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Analysis of synthetic phenolic antioxidants in edible oils by micellar electrokinetic capillary chromatography

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Abstract

Synthetic antioxidants most used in oil-based food to avoid oxidation processes, butylated hydroxyanisole (BHA), butylated hydroxytolueno (BHT), and dodecyl gallate (DG), were analyzed in edible oils using micellar electrokinetic capillary chromatography (MECK) with bis-(2-ethylhexyl) sodium sulfosuccinate as the pseudostationary phase.

Studies involving solid-phase and liquid–liquid extraction were performed to find the best sample treatment before injection into the electrophoretic system. The best methodology for the isolation of antioxidants was extraction with acetonitrile from edible oil diluted with hexane. A method that allows the determination of the antioxidants present in these samples was proposed. With this method BHA, BHT and DG were evaluated at levels permitted in the European Union.

Keywords: Butylated hidroxyanisole; Butylated hydroxytoluene; Edible oils; Micellar electrokinetic capillary chromatography (MEKC); Bis- (2-ethylhexyl) sodium sulfosuccinate

1. Introduction

The synthetic compounds butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and dodecyl gallate (DG) are the antioxidants most frequently used as additives in lipid-containing foods (Primo Yúfera, 1997). They have been used both alone and in mixtures in oils, especially in corn oil or sunflower oil.

The conclusions of research carried out on the effects of these substances on consumer health are contradictory. On one hand, it has been found that these synthetic antioxidants exert toxic effects in some animal tissues (Horvathova, Slamenova, Bonatti, & Abbondandolo, 1999;Yu, Mandlekar, & Kong, 2000) and that even at low dose levels phenolic compounds may produce additive/synergistic effects as regards carcinogenesis (Hirose et al., 1998) and therefore their use is being questioned (Valentao et al., 2002). By contrast, these substances have also been found to have anti-mutagenic and anti-tumour properties (Iverson, 1999; Kato, Harashima, Moriya, Kikugawa, & Hiramoto, 1996; Talalay, Fahey, Holtzclaw, Prestera, & Zhang, 1995). Consequently, the analytical control of these compounds in foods is of considerable importance. The use of synthetic antioxidants is regulated in the legislation of most countries, the limit amounts that can be added to foods being strictly defined. In the European Union, for example, the amount of synthetic antioxidants is limited to 0.01% (0.1 g/kg) for each antioxidant if used individually and 0.02% of the total amount if the antioxidants are used in mixtures (*Diario Oficial de las Comunidades Europeas*).

Micellar electrokinetic capillary chromatography (MEKC) has been established as a powerful technique for the separation of a large variety of neutral and charged analytes, with higher peak efficiency and resolution than high performance liquid chromatography and comparable to those of gas chromatography. Additionally, MEKC

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offers fast analysis times and is less costly. Unfortunately, the disadvantages of MEKC include detection sensitivity, as a result of the capillary dimensions, and the problems related to application of the separation to real samples. In most cases, direct injection of real samples, such as oils or pharmaceuticals, into capillary electrophoresis systems cannot be accomplished and sample treatment is necessary. Matrix effects are related to differences in electric strength between the sample and the separation buffer, disturbed equilibrium between analyte micelle, the absence of micelle formation in the sample zone, and modifications in the capillary wall surface. These effects are reflected in peak broadening, distorted peaks and migration times (Pyell, 2001).

Capillary electrophoresis (CE) has been applied to the analysis of food substances containing preservatives (Hall, Zhu, & Zeece, 1994;Kuo & Hsieh, 1997) and MEKC has been employed to resolve mixtures of food antioxidants (Summanen, Vuorela, Hiltunen, Sirén, & Riekkola, 1995). In all the above cases, one outstanding aspect is the difficulty involved in correctly quantifying BHT and DG, mainly due to the appearance of low signals and very broad peaks. The surfactant most widely employed is SDS, although other surfactants such as bile salts or mixed micelles formed by two combined surfactants have also proved to be very useful in the separation of food antioxidants. SDS as the pseudophase failed to resolve BHT and DG and α-tocopherol mixtures, but using a mixed micellar phase of SDS and sodium cholate in the presence of 10% methanol resolution was possible (Boyce, 1999, 2001).

The application of CE to synthetic antioxidants in food analysis has been limited. In a previous work (Delgado-Zamarreño, Sánchez-Pérez, González-Maza, & Hernández-Méndez, 2000), the separation of six synthetic antioxidants using an electrophoretic system with bis-(2-ethylhexyl) sodium sulfosuccinate vesicles were carried out. The aim of the present work was to apply the previously developed method to the detection and analysis of three antioxidants (BHA, BHT and DG) in oil samples. In order to find the best treatment of the sample before injection into the electrophoresis system, studies involving solid-phase and liquid-liquid extraction, to isolate the antioxidants from the oil samples, were carried out.

2. Experimental

2.1. Apparatus

Experiments were carried out using a P/ACE 2200 capillary electrophoresis system equipped with a UV detector, permitting measurements at 200, 214, 254 and 280 nm (Beckman, Fullerton, CA, USA). A fused-silica capillary with 57 cm × 75 μm i.d. was used. Detection at 280 nm was accomplished at 50 cm. Data were recorded on a computer with Beckman System Gold™ software.

Silica (Whatman SPE SIL 360 mg), C_{18} (Waters tC18, 400 mg) and polymeric-C_{18} (Waters OASIS HLB 60 mg) cartridges were used when the isolation of antioxidants was carried out using solid-phase extraction. A Büchi (Flawil, Switzerland) RE 121 rotavapor with a Büchi 461 water-bath was used. Water was purified in an ElgaStat water-purification system (Elga, High Wycombe, UK).

2.2. Reagents

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and dodecyl gallate (DG) were supplied by Sigma. Bis-(2-ethylhexyl) sodium sulfosuccinate was from Fluka. Special HPLC quality acetonitrile (Merck, Darmstadt, Germany), special HPLC quality methanol (BDH, Poole, England), special HPLC quality *n*-hexane (Merck) were used. The other reagents employed were supplied by Panreac (Barcelona, Spain) and were of analytical-reagent grade.

2.3. Samples and procedure

All assays were carried out using commercial sunflower oil or olive oil, purchased from Spain market, which were spiked with the antioxidants BHA, BHT and DG, dissolving these directly in the oil. All assays were realised in triplicate. The sample treatment followed in each case depended on the type of assay conducted before injection into the electrophoretic system.

The electrophoretic system employed consisted of an aqueous solution with 20% acetonitrile, 20 mM boric–borate buffer (pH = 9.2) and 20 mM bis-(2-ethylhexyl) sodium sulfosuccinate surfactant. Conditioning of the capillary was used in a previous work (Delgado-Zamarreño et al., 2000) and consisted of 5 min with water and then a further 2 min with 0.1 mol/L sodium hydroxide. Finally, the capillary was rinsed with the separation solution for 2 min. Before each run the capillary was rinsed for 1 or 2 min with the buffer solution. Injection was accomplished under pressure over 5 s and the voltage applied for separation was 24 kV. UV detection was carried out at 280 nm and temperature was maintained at 25 °C.

3. Results and discussion

3.1. Sample treatment

In most cases, direct injection of real samples into capillary electrophoresis systems cannot be accomplished and it is necessary to perform sample treatment in order to isolate the analytes in a suitable medium before their injection into the electrophoresis system. Accordingly, different assays were carried out using the separation buffer as the extractant solution; that is, boric-sodium borate buffer solution in acetonitriles-water medium in the presence of bis-(2-ethylhexyl) sodium sulfosuccinate. This would be a very simple sample treatment, direct injection after the extraction. However, the results obtained were not reproducible because even small amounts of oil produce changes in the capillary wall and hence the electropherograms

change with consecutive injections. Accordingly, a study of the extraction of the antioxidants from oil was carried out using the classic treatments of liquid–liquid or solid-phase extraction in order to find the best conditions for their isolation.

The study was carried out on samples spiked with synthetic antioxidants, the assays were realised in triplicate. Although any extraction step should be evaluated on natural samples that include the natural behaviour of the analyte/property in the original sample, the use of spiked samples is acceptable because these antioxidants are food additives and do not occur naturally.

3.1.1. Solid-phase extraction

Solid-phase extraction using C18 or silica sorbents was studied using sunflower oil spiked with 1 g/kg of each antioxidant. The oil sample (2 mL) was loaded through the C18 cartridge after the solid phase had been conditioned with methanol (5 mL). Finally, elution was carried out using 2 mL of methanol or acetonitrile. Two hundreds microliters of these extracts were diluted with 4.0 mL of separation buffer and injected into the electrophoretic system. Injection of eluate into the electrophoresis system was impossible because the electropherogram was disrupted.

Beside, there was other inconvenience; the viscosity of the oil hinders its passage through the cartridge. In order to avoid this effect and to improve contact between the sample and solid phase, these procedures were modified, using oil samples diluted with hexane (1:3) and elution with methanol or acetonitrile was carried out in the same way. Study of the silica solid phase was similar to that performed for the C18 phase, except hexane was used (5 mL) for the conditioning.

The recoveries obtained after application of the procedures are shown in Fig. 1. The polarity of each analyte was different and hence the recovery values also differed. For example, BHT was not extracted from the samples when were diluted with hexane; in case of BHA and DG the recoveries also were smaller using hexane. The best recovery value of BHA was obtained using C18 as the solid phase with oil samples not diluted with hexane and methanol as eluent. However, for DG the best value was obtained with the silica sorbent with acetonitrile as the eluent. In order to analyze BHA, BHT and DG in oil samples it was necessary to use an oil sample C18 sorbent and acetonitrile as eluent as an agreement procedure. At these concentration levels, the absorbance values were too low and for this reason a liquid-liquid extraction study was carried out.

3.1.2. Liquid–liquid extraction

Owing to the hydrophobic characteristics of BHA, BHT and dodecyl galate, it may be deduced that the isolation of these compounds from oil samples should be accomplished by extraction with an organic solvent. Therefore, methanol and acetonitrile were used because these solvents have been used for the extraction of antioxidants in other matrices,

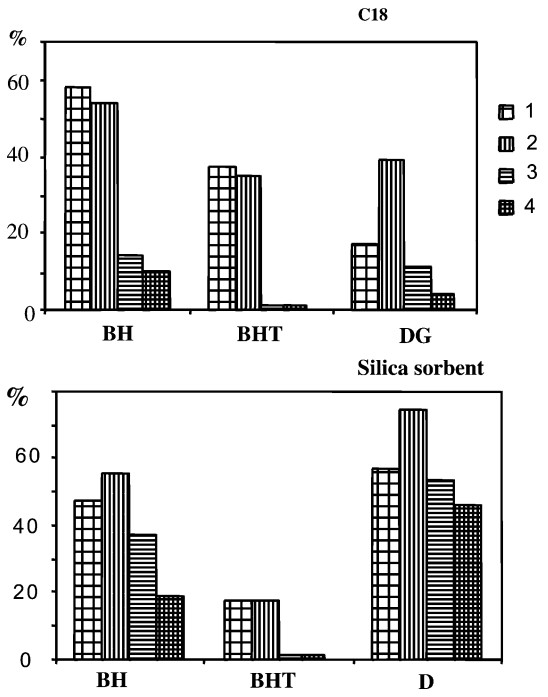

Fig. 1. Recoveries obtained when solid-phase extraction was used. Sunflower oil spiked with 1 g of each antioxidant/kg of oil. (1 and 2) samples of spiked oil; (3 and 4) samples of spiked oil diluted with hexane. Elution from solid phase, methanol: (1 and 3) samples; acetonitrile: (2 and 4) samples.

such as biscuits and chewing gum, before their determination by GC (Gonzalez, Ballesteros, Gallego, & Valcarcel, 1998; González, Gallego, & Valcarcel, 1999). A systematic study of the extraction was performed in order to obtain the best signal possible as well as the highest "cleanliness" of the extract, using oil spiked with a 1 g/kg concentration of each antioxidant.

One way of optimising extraction consists in diluting the oil in an apolar solvent such as hexane and then perform-

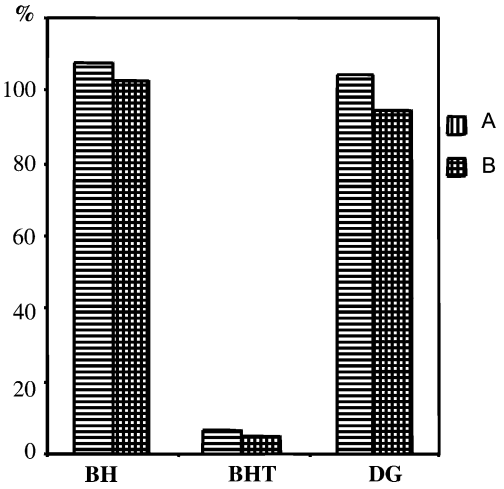

Fig. 2. Recoveries obtained when liquid–liquid extraction was used. Sunflower oil spiked diluted with hexane. Extractant: methanol (A) and acetonitrile (B).

ing the extraction. In fact, the literature contains references in which the fats are dissolved in hexane before carrying out extraction with more polar solvents (Official Methods of Analysis of the AOAC, 1990; Page & Charboneau, 1989). It is possible that the change in the viscosity of the sample might increase the contact surface between phases and hence it was first necessary to check that dilution in hexane would offer the best possible signal. The best signals were obtained for a dilution in hexane between 60% and 80% and hence the oil was diluted with hexane up to 1:3 (75%) because at this percentage phase separation is good and losses in the absorbance signal are not very important.

L–L extraction was applied to 25 mL of spiked sunflower oil after dilution with hexane (1:3). Extraction was carried out three times with methanol (50 mL). After evaporation in a rotavapor, the extracts were dissolved in 2.0 mL of methanol; 500 μL of this solution diluted with 4.0 mL of separation buffer was injected into the electrophoretic system. Acetonitrile, instead of methanol, was also used as extraction solvent. The hexane used in the dilution of oil was saturated with methanol or acetonitrile, depending on the solvent used in the extraction. The recoveries obtained are plotted in Fig. 2. As in solid-phase extraction, BHT was the analyte with the lowest recovery.

The most favourable extraction procedure would be the one carried out with acetonitrile, since this compound elicited a better phase separation and afforded a "cleaner" extract. It should also be noted that the electrophoretic peak of BHT had poor resolution, and should be considered as limit peak. Moreover when acetonitrile was used,

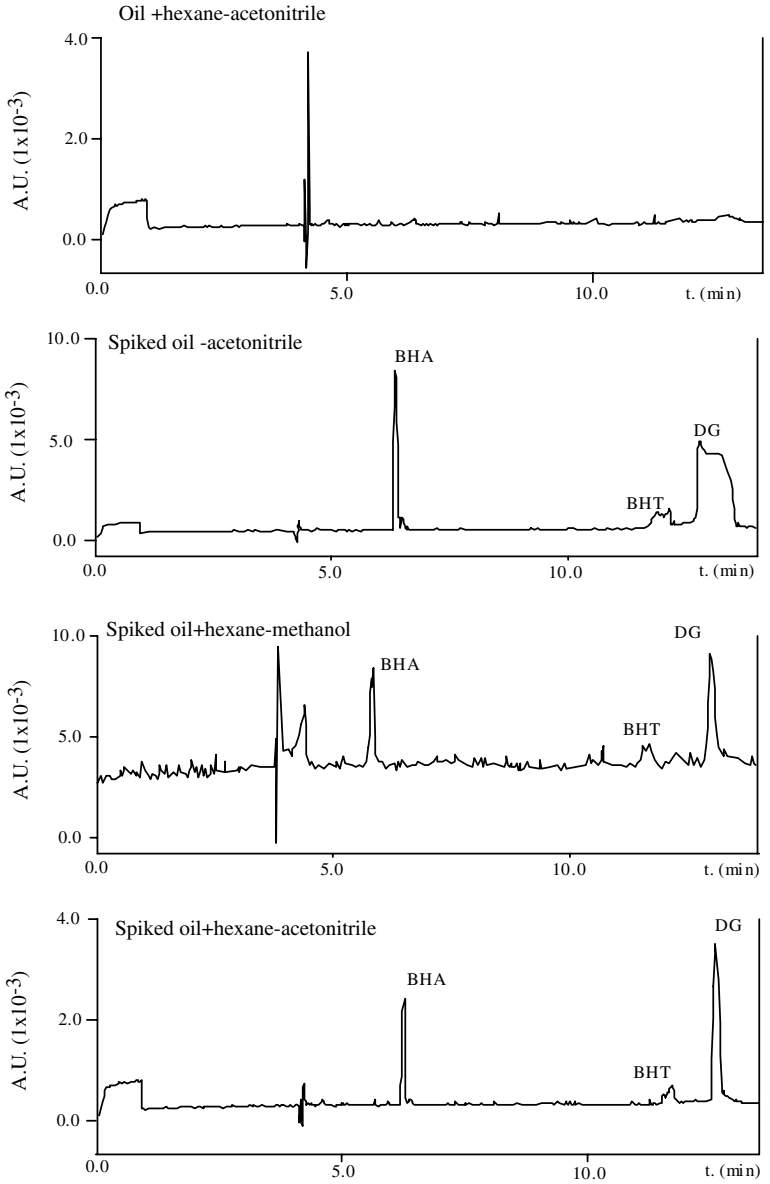

Fig. 3. Electropherograms obtained from different samples (oil, spiked oil, and spiked oil diluted with hexane) treated with L–L extraction procedures, indicated above the corresponding electropherogram. Conditions: hydrodynamic injection (5 s); applied voltage 24 kV; 20 mM borate buffer (pH 8, 4); 20% acetonitrile and 20 mM of surfactant bis-(2-ethylhexyl) sodium sulfosuccinate. Detection UV: 280 nm.

the BHT signal had a better peak shape. A loss in the DG signal is acceptable if BHT can be measured. From the experimental data obtained, it may be deduced that the best choice was extraction with acetonitrile after dilution with hexane. This type of extraction afforded a "cleaner" final extract, implying an improvement in the shape and resolution of the electrophoretic peaks (Fig. 3).

3.1.3. Analytical data

The parameters addressed below were investigated in the validation study of the method. Selectivity, generally defined as the absence of interfering peaks at the retention times of the analytes in the electropherograms, was tested by the injection of extracts of blank oil samples. Five samples of blank oil (olive, sunflower, corn and mixture of seeds oil samples) were analysed and no interfering peaks were observed in any case (Fig. 3).

Calibration was performed with the linear regression method. Three calibration graphs were obtained using standards dissolved in methanol, in sunflower oil and in olive oil. When samples of oils were used, standards were prepared by dissolving the antioxidants at different concentrations ranging between 0.01 and 1 g/kg in sunflower oil and olive oil. With each standard, analyte isolation was carried out using L–L extraction with acetonitrile, described as procedure B. Electrophoretic separation of the analytes was carried out in 20 mM boric–borate buffer, 20% acetonitrile, and 20 mM of the bis-(2-ethylhexyl) sodium sulfosuccinate surfactant, applying 24 kV and detection at 280 nm, like the procedure described in Section 2. The peaks thus obtained were then integrated. In the range studied, the calibration curves obtained were linear. The calibration curve parameters are given in Table 1. The correlation coefficients indicated linearity throughout the calibration range.

Detection limits for a signal-to-noise ratio of 3, using standards in methanol, were 0.45, 1.47 and 0.27 ppm for BHA, BHT and DG, respectively.

To determine the intra-day precision of the method, or repeatability, 10 samples of sunflower oil spiked at a concentration of 0.8 g/kg of each antioxidant were analysed. The variation coefficient for migration times were 1.7%, 2.8% and 3.0% and for corrected areas were 3.0%, 10% and 7.0% for the antioxidants BHA, BHT and DG, respectively.

Olive, sunflower, corn and mixture of seeds oil samples were analyzed and none of these antioxidants were found. As a consequence, the methodology developed was applied to samples of sunflower oil spiked with these analytes. Two levels of concentration were used 0.8 and 0.08 g/kg, one of them lower than the level accepted for the legislation (0.1 g/kg, 0.01%) for each synthetic antioxidant.

The samples were analysed in triplicate, each extract was injected three times, and the concentrations were calculated using the different calibration curves (Table 2). The consequence of the high percentages extracted of BHA and DG from the samples (Fig. 2) was the similarity of slopes of the calibration graphs in methanol (no extraction step) and in oils; for this reason the results for BHA and DG were acceptable when any of the calibration curves were used. However, the results for BHT only were acceptable when the calibration graph used was the same kind of oil.

Table 1
Calibration curves for standards in different matrices

Standards in methanol[a]	BHA Area units = $(-0.01 \pm 2) + (37.2 \pm 1)$ C (g/kg)	$R^2 = 0.999$
	BHT Area units = $(0.044 \pm 0.03) + (13.4 \pm 0.9)$ C (g/kg)	$R^2 = 0.998$
	DG Area units = $(-0.02 \pm 2) + (61.9 \pm 3)$ C (g/kg)	$R^2 = 0.998$
Standards in sunflower oil	BHA Area units = $(-0.4 \pm 1) + (40.2 \pm 4)$ C (g/kg)	$R^2 = 0.995$
	BHT Area units = $(0.04 \pm 0.03) + (0.85 \pm 0.06)$ C (g/kg)	$R^2 = 0.997$
	DG Area units = $(-0.02 \pm 2) + (65.0 \pm 5)$ C (g/kg)	$R^2 = 0.996$
Standards in olive oil	BHA Area units = $(-0.69 \pm 2) + (40.5 \pm 3)$ C (g/kg)	$R^2 = 0.997$
	BHT Area units = $(0.07 \pm 0.04) + (1.21 \pm 0.07)$ C (g/kg)	$R^2 = 0.998$
	DG Area units = $(-0.61 \pm 3) + (57.3 \pm 5)$ C (g/kg)	$R^2 = 0.996$

[a] The standards in methanol were only diluted with separation buffer.

Table 2
Amounts found using the calibration curves after application of the proposed method to a sunflower sample spiked at two concentration levels (three replicates)

	Antioxidants	Added: 0.8 g/kg found	Added: 0.08 g/kg found
Calibration curve Standards in Methanol	BHA	0.75 ± 0.02	0.064 ± 0.002
	BHT	0.064 ± 0.004	0.007 ± 0.002
	DG	0.77 ± 0.03	0.084 ± 0.004
Calibration curve Standards in Sunflower oil	BHA	0.79 ± 0.01	0.065 ± 0.01
	BHT	0.99 ± 0.08	0.067 ± 0.04
	DG	0.75 ± 0.05	0.087 ± 0.02
Calibration curve Standards in Olive oil	BHA	0.70 ± 0.06	0.075 ± 0.01
	BHT	0.64 ± 0.004	0.037 ± 0.02
	DG	0.82 ± 0.05	0.081 ± 0.02

4. Conclusions

The results of the present work indicate that it is possible to analyze synthetic food antioxidants in oil samples with a rapid methodology that includes L–L extraction and micellar electrokinetic capillary chromatography. The proposed procedure for the control of synthetic antioxidants in edible oils, up to authorized levels, is suitable as regards sensitivity and reproducibility for BHA and DG. In the case of BHT the method could be suitable if calibra-

tion curves with same kind of oil under study can be obtained.

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