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LIQUID CHROMATOGRAPHIC DETERMINATION OF SYNTHETIC ANTIOXIDANT CONTENT OF SOME VEGETABLE OILS PRODUCED IN TURKEY

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Abstract

In this work, the synthetic antioxidant contents of some vegetable oils produced in Turkey were determined by using liquid chromatography with ultraviolet detector. Butylated hydroxy anisole, butylated hydroxy toluene, and *tert*-butyl hydroquinone were the synthetic antioxidants selected. The extraction of antioxidants from oil samples was carried out with vortex-assisted liquid-liquid microextraction prior to chromatographic analysis. Some parameters that affect the extraction efficiency, such as the type of extraction solvent, the volume of solvent, and extraction time were optimized. Under the optimized conditions, the limits of detection and limits of quantification for the antioxidants were in the ranges 0.12-0.28 µg/g and 0.40-0.92 µg/g, respectively. The intra-day and inter-days precisions were calculated in terms of relative standard deviation and found to be less than 6.5% and 8.4%, respectively. Totally, 16 vegetable oil samples produced in Turkey were analyzed to detect the presence of the synthetic antioxidants and the concentrations of antioxidants in samples were found below the maximum permitted levels defined in Turkish Food Codex Regulation.

Keywords: Synthetic antioxidants, vegetable oils, microextraction, liquid chromatography.

TÜRKİYE'DE ÜRETİLEN BAZI BİTKİSEL SIVI YAĞLARIN SENTETİK ANTİOKSİDAN İÇERİKLERİNİN SIVI KROMATOGRAFİSİ İLE BELİRLENMESİ

Özet

Bu çalışmada, Türkiye'de üretilen bazı bitkisel sıvı yağların sentetik antioksidan içerikleri ultraviyole detektörlü sıvı kromatografisi ile belirlenmiştir. Butil hidroksi anisol, butil hidroksi toluen ve *tert*-butil hidrokinon seçilen antioksidanlardır. Kromatografik analiz öncesinde yağ örneklerindeki antioksidanların ekstraksiyonu vorteks-karıştırma destekli sıvı-sıvı mikroekstraksiyon tekniği ile yapılmıştır. Ekstraksiyon çözücüsünün türü, çözücü hacmi ve ekstraksiyon süresi gibi ekstraksiyon verimini etkileyen bazı parametreler optimize edilmiştir. Optimize edilen koşullarda antioksidanlar için metodun algılama ve belirleme sınırlarının sırasıyla 0.12-0.28 µg/g ve 0.40-0.92 µg/g aralığında olduğu tespit edilmiştir. Gün içi ve günler arası kesinlik, bağıl standart sapma olarak hesaplanmış ve sırasıyla % 6.5 ve % 8.4'den daha düşük bulunmuştur. Türkiye'de üretilen toplam 16 bitkisel yağ örneği sentetik antioksidanların varlığını tespit etmek için analiz edilmiş ve örneklerdeki antioksidanların konsantrasyonlarının Türk Gıda Kodeksi Yönetmeliğinde tanımlanan maksimum izin verilen seviyelerin altında kaldığı bulunmuştur.

Anahtar kelimeler: Sentetik antioksidanlar, bitkisel yağlar, mikroekstraksiyon, sıvı kromatografisi.

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INTRODUCTION

Synthetic antioxidants (SAs) have been widely used for many years as antioxidants to preserve and stabilize the freshness, nutritive value, flavour and colour of foods and animal feed products (1). Butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), tert-butyl hydroquinone (TBHQ) and propyl gallate (PG) are the most commonly used SPAs because of their chemical stability, low cost and availability (2). In several countries, the use of these antioxidants is regulated by various legislating authorities such as European Union Directives and Regulations, the FDA in the United States, Food Standards Australia New Zealand for Australia and New Zealand, Joint FAO/WHO Expert committee on food additives (3). According to Turkish Food Codex, in compliance with European Union Directives, the antioxidants mentioned above are permitted for use, individually or in combination, in oils, fats and lipid containing foods usually at concentrations up to 100 – 200 mg/ kg (4). The SPAs provide a high level of protection in maintaining food product quality but an excess of antioxidants is a health risk, since they may cause allergen reactions, including asthma and hives, in sensitive subjects (5). BHA and BHT have been suspected of being responsible for liver damage, and carcinogenesis when used at high levels in laboratory animals (6). However, there is no clear evidence that SAs intake might cause cancer among humans. Although SPAs are considered safe for human health at recommended doses, the concentration of these compounds in foods is strictly regulated by governments and requires monitoring.

Several analytical methods are available for the determination of SAs. These include liquid chromatography (LC) (7-9), gas chromatography (10-12), and micellar electrokinetic chromatography (13, 14). LC with UV is the most used technique (3). Prior to chromatographic analysis, the classical liquid–liquid extraction (LLE) (7, 15) and solid-phase extraction (SPE) (16, 17) are the most commonly used techniques for the preconcentration and cleanup of SPAs. Recently, some microextraction techniques, such as dispersive liquid-liquid microextraction (DLLME) (8), vortex-assisted liquid-liquid microextraction (VALLME) (9) and ultrasonic-assisted liquid-liquid microextraction (UALLME) (10) were introduced as the simple, low cost, fast, and environmental-friendly enrichment techniques.

Herein, an optimized and validated LC-UV method for the determination of SAs in some selected vegetable oils consumed in Turkey has been presented. SAs in vegetable oils were extracted with only 500 µL ethanol and then injected into LC-UV directly without clean-up or preconcentration, and the analytes were readily and sensitively detected. The best extraction and analysis conditions for the SAs were optimized and validated in relation to the following figures of merit: accuracy, precision, detection and quantification limits, linearity, and sensitivity. Finally, the methodology was applied to the analysis of vegetable oils obtained from local markets in Zonguldak, Turkey.

MATERIALS AND METHODS

Reagents and solutions

All of the reagents used in the experiments were of analytical grade. Butylated hydroxy toluene (BHT) was purchased from Supelco (Bellefonte, PA, USA). Butylated hydroxy anisole (BHA) and tert-butyl hydroquinone (TBHQ) were obtained from Sigma–Aldrich (Steinheim, Germany). HPLC grade acetonitrile were also obtained from Sigma–Aldrich (Steinheim, Germany). Acetone, methanol, and ethanol were purchased from Merck (Darmstadt, Germany). Water was purified with a Direct-Q3 water purification system (Millipore, Bedford, MA, USA).

The stock solutions of the antioxidants (1000 µg/ mL) were prepared by dissolving each individual standard of antioxidants in acetonitrile and stored at 4°C. Working solutions were obtained by appropriate dilution of the stock standard solutions. Total 16 vegetable oil samples including sunflower, virgin olive, refined olive, and corn oil were collected from local supermarkets in Zonguldak, Turkey during a period of January to March in 2016. The collected oil samples were packed in screw–cap glass tubes and stored at room temperature until analyzed.

Instrumentation and chromatographic conditions

The chromatographic analysis was performed by Thermo Finnigan LC system (San Jose, USA) consisting of a P1000 pump, a AS3000 automatic

injector system, a SCM 1000 degasser and a UV1000 UV detector. Separation was performed by means of a Phenomenex Fusion-RP column (250x4.6 mm i.d., 4.0 µm) protected by a C12 guard column (4x3 mm i.d., Phenomenex). A gradient elution program was optimized by using the mobile phases of acetonitrile and distilled deionized water (0.1% trifluoroacetic acid). The separation was performed at room temperature with a constant flow-rate of 1.0 mL/min by employing the elution program as follows; 0–2 min acetonitrile–water 30:70 (v/v) and then a linear gradient elution from 30% acetonitrile at 2 min to 100% acetonitrile at 15 min, followed by isocratic elution with acetonitrile for 5 min. Finally, 10 min was necessary in re-establishing the initial conditions. The UV detector was set at 280 nm. A vortex shaker (Velp Scientifica, Milan, Italy) and a NF 200 centrifuge (Nüve, Ankara, Turkey) were used in the sample preparation step.

Extraction procedure

Oil samples (1g) were weighed into a 12 mL screw–cap glass test tube. Ethanol (500 µL) was added, and the mixture was then vigorously shaken on a vortex agitator at 3000 rpm for 3 min. The mixture was next centrifuged at 3500 rpm for 3 min. The upper clear portion was removed with a micro-syringe and 20 µL of this solution was injected into the LC by using an automatic injector.

RESULTS AND DISCUSSION

Optimization of the extraction conditions

In order to improve extraction efficiency, several parameters were studied and optimized, such as the type of extraction solvent, the volume of solvent, and the extraction time. Optimization of vortex-assisted microextraction was carried out by using sunflower oil sample which was free of the analytes. The samples were spiked with the all SAs at a concentration of 25 µg/g each by adding minute volume (25 µL) of standard solution in acetonitrile. Spiked samples were vortex mixed for a few seconds and left to stand for 30 min at room temperature for equilibration between analyte and matrix. The optimal conditions were selected based on the peak areas obtained. All the experiments were carried out in triplicate.

The selection of an appropriate extraction solvent is a crucial parameter for a liquid-liquid extraction process. The extraction solvent has to meet the following requirements such as (a) good chromatographic behavior at optimized LC conditions, (b) low solubility in oil, and (c) high extraction capability for target compounds. In some preliminary studies, it has been reported that acetonitrile, methanol, ethanol or their different mixtures were suitable for extracting phenolic antioxidants from fats (10, 11). Based on these criteria, three extraction solvents including acetonitrile, methanol and ethanol were studied for the extraction of the SAs. A series of experiments were performed by using 500 µL of the selected extraction solvents and 1 min of vortex mixing time. The results shown in Fig. 1, indicated that ethanol provided the best analytical response among the other solvents; therefore, ethanol was selected as the suitable extracting solvent for subsequent experiments.

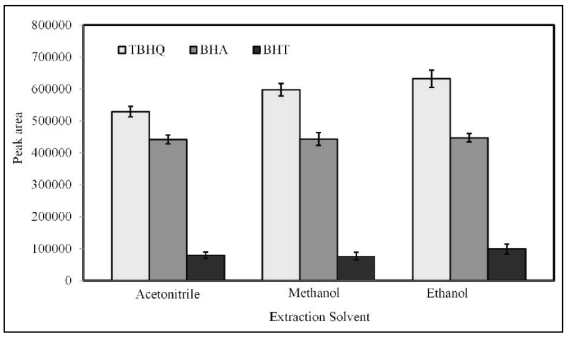

Fig. 1. Effect of type of extraction solvent. Extraction solvent volume, 500 µL; sample volume, 1 g; vortex mixing time, 1 min; spiked concentration, 25 µg/g.

In order to examine the effect of the extraction solvent volume, experiments were conducted in which the ethanol volume was varied in the range of 400 – 800 µL and the vortex mixing time fixed at 1 min. The obtained results are presented in Fig. 2. With the increase of the extraction solvent volume from 400 µL to 800 µL, the final organic phase increased, resulting in a decrease of peak response due to the dilution effect. In addition, 400 µL of extraction solvent resulted in less floating volume after centrifugation and led to collection difficulties. Therefore, 500 µL of ethanol was selected as the volume of extracting solvent.

The effect of the extraction time was examined under the constant experimental conditions. Following the addition of 500 µL ethanol, the sample solution was shaken by a vortex mixer (at a maximum vibration motion of 3000 rpm) for a series of extraction times in the range of 0–8

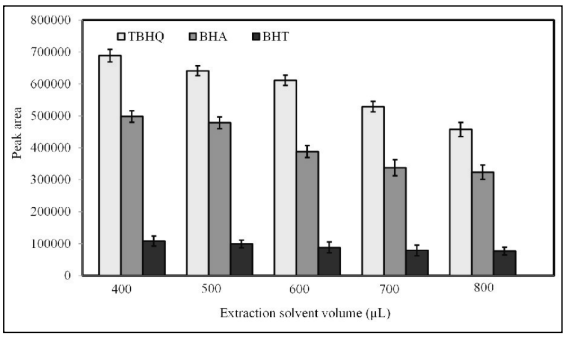

Fig. 2. Effect of volume of extraction solvent. Extraction solvent, ethanol; sample volume, 1 g; vortex mixing time, 1 min; spiked concentration, 25 µg/g.

min. The experimental results are presented in Fig.3. Lower efficiency was obtained when manual shaking was employed for a few seconds (0 min). The results showed that by increasing the vortex time, the extraction efficiency increased, reaching the maximum value at 3 min, and remaining constant after that. Thus, a vortex mixing time of 3 min at 3000 rpm was chosen as the optimal extraction time.

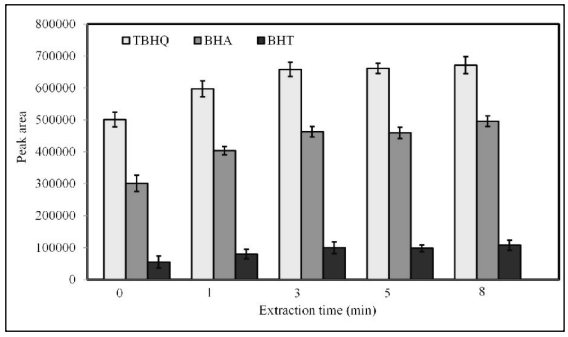

Fig. 3. Effect of extraction time. Extraction solvent, ethanol; extraction solvent volume, 500 µL; sample volume, 1 g; spiked concentration, 25 µg/g.

Method performance

Under the optimal experimental conditions, a series of experiments were performed for obtaining linear ranges, precision, and the limits of detection (LODs) and quantification (LOQs). The results are summarized in Table 1. The calibration curves were constructed by plotting the peak areas measured versus the concentrations of analytes in the samples. Linearity was observed in the range of 0.5 - 400 µg/g, with the square of correlation coefficients (r^2) ranging from 0.9992 to 0.9997. The LODs and LOQs were calculated, for each analyte, on the basis, respectively, of 3 and 10 times the concentration corresponding to the standard deviation of the signal obtained from six independent complete analyses of the blank sample. LODs ranged from 0.12 to 0.28 µg/g. The precision was evaluated by investigating the intra-day and inter-days precisions. For this purpose, oil samples spiked at a concentration level of 25 µg/g were analyzed five times in the same day (intra-day) and in the four consecutive days (inter-days). The relative standard deviations (RSDs) were satisfactory, remaining below 6.5% (intra-day) and 8.4% (inter-days) for all compounds.

As no certified reference materials for SAs in oil were available, recovery experiments were performed at spiked concentration levels of 25 µg/g and 100 µg/g by adding standard solution into oil samples. Recoveries were between 84–112% and 87–105% for the low and the high spiked levels, respectively. Fig. 4 shows the LC-UV chromatograms of SAs at the concentration level of 25 µg/g in sunflower oil and refined olive oil sample before and after spiking.

Analysis of commercial vegetable oils

The validated method was applied to detect the presence of the SAs in vegetable oils (sunflower, olive and corn oils) produced in Turkey by different companies. Totally, 16 vegetable oil samples were purchased from local supermarkets in Zonguldak and analyzed according to the procedure described above. Some of the SAs were found in seven of the samples, as shown in Table 2. TBHQ remained

Table 1. Analytical performance of the method for the determination of synthetic antioxidants in vegetable oil samples.

Analyte	LR[a]	r^{2b}	LOD[c]	LOQ[d]	RSD %[e]	RSD %[f]
TBHQ	0.5 – 400	0.9992	0.12	0.40	4.3	5.2
BHA	0.5 – 400	0.9997	0.14	0.48	3.2	4.8
BHT	1 – 400	0.9995	0.28	0.92	6.5	8.4

[a]Linear range (µg/g).
[b]Square of correlation coefficient.
[c]Limit of detection (µg/g, S/N = 3).
[d]Limit of quantification (µg/g, S/N = 10).
[e]Intra-day relative standard deviation (C = 25 µg/g, n = 5).
[f]Inter-days relative standard deviation (C = 25 µg/g, n = 4).

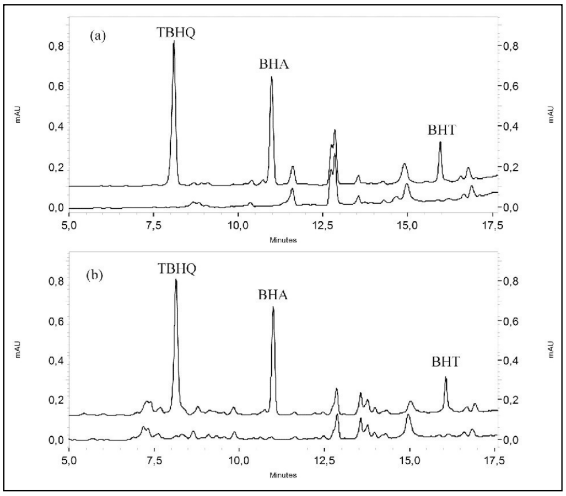

Fig. 4. LC-UV chromatograms of SAs at the concentration level of 25 µg/g in (a) sunflower oil and (b) refined olive oil sample before and after spiking.

under quantification limit of the method in all samples, whereas concentrations up to 3.48 µg/g for BHA and 3.87 µg/g for BHT were found in some of the samples. In most cases, BHA and BHT were found at concentration levels near the quantification limits. All the concentrations found for SAs were far below the maximum permitted levels defined in Turkish Food Codex Regulation (4).

CONCLUSIONS

In this study, an optimized and validated vortex-assisted liquid-liquid microextraction method combined with LC-UV has been proposed for the determination of synthetic antioxidant in vegetable oil samples. The method provided satisfactory validation parameters in terms of linearity, accuracy, precision and sensitivity, requiring only 500 µL of organic solvent per sample. Finally, this method was applied for monitoring of SAs in same selected vegetable oils produced in Turkey. TBHQ was not detected in any of samples. Although BHA and BHT were found in some samples, their levels were far below the maximum permitted levels defined in Turkish Food Codex Regulation.

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Table 2. Synthetic antioxidant contents of commercial vegetable oils.

Oil Sample	Concentration of antioxidants ± SD (µg/g)		
	TBHQ	BHA	BHT
Sunflower-1	nd	1.56 ± 0.21	1.15 ± 0.15
Sunflower-2	nd	0.76 ± 0.10	3.63 ± 0.53
Sunflower-3	nd	nd	nd
Sunflower-4	nd	3.48 ± 0.35	nd
Sunflower-5	nd	nd	nd
Sunflower-6	nd	nd	nd
Sunflower-7	nd	nd	nd
Sunflower-8	nd	2.18 ± 0.18	2.17 ± 0.32
Virgin Olive-1	nd	nd	nd
Virgin Olive-2	nd	nd	nd
Refined Olive-1	nd	nd	nd
Refined Olive-2	nd	3.61 ± 0.40	nd
Refined Olive-3	nd	nd	nd
Corn-1	nd	0.85 ± 0.10	3.87 ± 0.40
Corn-2	nd	3.46 ± 0.43	nd
Corn-3	nd	nd	nd

nd: Not detectable.
SD: Standard deviation (n=3).

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