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Short communication

Liquid chromatographic determination of phenolic antioxidants in bakery products

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Abstract

A simple and rapid method for the determination of phenolic antioxidants (propyl gallate, octyl gallate, dodecyl gallate, butylated hydroxyanisole and butylated hydroxytoluene) in bakery products is described. The method involves direct extraction and liquid chromatography–UV determination. The linearity (0.9992–0.9999), resolution, precision (coefficients of variation (%)=3.5–5.9) and recovery (40.2–95.1%) of this method are good.

Keywords: Extraction methods; Antioxidants; Propyl gallate; Octyl gallate; Dodecyl gallate; Butylated hydroxyanisole; Butylated hydroxytoluene; Phenolic compounds

1. Introduction

Antioxidants are used as food additives in order to prevent the oxidative deterioration of the lipid fraction during storage and processing. The antioxidants to be used are determined by various factors including legislation, cost, stability, effectiveness and the consumer preference for natural antioxidants. However, when permitted, synthetic antioxidants are widely used because of their low cost and high stability and effectiveness. Their use is especially widespread in oils and fats destined to be subjected to high temperatures during processing (e.g. frying, baking process). Thus, fats and oils used as ingredients for bakery products are usually stabilized by phenolic antioxidants.

Determination of phenolic antioxidants in edible fats and oils is well established, whereas determination in food products with a complex matrix (e.g. bakery products), where higher selectivity and sensitivity are required, has been studied less (reviewed by Rajalaksmi and Narasimhan [1] and Robards and Dilli [2]). Determination of phenolic antioxidants in foods is usually carried out by liquid chromatography (LC) [3–8] or gas chromatography (GC) [3,9,10]. Due to the wide polarity range of phenolic antioxidants and the polarity of the extracting solvent, a low recovery for some of these compounds is often obtained using these methods. Thus, there are few methods that use a direct extraction with a single solvent for the determination of various antioxidants with quite different polarities in foods with a complex matrix [4,6].

LC–UV seems to be the most popular method for phenolic antioxidant determination because it does not require previous derivatization and it has been

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adopted as the official method by various analytical associations (e.g. AOAC, IUPAC) [3]. Therefore, the main objective of our work was to develop a direct extraction method to enable the LC–UV determination of all phenolic antioxidants normally found in bakery products. The choice of the final method was based on comparisons of methods' performance characteristics for the synthetic antioxidants (propyl gallate, octyl gallate, dodecyl gallate, butylated hydroxyanisole and butylated hydroxytoluene) that are permitted by the European Union [11] in bakery products.

2. Experimental

2.1. Reagents, standards

The organic solvents used were all LC grade and were supplied by Romil (Cambridge, UK). Acetic acid was LC quality and was supplied by Montplet and Esteban (Barcelona, Spain). LC water was obtained from a Milli-Q plus Millipore system (Milford, MA, USA). 2,4,6-Trimethylphenol (97%), used as an internal standard (I.S.), was from Aldrich (Steinheim, Germany). Propyl gallate (PG, ≥98%) and butylated hydroxytoluene (BHT, ≥99%) were from Sigma (St. Louis, MO, USA). Octyl gallate (OG, ≥99%), dodecyl gallate (DG, ≥99%) and butylated hydroxyanisole (BHA, ≥98%) were from Fluka (Buchs, Switzerland).

2.2. Samples

Fifteen samples of industrial bakery products commercially available in Spain were used for the study. Samples included: doughnuts, biscuits and various types of cake.

2.3. Antioxidant extraction

A 1-mg amount of 2,4,6-trimethylphenol (I.S.) was added to 10 g of a minced sample. After 5 min, 75 ml of acetonitrile–isopropanol (1:1, v/v) was added and the mixture was homogenized for 1 min at 20 000 rpm. The liquid phase was separated by filtering under vacuum and the residue was reextracted twice with the same solvent mixture. The three extracted phases were collected in a round-bottomed flask and concentrated to 3–4 ml by a vacuum rotary evaporator at 33°C. This solution was quantitatively transferred to a 10-ml volumetric flask using acetonitrile–isopropanol–dichloromethane (1:1:1, v/v/v) and the volume was made up to 10 ml using acetonitrile–isopropanol (1:1, v/v).

2.4. LC determination of antioxidants

LC was carried out in a Series 3 Perkin-Elmer liquid chromatograph equipped with a Rheodyne manual injector and a Perkin-Elmer LC-100 oven (21°C). A C_{18} column (25×0.46 cm) packed with 5 μm-80 Å Extrasil ODS2 and a C_{18} pre-column packed with 5 μm-100 Å Kromasil ODS2 were used (Teknokroma, Barcelona, Spain). Detection was carried out at 280 nm, using a Shimadzu SPD-10AV spectrophotometric detector. The elution gradient was made from two solutions: solution A, acetic acid–methanol (5:95, v/v), and solution B, acetic acid–water (5:95, v/v). The elution programme was linear from 50 to 85% of A in 3 min, and then isocratic for 15 min (flow-rate, 1.5 ml/min). A 10-μl volume of the sample extract was injected. Antioxidants were identified by their retention times and by the addition of standards.

2.5. Lipid extraction

A 50-g amount of sample was homogenized in a mincer and 100 ml of chloroform–methanol (2:1, v/v) was added. The extraction was performed by magnetic stirring of the mixture for 30 min. Then the solid residue was separated by paper filtering, and was reextracted for 30 min with the same volume of the solvent mixture. The residue was separated again by filtering and then 50 ml of the solvent was added to rinse it. The three extracted fractions were collected in a separatory funnel and 35 ml of a saturated sodium chloride solution was added to help the separation of the chloroform phase. This phase was filtered through anhydrous sodium sulphate and recovered in a 250-ml round-bottomed flask and the solvent was evaporated by a vacuum rotatory evaporator at 33°C. Removal of solvent was completed in a slight nitrogen stream and by keeping the flask in a vacuum desiccator at 10 mmHg for 16 h.

The lipid residue was weighed and the percentage of fat in the sample was calculated.

2.6. Peroxide value and fatty acid determination

The AOAC method (965.33) [3] was used with a few modifications to determine the peroxide value in the extracted lipid fraction. Results were expressed as milliequivalents peroxide/kg fat. Fatty acid composition was determined following the method proposed by Guardiola et al. [12].

3. Results and discussion

3.1. Linearity of response

Calibration curves were obtained from methanol solutions of the I.S. (100 μg/ml) and antioxidants (BHT, BHA, DG, OG and PG; 2–100 μg/ml). Solutions were prepared in quintuplicate and injected in duplicate. The linearity of the response was good for all antioxidants, as indicated by the determination coefficients (0.9992–0.9999).

3.2. Recovery and precision

In previous studies, solvents with a wide range of polarities (from methanol or acetonitrile to hexane or petroleum ether) have been used to extract phenolic antioxidants from food samples [2]. As bakery products have a complex matrix, the choice of the extraction solvent was based on obtaining good recovery values for all antioxidants considered and also an effective removal of impurities. Although different purification systems have been proposed for the purification of antioxidant extracts from complex matrixes, we wanted to develop a direct extraction procedure. So, we decided to test the direct extraction procedure proposed by Gertz and Herrmann [4], which uses acetonitrile–isopropanol–0.2% (p/v) oxalic acid in ethanol (50:25:25, v/v/v). For comparison, the extraction procedure proposed by Page and Charbonneau [8] in which the antioxidants are extracted with acetonitrile after mixing the sample with hexane was also tested. The latter method is one of the most widely accepted methods for phenolic antioxidant extraction and determination and it achieved better recoveries, but it is much more time-consuming (see Table 1). This led us to modify Gertz and Herrmann's extraction in order to obtain better recoveries. Finally, instead of acetonitrile–isopropanol–0.2% (p/v) oxalic acid in ethanol (50:25:25, v/v/v), we used acetonitrile–isopropanol (50:50, v/v), which led to higher antioxidant recoveries, mainly for BHT and DG (Table 1). Recovery values were obtained by addition to the same bakery sample of two levels of each antioxidant, 10 and 30 μg/g. Analysis using the Student–Fisher's *t*-test showed that there were no significant differences ($\alpha=0.05$) between the recoveries obtained for the two levels of addition. So, we calculated the global mean recoveries for the five antioxidants, which were similar to recoveries obtained using Page and Charbonneau's extraction (Table 1). Global mean recoveries were used for the calculation of

Table 1
Recovery values of various extraction procedures

Extraction procedure	Level of addition[a]	Antioxidant recovery (%)				
		PG	BHA	OG	BHT	DG
Page and Charbonneau [8]	100	88.5[b]	93.6	85.0	75.7	58.2
Gertz and Herrmann [4]	100	78.4	75.1	70.2	57.8	28.3
Modification of Gertz and Herrmann's procedure	10	89.5	94.5	77.9	85.7	40.7
	30	88.9	95.8	79.7	89.4	39.8
	Global mean[c]	89.2	95.1	78.8	87.6	40.2

[a] Micrograms of antioxidant added per gram of sample.
[b] Mean values ($n=3$ for level of addition 100, $n=5$ for levels of addition 10 and 30, and $n=10$ for global mean).
[c] Global mean recovery obtained from values corresponding to levels of addition 10 and 30.

antioxidant content in the samples. Recovery was also calculated for the internal standard, and the mean value obtained ($n=10$) was 94.6%.

To determine the precision of the method finally chosen, we made a cake in which we included the five antioxidants at a concentration of 16 μg/g. Antioxidant determination was performed on ten aliquots of this sample and the C.V.s (%) of the values obtained were: 5.3 for PG, 3.5 for BHA, 5.7 for OG, 4.9 for BHT and 5.9 for DG. In addition, as can be seen in the chromatogram corresponding to this sample (Fig. 1), the method shows a good resolution.

3.3. Content of phenolic antioxidants in bakery samples

Nine of the fifteen samples analyzed contained BHA and/or BHT, while no gallates were detected in any sample. However, although phenolic antioxidant labelling is mandatory in the EU [13], only BHA was mentioned on the label of three of these samples. Antioxidant content expressed as μg/g of sample ranged from 3.9 to 10.7 for BHA (detected in eight samples) and from 3.0 to 17.0 for BHT (detected in seven samples). Both antioxidants were found in six samples, while the combination of BHA plus BHT is not permitted by the EU food law for additives [11]. Moreover, if results are expressed as antioxidant content over the fat fraction, quite a high use of these phenolic antioxidants is indicated. BHA ranged from 17.5 to 55.6 and BHT from 14.9 to 90.0 μg/g of fat, while maximum levels set by the EU food law for additives [11] are 200 for BHA (separately or combined with gallates) and 100 for BHT (no combinations permitted).

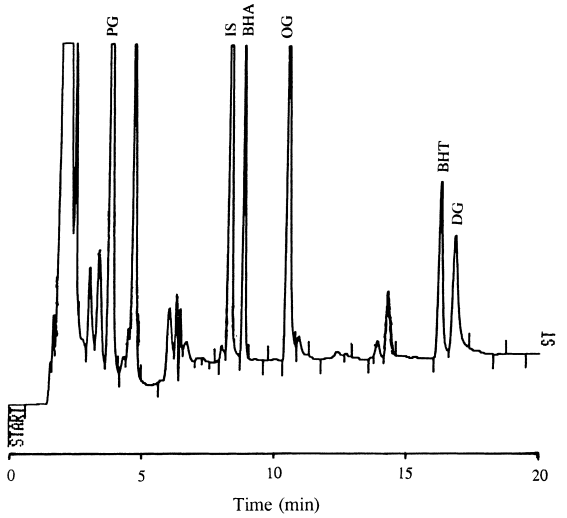

Fig. 1. Chromatogram corresponding to the sample used to determine the precision of the method. Concentations are 16 μg/g for each of the five antioxidants.

3.4. Fat content, peroxide value and fatty acid composition in bakery samples

Fat content (%) ranged from 13.2 to 21.0 with a mean value of 19.4, peroxide value (mequiv. peroxide/kg fat) from 0.9 to 6.3 with a mean value of 2.2 and percentage of polyunsaturated fatty acids (PUFA) over total fatty acids from 7.7 to 51.6 with a mean value of 18.0.

No significant correlations were observed between the level of antioxidants expressed as μg/g of fat (BHA+BHT) and fat content ($P=0.735$), nor between the level of antioxidants and peroxide values ($P=0.316$). In contrast, as could have been expected, a significant correlation was observed between the level of antioxidants and the percentage PUFA in the fat ($P=0.026$).

4. Conclusions

The method proposed shows good linearity, good resolution, good precision and similar recoveries to one of the most accepted methods for phenolic antioxidant determination in foods [8]. In addition, this method is much less time-consuming.

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