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Article

Vortex-Assisted Hollow-Fiber Liquid-Phase Microextraction Coupled with High Performance Liquid Chromatography for the Determination of Three Synthetic Endocrine Disrupting Compounds in Milk

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Neste estudo, uma microextração em fase líquida suportada em fibra oca e com agitação vórtex (VA-HF-LPME) acoplada à cromatografia líquida de alta performance (HPLC) foi desenvolvida para a determinação de três compostos endócrinos sintéticos desreguladores [EDCs, bisfenol-A (BPA), bisfenol-AF (BPAF) e tetrabromobisfenol-A (TBBPA)] em amostras de leite. A agitação vórtex forneceu uma mistura efetiva e moderada das soluções de amostra e aumentou o contato entre os analitos e as camadas de fronteira da fibra oca, aumentando assim a razão de transferência de massa e levando à uma alta eficiência de extração dos analitos alvo. Os parâmetros que influenciaram o método de preparação da amostra do VA-HF-LPME, como os solventes orgânicos (fase aceptora), pH da solução da amostra (fase doadora), volume da amostra, concentração de NaOH, tempo de extração e força iônica, foram sistematicamente otimizados. As curvas de calibração instrumental do BPA, BPAF e TBBPA mostraram boas relações lineares ($R^2 > 0,9988$) na faixa de concentração de 0,5-200, 0,5-200 e 1,0-250 μg L^{-1}, respectivamente. O desvio relativo padrão (RSD, n = 5) estava entre 1,3-3,7%. Os limites de detecção (LOD, S/N = 3) estavam na faixa de 0,16-0,35 μg L^{-1} e os limites de quantificação (LOQ, S/N = 10) na faixa de 0,51-1,12 μg L^{-1}. O método proposto foi aplicado com sucesso na extração de EDCs em amostras de leite.

In this study, a vortex-assisted three-phase hollow fiber liquid-phase microextraction (VA-HF-LPME) coupling with high performance liquid chromatography (HPLC) method was developed for determination of three synthetic endocrine disrupting compounds[EDCs, bisphenol-A (BPA), bisphenol-AF (BPAF) and tetrabromobisphenol-A (TBBPA)] in milk samples. Vortex provided effective and mild mixing of the sample solution and increased the contact between analytes and boundary layers of the hollow fibre, thereby enhancing mass transfer rate and leading to high extraction efficiency of the target analytes. The influencing parameters of VA-HF-LPME sample preparation method, such as organic solvents (acceptor phase), pH of sample solution (donor phase), sample volume, concentration of NaOH, extraction time, and ionic strength were systematically optimized. The instrumental calibration curves of BPA, BPAF and TBBPA show good linear relations ($R^2 > 0.9988$) in the concentration range of 0.5-200, 0.5-200 and 1.0-250 μg L^{-1}, respectively. The relative standard deviations (RSD, n = 5) were 1.3-3.7%. The limits of detection (LOD, S/N = 3) were in the range of 0.16-0.35 μg L^{-1} and the limits of quantification (LOQ, S/N = 10) were in the range of 0.51-1.12 μg L^{-1}. The proposed method was successfully applied to the extraction of EDCs in milk samples.

Keywords: hollow-fiber vortex-assisted liquid-phase microextraction, bisphenol-A, bisphenol-AF, tetrabromobisphenol A, milk, high performance liquid chromatography

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Introduction

During the past years, endocrine disruptors (EDs) have been attracting much more attention because of their possible negative effects on human health.[1] The synthetic endocrine disrupting compounds (EDCs) such as bisphenol-A (BPA), bisphenol-AF (BPAF) and tetrabromobisphenol A (TBBPA) have been reported that have endocrine disruption properties and become important environment pollutants.[2-4] BPA, BPAF and TBBPA levels found in the aquatic environment were relatively low; hence, a simple, fast, sensitive and selective analytical method is very important to assess their risks.

So far, various methods have been developed for the analysis of EDCs such as liquid chromatography-mass spectrometry (LC-MS or LC-MS-MS),[5] gas chromatography-mass spectrometry (GC-MS or GC-MS-MS).[6-8] High performance liquid chromatography (HPLC) and capillary electrophoresis (CE) with different detectors have extensive applications for simultaneous determination of various EDCs in liquid samples.[9,10]

Owing to their low concentrations and complicated matrix effects, an effective pretreatment approach is very important for the analysis of these compounds. Therefore, several extraction approaches have been developed such as solid-phase extraction (SPE),[11] accelerated solvent extraction (ASE),[12] vortex-assisted liquid-liquid microextraction (VALLME)[13] and dispersive liquid-liquid microextraction (DLLME).[14] Traditional liquid-liquid extraction (LLE) and SPE approaches uses large amount of toxic organic solvents. Compared with conventional extraction methods, microextraction approaches are much simpler, more rapid and environment friendly.[15-19]

The application of hollow fiber liquid-phase microextraction (HF-LPME) technique was first introduced by Pedersen-Bjergaard and Rasmussen in 1999.[20] There are two modes of HF-LPME: three- and two-phase HF-LPME. In the two-phase HF-LPME, the analytes are directly extracted from the sample into the organic phase supported by the fiber. In three-phase mode, three liquid phases participate in analyte extraction: (*i*) the sample solution, with a pH that is adjusted to keep analytes neutrally charged, (*ii*) the water-immiscible organic extractor phase, which is immobilized in the wall pores of the hollow fiber, and (*iii*) the aqueous acceptor phase, with a pH that is adjusted to ionize the analytes. The major advantages of HF-LPME are high enrichment factor, strong purification ability, simple and rapid operation, low organic solvent consumption and cheap equipment. Thus, HF-LPME showed the potential for extraction of analytes from complex matrices such as biological and environmental samples.[21-27]

The main goal of this work was to develop and optimize a vortex-assisted hollow-fiber liquid-phase microextraction(VA-HF-LPME) procedure for extraction and determination of BPA, BPAF and TBBPA. The VA-HF-LPME experimental conditions and the chromatographic separation were optimized. The proposed analytical method was successfully applied to the determination of the compounds in milk samples.

Experimental

Apparatus

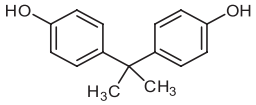
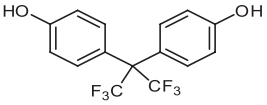
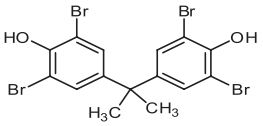
Chromatographic evaluation and separation were performed on an HPLC system (consisting of a quatpump, an auto sampler, a vacuum degasser, and a diode-array detector; Agilent 1100 Series, Agilent Technologies, Palo Alto, Calif., USA) equipped with a analytical column of reversed phase C18 (150 mm × 4.6 mm, 5 μm particle size) (Agilent TC-C18). Empower software was used for spectra recording of the studied EDCs and used for spectra confirmations of peaks in the studied samples. A vortex agitator (Jiangsu, China) was used for vortex-assisted extraction. A centrifuge (Shanghai, China) was used for complete phase separation. An ultrasonic clear with temperature control (Shanghai, China) was used for ultrasonic extraction.

Reagents

BPA, BPAF and TBBPA (analytical standard) were purchased from Aladdin (Shanghai, China). Standard stock solutions of BPA, BPAF and TBBPA were prepared in methanol at a concentration of 20 μg mL^{-1}. Working solutions were prepared daily by an appropriate dilution of the stock solutions. The chemical structures of these compounds are depicted in Table 1.

The porous hollow fiber used to support the organic phase was Q3/2 polypropylene (Wuppertal, Germany) with 600 μm inner diameter, 200 μm of wall thickness and pores of 0.2 μm. A 1.0 mL microsyringe (model 702SNR) with a sharp needle tip was used. HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany). Pure analysis methanol was purchased from Aladdin (Shanghai, China). Octanol (98%) was purchased from Kedi (Tian Jin, China). Phosphoric acid, acetic acid and boric acid were purchased from ZhiYuan (Tian Jin, China). All the other solvents were analytical reagent grade unless stated.

Table 1. Chemical and physical parameters of BPA, BPAF and TBBPA considered in this work

Analyte	Structure	Formula	Molecular weight / (g mol^{-1})
BPA		$C_{15}H_{16}O_2$	228.29
BPAF		$C_{15}H_{10}F_6O_2$	336.23
TBBPA		$C_{15}H_{12}Br_4O_2$	543.87

HPLC conditions

The HPLC separation was performed on a reversed-phase system with the gradient elution using acetonitrile and water. The gradient elution was performed as follows: 0-8.0 min, 42:50; 8.0-15 min, 50:85; acetonitrile: water, v/v. The injection volume was 10 µL. The flow rate was set at 1 mL min^{-1} and the column temperature was maintained at 30 °C. BPA, BPAF and TBBPA were recorded at the wavelength of 280 nm.

Sample preparation

Milk samples were purchased from a local supermarket (Kunming, China). In order to reduce the viscosity of the sample and be convenient for experimental operation, milk samples were diluted 1:1 with pure water. Then the samples were filtered through 0.45 µm filters and the resulting solutions were referred to as sample solutions.

VA-HF-LPME procedure

The hollow fiber was cut manually into 8 cm length pieces. Before using them, each piece was ultrasonically cleaned in acetone for 5.0 min in order to remove any contaminants and then dried in air. For each experiment, a 8 mL aqueous sample solution (pH = 3.0) containing 40 µg L^{-1} of each EDC was placed in a 10 mL vial. Before extraction, the syringe was rinsed with acetone followed by octanol, to avoid carryover and air bubble formation. Then 30 µL of the acceptor phase was withdrawn into the microsyringe and its needle was inserted into the lumen of the hollow fiber. The hollow fiber was immersed in the organic solvent in order to impregnate its pores with organic solvent. Then it was inserted into the water for 15 s to wash the excess organic solvent from the surface of the hollow fiber. The acceptor phase in the syringe was injected into the lumen of the hollow fiber and the ends of the fiber were sealed by a piece of aluminum foil. The impregnated and filled fiber was then immersed in the sample for immediate extraction. The extraction was carried out at room temperature (approximately 20 °C) with a vortex mixing at 3000 rpm for 6.0 min. At the end of the extraction time, the fiber was removed from the sample and its closed end was cut, the lumen of the hollow fiber was washed with 200 µL of methanol, and 10 µL of the filter liquid was injected into the HPLC system for analysis. Operation process is shown in Figure 1.

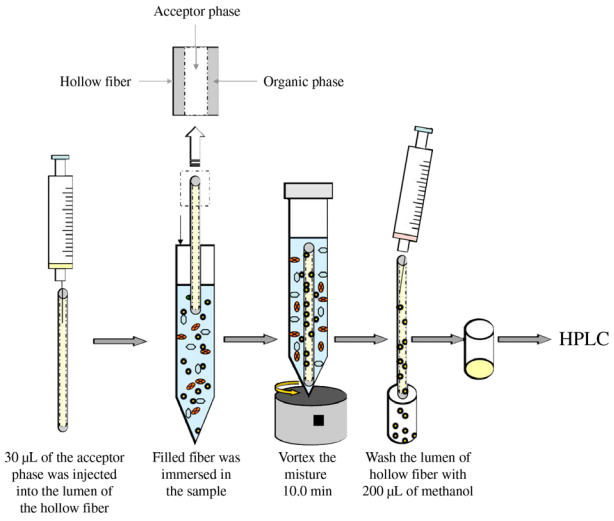


Figure 1. The VA-HF-LPME procedure.

Analytical performance of the method

In order to determine the recovery, repeatability, and reproducibility of this method, EDCs were spiked into blank milks at three different concentrations (1.0, 5.0, and 12.0 µg L^{-1}), and five replicates were analyzed *per*

concentration level in two independent analytical runs under the established chromatographic conditions.

Results and Discussion

Optimization of the VA-HF-LPME procedure

For optimization of VA-HF-LPME, factors that affect sample extraction in LPME, such as organic solvent type, sample volume, concentration of NaOH in acceptor phase, ionic strength in sample, pH of donor phase, extraction time were studied.

In this experiment, 8.0 mL of prepared milk spiked with 5 μg mL^{-1} of each EDCs were used for the study. All the experiments were performed five times and the averages of the results were used for optimization.

Recovery (R) was calculated as $R = (C_{measured} - C_{sample})/C_{spiked}$, where $C_{measured}$ is the concentration in a spiked sample, C_{sample} is the concentration in the sample prior to spiking and C_{spiked} is the concentration of added standard.

Selection of the organic extraction solvent

It was important to choose a suitable organic extraction solvent in the HF-LPME method. Firstly, organic extraction solvent should have a good affinity with the fiber, and should be insoluble in water. Also, the ideal organic extraction solvent should have an appropriate viscosity and a low volatility to prevent volatile loss and diffusion. Compared with the other extraction solvent, long chain alcohols and acids have some particular properties, which have special extraction efficiency for the analytes. Thus, we chose nonanoic acid, octanoic acid, heptanol and octanol as final organic extraction solvents in this study.

From Figure 2, it was found that the tested solvents of octanol get the best recoveries. Octanol possesses an active hydrogen atom (hydroxyl group), which make it easier to form hydrogen bond with analytes. In addition, octanol has other better characteristics, such as larger viscosity and less volatility, those attributed to lower solvent loss. Based on these results, octanol was chosen as extraction solvent for further experiments.

Optimization of donor phase pH

It is widely known that donor and acceptor pH optimizations are usually the more critical steps to establish the optimal experimental conditions for a three phase HF-LPME. Substances to be extracted must be in non-ionized form in the donor phase to cross the organic liquid membrane. pH values range from 1.0 to 9.0 were

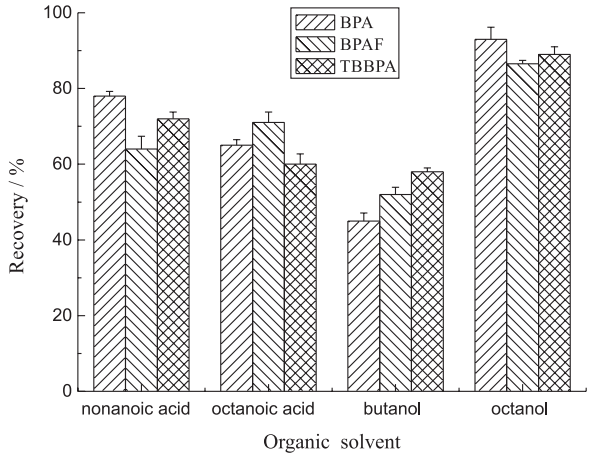


Figure 2. Effect of organic extraction solvent on the extraction of BPA, BPAF and TBBPA. Extraction conditions: sample pH: 3.0; extraction time: 6.0 min; concentration of NaOH in acceptor phase: 0.3 mol L^{-1}; sample volume: 8 mL.

investigated to study their influence on the extraction efficiency. The Britton-Robinson buffer solution make up of phosphoric acid, acetic acid and boric acid was used to adjust the sample pH. Figure 3 shows that the extraction efficiency is the highest when the pH value is 3.0. The three phenols in this study are acidic because they contain 2 phenolic hydroxyl groups. Therefore, changes in pH could change their existing forms. Consequently, the donor phase should be acidified to deionize the analytes and increase their transfer from the donor phase into the organic phase. Thus, pH 3.0 was selected as optimum for donor phase.

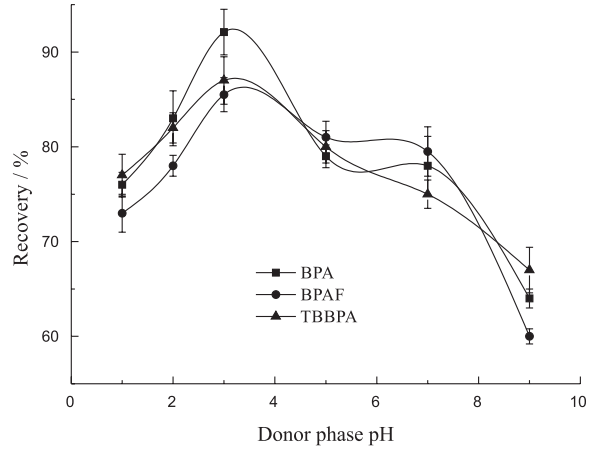


Figure 3. Effect of donor phase pH on the extraction of BPA, BPAF and TBBPA. Extraction conditions: organic solvent: octanol; extraction time: 6.0 min; concentration of NaOH in acceptor phase: 0.3 mol L^{-1}; sample volume: 8 mL.

Effect of concentration of NaOH in acceptor phase

The impact of concentration of NaOH in acceptor phase on the extraction efficiency was studied. The extraction

results at different concentrations of NaOH (0.1, 0.3, 0.5, 0.7. and 0.9 mol L^{-1}) were shown in Figure 4. It was proposed that the extraction process should be as follows: first, BPA, BPAF and TBBPA gathered on the surface of fiber; second, BPA, BPAF and TBBPA were extracted into organic solvents; third, BPA, BPAF and TBBPA were transferred from organic solvents to acceptor phase (NaOH solution). Changing the concentration of NaOH can provide high solubility for the acidic analytes and ionize them. This makes it easier to transfer into the acceptor phase and improve the efficiency of extraction. The highest extraction efficiency of BPA could be achieved in 0.3 mol L^{-1} NaOH. Therefore, 0.3 mol L^{-1} NaOH was selected as acceptor media which could provide relative high recovery for the target analytes.

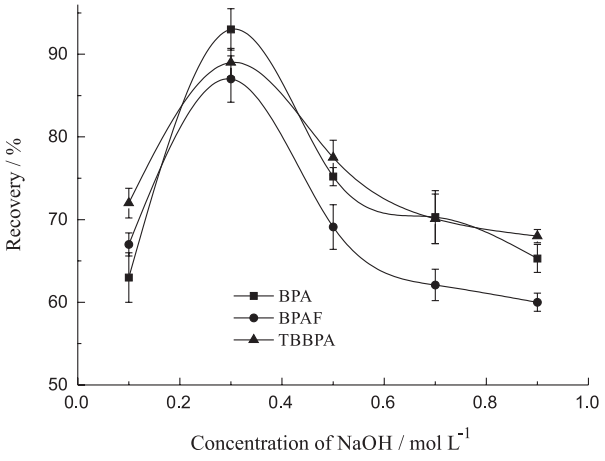


Figure 4. Effect of concentration of NaOH in acceptor phase on the extraction of BPA, BPAF and TBBPA. Extraction conditions: organic solvent: octanol; sample pH: 3.0; extraction time: 6.0 min; sample volume: 8 mL.

Effect of donor phase volume

Generally, as the volume of the sample enhanced, the preconcentration factor also enhances.[28,29] However, a larger sample volume can be disadvantageous due to poorer mass transfers kinetics that result in a poor extraction efficiency. This would ultimate to a decrease in the microextraction output.[30,31] So, in our experiment, the volume of donor phase from 4 to 10 mL with the same concentration of each analyte was investigated. The results are displayed in Figure 5, According to the results, 8 mL was therefore selected as the optimum sample volume.

Effect of vortex-mix time

Extraction time can affect extraction efficiency. In order to study the effect of vortex-mix time on the extraction

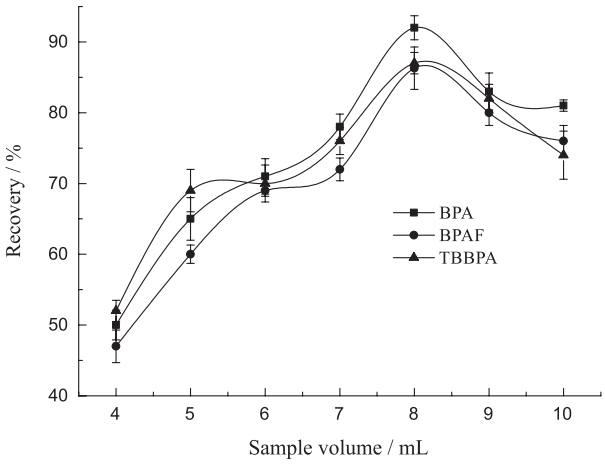


Figure 5. Effect of donor phase volume on the extraction of BPA, BPAF and TBBPA. Extraction conditions: organic solvent: octanol; sample pH: 3.0; extraction time: 6.0 min; concentration of NaOH in acceptor phase: 0.3 mol L^{-1}.

recoveries, the effect of extraction time was investigated by conducting experiments for 3.0, 6.0, 10.0, 14.0, and 20.0 min at a vortexing speed of 3000 rpm. As can be seen from Figure 6, the recoveries of the analytes increase significantly when the extraction time increase from 3.0 to 6.0 min, and after 6.0 min the recoveries appear a slow descent. Therefore, 6.0 min was chosen for the following investigation.

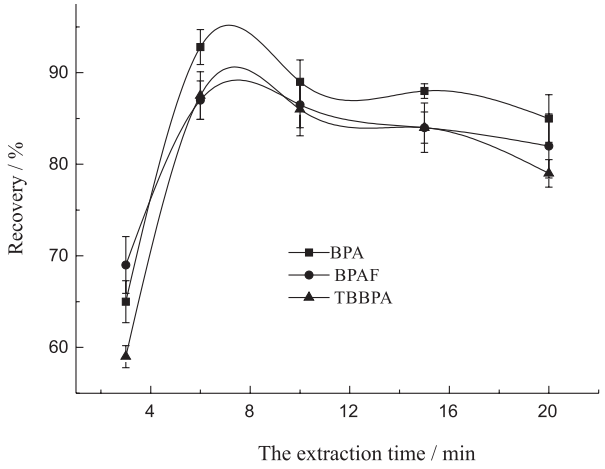


Figure 6. Effect of vortex-mix time on the extraction of BPA, BPAF and TBBPA. Extraction conditions: organic solvent: octanol; sample pH: 3.0; concentration of NaOH in acceptor phase: 0.3 mol L^{-1}; sample volume: 8 mL.

Effect of ionic strength

The effect of salt addition on the extraction efficiency of BPA, BPAF and TBBPA by the VA-HF-LPME method was examined by adding NaCl to 8 mL aqueous samples at 0, 2, 4, 6 and 8% (w/v). The addition of salt to the

sample will lead to a higher ionic strength in the sample and decrease the solubility of three EDCs in the aqueous solution. In addition, electrostatic inter action will resist organic solvent extraction. Therefore, the effect of salt on extraction is indefinite. Figure 7 shows that the addition of NaCl decreased the extraction efficiencies of the three EDCs. Hence, NaCl was not added in further study.

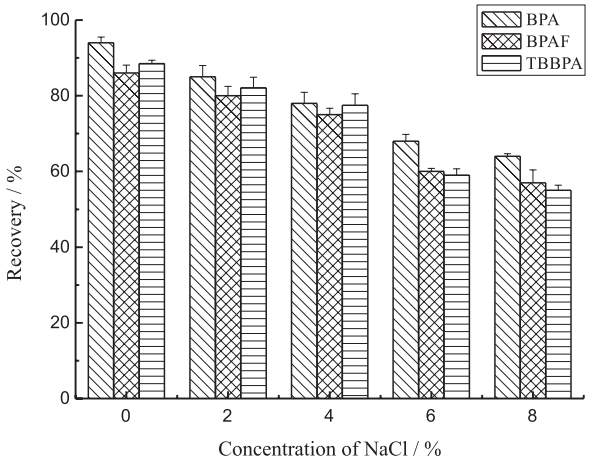


Figure 7. Effect of ionic strength on the extraction of BPA, BPAF and TBBPA. Extraction conditions: organic solvent: octanol; sample pH: 3.0; extraction time: 6.0 min; concentration of NaOH in acceptor phase: 0.3 mol L^{-1}; sample volume: 8 mL.

Method validation

A series of experiments with regard to the linear equation, linearity, squared regression coefficients (R^2), limit of detection (LOD) (S/N = 3), limit of quantification (LOQ), R% and relative standard deviations (RSD) were performed to validate the proposed method under the optimized extraction condition. The results are listed in Table 2. The linearity of the method was explored at EDCs concentrations from 0.5 to 250 μg L^{-1} with good R^2 ranging from 0.9988 to 0.9992. The LODs (S/N = 3) ranged between 0.16 and 0.35 μg L^{-1} and the LOQs ranged between 0.51 and 1.12 μg L^{-1}. Reproducibility studies (five replicates) were performed by samples spiked with three different concentrations (1.0, 5.0, and 12.0 μg L^{-1}) of EDCs and the RSDs were in the range 2.3-3.5%. Under the optimized extraction condition, the extraction recoveries were 94%, 87% and 89% for BPA, BPAF and TBBPA in milk, respectively.

Comparison of VA-HF-LPME with other methods

In this procedure, 8.0 ml of prepared milk spiked with 2.0 μg L^{-1} of each EDCs were used for the study. It is very useful to develop at least a brief comparison with other methods published in the literature. Some other methods reported in literature, such as ultrasound-dispersive liquid-liquid microextraction (US-DLLME),[32] stir bar sorptive extraction (SBSE),[33] reversed-phase dispersive liquid-liquid microextraction (RP-DLLME),[34] hollow fiber liquid-phase microextraction (HF-LPME)[35] were compared with the present method, and the results are presented in Table 3. The proposed method had lower RSDs and shorter extraction time compared with other methods. The LODs (S/N = 3) are close to SBSE/HPLC, and lower than US-DLLME/HPLC, RP-DLLME /HPLC and HF-LPME/HPLC.

Analysis of real samples

In order to validate the precision, accuracy, and reproducibility of the proposed method, it was applied successfully for the determination of EDCs in real milk

Table 2. Quantitative results of the proposed HF-VA-LPME method

Analyte	Regression equation	Linear range / (μg L^{-1})	R^2	R%	RSD / % (n = 5)	LOD / (μg L^{-1})	LOQ / (μg L^{-1})
BPA	y = 32.252x – 2.1	0.5-200	0.9992	94	2.3	0.16	0.51
BPAF	y = 56.856x – 5.575	0.5-200	0.9989	87	3.5	0.18	0.56
TBBPA	y = 79.295x + 13.204	1.0-250	0.9988	89	2.9	0.35	1.12

Table 3. Characteristic performance data obtained by using this method with other extraction methods for determination of BPA

Matrix	Sample preparation	Detection	LODs	Recovery / %	RSD / %(n = 5)	Ref.
Water	US-DLLME	HPLC	0.34 (μg L^{-1})	82.7-92.5	4.5	29
Water	SBSE	HPLC	0.15 (μg L^{-1})	77.6-124.4	3.7	30
Edible oil	RP-DLLME	HPLC	2.5 (μg kg^{-1})	89.5-99.7	1.9-5.9	31
River water	HF-LPME	HPLC	0.2 (μg L^{-1})	92.8-101.9	2.2-3.2	32
Milk	VA-HF-LPME	HPLC	0.16 (μg L^{-1})	86.7-97.5	1.4-3.5	This work

Table 4. Determination of BPA, BPAF and TBBPA in milk samples

Milk sample	Added / (μg L^{-1})	Found / (μg L^{-1}) (RSD / %)[a] (n = 5)			Recovery / %		
		BPA	BPAF	TBBPA	BPA	BPAF	TBBPA
Milk 1	0	–	–	–	–	–	–
	1.2	1.35(1.6)	1.05(2.7)	1.06(1.5)	97.5	87.5	88.3
	5.6	5.41(3.4)	4.88(2.8)	4.86(2.9)	93.4	87.1	86.7
Milk 2	0	–	–	–	–	–	–
	1.2	1.13(3.1)	1.04(1.8)	1.05(3.7)	94.2	86.7	87.5
	5.6	5.22(2.3)	4.87 (2.9)	4.90(2.5)	93.2	86.9	87.5
Milk 3	0	–	–	–	–	–	–
	1.2	1.44(1.3)	1.07(2.4)	1.1(2.7)	94.1	89.1	91.6
	5.6	5.46(2.7)	4.96(1.5)	5.02(2.6)	92.0	88.6	89.6

[a]Data were calculated based on five-replicate experiments.

samples and spiked of EDCs in milk samples. The results are listed in Table 4. The chromatograms of milk samples and the milk samples spiked with BPA, BPAF and TBBPA were shown in Figure 8. According to the results, BPA, BPAF and TBBPA were not found in the milk analysed. Some impurities were found in milk samples but did not interfere with the determination of BPA, BPAF and TBBPA. The spiking concentrations of BPA, BPAF and TBBPA were 0, 1.2 and 5.6 μg L^{-1}, respectively. The relative recoveries for the analytes were in the range of 86.7-97.5% and the RSDs (n = 5) ranged from 1.3% to 3.7% in milk samples. The experimental results proved that the matrices of milk samples did not make significant impact on the determinations of BPA, BPAF and TBBPA in milk.

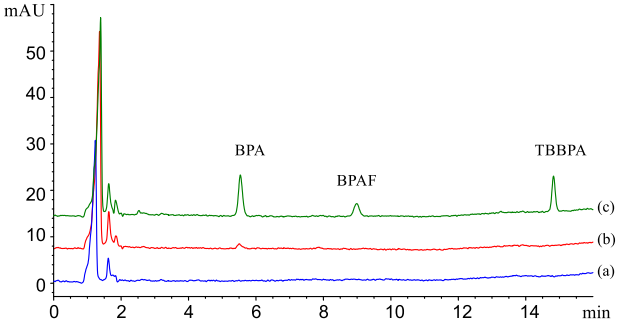


Figure 8. Typical chromatogram of milk: (a) blank milk; (b) milk after HF-VALPME; (c) milk spiked with BPA, BPAF and TBBPA (5 μg L^{-1}) after HF-VALPME.

Conclusions

A three-phase HF-VALPME procedure combined with HPLC method was developed for the analysis of trace level of three synthetic endocrine disrupting compounds (BPA, BPAF and TBBPA) in milk samples. Various influencing parameters, including organic solvent type, sample volume, concentration of NaOH, ionic strength, pH of sample, extraction time of HF-VALPME sample preparation method were optimized. The results demonstrated that the proposed VAHF-LPME method has many advantages, such as environmental friendly, easy to operate, strong purification ability, short extraction time and high enrichment efficiency. The HF-VALPME sample preparation method coupled with HPLC detection method can be applied for the determination of EDCs at trace levels in different kinds of real milk samples.

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