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Determination of Bisphenol A in Packed Milk and Mineral Water Samples Marketed in Tabriz (Iran) in 2020 Using High-performance Liquid Chromatography-ultraviolet Detector

Amin Rostamzadeh[a], Mahboob Nemati[b,c,*], Mir Ali Farajzadeh[d,e] and Mohammad Reza Afshar Mogaddam[b,f,*]

[a]*Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

[b]*Food and Drug Safety Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

[c]*Department of Pharmaceutical and Food Control, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*

[d]*Department of Analytical Chemistry, Faculty of Chemistry, University of Tabriz, Tabriz, Iran*

[e]*Engineering Faculty, Near East University, 99138 Nicosia, North Cyprus, Mersin 10, Turkey*

[f]*Pharmaceutical Analysis Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

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Bisphenol A (BPA) is a monomer used to produce several plastic resins in food storage containers. Residual BPA in plastics can be found in filled foods in containers. Because BPA is an endocrine disruptor, its dietary exposure must be estimated. In this method, the analyte was extracted using a dispersive liquid-liquid microextraction procedure from water and milk samples. For this purpose, a mixture of acetone and chloroform (142 µl) was dispersed in the water sample or in the aqueous phase obtained from milk after its deproteinization with trichloroacetic acid. The obtained cloudy solutions were centrifuged in both samples and the sedimentation phase was analyzed by a high-performance liquid chromatography-ultraviolet detector. Analytical features of the method consist of limits of detection (0.44 ng ml^{-1} in water and 0.51 ng ml^{-1} in milk samples) and quantification (1.47 ng ml^{-1} in water and 1.72 ng ml^{-1} in milk samples), linearity (r^2 = 0.992), precision (RSD ≤ 11.1%), and accuracy (RSD ≤ 3%) were studied under final conditions. The results showed that the obtained results are acceptable. Different milk and water samples were analyzed using the developed method, and BPA was found in several samples in the ranges of 11.2-32.6 and 9.6-23.5 ng ml^{-1} in milk and water samples, respectively. Comparison of the results with the maximum residue limit established by the European Commission showed that the BPA content exceeded the authorized content.

Abbreviations: BPA, Bisphenol A; DLLME, Dispersive liquid-liquid microextraction; RSD, Relative standard deviation; HPLC, High performance liquid chromatography; LPME, Liquid phase microextraction; LOD, limit of detection; LOQ, limit of quantification

Keywords: Bisphenol A, High-performance liquid chromatography, Water, Milk, Dispersive liquid-liquid microextraction

INTRODUCTION

Bisphenol A (BPA) is an organic compound broadly used as a monomer in the manufacture of different plastics [1]. It is consumed in producing other products like textiles, wood, pesticides, leathers, epoxy resins, pharmaceuticals, polycarbonate sheets and bottles [2]. However, it can be easily transferred to the environment by degrading plastics and household products, industrial waste, and landfill leaching [3]. The literature showed that BPA residues are found in polycarbonate containers due to incomplete polymerization. Since epoxy resins and polycarbonates are used to produce beverage cans and feeding bottles, they can

*Corresponding authors. E-mail: nematim@tbzmed.ac.ir; afsharmogaddam@tbzmed.ac.ir

migrate into drinks or foods filled into the containers [4]. Since BPA is an estrogenic compound, it can disrupt the normal function of the endocrine system [5]. Also, it can cause breast cancer in some humans. As a result, BPA levels must be controlled at trace levels in food or environmental samples to prevent accumulation in the human body [6]. According to the literature, chromatographic-based techniques like high-performance liquid chromatography (HPLC) equipped with different detectors like mass spectrometry [7], tandem mass spectrometry [8], fluorescence detector [9] and gas chromatography equipped with mass spectrometry [6], flame ionization detector [10], and tandem mass spectrometry [11] have been developed for the determination of BPA in different samples. Despite the sufficient sensitivity of these instruments, the need for removing interference leads to perform sample preparation methods before instrumental analysis [12]. Different sample preparation methods such as liquid-liquid extraction [13], solid-phase microextraction [14], solid-phase extraction [15-18] and liquid phase microextraction (LPME) have been developed in preparation of different samples [19]. LPME is a known method that has been developed for the efficient extraction and separation of analytes from the sample matrix. It has attracted outstanding benefits owning to its sensible advantage on low consumption of toxic solvents. Up to now, different modes of LPME were developed and used in the extraction of BPA from different samples. DLLME is a well-known LPME method that consists of a ternary solvent system, including an aqueous solution containing the analytes, a water-immiscible extraction solvent, and a disperser solvent. In this method, a mixture of dispersive and extraction solvents is prepared and dispersed into the aqueous solution by a syringe. Thus, an emulsion is formed by the dispersion of the extraction solvent into the aqueous solution. Due to distinguished advantages of DLLME such as rapidity, expensiveness, high enrichment factors (EFs), and low consumption of organic solvents, considerable attention was focused on DLLME, and it was proposed to use in the extraction of different compounds [20].

The main goal of this study was the use of the DLLME procedure for the extraction of BPA from mineral water and milk samples marketed in Tabriz (Iran) in 2020 before its determination by HPLC-ultraviolet detector (UV). For this purpose, a previously reported DLLME method was performed on milk and water samples. The method was completely validated and used for the determination of several samples. The method was performed on milk samples with several modifications after achieving optimal conditions.

MATERIAL AND METHODS

Devices

A Smartline HPLC (Smartline®S-1000, Knauer, Berlin, Germany) equipped with a UV (S-2500) detector and a manual injection valve was used to separate and quantify BPA. The analyte was separated on an STR-ODS C_{18} analytical column (250 mm, 4.6 mm id, particle size of 5 μm) (Knauer, Berlin, Germany), and it was eluted by a mixture of acetonitrile: water (46:55, v/v) at a flow rate of 1.0 ml min^{-1} in an isocratic elution mode. The analyte was detected at a wavelength of 224 nm. An Eppendorf centrifuge (Hamburg) was used for the phase separation. A vortex mixer (Scientific Industries Inc., Bohemia, NY) was used for vortexing.

Chemicals and Solutions

BPA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetone, methanol, acetonitrile (ACN), ethanol, chloroform, carbon tetrachloride (CT), 1,1,1-trichloroethane (1,1,1-TCE), dichloromethane (DCM) and trichloroacetic acid (TCA) were prepared from Merck (Darmstadt, Germany. Acetonitrile and water (HPLC-grade) were obtained from Carlo-Erba Company (Barcelona, Spain). BPA was dissolved in ACN at 100 mg l^{-1}, which was used to prepare working solutions by appropriate dilution with deionized water.

Real Samples

Ten packed mineral waters were randomly collected from supermarkets (Tabriz, East Azarbaijan, Iran). Also, ten milk samples packed in tetrapack boxes and polymeric bottles were purchased from local vendors. All samples were stored in a refrigerator at 4 ºC before analysis.

Sample Preparation Method for Water Samples

The procedure was done according to the previously published method [21]. Briefly, 10 ml of water sample containing the analyte was transferred into a conical bottom glass test tube. Then, 2 ml of acetone was mixed with 142 µl chloroform, and the mixture was injected into the aqueous solution, and the obtained cloudy solution was centrifuged for 5 min at 5000 rpm. The sedimented phase was completely taken, and after evaporation under a nitrogen stream, the residue was dissolved in 50 µl of the mobile phase and injected into the separation system.

Sample Preparation Method for Milk Samples

For this purpose, 15 ml of milk sample was poured into a glass test tube, and then 200 mg TCA was added to the sample and vortexed for 5 min. After centrifugation of the mixture at 5000 rpm for 10 min, the obtained supernatant phase was withdrawn and transferred into another tube. Subsequently, 1.5 ml of ACN was mixed with 75 µl chloroform, and the mixture was quickly injected into the solution. The collected phase at the bottom of the tube was transferred into a Microtube and evaporated under a nitrogen stream. The residue was dissolved in 50 µl of the mobile phase and injected into the separating system.

RESULTS AND DISCUSSION

Optimization of DLLME Parameters Used in the Milk Sample

In this approach, BPA was extracted from the milk sample by using a DLLME procedure. The effective parameters for this step, including dispersive solvent type and volume, extraction solvent type and volume, salt addition and pH, must be optimized. Also, the amount of protein precipitation agent should be optimized.

In this method, TCA was used for removing milk proteins to prevent their interference with the analyte during extraction. TCA forces the proteins to be precipitated by sequestering the protein-bound water. The quantity of TCA was investigated by dissolving it in the range of 75-300 mg in a 10 ml milk sample. The results (Fig. 1a) show that the method efficiency increases up to 200 mg TCA and then remains constant. At amounts less than 75 mg TCA, the milk proteins were not precipitated completely, and the method became useless. Therefore, 200 mg of TCA was used for the next steps.

The type of extraction solvent was investigated by choosing four organic solvents with a density higher than water, including CT, chloroform, 1,1,1-TCE, and DCM. All experiments were done with 150 µl of each solvent. The results obtained using chloroform lead to better efficiency compared to other solvents (Fig. 1b). Thus, chloroform was selected for the next experiments.

The extraction solvent volume effect on the method efficiency was tested by adding different volumes of chloroform in the range of 75-200 µl to 1.0 ml of acetone (as a disperser). The obtained results are shown in Fig. 1c. It is clear that chloroform volume has no remarkable effect on the method performance, and 75 µl was selected for the next experiments to obtain high EF and low limit of detection (LOD).

The type of dispersive solvent was investigated by performing different experiments with acetone, methanol, ACN and ethanol independently. In all tests, 1.0 ml of each solvent was used. The obtained results are shown in Fig. 1d. The data depict that acetone is most effective than other solvents, and it was selected for the next experiments.

The dispersive solvent volume was evaluated by performing different experiments with acetone in the range of 0.5-2.0 ml. The other experimental conditions were kept constant. The results (Fig. 1e) show that the method efficiency increases up to 1.5 ml and then decreases. Therefore, 1.5 ml was selected as the suitable volume for dispersive solvent.

The effect of salt addition on the method efficiency was assayed by dissolving sodium chloride at different concentrations in the range of (0-10%, w/v) into the aqueous phase. The obtained extraction recovery (ER) for each concentration (Fig. 1f) shows that the addition of salt has an adverse effect on the method efficiency. Therefore, the experiments were done in the absence of salt.

As BPA has acidic nature, the pH of the aqueous phase used in DLLME can affect the efficiency of the method by ionization of the analyte and its converting to the related deprotonated form. Therefore, the pH of the aqueous phase was adjusted in the range of 2-12 by adding appropriate volumes of 0.1 M NaOH or HCl solution. The results (Fig. 1g) show that the extraction efficiency of the method

is almost constant in the range of 2-8. Because the aqueous solution pH was in the mentioned range, there was no need for pH adjustment in this study.

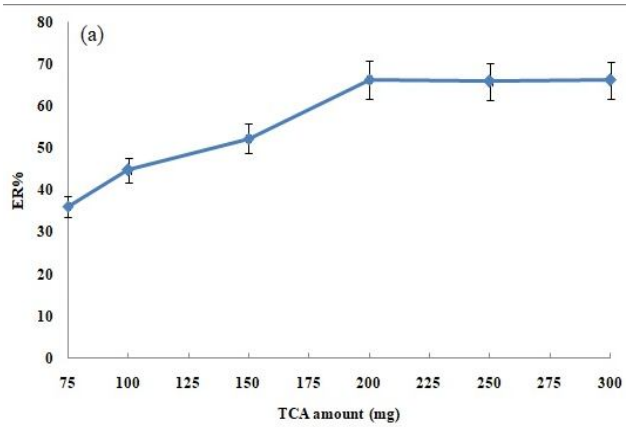

Fig. 1a. Optimization of TCA amount.
Extraction conditions: sample, 10 ml milk containing 50 ng ml^{-1} of BPA; vortex time, 5 min; dispersive solvent type (volume), acetone (1.0 ml); extraction solvent type (volume), carbon tetrachloride (76 µl), centrifugation rate (time), 5000 rpm (5 min). The error bars show the minimum and maximum of three repeated determinations.

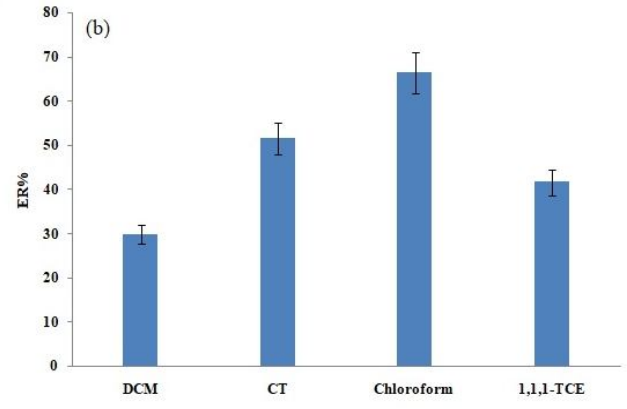

Fig. 1b. Selection of extraction solvent type.
Extraction conditions are the same as those used in Fig. 1a, except 200 mg TCA was selected as a protein precipitation agent.

Method Validation

The used approach for extracting BPA from milk and water samples was validated by calculating several parameters consisting of LOD and the limit of quantification (LOQ), accuracy and precision, linearity, and ER using international guidelines and protocols [22].

LOD and LOQ. The lowest concentration of an analyte that can be consistently determined by an analytical

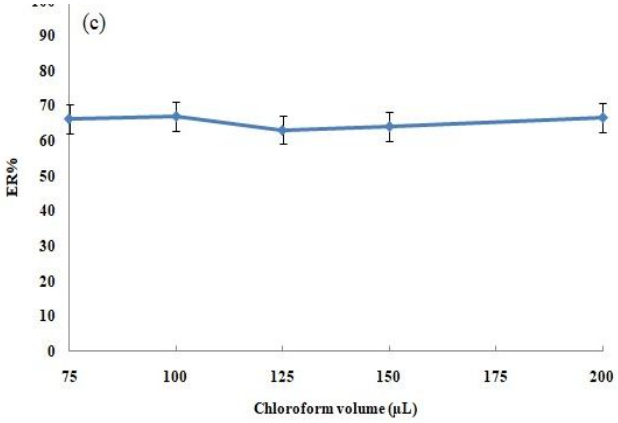

Fig. 1c. Optimization of extraction solvent volume.
Extraction conditions are the same as those used in Fig. 1b, except chloroform was selected as an extraction solvent.

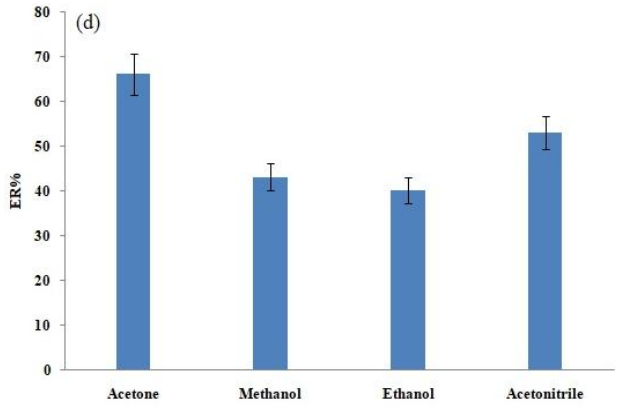

Fig. 1d. Selection of a dispersive solvent kind.
Extraction conditions are the same as those used in Fig. 1c, except 75 µl chloroform was used as an extraction solvent.

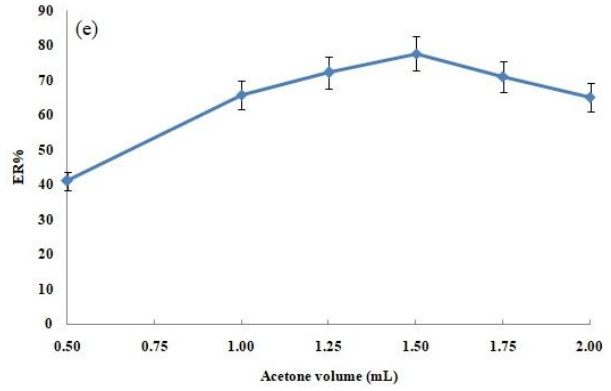

Fig. 1e. Selection of dispersive solvent volume. Extraction conditions are the same as those used in Fig. 1d, except acetone was used as a dispersive solvent.

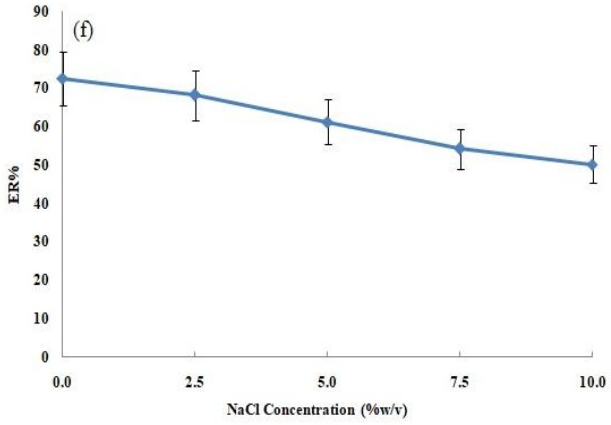

Fig. 1f. Salt addition effect. Extraction conditions are the same as those used in Fig. 1e, except 1.5 ml acetone was used as a dispersive solvent.

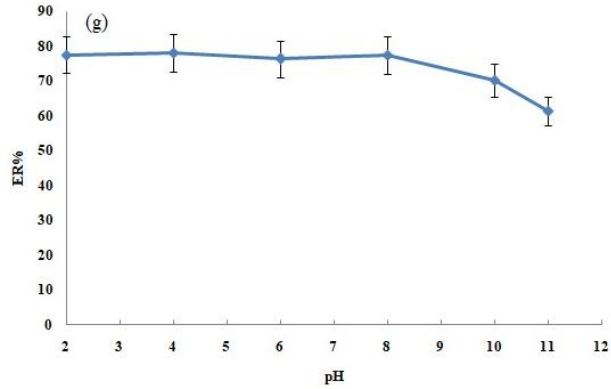

Fig. 1g. pH effect. Extraction conditions are the same as those used in Fig. 1f, except the experiments were done in the absence of salt.

approach is considered to be LOD. Generally, the LOD is measured as a concentration in where the peak height (S) of an analyte to noise (N) ratio is equal to 3 (S/N = 3). The LOQ is the lowest concentration which can be measured quantitatively with acceptable accuracy and precision and is calculated based on S/N = 10. The results showed that LOD and LOQ for BPA were 0.44 and 1.47 ng ml^{-1} in water and 0.51 and 1.72 ng ml^{-1} in milk, respectively.

Linearity. The linearity of a calibration graph is evaluated by calculating the square of the correlation coefficient (r^2). The calibration graph was plotted as the analyte concentration versus analytical signal (peak area) after performing the method. In this work, six different concentrations of BPA (5, 50, 125, 250, 500 and 5000 ng ml^{-1}) were spiked into an analyte-free water sample, and after performing the method, analytical signals (peak areas) were plotted versus concentration. The data showed good linearity with r^2 = 0.991 and 0.992 for water and milk samples, respectively.

Repeatability. The repeatability of a method is defined as the closeness of agreement between independent test results from the same method. Repeatability is considered the precision of an analytical method, and it is expressed in the relative standard deviation (RSD) for repeated analyses. The RSD was calculated by analyzing blank water and the milk sample was spiked at two different concentrations, including 5 and 50 ng ml^{-1} with respect to BPA. It was found that the RSDs were in the ranges of 6.6-8.9% and 7.3-11.1% for intra- (n = 5) and inter-day (n = 3) precisions. The results showed that the method is sufficiently repeatable for reliable determination of BPA in the studied samples.

Accuracy. The nearness of the mean of the experimental results calculated by an analytical method to an actual concentration is expressed in term of the accuracy. The best approach to evaluate the method's accuracy is to perform the method on a Certificated Reference Material (CRM). In this case, there is no CRM for BPA in water or milk samples, so the added method was used to assess accuracy. In this method, five water and milk samples were spiked with BPA at two concentrations, including 50 and 125 ng ml^{-1} and the method was applied to these samples. The resulting mean values (50 ± 1.5 or 125 ± 3.7) showed that RSDs were less than 3% for BPA for all samples.

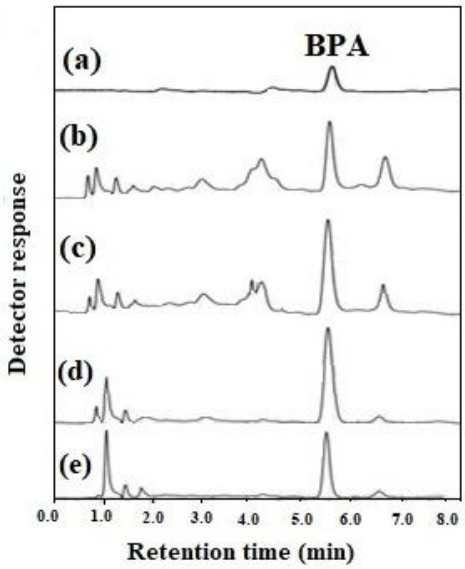

Fig. 2. Typical HPLC-UV chromatograms of (a) a standard solution at a concentration of 5 mg l^{-1} of BPA (b), unspiked milk, (c) spiked milk at a concentration of 10 ng ml^{-1}, (d) unspiked, (e) and spiked water sample at a concentration of 10 ng ml^{-1}. All of the chromatograms were obtained after performing the developed method, except chromatogram (a) obtained after direct injection of the standard solution.

Real Samples Analysis

The method was used for the analysis of ten water samples and ten milk samples of various brands. All samples were packed in plastic bottles. The results showed that BPA was found in all samples, except for three milks and four mineral water samples. BPA was found to be in the range of 11.2-32.6 and 9.6-23.5 ng ml^{-1} in water and milk samples, respectively. Unfortunately, its concentration was completely higher than the specific migration limit of 0.6 μg g^{-1} for BPA in foods and food stimulants established by the European Commission [23]. Figure 2 shows typical HPLC-UV chromatograms of a standard solution at a concentration of 5 mg l^{-1} (BPA), unspiked and spiked, and unspiked and spiked water samples after performing the developed method. The effect of non-analyte compounds on the method efficiency in milk samples were studied by analyzing four milk samples spiked with the analytes at three concentrations (5, 10 and 25 ng ml^{-1}) and comparing the results with those obtained for deionized water spiked at the similar concentrations. The results are reported as mean relative recoveries in Table 1. They demonstrate that the sample matrices have no significant effect on the efficiency of the method.

Comparison of the Method with UNE-EN: 13130-13:2005 Method

The efficiency of the present study was compared with the developed method UNE-EN: 13130-13:2005 [24] for the determination of BPA. For this purpose, three samples were spiked with BPA, three concentrations, and the methods were performed synchronously. The obtained results were calculated as mean relative recoveries, and they were analyzed statistically with respect to accuracy (t-test). The resulting data can be found in Table 2. According to the obtained results, there is no significant difference between both approaches as $t_{statistics} < t_{critical}$.

Comparison of the Method with other Approaches

The efficiency of this study was compared with previously reported methods used in the determination of BPA considering different terms of features such as LOD, LR, RSD, extraction time and ER. The results are listed in

Table 1. Results of Assays to Check the Sample Matrix Effect for BPA. It is Noted that the BPA Contents of Samples were Subtracted

Analyte	Mean relative recovery ± standard deviation (n = 3)							
	Milk #1	Milk #2	Milk #3	Milk #4	Mineral water #1	Mineral water #2	Mineral water #3	Mineral water #4
	All samples were spiked with BPA at a concentration of 5 ng ml^{-1}.							
BPA	85 ± 3	89 ± 3	82 ± 5	86 ± 5	92 ± 5	92 ± 3	97 ± 4	93 ± 6
	All samples were spiked with BPA at a concentration of 10 ng ml^{-1}.							
BPA	84 ± 4	90 ± 5	81 ± 6	83 ± 4	96 ± 3	93 ± 4	92 ± 5	94 ± 3
	All samples were spiked with BPA at a concentration of 25 ng ml^{-1}.							
BPA	90 ± 3	86 ± 3	92 ± 4	96 ± 7	90 ± 6	94 ± 4	96 ± 3	97 ± 4

Table 2. The Obtained Concentrations of the Analytes in the Spiked and Unspiked Samples and their Comparison with other Method

Analyte	Added (ng ml^{-1})	Mean recovery (%) ± standard deviation (n =3)								
		Water sample #1			Water sample #2			Milk sample #1		
		DLLME-HPLC-UV	UNE-EN: 13130-13:2005	t-static[a]	DLLME-HPLC-UV	UNE-EN: 13130-13:2005	t-static	DLLME-HPLC-UV	UNE-EN: 13130-13:2005	t-static
BPA	5	92 ± 3	86 ± 5	1.89	93 ± 6	94 ± 6	1.74	91 ± 4	92 ± 3	2.11
	10	91 ± 4	92 ± 6	2.06	89 ± 4	93 ± 5	1.96	92 ± 5	88 ± 4	1.39
	50	84 ± 5	97 ± 4	1.98	85 ± 5	95 ± 4	2.05	94 ± 6	97 ± 5	1.74

[a]t-Critical = 2.23 for n = 4, p = 0.05. [b]Not detected. [c]The initial concentration of the analyte was subtracted.

Table 3. The relatively short extraction time and better ERs are other characteristics of the proposed method compared to others. The LODs are comparable or lower than those of the mentioned techniques except DLLME-HPLC-UV method performed on water samples [21]. The RSDs for this method is comparable or better than the other mentioned methods. All these results reveal that the present method is a sensitive, rapid and repeatable technique that can be used as an alternative method for the extraction, preconcentration and determination of BPA.

CONCLUSIONS

In this work, a successful DLLME approach was conducted on milk and water samples to extract BPA prior to its HPLC-UV determination. The method was optimized and validated, and the obtained results showed lower LODs and LOQs, and acceptable precisions, accuracy, and recoveries. Finally, the method was performed on various milk and water samples packaged in plastic bottles, and BPA was determined by them. Unfortunately, it was found that BPA was higher than the level established by the European Commission in most samples.

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Table 3. Comparison of the Proposed Method with other Methods in the Extraction and Determination of BPA

Method	Sample	LOD[a]	LR[b]	RSD (%)[c]	Extraction time (min)	ER (%)[d]	Ref.
QuEChERS -DLLME-GC-MS[e]	Canned seafood	0.2-0.4 ($ng\ g^{-1}$)	1-150 ($ng\ g^{-1}$)	11-19	> 30	-	[25]
IL-VA-LLE-GC-MS[f]	Thermal paper	1.25 ($ng\ g^{-1}$)	4.0-100 ($ng\ g^{-1}$)	10	16	99	[26]
DLLME-GC-FID[g]	Honey sample	16000 ($ng\ ml^{-1}$)	48000 ($ng\ ml^{-1}$)	18.6	10	93	[10]
DLLME -GC-MS[h]	Beverages	7-10 ($ng\ ml^{-1}$)	20-1000 ($ng\ ml^{-1}$)	7-9	5	-	[27]
VALLE-AALLME-HPLC-VWD[i]	Canned doogh sample	0.54-0.82 ($ng\ ml^{-1}$)	2.7-100 ($ng\ ml^{-1}$)	5-6	10	81-86	[4]
DLLME-HPLC-UV[j]	Water	0.07	0.5-1000	6.0	3.0	48	[21]
DLLME-HPLC-UV	Water	0.44 ($ng\ ml^{-1}$)	1.47-5000 ($ng\ ml^{-1}$)	6.6	~5	82	Present work
	Milk	0.51 ($ng\ ml^{-1}$)	1.72-5000 ($ng\ ml^{-1}$)	8.9	~15	73	

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