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Determination of Synthetic Phenolic Antioxidants in Biological Fluids Based on Air-assisted Liquid-liquid Microextraction Followed by Gas Chromatography-flame Ionization Detection

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An air-assisted liquid-liquid microextraction method for the extraction and preconcentration of trace amounts of some synthetic phenolic antioxidants in biological fluids followed by their determination by gas chromatography-flame ionization detection has been reported. In this method the target analytes are extracted into a few microliters of carbon tetrachloride (extraction solvent) from an aqueous solution by aspirating and dispersing the extraction solvent and sample solution mixture by a syringe. After extraction, phase separation is performed by centrifugation and the enriched analytes in the sedimented phase are determined. The parameters affecting the extraction efficiency including the type and volume of extraction solvent, salt addition, extraction times, and pH are investigated in details. Under the optimum extraction conditions, the method shows low limits of detection and quantification between 0.8-1.8 and 2.7-5.6 ng ml^{-1}, respectively. The method is applied to determine some phenolic antioxidants in biological samples and extraction recoveries are ranged from 63-81%. Enrichment factors are obtained between 315 and 405. The method shows good linearities in the range of 3-6000 ng ml^{-1} with the correlation coefficients higher than 0.996. Relative standard deviations are lower than 8% for intra-day (n = 6) and inter-day (n = 4) precisions. Finally the proposed method is successfully used for determination of the analytes in urine and plasma samples.

Keywords: Air-assisted liquid-liquid microextraction, Synthetic phenolic antioxidants, Biological fluids, Gas chromatography

INTRODUCTION

Phenolic compounds are powerful antioxidants which prolong the shelflife of foodstuffs by protecting them against deterioration caused by oxidation such as fat rancidity and color changes [1]. These compounds are mostly employed in chemicals with antioxidant activities, and sometimes appear in food alone. It is more effective to use a mixture of two or more antioxidants rather than a single compound in a practical use [2]. Antioxidants include natural antioxidants such as ascorbic acid and tocopherols, as well as synthetic antioxidants. Due to poor stability of natural antioxidants, manufacturers prefer to use synthetic antioxidants which are stable, efficient, pure, relatively cheap, and easily available [3]. One of the major classes of the synthetic antioxidants is phenolic compounds which are more effective than other antioxidants. Antioxidant action of the phenolic compounds is due to their high tendency to chelate metallic ions. Although the synthetic phenolic antioxidants (SPAs) provide a high level of protection in maintaining food products quality, an excess of antioxidants has a health risk [4,5], and the concentration of these additives is strictly monitored in most countries [6]. Among the available SPAs, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are the most used antioxidants. In the literature, there are various methods for antioxidants' determination in different samples that use techniques such as high-performance liquid chromatography (HPLC) coupled with different detection systems [7-9], gas chromatography [1,10-12], micellar electrokinetic capillary

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chromatography [13], voltammetry [14-23], and amperometry [24]. SPAs are not completely metabolized by liver; therefore, an amount of the unchanged parts of them is excreted in biological fluids [25]. In general, BHA is rapidly absorbed from the gastrointestinal tract, metabolized and excreted in the form of metabolites in urine and/or feces. The major metabolites of BHA are the glucuronides, sulphates and free phenols, including *tert*-butylhydroquinone. The contribution of different metabolites vary in different species and also in different dose levels [26]. Absorption, distribution, metabolism and excretion of BHT have been studied in mice, rates, rabbits, chickens, monkeys, and human. Overall, these studies show that BHT is rapidly absorbed from the gastrointestinal tract. Upon absorption, BHT is distributed to the liver and body fat, while excretion takes place mainly *via* urine and feces. The metabolism of BHT is complex. It is un-known, for example, whether humans are capable of forming the quinone methids (metabolites found in rats and mice). In addition, biliary excretion seems not to be a significant in man as it is in rats, rabbits, and dogs [27]. However, there are relatively few studies on the determination of antioxidants in biological samples. Most of the methods described in literature for the quantitative analysis of antioxidants or antioxidant mixtures have been developed for their analysis in foodstuffs and food packaging [28]. In these cases, liquid chromatography with ultraviolet detection (LC-UV) is the most common determination technique after extraction of the analytes by liquid-liquid extraction (LLE) or solid-phase extraction (SPE). However, these methods are not applicable for trace analysis of the target analytes in biological samples. Biological samples are characterized by their complexity and the low concentration of the target analytes, which make difficult their direct analysis [29]. In order to overcome these drawbacks, many efforts have been done towards the designing efficient extraction procedures that permit the isolation and preconcentration of the analytes before their analysis. Therefore, much efforts have been made to develop simple, highly sensitive, and environmentally friendly sample preparation methods that consume low volumes of samples and reagents, such as solid-phase microextraction (SPME) [30,31] and liquid-phase microextraction (LPME) [32,33]. SPME is based on partitioning the analytes between the sample matrix and a fused-silica fiber coated with an extractive phase. Despite simplicity of the method, most commercial extractive fibers used in SPME are relatively expensive, fragile, and have a limited lifetime. Furthermore, sample carry-over is also a problem [34,35]. LPME as a solvent-minimized sample preparation procedure is inexpensive, and only several microliters of an extracting solvent are used. In 2006, Assadi and coworkers introduced a novel LPME technique as an efficient and powerful preconcentration method for the analysis of polycyclic aromatic hydrocarbons which was termed dispersive liquid-liquid microextraction (DLLME) [36]. In this method dispersion an extraction solvent into an aqueous solution having the analytes is performed with the aid of a disperser solvent. The dispersive solvent is miscible with both extraction solvent and water. Analytes are enriched in the dispersed fine droplets of the extraction solvent, which is then separated by centrifugation. The advantages of DLLME method are short extraction time, low cost, simplicity of operation, relatively high extraction recoveries, and high enrichment factors [37-41]. Like other methods, DLLME also has its own drawbacks. As mentioned above, in the conventional DLLME the extraction solvent is dispersed into the aqueous sample solution using a disperser solvent. The use of relatively larger volumes of a disperser solvent is the most significant drawback of DLLME, because it causes a partial dissolution of the target analytes in the aqueous sample. In order to eliminate above mentioned drawback, some techniques such as ultrasound-assisted dispersive liquid-liquid microextraction (USADLLME) [42,43], vortex-assisted liquid-liquid microextraction (VALLME) [44,45], and air-assisted liquid-liquid microextraction (AALLME) [46-48] have been developed in which no disperser solvent is used. In AALLME, a few microliters of an extraction solvent is transferred into an aqueous sample solution and then the mixture is repeatedly aspirated into a glass syringe and expelled to a conical test tube. Fine droplets of the extraction solvent are formed by this action and the solution becomes turbid. After centrifuging the cloudy solution, the extractant is settled down in the bottom of the centrifuge tube and used for the further analysis. This method has been proved to be simple, rapid, efficient, and environmentally friendly.

The aim of this study is to develop an AALLME method for the extraction and preconcentration of the most used SPAs, *e.g.* BHT and BHA, from biological fluids. Along with these antioxidants methyl *tert*-butyl phenol (MTBP) is also monitored. MTBP is used as an intermediate for preparation of both target antioxidants and may exist as an impurity in BHT and BHA. Based on our knowledge, no LPME method has been reported previously on the extraction of BHT and BHA from biological samples. Some experimental parameters, including the type of extraction solvent and its volume, salt addition, extraction times, and pH are studied and optimized. The analytical performance and application of the proposed method in real samples are explored.

EXPERIMENTAL

Chemicals and Materials

All used analytes (BHT, BHA and MTBP) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). The tested extraction solvents were obtained from the following supplies: carbon tetrachloride, 1,2-dichloroethane (1,2-DCE), and 1,1,1-trichloroethane (1,1,1-TCE) were from Merck (Darmstadt, Germany), and 1,1,2,2–tetrachloroethane (1,1,2,2-TCE) was from Janssen Chimica (Beerse, Belgium). HPLC-grade methanol, sodium chloride, hydrochloric acid, and sodium hydroxide were purchased from Merck. Deionized water (Ghazi Company, Tabriz, Iran) was used for the preparation of aqueous solutions.

Standard Solutions and Samples

The individual stock solutions were prepared by dissolving an appropriate amount of each analyte in methanol (1000 mg l^{-1}). Working standard solutions were prepared daily by appropriate diluting the stock solutions with deionized water. Another standard solution was prepared in carbon tetrachloride at a concentration of 250 mg l^{-1} (each analyte) to quality control of the separation system This solution was injected into the separation system (there times in a day) and the obtained peak areas were used in the calculation of enrichment factors and extraction recoveries. Plasma samples were obtained from the Iranian Blood Transfusion Organization (Tabriz, Iran). Due to high protein contents of the plasma samples, some pretreatments were required to eliminate them. For this purpose, 1 ml plasma sample was mixed with 2 ml methanol to precipitate proteins and the obtained solution was centrifuged for 5 min at 6000 rpm. Then, 1 ml of the supernatant phase was removed and diluted at a ratio of 1:4 with deionized water. Two distinct human urine samples were obtained from the volunteers in our laboratory (Tabriz University, Tabriz, Iran). The urine samples were diluted at a ratio of 1:2 with deionized water before their analysis.

Instrumentation

Chromatographic analysis of the selected analytes was performed on a gas chromatograph (GC-2014, Shimadzu, Japan) equipped with a splitless/split injector and a flame ionization detector (FID). Helium (99.999%, Gulf Cryo, United Arabic Emirates) was used as the carrier gas at a constant linear velocity of 30 cm s^{-1} and make up gas (40 ml min^{-1}). Injections were carried out in a splitless mode with a sampling time of 1 min. The analytes were separated on an SP-2380 fused silica capillary column (90% biscyanopropyl, 10% cyanopropylphenyl siloxane) (60 m × 0.25 mm i.d., and film thickness of 0.2 µm) (Supelco, Bellefonte, USA), with the following oven temperature programming: initial temperature 80 °C (held 3 min), and then was raised to 240 °C at a rate of 12 °C min^{-1}, and held at 240 °C for 2 min. The temperature of detector and injection port was set at 260 °C. For FID, hydrogen gas was generated with a hydrogen generator (OPGU-1500S, Shimadzu, Japan) at a flow rate of 40 ml min^{-1}. The flow rate of air for FID was 300 ml min^{-1}. Gas chromatography-mass spectrometry (GC-MS) analysis was carried out on an Agilent 7890A gas chromatograph equipped with a 5975C mass-selective detector (Agilent Technologies, CA, USA). The separation was carried out on an HP-5 MS capillary column (30 m × 0.25 mm i.d., and film thickness of 0.25 µm) (Hewlett-Packard, Santa Clara, USA). Helium was used as the carrier gas at a flow rate of 1.0 ml min^{-1}. The oven temperature programming was the same as used in GC-FID analysis mentioned above. A Hettich centrifuge, model D-7200 (Germany) was used for accelerating phase separation. pH measurements were performed with a Metrohm pH meter model 654 (Herisau, Switzerland).

AALLME Procedure

Five ml deionized water spiked with 1 mg l^{-1} of each analyte or diluted plasma and urine samples (*see section 2.2*) was placed into a 10-ml glass test tube with conical bottom. Carbon tetrachloride (32 µl) as an extraction solvent was added to the tube and then the mixture was repeatedly aspirated into a 5-ml glass syringe and then expelled into the tube. The procedure was repeated for 4 times. Then the mixture was centrifuged at 4000 rpm for 6 min and the fine droplets of the extractant were settled down in the bottom of the tube (10 ± 1 µl). Finally, 1 µl of the extractant was injected into the separation system for analysis.

Calculation of Enrichment Factor and Extraction Recovery

The enrichment factor (EF) is defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration of analyte (C_0) in the sample.

$$EF = \frac{C_{sed}}{C_0} \quad (1)$$

C_{sed} is obtained from a calibration graph plotted by direct injection of the standard solutions of the analytes prepared in the extraction solvent. Extraction recovery (ER) is defined as the percentage of the total analyte amount (n_0) which is extracted into the sedimented phase (n_{sed}).

$$ER = \frac{n_{sed}}{n_0} \times 100 = \frac{C_{sed} \times V_{sed}}{C_0 \times V_{aq}} \times 100 = EF \times \frac{V_{sed}}{V_{aq}} \times 100 \quad (2)$$

where V_{sed} and V_{aq} are the volumes of the sedimented phase and sample solution, respectively.

RESULTS AND DISCUSSION

To obtain the high extraction efficiency, effect of various factors, such as type and volume of extraction solvent, number of extraction cycles, pH, and ionic strength, was investigated systematically. The experiments were performed by variation of one parameter at a time while the rest of the parameters were kept fixed.

Selection of Extraction Solvent

Selecting an appropriate extraction solvent is critical in all extractive methods to achieve high extraction efficiency. In this study four organic solvents including: 1,1,2,2-TCE, carbon tetrachloride, 1,1,1-TCE and 1,2-DCE were chosen. To achieve a same volume of the sedimented organic phase (10 ± 1 µl) after performing the extraction method, 65 µl of 1,2-DCE, 42 µl of 1,1,2,2-TCE, 38 µl of 1,1,1-TCE and 32 µl of carbon tetrachloride were used for extraction of the analytes from 5 ml deionized water spiked with 1 mg l^{-1} of each analyte. The obtained results are shown in Fig. 1. The results show that among the examined solvents, carbon tetrachloride has the highest analytical signals for all analytes with the lowest consumption of the organic solvent. Therefore, carbon tetrachloride was selected as an optimum extraction solvent for the further experiments.

Extraction Solvent Volume

In each microextraction procedure, volume of extraction solvent is another crucial parameter with a significant effect on the extraction efficiencies and analytical signals. Although volume of the extraction solvent is taken as small as possible to reach the higher EFs and the low toxicity for environment, it should be enough to extract the analytes as much as possible and to ensure that the sufficient sedimented phase volume is collected for the further chromatographic analysis. To investigate the effect of extraction solvent volume on the performance of the presented AALLME procedure, experiments involving different volumes of carbon tetrachloride (32, 40, 50 and 60 µl) were performed. The results in Fig. 2 indicate that the analytical signals decrease gradually by increasing extraction solvent volume in the range examined. However, volume of the sedimented phase at the bottom of the tube also increases from 10 to 36 µl by increasing volume of the extraction solvent from 32 to 60 µl. Dilution effect of the target analytes on the sedimented phase in high volumes of the extraction solvent is the reason for decreasing analytical signals. The volumes less than 32 µl were not tested due to the low volume or lack of the sedimented phase. Thereby, the further experiments were carried out with 32 µl of the extraction solvent.

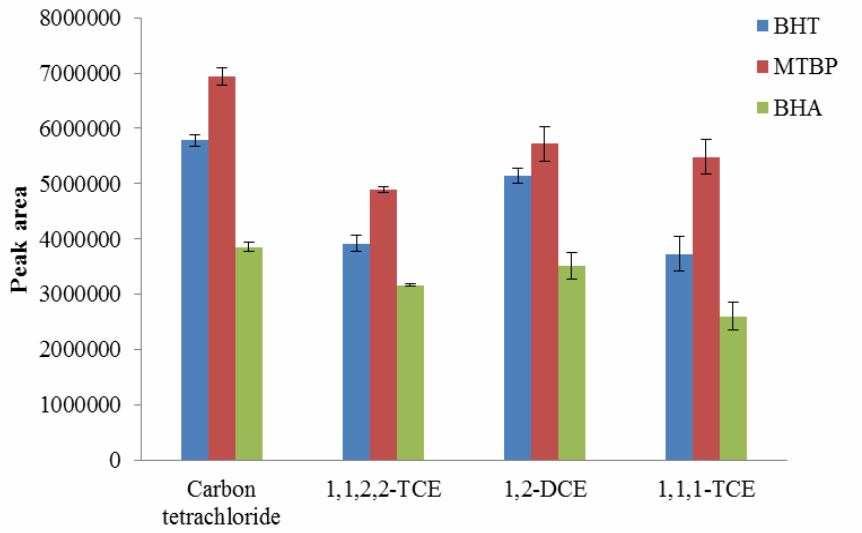

Fig. 1. Selecting extraction solvent type. Extraction conditions: sample, 5 ml deionized water spiked with the analytes (1 mg l^{-1}, each analyte); extraction solvent, 1,1,1-TCE (38 µl), 1,1,2,2-TCE (42 µl), carbon tetrachloride (32 µl), and 1,2-DCE (65 µl); extraction cycle numbers, 6 times; centrifuge rate, 4000 rpm; and centrifuge time, 5 min. The error bars indicate standard deviations of three repeated determinations.

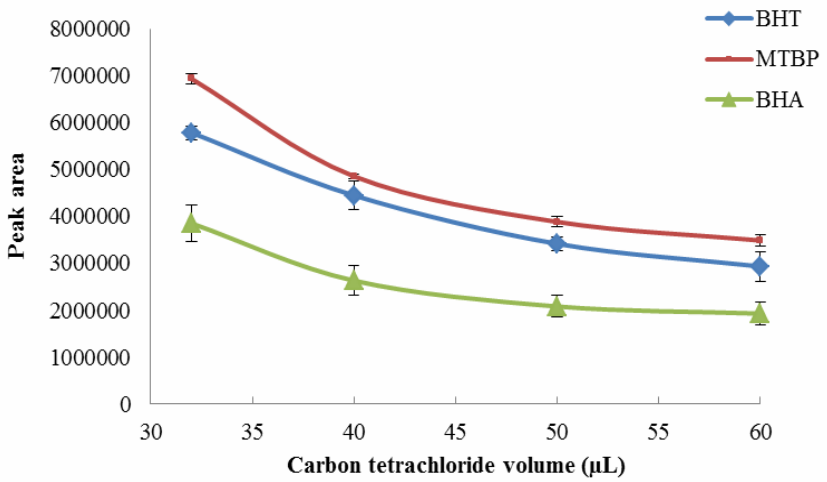

Fig. 2. Optimization of extraction solvent volume. Extraction conditions: extraction solvent, carbon tetrachloride. Other conditions are the same as used in Fig. 1. The error bars indicate standard deviations of three repeated determinations.

Effect of Salt Addition

Salting out effect has been widely used in LPME methods. Addition of a salt to the sample solution can increase ionic strength of the aqueous phase, leading to reducing the solubility of the analytes in the sample solution. On the other hand, the presence of a salt increases viscosity of the aqueous phase which affects mass transfer rate of the analytes into the aqueous phase. This can lead to

reducing extraction efficiency. In this work, the effect of salt addition on the performance of the extraction procedure is investigated by adding different concentrations of NaCl (0-15%, *w/v*) into the aqueous phase whereas the other experimental conditions were kept constant. The experiments were performed using different volumes of the extraction solvent to achieve 10 µl of the sedimented phase volume (32, 29, 26, 21 and 17 µl for 0, 2.5, 5.0, 10 and 15 %, *w/v*, NaCl, respectively). Plots of analytical signals (peak areas) *vs.* NaCl concentration showed that the addition of salt had no significant effect on the analytical signals (data not shown here). Considering the experimental results, the subsequent works were performed in the absent of NaCl.

Study of Extraction Cycles Number

In an AALLME method, formation of the fine droplets of an extraction solvent dispersed into an aqueous phase is performed by repeatedly aspirating/dispensing of the extraction solvent and sample solution using a glass syringe. Through this action, the contact area of the phases and mass transfer rate of the analytes between aqueous and extracting phases are increased during cycles. These cycles are defined as the extraction cycle number in AALLME. It is expected that with increasing the extraction cycles, ERs and EFs are increased and then remained constant. Therefore, to obtain the high extraction efficiency at the least time, this parameter should be in the optimum level. To reach the optimum state, the extraction cycles were investigated in the range of 1-6 times. The results in Fig. 3 show that analytical signals increase with increasing the extraction cycles to 4 and then remain constant. Consequently, 4 cycles of extraction were selected for the following studies. It is noted that in high extraction cycle numbers vaporization of the extraction solvent would be significant.

Effect of pH

Sample pH value is a key factor in most sample pretreatment techniques, which determines the present form of the analytes (protonated, neutral or ionized) and hence affects extraction efficiency. Neutral forms of the analytes are often extracted into an organic solvent. With respect to acidic property of the analytes, to ensure the efficient extraction, pH of sample solution must be adjusted at pH < pk_a of the analytes to prevent their ionization. In this work, pH study was carried out in the range of 2-12 (Fig. 4). The obtained results show that the peak areas remain constant in the studied range, except for BHA that its analytical signal decreases at pH >10, due to the ionization of BHA at those pHs. These results can be confirmed by pK_a values of the target analytes (12.75 for BHT, 11.72 for MTBP, and 10.75 for BHA). It should be noted that the pH of all samples used in this study was less than 10; therefore no attempt was made to adjust pH in the following studies.

Optimization of other Parameters

Centrifugation is a critical step to obtain two distinguishable phases, so it affects size of the settled phase and concentration of the analytes in the extracting phase. The centrifugation time was checked in the range of 3-10 min at a constant centrifugation rate of 4000 rpm. The results indicated that the peak areas of the analytes are increased as the centrifugation time increases up 6 min, and then remains constant after 6 min. To optimize centrifugation speed, some experiments were carried out in the range of 1000-5000 rpm at a constant centrifugation time of 6 min. The obtained extraction efficiencies according to chromatographic peak areas as a function of centrifugation speed revealed that the extraction efficiencies are increased with increasing centrifuging speed and reach to a plateau after 4000 rpm. Consequently, 4000 rpm and 6 min were selected as centrifugation rate and time, respectively, for the subsequent experiments. Stability of the analytes is another parameter which was studied. It was assessed by triplicate analyzing of the quality control samples exposed to different storage conditions including room temperature (25 ± 2 °C) for 12 h and three freeze-thaw (-20 to 25 °C) cycles. No significant degradation of the studied analytes was observed under various storage conditions.

Analytical Performance

Quantitative characteristics of the proposed method were obtained under the optimized experimental conditions. Analytical performance of the presented method was evaluated by obtaining some quantitative parameters including limit of detection (LOD), limit of quantification (LOQ), linear range (LR) of the calibration graphs, correlation coefficient (r), relative standard deviation

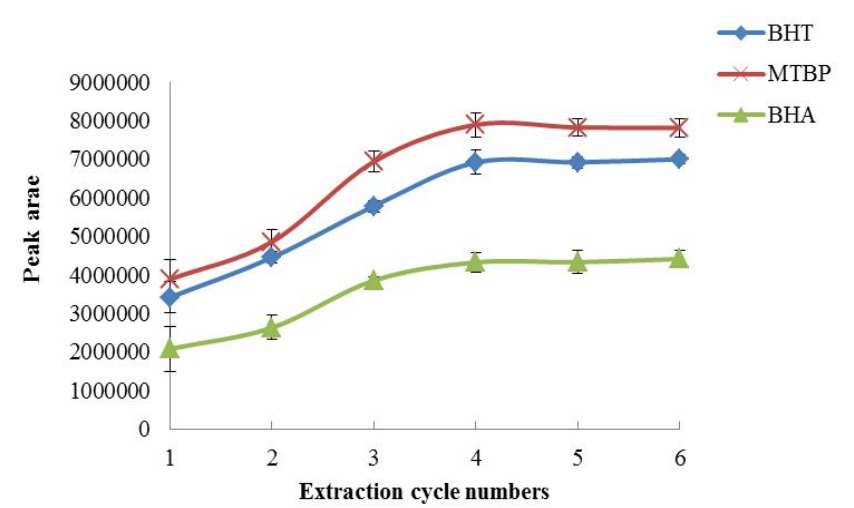

Fig. 3. Optimization of the number of extraction cycles. Extraction conditions: the same as used in Fig. 2, except 32 µl carbon tetrachloride was used as the extraction solvent. The error bars indicate standard deviations of three repeated determinations.

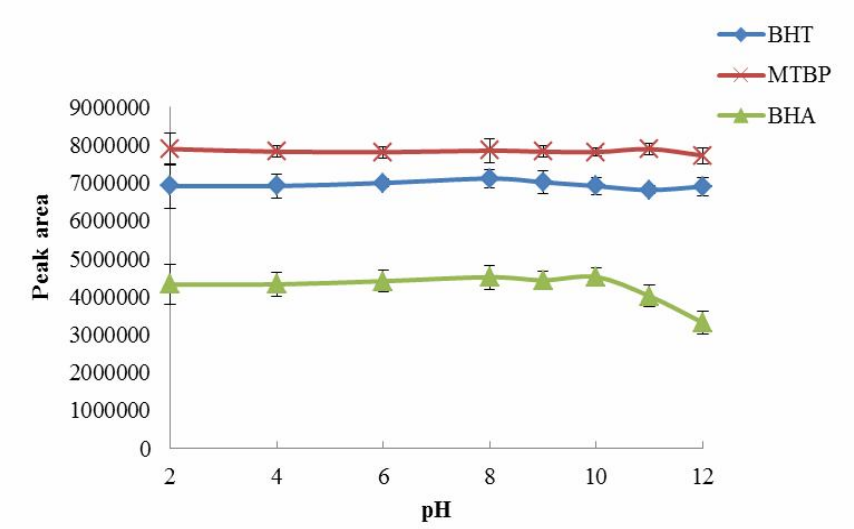

Fig. 4. Selection of solution pH. Extraction conditions: the same as used in Fig. 3, except extraction numbers which is 4. The error bars indicate standard deviations of three repeated determinations.

(RSD), EF and ER. The data are summarized in Table 1. Linearity of the method was evaluated using a series of standard solution with seven different concentrations extracted by the developed method. Good linearities with correlation coefficients ranging from 0.996-0.999 were obtained for all analytes. The LODs, based on signal-to noise ratio (S/N) of 3, ranged from 0.8 to 1.8 ng ml^{-1}, and the LOQs, based on signal-to-noise ratio (S/N) of 10, ranged from 2.7-5.6 ng ml^{-1} which are completely low. Intra-day (n = 6) and inter-day (n = 4) precision studies were carried out by analyzing the standard solution of 50 ng ml^{-1} of each analyte. The results (expressed as RSD%) ranged from 3.7-7.1% and 4.1-8.0%, respectively. The EFs and ERs for the selected analytes were in the ranges 315-405 and 63-81%,

Table 1. Quantitative Features of the Proposed AALLME Method Followed by GC-FID Determination

Analyte	LOD[a]	LOQ[b]	LR[c]	r [d]	RSD (%)[e]		EF ± SD[f]	ER ± SD[g]
					Intra-day (n = 6)	Intra-days (n = 4)		
BHT	0.8	2.7	3-6000	0.998	3.7	4.5	405 ± 10	81 ± 2
MTBP	1.2	3.4	3-6000	0.999	7.1	8.0	370 ± 15	74 ± 3
BHA	1.8	5.6	6-6000	0.996	3.9	4.1	315 ± 10	63 ± 2

[a]Limit of detection (S/N = 3) (ng ml^{-1}). [b]Limit of quantification (S/N = 10) (ng ml^{-1}). [c]Linear range (ng ml^{-1}). [d]Correlation coefficient. [e]Relative standard deviation (C = 50 ng ml^{-1}). [f]Enrichment factor ± standard deviation (n = 3). [g]Extraction recovery ± standard deviation (n = 3).

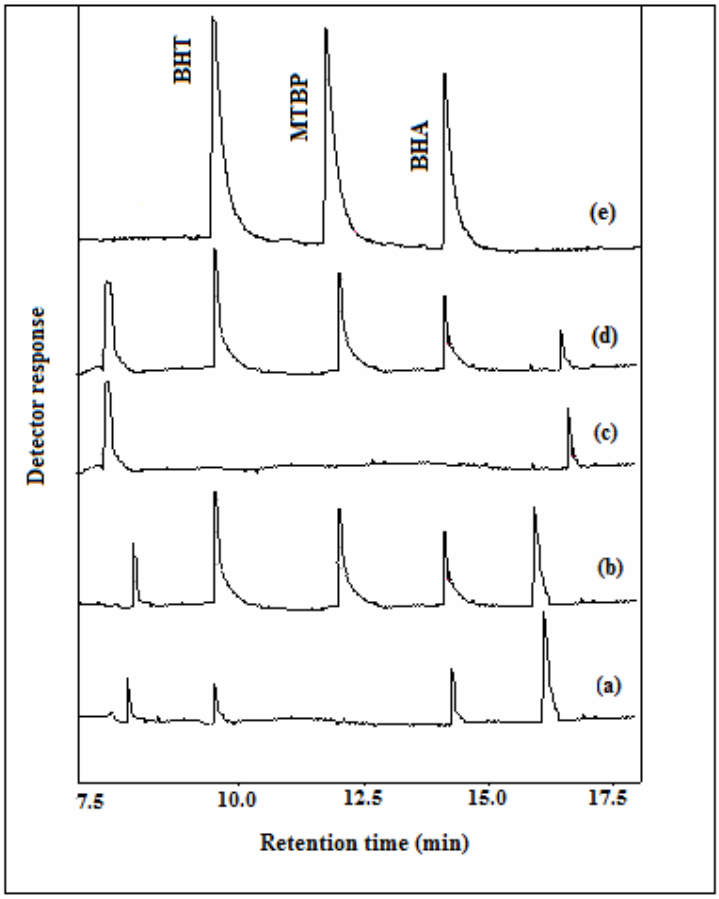

Fig. 5. GC-FID chromatograms of: (a) urine, (b) the urine spiked with 50 ng ml^{-1} of each analyte, (c) plasma, (d) the plasma spiked with 50 ng ml^{-1} of each analyte, and (e) standard solution (100 mg l^{-1} of each analyte) prepared in carbon tetrachloride. Chromatogram (e) was obtained by direct injection whereas in the cases of other chromatograms the proposed AALLME method was carried out on the samples and 1 µl of the sedimented phase was injected into the separation system.

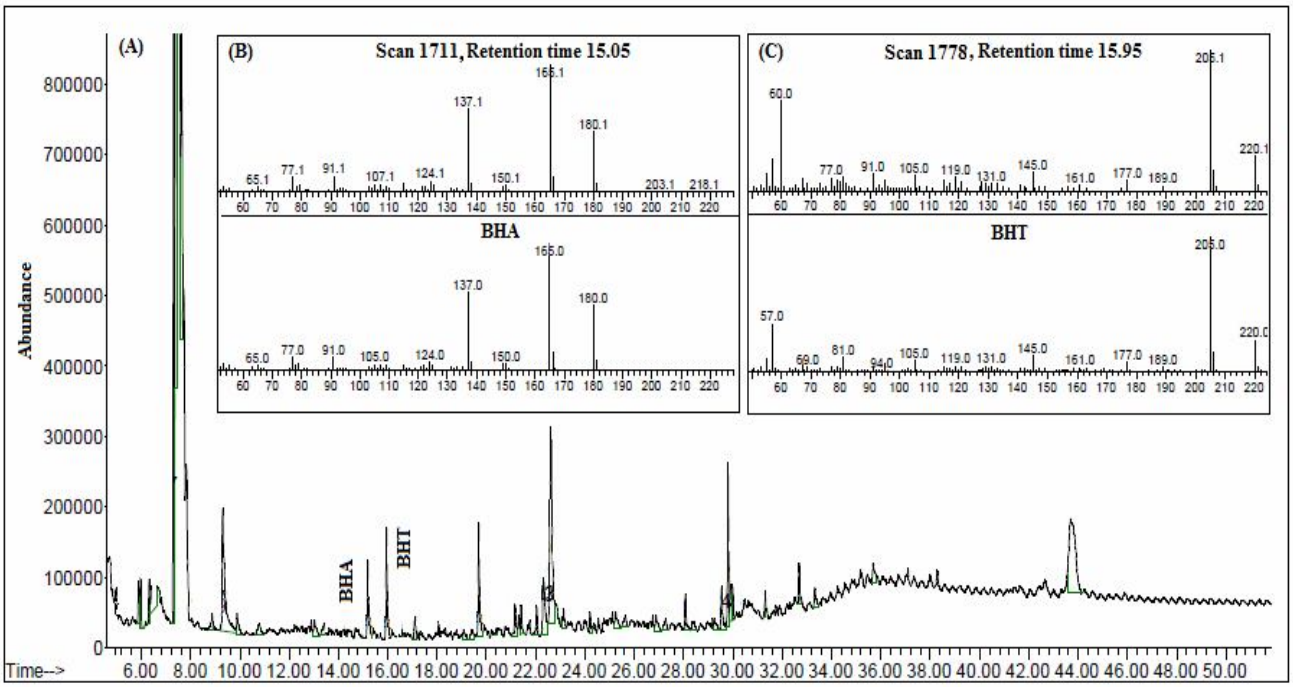

Fig. 6. (A) GC-total ion current (TIC) –MS chromatogram of urine sample, (B) mass spectrum of BHA and scan 1711 (retention time 15.05 min), and (C) mass spectrum of BHT and scan 1778 (retention time 15.95 min).

Table 2. Concentrations of BHT, MTBP and BHA (ng ml^{-1}) in Human Urine and Plasma after Performing the Developed Method on them

Analyte	Urine 1	Urine 2	Plasma 1	Plasma 2
BHT	74 ± 6[a]	36 ± 3	ND[b]	ND
MTBP	ND	ND	ND	ND
BHA	106 ± 4	79 ± 3	ND	ND

[a]Mean concentration ± standard deviation (n = 3). [b]Not detected.

respectively. It is noted that based on the dilution factors for plasma and urine samples, the following EFs are obtained in those samples: in plasma, 27, 25 and 21; and in urine 202, 185 and 158 for BHT, MTBP and BHA, respectively. High EFs and ERs, low LODs and LOQs, and good repeatability are the main advantages of the presented method.

Samples Analysis

To assess performance of the proposed method, extraction and determination of the target analytes in different plasma and urine samples were carried out under the optimum experimental conditions established above. Figure 5 shows the typical GC-FID chromatograms of two plasma and two urine samples before and after spiking with 50 ng ml^{-1} of each phenolic compound studied. Considering the chromatograms, there are some suspected peaks eluted in the retention times belong to BHT and BHA in urine samples. To identify these compounds, the urine samples

Table 3. Relative Recoveries Obtained by the Developed Method in Human Plasma and Urine Samples Spiked at Different Concentrations Compared to De-ionized Water Spiked at the same Concentrations

Analyte	Standard deviation (n = 3) ± (%) Mean recovery			
	Urine 1	Urine 2	Plasma 1	Plasma 2
	All samples were spiked with each analyte at a concentration of 100 ng ml^{-1}			
BHT	107 ± 4	106 ± 1	99 ± 1	98 ± 1
MTBP	94 ± 4	93 ± 1	111 ± 3	95 ± 2
BHA	109 ± 1	106 ± 2	106 ± 2	102 ± 4
	All samples were spiked with each analyte at a concentration of 250 ng ml^{-1}			
BHT	104 ± 2	105 ± 1	97 ± 1	91 ± 4
MTBP	94 ± 1	89 ± 2	93 ± 3	96 ± 3
BHA	102 ± 1	108 ± 3	95 ± 5	95± 2
	All samples were spiked with each analyte at a concentration of 500 ng mL^{-1}			
BHT	106 ± 5	103 ± 1	102 ± 5	91 ± 1
MTBP	97 ± 2	88 ± 2	91 ± 3	96 ± 4
BHA	108 ± 4	106 ± 4	92 ± 1	92 ± 2

were injected into GC-MS after performing the proposed microextraction/preconcentration method (Fig. 6). The presence of BHT and BHA in the urine samples was verified by comparison of mass data for scans 1711 and 1778 (retention times 15.05 and 15.59 min, respectively) with those of the studied analytes. The obtained concentrations for the analytes on the basis of GC-FID data in the urine samples along with their standard deviations are given in Table 2. The plasma samples were free of the target analytes. To evaluate accuracy of the method and matrix effect in the selected samples, added-found method was used. The relative recoveries obtained for the target analytes in the samples compared with deionized water spiked at the same three concentration levels (100, 250 and 500 ng ml^{-1} of each analyte) are listed in Table 3. According to the obtained results, matrices of the selected samples have no significant effect on the performance of the presented method.

Comparison of the Proposed Method with other Methods

Table 4 summarizes the values of LR, RSD, LOD and LOQ of some analytical methods along with the proposed method for the extraction and determination of the selected analytes in different samples. The repeatability of the method is good and the RSDs for the proposed method are lower than or comparable with those of the mentioned methods. The LODs of the method are low and are comparable with that of the other methods. It should be noted that in some methods, a high sensitive detection system (mass spectrometry) has been used which is inherently more sensitive than FID. The wide LRs of the method are another advantage with respect to the others. These results reveal that the presented AALLME-GC-FID

Table 4. Comparison of the Presented Method with other Methods used in Preconcentration and Determination of the Target Analytes

Analyte	Sample	LOD ($ng\ ml^{-1}$)[a]	LOQ ($ng\ ml^{-1}$)[b]	LR ($ng\ ml^{-1}$)[c]	r [d]	RSD (%)[e]	Method	Ref.
BHA	Soybean and biodiesel samples	0.2	0.8	0.006-0.28	0.999	-	HPLC-DAD[f]	[8]
BHT	Edible oils	1	-	10-20000	0.9992	-	GC-MS[g]	[12]
BHA		2	-	10-20000	0.9998	-		
BHT	Water samples	0.2	0.6	2-2000	0.998	4	SPE-GC-MS[h]	[1]
BHA		0.8	2.7	2-2000	0.997	3		
BHT	Milk samples	0.93	3.24	3.24-2000	0.998	6.1	AALLME-GC-FID[i]	[10]
BHA		1.16	3.96	3.96-2000	0.997	5.1		
BHT	Biological samples	0.8	2.7	3-6000	0.998	3.7	AALLME-GC-FID	This method
BHA		1.8	5.6	6-6000	0.996	3.9		

[a]Limit of detection. [b]Limit of quantification. [c]Linear range. [d]Correlation coefficient. [e]Relative standard deviation. [f]High performance liquid chromato graphy-diode array detection . [g]Gas chromatography-mass spectrometry. [h]Solid phase extraction-gas chromatography-mass spectrometry. [i]Air-assisted liquid-liquid microextraction-gas chromatography-flame ionization detection.

method is sensitive, simple, rapid, and repeatable technique and can be used for the preconcentration and determination of the synthetic phenolic antioxidants from biological samples.

CONCLUSIONS

In this study, for the first time, an AALLME procedure was presented for the extraction and concentration of SPAs and MTBP from biological fluids. This method provides high extraction recovery, wide linearity, and good repeatability with a short extraction time. In comparison with other conventional extractive methods, this method has advantages such as rapidity, simplicity, ease of operation, and low consumption of organic solvent. Finally the proposed AALLME method combined with GC-FID analysis was successfully applied to determine the target analytes in biological samples at $ng\ ml^{-1}$ level.

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