) is a particular unsaturated fatty acid (2,4-hexadienoic acid) which, in the lactone state (parasorbic acid), is also found in berries of some other genera of the Rosaceae family. Sorbic acid and its sodium, potassium and calcium salts are food preservative additives which counteract microbiological alterations by inhibiting the action of moulds and yeasts. Gooding [1] was the first to confirm its fungistatic properties and proposed its use to prevent the growth of moulds in foods and packaging materials. Today sorbic acid is one of the most widely used additives. It is allowed (Italian regulations [2]) at various concentrations (50–2000 mg/kg) in a wide range of foods.

Extraction followed by determination is the procedure generally reported in the literature to determine the sorbic acid content in foods. It can easily be isolated from complex food matrices by steam distillation [3–5], diethyl ether extraction of the food mixed with sand [6] and extraction with an aqueous solution of metaphosphoric acid followed by partitioning into a diethyl ether–light petroleum mixture [7,8].

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Leuenberger *et al.* [9] and Coelho and Nelson [10] used an Extrelut prepacked column (Merck, Darmstadt, Germany) containing silica with a large-pore granular structure which permits the extraction of lipophilic substances from an aqueous phase by liquid–liquid partition chromatography. Extraction by ion-pair formation with tertiary amines and their salts was used by Puttemans *et al.* [11] and Terada *et al.* [12].

For determination the first methods used UV and visible spectrophotometry. The former is based on absorption at *ca.* 260 nm due to an extensive conjugated system of three double bonds, one of which originated from a carboxyl group. The second (oxidation method) is based on absorption at 532 nm due to a red pigment derived from the reaction of malonaldehyde, a product of the oxidation of sorbic acid, with 2-thiobarbituric acid [13]. Some spectrophotometric procedures [3–5,8] were adopted as AOAC methods [14] for the determination of sorbic acid in cheeses, wine and dairy products.

The usefulness of thin-layer chromatography (TLC) should be noted with respect to qualitative and semi-quantitative research [15,16]; it can also permit a quantitative evaluation if a reflectance spectrophotometric determination is performed [17–19]. High-performance TLC with fluorescence detection [20,21], used to determine propionic, sorbic and benzoic acids, requires a derivatization procedure with dansylsemipiperazide in the presence of N,N′-dicyclohexylcarbodiimide. Determination of the fluorescent amidic derivatives of the acids is performed using a TLC plate scanner ($\lambda_{ex.} = 366$ nm).

Determination of sorbic acid in foods by gas chromatography [6,22–25] can be achieved directly by injecting the extract obtained from the sample or after its derivatization.

In recent years, high-performance liquid chromatography (HPLC) has been used for the determination of sorbic acid in a number of different food systems. Some workers employed anion-exchange chromatography [26–28] and others reversed-phase (RP) HPLC [12,29–36], including ion-pair chromatography [11,37]. Most of these methods were also used for the determination of other preservatives in the eluted sample and detection was always performed by means of UV spectrophotometry.

This present paper describes an RP-HPLC procedure with fluorimetric detection which offers high sensitivity and specificity in the detection of sorbic acid as a derivative of 4-bromomethyl-6,7-dimethoxycoumarin. This procedure allows the identification, separation and determination of sorbic acid isolated from commercial samples of margarine and butter by steam distillation. The derivatization with 4-bromomethyl-6,7-dimethoxycoumarin [38], which converts sorbic acid into a fluorophore, and the chromatographic parameters were optimized. The method was compared with the AOAC UV and visible spectrophotometric procedures [14].

EXPERIMENTAL

Apparatus

The separation of the sorbic acid from the samples was achieved with a steam distillation apparatus similar to that reported [14]. A Varian (Palo Alto, CA, U.S.A.) Model 5000 liquid chromatograph equipped with a Varian Fluorichrom fluorescence detector was used at the following settings: gain and lamp, LO; attenuator, $\times 20$; excitation filters, CS 7-60/CS 7-54 (maximum wavelength transmission at 355 nm); emission filters, CS 3-73/CS 4-76 (wavelength emission >420 nm).

Separation was performed by RP-HPLC on a Spherisorb ODS-2 (5 μm) column (250 × 4.6 mm I.D.) (Custom LC, Houston, TX, U.S.A.). The system was interfaced with a Varian 4270 computing integrator: attenuation × 2, chart speed 0.25 cm/min.

The spectrophotometric analyses were performed on a Varian DMS Model 200 UV–visible spectrophotometer.

Reagents

Potassium sorbate (99%), analytical-reagent grade magnesium sulphate heptahydrate, enanthic acid, 2-thiobarbituric acid (TBA), potassium dichromate, sulphuric acid, hydrochloric acid and all the solvents for HPLC, such as acetone, water and methanol, were purchased from Carlo Erba (Milan, Italy). 4-Bromomethyl-6,7-dimethoxycoumarin (4-Brmdmc) and 18-crown-6 were purchased from Sigma (St. Louis, MO, U.S.A.).

A 2 *M* methanolic potassium hydroxide solution was prepared by dissolving 5.6 g of potassium hydroxide in 50 ml of methanol. Acetone solutions of 4-Brmdmc (0.7 mg/ml) and 18-crown-6 (0.63 mg/ml) were prepared.

Samples

Commercial samples of margarine and butter were used.

Preparation of standard solutions

A solution was prepared by dissolving 134 mg of potassium sorbate (equivalent to 100 mg of sorbic acid) in 100 ml of distilled water. The internal standard solution was prepared by adding 0.4 ml of 2 *M* methanolic potassium hydroxide solution to 100 mg of enanthic acid in a 100-ml volumetric flask and diluting to volume with distilled water. Stock solutions (100 μg/ml) were obtained diluting these two solutions 1:10 with distilled water. Aliquots of 0.5–4 ml of sorbic acid stock solution were mixed with 1 ml of internal standard stock solution in separate 100-ml volumetric flasks, containing 50 μl of 2 *M* methanolic potassium hydroxide solution, and diluted to volume with distilled water. These standard solutions, containing 0.5–4 μg/ml of sorbic acid and 1 μg/ml of the internal standard, were then derivatized and used to obtain the calibration graph by HPLC analysis. When refrigerated, the standard solutions were stable for several days.

Derivatization and calibration

The derivatization is based on the reaction between fatty acid potassium salts and 4-Brmdmc in the presence of 18-crown-6, which yields a fluorescent derivative as shown in Fig. 1.

After preliminary studies the following derivatization procedure was adopted throughout. A 0.5-ml volume of each standard solution was pipetted into a 10-ml emery-cap test-tube and evaporated using a Rotavapor at 50–55°C under reduced pressure. The dry residue was treated with 100 μl of the 4-Brmdmc solution, 100 μl of 18-crown-6 solution and 300 μl of acetone. The test-tube was closed and placed in a water-bath at 80°C for 15 min, then cooled to room temperature. The relative response factors (RRF) of sorbic acid were obtained from separate injections (10 μl) of their derivatized standard solutions at various concentrations [mean RRF = 1.26, relative standard deviation (R.S.D.) = 3.5%]. The calibration graph was obtained by

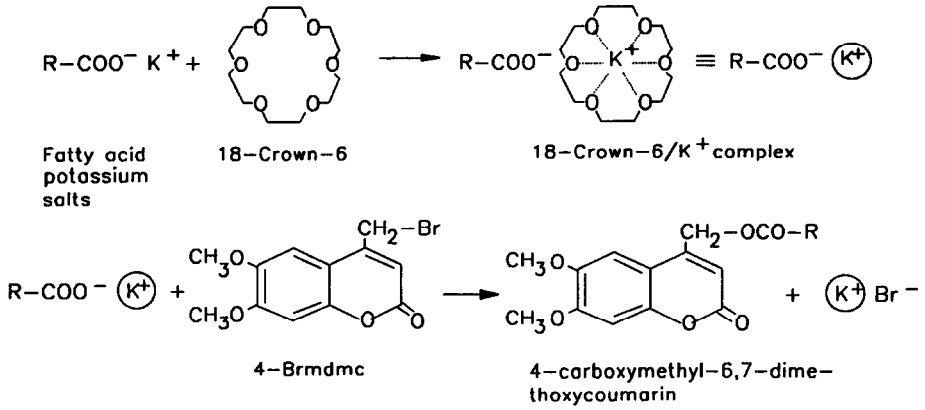

Fig. 1. Derivatization reaction.

plotting the concentration of sorbic acid *versus* the peak-area ratio of sorbic acid to the internal standard. Fig. 2 shows a chromatogram of the derivatized standard solution containing 1 μg/ml of sorbic acid and 1 μg/ml of enanthic acid. The derivatized solutions remain stable for several days if stored in the dark.

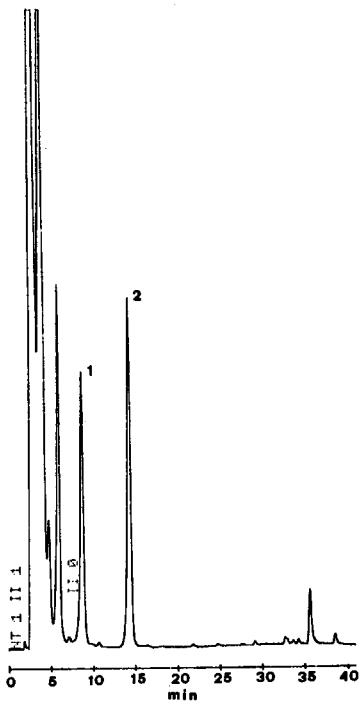

Fig. 2. Standard chromatogram of (1) sorbic and (2) enanthic acid derivatives (10 ng of each). Retention times: (1) 8.23 min; (2) 13.54 min.

Chromatographic conditions

The chromatographic conditions used were as follows: solvent A, water; solvent B, methanol; elution gradient programme, starting composition 30% of A and 70% of B, linear gradient from 70 to 100% of B in 25 min (1.2%/min), isocratic flow of 100% B for 15 min, return of the system to 30% of A and 70% B in 5 min; flow-rate, 1 ml/min; column pressure, initial 152 bar, final 65 bar; column, Spherisorb ODS-2 (5 μm) (250 × 4.6 mm I.D.); fluorimetric detection.

Preparation of samples and standards

A 1-g amount of margarine or butter, 10 ml of 1 *M* sulphuric acid, 10 g of magnesium sulphate heptahydrate and 2 ml of a 100 μg/ml aqueous solution of internal standard were placed in a 250-ml flask. Steam distillation afforded 130 ml, which were collected into a 200-ml volumetric flask containing 0.5 ml of 0.1 *M* hydrochloric acid. The condenser was washed with distilled water and the distillate was diluted to volume and mixed. Aliquots of 1, 2 and 4 ml of 100 μg/ml stock solution of sorbic acid were similarly distilled.

Determination by RP-HPLC

A 50-μl volume of 2 *M* methanolic potassium hydroxide solution were added to 50 ml of the distillates obtained from pure standards and from samples and 0.5-ml aliquots were then derivatized as described for the standard solutions (see *Derivatization and calibration*). A 10-μl volume of each solution was then injected in duplicate into the LC apparatus and the peak-area ratio of sorbic acid to internal standard was integrated on the calibration graph in order to obtain the sorbic acid concentration in samples and in pure standards. Fig. 3 shows typical chromatograms of commercial samples of margarine and butter.

Determination by UV and visible spectrophotometric methods

The distillate was treated according to the AOAC methods [14]. The preparation of the calibration graph and the determination of sorbic acid with the spectrophotometric method was simplified as follows: 2 ml of each solution containing 0, 1, 2 and 3 μg/ml of sorbic acid were pipetted into a 15-ml test-tube, 1 ml of 0.15 *M* sulphuric acid, 1 ml 0.15% potassium dichromate solution and 2 ml of 0.5% TBA solution were added and the tube was placed in a boiling water-bath for exactly 10 min. The solution was then cooled to room temperature and the absorbance was measured at 532 nm against the reagent blank. Fig. 4 shows the calibration graphs obtained plotting absorbance at 260 and 532 nm against sorbic acid concentration; the correlation coefficients were 0.9992 and 0.9995, respectively.

RESULTS AND DISCUSSION

After various trials, the RP-HPLC procedure proposed for the determination of sorbic acid in margarine and butter was adjusted so as to establish the optimum derivatization conditions for the internal standard to be used and to determine the chromatographic parameters that would provide the best separation results and reproducibility.

The concentrations of 4-Brmdmc and 18-crown-6 in the derivatization reaction

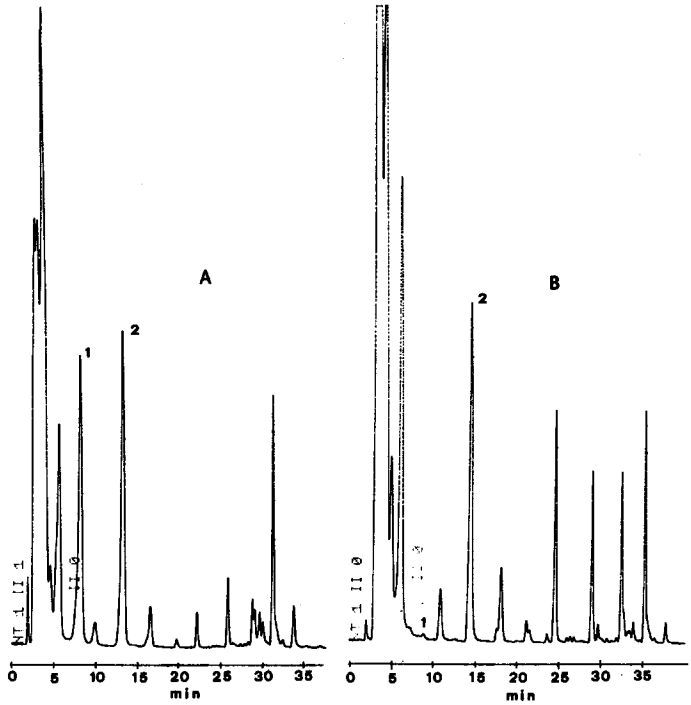

Fig. 3. Typical chromatograms of commercial samples of (A) margarine and (B) butter. Amount of sorbic acid (1) found: in margarine, 12.16 ng; in butter, not detected. Amount of enanthic acid (2) taken: 10 ng in both margarine and butter.

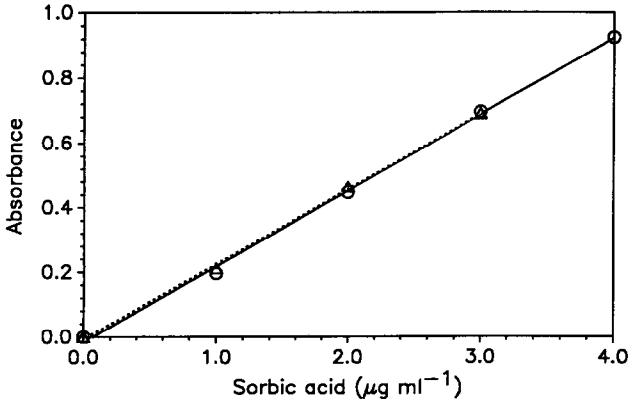

Fig. 4. Calibration graphs for (○) UV (260 nm) and (△) visible (532 nm) spectrophotometric determination of sorbic acid.

were equimolar. When the concentration of the sorbic acid in the sample was 500 mg/kg (the maximum amount permitted by Italian law) and the concentrations of the sorbic acid added and the enanthic acid, used as the internal standard, were 400 and 200 mg/kg, respectively, the concentrations of 4-Brmdmc and 18-crown-6 were ten times greater than the total concentration of sorbic and enanthic acids.

The use of a catalytic amount of 18-crown-6 in the derivatization reaction did not lead to the formation of fluorophores of the acids. Enanthic acid, a C_7 saturated fatty acid, was chosen as the internal standard because it was absent from or present in only trace amounts in the samples. The addition of the internal standard before steam distillation eliminates many causes of error during the separation of the sorbic acid and the subsequent derivatization and does not invalidate determinations which use UV and visible spectrophotometric procedures. The calibration graph obtained by plotting sorbic acid concentrations *versus* the peak-area ratio of sorbic acid to internal standard showed good linearity with a correlation coefficient of 0.9994 and an R.S.D. of 3.5%. The linearity was verified for sorbic acid concentrations up to four times greater than the internal standard concentration (200 mg/kg). This allowed the stringent requirement of employing an internal standard with a concentration similar to that of the peak of interest to be avoided. Therefore, 200 mg/kg of internal standard were added to the sample.

Using water–methanol as eluent, the carboxylic acids showed a considerable increase in retention time which paralleled the increase in the number of carbon atoms; as a consequence, it was necessary to use an elution gradient. As can be seen, the other carboxylic acids were clearly separated and therefore did not cause interference (Fig. 3).

By examining the mean values (bold numbers) in Tables I–III, a comparison can be made between the three different procedures used to determine the sorbic acid contents in the margarine and butter samples. It can be seen that the UV and visible spectrophotometric methods gave considerable differences in the mean values for all the samples examined when compared with RP-HPLC. In butter, the data obtained with UV and visible spectrophotometric procedures (Tables II and III) show average values of 9 and 5 mg/kg of sorbic acid, respectively, although sorbic acid was absent. In order to study this problem, experiments were carried out on pure standard solutions of sorbic acid. These solutions were distilled as described under *Preparation of samples and standards* and analysed by RP-HPLC and UV and visible spectrophotometry. The results obtained showed good agreement between the mean values and standard deviations in the three different methods used (Table IV). The reliability suggests that the real source of the discrepancies mentioned above can be explained by the presence of interfering substances in margarine and butter.

The precision of the methods was assessed by submitting each sample of five runs; in addition, the percentage recovery of sorbic acid was verified by adding different amounts of sorbic acid to three samples of margarine and butter (Tables I–III). The R.S.D. values show reliability regarding the reproducibility of the methods; the R.S.D. values were below 5.6% in each instance except for the values (Tables II and III) for the butter samples without the addition of sorbic acid.

The validity of the procedures is demonstrated by the recovery of sorbic acid from margarine and butter samples spiked with known amounts. The recovery using the proposed procedure ranged from 93.5 to 103.5% and using the UV and visible

TABLE I

RESULTS OF FIVE REPLICATE ANALYSES ON FIVE COMMERCIAL MARGARINE AND BUTTER SAMPLES USING THE PROPOSED RP-HPLC METHOD

For bold values, see text.

Sample No.	Sorbic acid (mg/kg)										
	Added	Margarine					Butter				
		Range	Mean	S.D.	R.S.D. (%)	Recovery (%)	Range	Mean	S.D.	R.S.D. (%)	Recovery (%)
1	0	90.6–101.3	**96.2**	4.6	4.8	–	n.d.[a]	–	–	–	–
	100	193.0–200.5	198.0	2.7	1.4	98.8–103.5	94.5–99.5	96.9	2.1	2.2	94.5–99.5
	200	276.1–297.4	285.3	7.1	2.5	94.8–98.7	193.4–200.2	196.7	2.7	1.4	96.7–100.1
	400	488.0–503.2	498.6	5.5	1.1	99.5–101.8	380.0–394.6	387.7	4.9	1.3	95.0–98.7
2	0	124.4–130.9	**127.8**	2.2	1.7	–	n.d.	–	–	–	–
	100	212.6–233.4	224.7	6.8	3.0	94.7–101.1	93.5–100.1	97.7	2.3	2.4	93.5–100.1
	200	312.1–330.5	321.0	6.9	2.1	96.2–98.7	192.0–201.5	197.2	3.9	2.0	96.0–100.8
	400	510.6–520.5	515.9	3.2	0.6	97.0–98.2	378.5–396.5	387.6	7.0	1.8	94.6–99.1
3	0	226.0–243.2	**233.8**	6.8	2.9	–	n.d.	–	–	–	–
	100	315.6–340.5	326.2	8.7	2.7	96.1–99.4	93.6–98.5	96.4	1.7	1.8	93.6–98.5
	200	422.7–430.5	426.0	3.4	0.8	97.0–99.4	185.0–197.2	190.0	4.0	2.1	92.5–98.6
	400	625.5–644.3	634.6	8.1	1.3	97.7–102.1	385.2–400.1	390.4	5.2	1.3	96.3–100.0
4	0	175.5–195.5	**180.9**	8.7	4.8	–	n.d.	–	–	–	–
5	0	108.2–120.5	**113.2**	5.1	4.5	–	n.d.	–	–	–	–

[a] n.d. = Not detected.

TABLE II

RESULTS OF FIVE REPLICATE ANALYSES ON FIVE COMMERCIAL MARGARINE AND BUTTER SAMPLES USING THE UV SPECTROPHOTOMETRIC METHOD

Sample No.	Sorbic acid (mg/kg)										
	Added	Margarine					Butter				
		Range	Mean	S.D.	R.S.D. (%)	Recovery (%)	Range	Mean	S.D.	R.S.D. (%)	Recovery (%)
1	0	114.0–129.0	**122.0**	5.6	4.6	–	7.8–10.6	**9.1**	1.2	13.2	–
	100	202.0–224.2	214.5	9.8	4.6	93.6–99.8	103.3–112.7	107.3	3.6	3.4	94.2–102.2
	200	297.4–304.2	302.0	2.4	0.8	92.5–95.5	191.6–215.7	203.1	8.5	4.2	92.2–102.4
	400	488.0–516.0	497.9	9.9	2.0	93.5–98.4	376.3–392.5	386.3	6.1	1.6	92.2–95.6
2	0	152.7–166.3	**161.7**	6.4	4.0	–	8.5–10.5	**9.2**	0.7	7.6	–
	100	239.5–264.0	251.6	8.3	3.3	93.2–100.2	102.2–106.0	104.3	1.5	1.4	93.5–97.7
	200	338.8–352.0	347.4	5.4	1.6	92.5–99.8	194.2–215.3	203.7	7.4	3.6	93.1–102.5
	400	530.7–542.5	535.0	4.4	0.8	93.0–97.2	397.0–402.1	391.2	9.4	2.4	92.8–98.0
3	0	234.3–253.1	**245.0**	7.4	3.0	–	7.1–11.5	**8.9**	1.5	16.9	–
	100	338.2–360.3	348.9	8.5	2.4	99.6–102.0	98.8–109.9	104.1	3.5	3.4	93.2–98.6
	200	438.0–459.1	446.7	7.5	1.7	99.0–101.3	192.8–210.0	202.6	6.6	3.3	95.3–100.2
	400	634.4–645.3	638.5	4.0	0.6	97.4–100.3	390.1–410.5	399.4	7.9	2.0	95.8–99.8
4	0	190.2–215.6	**201.7**	9.0	4.5	–	6.8–10.8	**8.6**	1.5	17.4	–
5	0	132.5–145.6	**136.8**	4.5	3.3	–	7.5–10.9	**9.2**	1.3	14.1	–

TABLE III

RESULTS OF FIVE REPLICATE ANALYSES ON FIVE COMMERCIAL MARGARINE AND BUTTER SAMPLES USING THE VISIBLE SPECTROPHOTOMETRIC METHOD

Sample No.	Sorbic acid (mg/kg)										
	Added	Margarine					Butter				
		Range	Mean	S.D.	R.S.D. (%)	Recovery (%)	Range	Mean	S.D.	R.S.D. (%)	Recovery (%)
1	0	114.0–128.2	**119.9**	4.7	3.9	–	0.0–13.0	**5.8**	5.3	91.4	–
	100	197.0–218.4	207.5	6.8	3.3	92.1–95.9	92.9–106.0	101.6	5.4	5.3	92.9–99.6
	200	293.5–312.5	302.8	6.6	2.2	93.5–96.6	185.0–204.0	195.2	6.8	3.5	92.5–98.8
	400	489.6–510.0	497.3	7.5	1.5	94.6–96.7	378.0–396.9	387.3	6.9	1.8	93.9–97.6
2	0	135.1–155.3	**143.5**	7.3	5.1	–	0.0–12.5	**5.1**	4.7	92.2	–
	100	228.8–247.0	236.1	6.3	2.7	94.8–99.4	93.5–110.0	102.6	5.7	5.6	93.5–99.1
	200	324.5–343.5	331.8	6.5	2.0	93.2–98.0	188.2–205.6	196.6	6.3	3.2	94.1–97.1
	400	522.7–540.1	534.3	6.4	1.2	96.8–100.2	375.0–400.0	389.8	9.1	2.3	93.8–98.5
3	0	230.0–247.8	**240.2**	6.2	2.6	–	0.0–10.9	**4.7**	4.2	89.4	–
	100	328.8–346.1	338.6	6.0	1.8	97.1–100.8	93.0–104.2	100.0	4.9	4.9	93.0–99.2
	200	425.3–444.6	436.8	8.3	1.9	97.1–100.5	190.3–207.8	197.5	6.0	3.0	95.2–100.9
	400	620.8–639.4	628.6	6.2	1.0	96.5–99.9	380.3–410.5	397.0	9.8	2.5	95.1–99.9
4	0	181.0–195.5	**187.4**	5.2	2.8	–	0.0–9.8	**5.1**	4.2	82.4	–
5	0	118.5–130.0	**123.7**	4.5	3.6	–	0.0–12.0	**4.4**	4.4	100.0	–

TABLE IV

RESULTS OF FIVE REPLICATE ANALYSES ON THREE PURE STANDARD SOLUTIONS OF SORBIC ACID

Sorbic acid (mg/kg)

Given	RP-HPLC method			UV spectrometric method			Visible spectrophotometric method		
	Found	Mean	S.D.	Found	Mean	S.D.	Found	Mean	S.D.
100	94.7–99.1	97.0	1.7	93.5–98.2	96.3	2.1	93.2–99.1	96.2	2.2
200	190.0–202.5	196.1	5.1	187.5–202.1	195.3	5.3	186.1–199.3	193.7	4.9
400	380.5–399.2	390.8	7.0	375.0–390.9	386.2	7.5	378.5–396.8	388.9	6.9

spectrophotometric methods from 92.2 to 102.5% and from 92.1 to 100.5%, respectively.

The determination of sorbic acid as the 4-Brmdmc derivative by HPLC with fluorescence detection offers some advantages over earlier HPLC determinations with UV detection: fluorescence is a more selective means of detection than absorption and, further, it is far more sensitive, allowing the detection of very low levels of sorbic acid.

The results of the proposed procedure for the determination of sorbic acid in margarine and butter samples, on comparison with those of UV and visible spectrophotometric methods, show that the most accurate data are obtained by steam distillation of the samples followed by RP-HPLC determination with fluorescence detection.

Research is in progress on benzoic acid, another preservative added to foods and beverages to prevent or inhibit microbial growth. Preliminary investigations show that benzoic acid reacts with 4-Brmdmc, under the derivatization conditions described, to produce a fluorescent derivative that can be separated by HPLC.

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