Supramolecular Solvent-Based Vortex-Assisted Hollow Fibre Liquid-Phase Microextraction Technique Combined With High Performance Liquid Chromatography for the Determination of Estrogens in Milk Samples

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(Received on 29th August 2023, accepted in revised form 18th April 2024)

Summary: A fast, simple, environmental friendly and strong sample clean-up ability method for the determination of three estrogens (17β-estradiol (17β-E2), estrone (E1), and diethylstilbestrol (DES)) in milk samples has been developed by using supramolecular solvent-based vortex-assisted hollow fibre liquid-phase microextraction (SS-VA-HF-LPME) and high performance liquid chromatography. Method is based on estrogens was extracted from aqueous samples into a supramolecular solvent which were made up of n-octanol and ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM] BF_4) impregnated in the wall pores and also filled inside the porous polypropylene hollow fiber membrane followed by vortex-mixing. The supramolecular solvent has better compatibility with the polarity of target analytes and the driving forces for the extraction include hydrogen bonding, hydrophobic and π-cation interactions between the analytes and the vesicular aggregates, thereby leading to high recoveries. Vortex can provide mild and effective mixing of sample solution and increase the contact between analytes and boundary layers of the hollow fibre. Therefore the mass transfer rate was enhanced. The parameters that affect the recoveries were studied and optimized. HF of 4 cm length was used and the pH 4.0 was employed. The percentages of n-octanol and [BMIM]BF4 were 10% and 3%, respectively. The extraction equilibrium time was 4 min. The method provided good linearity (>0.998), repeatability (RSD% = 1.24–4.36), low detection limit (0.10–0.22 ng mL^{-1}), and high enrichment factor (330).

Keywords: Supramolecular solvent; Vortex-assisted; Hollow fibre liquid-phase microextraction; Estrogens; ionic liquid; n-octanol.

Introduction

Estrogens are a large class of compounds with high estrogen activity, which can regulate the reproduction, metabolism and development, water, electrolyte and nutritional balance of the blood [1]. They can be divided into endogenous estrogens (17β-estradiol (E2), estrone (E1), etc.) and artificial estrogens (diethylstilbestrol (DES), etc) (Table 1) [2]. If these compounds remain in the body, they may have adverse effects on the endocrine system in humans and wildlife [3,4]. Several studies have shown that estrogens exposed to environment have given rise to decreased sperm counts, increased testicular, prostate, and reproduction disorders in females and males [5]. Endogenous estrogen levels have been positively correlated with an increased risk of breast cancer, particularly in postmenopausal women [6]. Recently, the abuse of estrogens in animal fattening or growth promotion is serious. Matrices such as muscle tissue, fat and milk are all considered as specimens for analysis. With regard to milk, hormones can pass from the bloodstream and can be finally excreted in milk by the mammary gland which synthesizes milk proteins and lactose during late pregnancy and lactation [7]. Therefore, developing a sensitive, accurate and selective analytical method for detecting estrogens in milk samples is important for the investigation of potential use of estrogens in food-safety area.

Various chromatographic analytical methods have been reported for the determination of estrogens, including micellar electrokinetic chromatography (MEKC) [8], high performance liquid chromatography (HPLC) [9], liquid chromatography with mass spectrometry (LC-MS) [10,11], gas chromatography (GC), gas chromatography with mass spectrometry (GC-MS) [12,13]. The pretreatment method prior to analytical methods for the sample is another important factor in the analysis of estrogens. At present, preconcentration and clean-

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up techniques for estrogens mainly focus on the classical liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid-phase microextraction (MSPE) [14], and microsolid-phase extraction (μ-SPE) [15]. However, these sample preparation techniques are somewhat time-consuming or environmentally unfriendly. Thus, an effective, environment-friendly and inexpensive method is still needed for the extraction of estrogens.

Supramolecular solvents (SUPRASs) are water-immiscible liquids formed by a sequential self-assembly of amphiphilic molecules with molecular or nanoscales [16]. The application of SUPRAS to analytical extraction was first proposed by Watanabe et al. in 1978 [17]. A unique characteristic of SUPRASs is that its polarity changes in different regions. This property enables SUPRAS to dissolve, concentrate, compartmentalize, organize and localize solutes in a very wide range of polarities, thereby creating specific media for separating target compounds [18]. We therefore can control self-assembly process according to different analytes by selecting different composition of supramolecular solvent.

Hollow fiber liquid-phase microextraction (HF-LPME) technique was originally proposed by Pedersen-Bjergaard and Rasmussen. It has aroused considerable interest in the analytical area and has been widely applied to several of biological and environmental samples [19]. It is an excellent pretreatment method and highly compatible with High-performance liquid chromatography. In HF-LPME procedure, analytes are extracted from an aqueous sample (donor phase) into an acceptor phase in the lumen of either organic (the so-called two-phase extraction) or aqueous nature (three-phase extraction) supported by a porous-walled polypropylene hollow fiber [20-22]. High enrichment factor can be obtained in HF-LPME because the sample volume can range between 5 mL and greater than 1 L, however, in most cases, the volume of the extraction solvent (acceptor phase) is in the range 2–30 μL [19]. The major advantages of HF-LPME are simplicity, negligible volume of solvents used, large pH tolerance range, high enrichment factor, low cost and excellent sample clean-up ability [23].

The aim of the present study is regulation of supramolecular self-assembly according to the structure of estrogens and the characteristics of HF-LPME. A novel supramolecular solvent-based vortex-assisted hollow fibre liquid-phase microextraction (SS-VA-HF-LPME) was applied to determinate estrogens in milk samples. In general, the reported extraction solvents used in HF-LPME are mostly 1-octanol, n-dodecane and ethyl acetate [24-27]. Supramolecular solvent-based hollow fiber liquid phase microextraction of benzodiazepines using classical SUPRAS produced from coacervation of decanoic acid aqueous vesicles in the presence of tetrabutylammonium (Bu_4N^+) has been reported by Fatemeh Rezaei [28]. A novel SUPRAS system comprised of organic alcohol and ionic liquid was firstly reported by our team for the determination of glucocorticoids in water samples demonstrating hydrogen bond plays a key role in the extraction and confirmed the formation of hydrogen bonds by ^{1}H and ^{2}H-MR [29]. In the present study, hydrophobic n-octanol, amphiphilic fluorinated ionic liquid [BMIM]BF_4 and water were used to prepare a new SUPRAS combined with vortex-assisted hollow fibre liquid-phase microextraction. This SUPRAS system has better compatibility in polarity with estrogens. Estrogens were expected to form hydrogen bonds with fluorine of ionic liquid [BMIM]BF_4. The regulation of supramolecular self-assembly process for the extraction is successful. The formation and role of hydrogen bond (H-bond) were confirmed using ^{1}H, ^{2}H-MR and infrared spectroscopy. Both the hydrophobic and hydrogen bond between analytes and SUPRAS contribute to the extraction. Vortex-mixing was used as an auxiliary method to accelerate mass transfer and minimize fluid loss. The extraction equilibrium is achieved within 4 minutes. Several parameters including the selection and optimization of SUPRAS, donor phase pH, vortex-mix time, the fiber length, and salt addition that affect the performance of the system were optimized.

Experimental

Chemicals and Reagents

Analytical standards (17β-E2, E1 and DES) were all purchased from Sigma (Sigma, USA). Standard stock solutions of 17β-E2, E1 and DES were prepared in methanol at a concentration of 500 μg mL^{-1}. Working solutions were prepared daily by an appropriate dilution of the stock solutions. Analytical standard of N-butanol, n-pentanol, n-hexanol, n-heptanol, n-octanol, n-nonanol, n-decanol, 1-undecanol were purchased from Aladdin Chemistry (Shanghai, China). 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4) was obtained from

Sigma (Sigma, USA). Acetonitrile of HPLC grade was purchased from Merck (Darmstadt, Germany).

Instrumentation and Materials

An HPLC system (containing a quatpump, a vacuum degasser, an auto sampler and a diode-array detector; Agilent 1200 Series, Agilent Technologies, Ca U.S.A.) equipped with a reversed phase C18 analytical column (150 × 4.6 mm, Agilent TC-C18) was performed for Chromatographic separation and evaluation. Empower software was used for spectra confirmations of peaks in the studied samples and spectra recording of the estrogens. The gradient program was as follows: 0–4.5 min, 35:65; 6.0–20 min, 55:45; acetonitrile:water, v/v. The detection wavelength was set at 280 nm. The injection volume was 20 μL and the flow rate was set at 1.0 mL min^{-1}. The column temperature was maintained at 25 □C.

A vortexer (Kylin-Bell Lab Instruments Co. Ltd., Jiangsu, China) was used for vortex-mixing. An ultrasonic instrument (Kunshan ultra-sonic instrument plant, Jiangsu, China) was used in the procedure. A centrifuge with centrifugal vials (Shanghai surgical instrument factory, 80-2, Shanghai, China) was used for SUPRAS production. 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 for the injection of the SUPRAS into the hollow fiber lumen.

SUPRAS production

0.15 mL of 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4) and 0.5 mL of n-octanol were added in a 10 mL centrifuge tube and diluted to 5 mL with distilled water. The mixture was vortex-mixed for 2 min, then the SUPRAS formed into the bulk solution spontaneously [17-18]. The turbid solution was centrifuged for 5 min at 3500 rpm, then the SUPRAS was formed which is less dense than water floating on the top of the sample. Then the aqueous phase was removed and the SUPRAS was transferred to a hermetically close storage glass vial to avoid octanol losses and stored at 4 ℃ until used.

SS-VA-HF-LPME extraction procedure

The hollow fibers were cut into 4cm long pieces and washed in an ultrasonic bath with acetone for 5 minutes, then dried in air to remove any pollutants from the fibers. The fiber was soaked in the SUPRAS for 30s to impregnate the pores, and the lumen of the prepared fiber piece was filled with 15 μL SUPRAS using a microsyringe very carefully. Both open ends of the fiber were sealed with an aluminum foil. Then the hollow fiber was bent to a U-shape and immersed in the 5 mL sample solution (pH 4.0, adjusted with Britton-Robinson buffer solution) containing 100 ng mL^{-1} of each estrogen. The sample was vortex-mixed for 4min. Remove the fiber from the sample solution and cut its closed end. To elute the acceptor phase, 100μL of methanol was injected into the lumen of sample-treated hollow fiber to eluent the SUPRAS containing extracted estrogens. The extract was stored in a sample vial. Then injecting extract (20 μL) into the HPLC system. A fresh hollow fiber was used for each extraction to decrease the memory effect.

Sample preparation

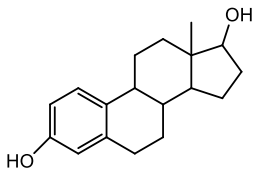
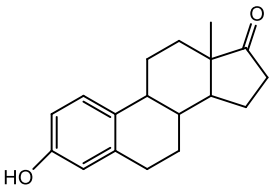
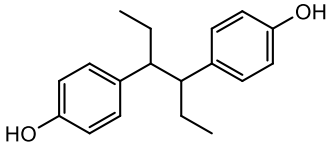
Two types of homogenized samples (whole milk and skimmed milk) were purchased from local supermarkets around Kunming, China. All samples were frozen at −18 ℃ until analysis. NaAc (0.1 g), $MgSO_4$ (0.2 g) and 2.0 mL acetonitrile were added into a centrifuge (10 mL) vial with milk (5 mL) sample continuously for protein precipitation [27]. Thus reduced protein interference in detection. Then the samples were centrifuged at 5000 rpm for 10 minutes. The supernatant was collected to a new vial. Then, the supernatant was diluted to 5 mL with Britton-Robinson buffer solution (pH 4.0) and subjected to SS-VA-HF-LPME procedure.

Results and Discussion

Optimization of the SS-VA-HF-LPME procedure

Spiked samples of estrogens (100 ng mL^{-1}) in aqueous solutions were used in the experiments. The influence of several different experimental parameters on the recovery rate was studied. Total of 5 replicates were performed to obtain a mean value. The optimization procedure was done as described in SS-VA-HF-LPME procedure.

Table-1: The structures of three estrogens.

17β-雌二醇 **$C_{18}H_{24}O_2$**	
雌 酮 **$C_{18}H_{22}O_2$**	
己烯雌酚 **$C_{18}H_{20}O_2$**	

Selection and optimization of SUPRAS

SUPRASs have exceptional properties for extraction processes, which derive from the special structure of the supramolecular assemblies making them up. The selection of SUPRAS was based on its capability to provide different type of interactions (e.g., electrostatics, π-cation, hydrogen bonds) with analytes. Because of very little amount of extraction solvent was used in this method, the ideal SUPRAS should provide high solubility for the target estrogens. Thus, various combinations of supramolecular solvents on the recovery effects were examined for the extraction of estrogens as shown in Fig. 1, including decanoic acid in tetrahydrofuran (THF) (Fig. 1, labeled as 1 on x axis), $[BMIM]BF_4$ in n-butanol (Fig. 1, labeled as 2 on x axis), $[BMIM]BF_4$ in n-pentanol (Fig. 1, labeled as 3 on x axis), $[BMIM]BF_4$ in n-hexanol (Fig. 1, labeled as 4 on x axis), $[BMIM]BF_4$ in n-octanol (Fig. 1, labeled as 5 on x axis), $[BMIM]BF_4$ in n-nonanol (Fig. 1, labeled as 6 on x axis), $[BMIM]BF_4$ in n-decanol (Fig. 1, labeled as 7 on x axis), and $[BMIM]BF_4$ in 1-undecanol (Fig. 1, labeled as 8 on x axis). The results showed that $[BMIM]BF_4$ in n-octanol is the most efficient system and the regulation of supramolecular self-assembly process for the extraction is successful. The system has larger viscosity, less volatility and better molecular recognition and molecular assembly, those characteristics attributed to higher recovery and lower solvent loss during extraction. This indicates that higher recoveries can be obtained at the existence of both the hydrophobic and hydrogen bond simultaneously.

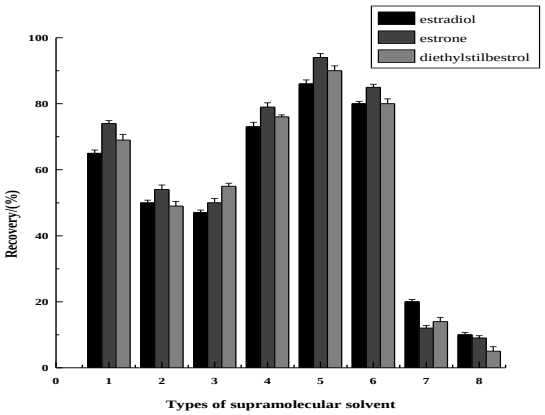


Fig. 1: Effect of supramolecular solvent types on the recovery.

Besides, SUPRASs containing different percentages of n-octanol (2–18%) and $[BMIM]BF_4$ (1.5–6%) were investigated as shown in Table 2, the highest recoveries were obtained as the percentages of n-octanol and $[BMIM]BF_4$ were 10% and 3%, respectively. The eluent used for elution of the extraction solvent after SS-VA-HF-LPME procedure can affect chromatographic behavior of the estrogens. Thus, methanol, ethanol and acetonitrile were studied, obtaining more reproducible results by using methanol.

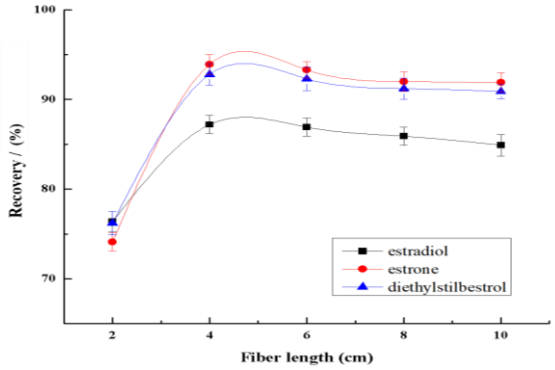


Fig. 2: Effect of fiber length on the recovery.

Effect of fiber length

In general, a short fiber length will provide a high enrichment factor for the concentration of trace analytes in HF-LPME procedure. However, too short fiber membranes cannot provide sufficient extractant to promote analytes transport to extraction solvent and decrease recoveries. Thus, membrane length was

optimized from 2 to 10 cm. The results (Fig. 2) indicated that 4 cm was sufficient, and no significant effect was found when the fiber length ranged from 6 to 10 cm. An HF length of 4 cm was maintained for further experiments.

Donor phase pH

The pH value of the donor phase plays an important role during the extraction and it can change the partition coefficient of analytes between the extraction solvent and sample solution. The effect of the pH on the recovery was studied in the range of 2.0–7.0. As shown in Fig. 3, the recoveries are the highest when the pH value is 4.0. The results can be explained by the principle that the lower pH value can inhibite the ionization of the estrogens, the target analytes will be in the neutral form, which facilitates the extraction from sample solution. However, when pH $<$ 4, the phenolic hydroxyl group of estrogens and H^+ may form oxonium ions which will increase the hydrophilicity of the estrogens, resulting in deceased recovery [30]. Based on the above results, pH 4.0 was employed in the remaining studies.

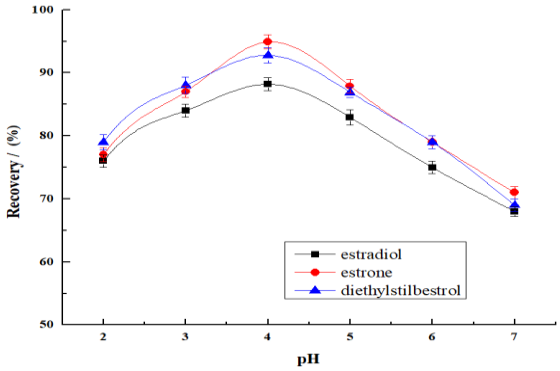


Fig. 3. Effect of donor phase pH on the recovery.

Effect of vortex-mix time

Vortex-mix time is a critical factor in SS-VA-HF-LPME. The extraction can be accelerated by vortex-mix of the sample solution. Vortex-mix facilitates analyte diffusion from donor phase into the acceptor phase and remarkably affects the recoveries of the established method. For the present study, the effect of the vortex-mix time was investigated in the range of 0.5 to 7 min (Fig. 4). The recoveries increased as the vortex-mix time increased from 0.5 to 4 min, beyond 4 min, the recoveries remained almost constant. Accordingly, 4 min was selected as the vortex-mix time.

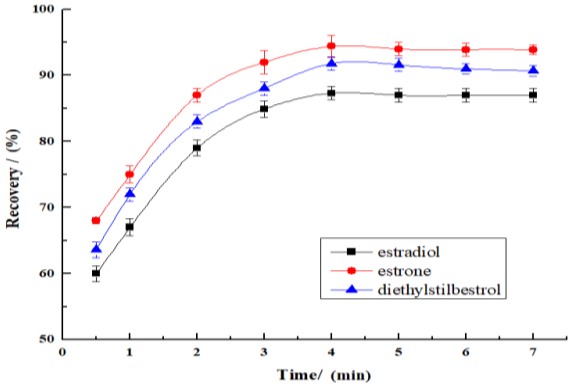


Fig. 4: Effect of vortex-mix time on the recovery.

Effect of salt addition

Generally, the salting-out effect can enhance the recovery because addition of salt can decrease the solubility of analytes in the aqueous solution. The effect of salt addition on recoveries was examined by adding sodium chloride to aqueous samples at the concentration levels of 0–15% (w/v). The results showed that the salt addition has a negative effect on the recoveries. This effect may be due to the increasing interactions between the analytes and salt in sample solution with increasing salt concentration which would tend to restrict movement of the analytes from the donor phase to the membrane solvent [31]. Thus, the salt was not added in the subsequent experiments.

Table-2: Effect of different percentage of n-butanol and [BMIM]BF_4 used for supramolecular solvent production on the recoveries (n = 3) and standard deviations of estrogens in milk.

n-octanol (%)	Recoveries ± standard deviations (%)											
	[BMIM]BF_4(%)											
	1.5%			3%			4.5%			6%		
	17β-E2	E1	DES	17β-E2	E1	DES	17β-E2	E1	DES	17β-E2	E1	DES
2%	62±3	66±1	65±2	70±3	78±2	77±2	67±3	69±1	65±2	60±2	63±1	62±3
6%	69±2	70±1	71±1	79±2	82±2	80±1	76±2	82±2	80±1	63±2	66±2	68±2
10%	74±3	79±2	76±2	85±3	93±1	89±2	82±2	89±1	87±1	69±1	73±2	70±2
14%	76±3	84±1	87±2	83±2	90±1	87±2	83±2	88±1	95±2	72±3	78±1	75±2
18%	81±2	89±2	82±2	79±2	85±2	83±1	80±3	85±1	82±2	78±4	86±2	84±1

Comparison with UA-HF-LPME and ST-HF-LPME

A comparison was made of VA-HF-LPME, UA-HF-LPME (ultrasound-assisted hollow fibre liquid-phase microextraction) and mostly reported ST-HF-LPME (string-assisted hollow fibre liquid-phase microextraction). The results are presented in Fig 5. It is evident that the proposed VA-HF-LPME method provides higher recoveries compared with UA-HF-LPME. This may be due to that some extractant overflowed from fiber membrane and emulsified the sample solution in powerful ultrasonic radiation, resulting in difficulty in mass transfer and decreased recovery. Moreover, the proposed method is much more efficient and provides high recovery in a much shorter time compared with traditional ST-HF-LPME method. This indicates the application of vortex-mixing as driving force facilitates mass transfer kinetics. The problem of prolonged extraction time of traditional HF-LPME procedure was solved by using vortex-mixing instead of stirring. Taking all of the above into account, vortex-mixing was proven to be a good approach in HF-LPME procedure.

Comparison of the proposed method with other reported HF-LPME methods

Table 3 provides a comparison between the proposed method and other reported methods for the extraction of estrogens from the viewpoint of extraction time, extraction solvent, linearity range, and LOD. SS-VA-HF-LPME had great advantage in extraction time compared with other methods, which indicated the efficient influence of vortex-mixing and supramolecular solvent on the extraction.

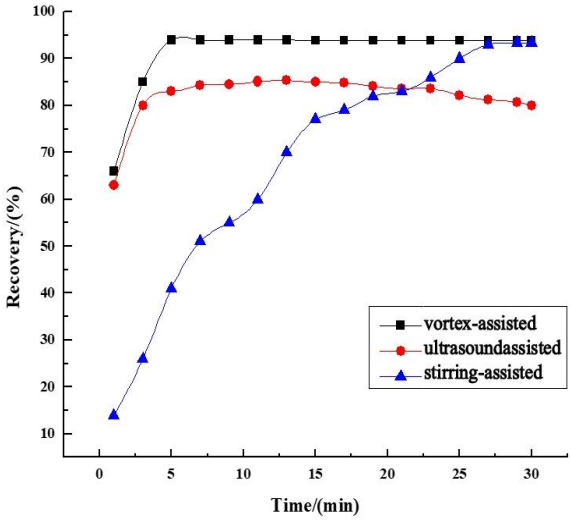


Fig. 5: Effect of time on the recovery of vortex-mixed, ultrasound, stirring assisted HF-LPME.

Table-3: Comparisons of the proposed method with other HF-LPME methods for the determination of estrogens

Method	extraction time	extraction solvent	LR[a] (ng mL^{-1})	LOD (ng mL^{-1})	Reference
HF-SEBLLME-GC/MS[b]	**> 20 min**	**ethyl acetate**	**0.2–20**	**0.02–0.06**	**[27]**
HF-LPME-HPLC[c]	**> 60min**	**1-octanol**	**20–300**	**0.18–5.97**	**[32]**
HF-LLLME-HPLC[d]	**> 50min**	**toluene:n-octanol /NaOH-β-CD**	**0.6–500**	**0.2–0.66**	**[33]**
UASEME- HPLC[e]	**> 10min**	**CCl_4/Triton X-100**	**10.0–1000**	**0.1–0.12**	**[34]**
DLLSME-HPLC[f]	**> 45min**	**n-hexane**	**0.5–100**	**0.08–0.25**	**[35]**
UVCPE- HPLC[g]	**> 60min**	**Tergitol TMN-6**	**5.0–1000**	**0.1–0.2**	**[36]**
DLLME-HPLC[h]	**> 10min**	**(CCl_4)**	**0.02–500**	**0.008–0.01**	**[37]**
SS-VA-HF-LPME-HPLC	**< 10min**	**supramolecular solvent**	**0.5–500**	**0.1–0.22**	**this work**

[a] Linear range

[b] HF-SEBLLME, hollow fiber-based stirring extraction bar liquid-liquid microextraction

[c] HF-LPME, hollow-fiber liquid-phase microextraction

[d] HF-LLLME, hollow fiber liquid-liquid-liquid microextraction

[e] UASEME, ultrasound-assisted surfactant-enhanced emulsification microextraction

[f] DLLSME, dynamic liquid–liquid–solid microextraction

[g] UVCPE, ultrasound-assisted cloud-point extraction

[h] DLLME, dispersive liquid liquid microextraction

Table-4: Analytical results for milk samples over 2 days after SS-VA-HF-LPME-HPLC/DAD method.

Analyte	sample	Spiked (ng mL^{-1})	Recovery (%) (%) Day1	RSD (%) (n=5)	Recovery (%) (%) Day2	RSD (%) (n=5)
17β-estradiol	Whole milk	10	87.29	4.36	85.68	3.37
		100	90.17	3.16	89.03	4.25
		500	92.16	1.98	91.92	3.09
	Skimmed milk	10	89.17	4.07	89.94	4.35
		100	92.03	2.59	90.25	2.48
		500	91.72	1.39	90.08	1.88
Estrone	Whole milk	10	92.92	4.01	91.26	3.04
		100	92.29	2.62	91.97	3.75
		500	89.24	1.24	91.05	1.69
	Skimmed milk	10	88.94	2.08	89.90	2.30
		100	93.75	3.19	89.88	2.75
		500	94.05	1.94	92.05	1.56
Diethylstilbestrol	Whole milk	10	90.05	3.36	91.06	2.74
		100	89.83	3.07	90.27	2.93
		500	90.08	2.19	88.28	2.67
	Skimmed milk	10	88.85	3.23	90.12	3.41
		100	91.72	4.28	90.37	3.49
		500	90.98	2.03	89.73	2.96

Table-5: Parameters of calibration plots.

Analyte	LR[a] (ng mL^{-1})	Regression equation	Correlation coefficient	LOD (ng mL^{-1})
17β-estradiol	0.5–500	y=903.40x + 51.04	0.9992	0.15
Estrone	0.5–500	y = 1003.64x + 43.96	0.9985	0.22
Diethylstilbestrol	0.5–500	y =899.37x + 87.33	0.9990	0.10

Method evaluation

To evaluate the application of the proposed SS-VA-HF-LPME method for the quantitative determination of estrogens, calibration plots over the concentration ranges were determined and summarized. For quantitative purposes, the linear dynamic range was assessed by plotting the peak area against the concentration of the respective compounds. A linear behaviour in the range of 0.5–500 ng mL^{-1} with correlation coefficients (r^2) values more than 0.998 was observed for all the estrogens. The limit of detection calculated from a signal/noise ratio (s/n=3) was 0.15 ng mL^{-1} for 17β-E2, 0.22 ng mL^{-1} for E1, and 0.10 ng mL^{-1} for DES, respectively. All the data were obtained under the optimized conditions (Table 4).

Analysis of real milk samples

To evaluate the effectiveness of the established method in larger range of milk samples, it was applied to the analysis of whole and skimmed spiked milk samples at three different levels over 2 days (Table 5). The recoveries are in the range of 85.68–94.05%, with RSD% (n = 5) of 1.24–4.36. Fig. 6 shows typical HPLC chromatograms of extracted and preconcentrated estrogens. Fig. 6a is the chromatogram of estrogens after extraction from milk spiked with SS-VA-HF-LPME, and Fig. 6b is the chromatogram of estrogens after supramolecular solvent-based vortex-mixed microextraction (SS-BVMME). The results show that the proposed method is effective for the determination of trace amounts of estrogens in the real milk sample. It has excellent sample clean-up ability compared with SS-BVMME.

Extraction mechanism

Hydrogen bond is very important in supramolecular chemistry, as well as in biochemical environments including protein folding [29]. Wan et al. reported the formation of hydrogen bonds between fluorine of [BMIM]BF4 and hydroxy of phenolic compounds during extraction [38].

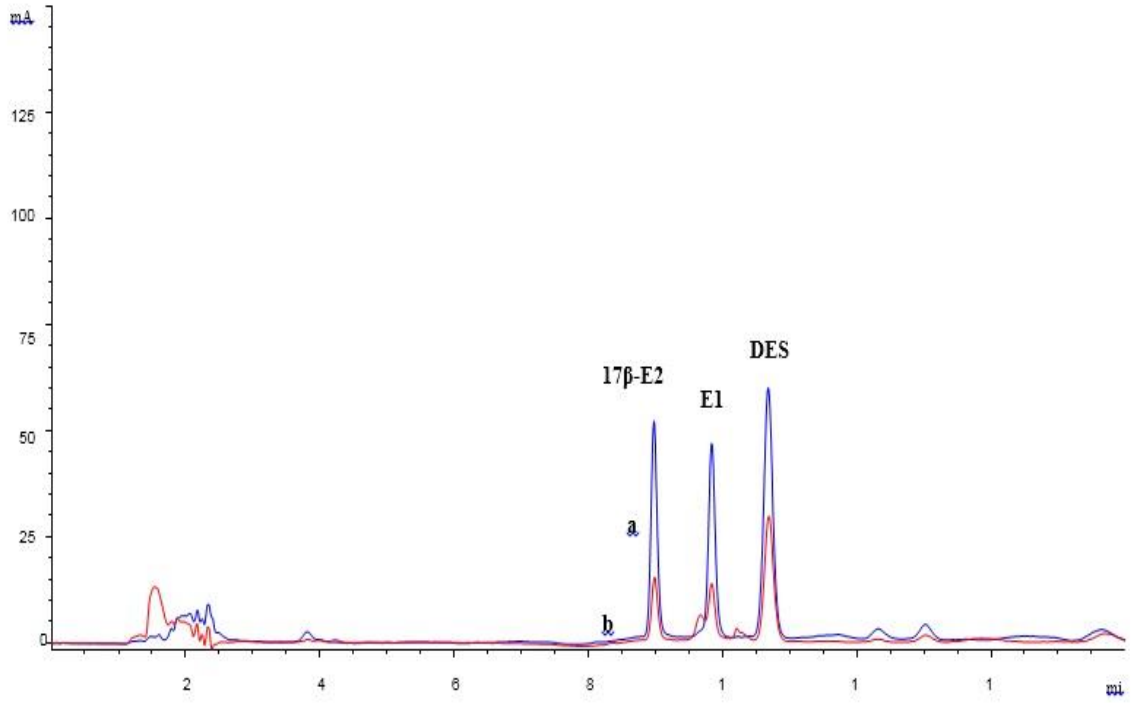
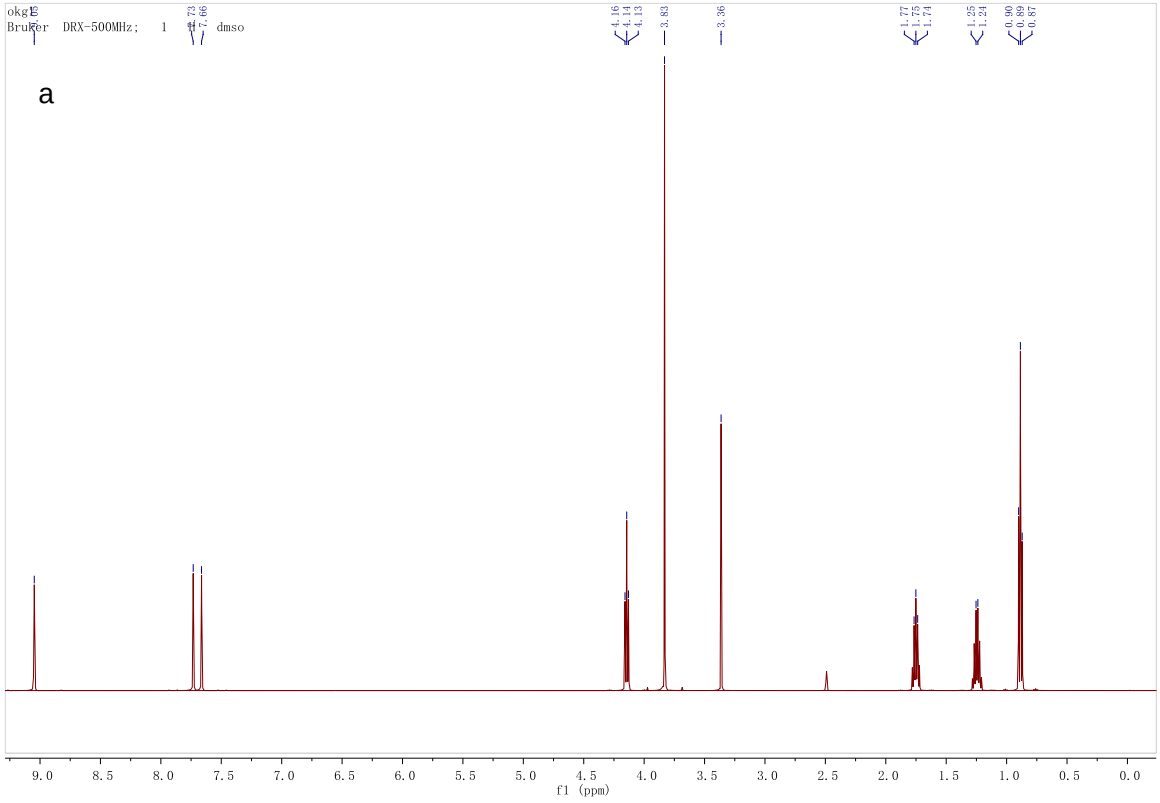


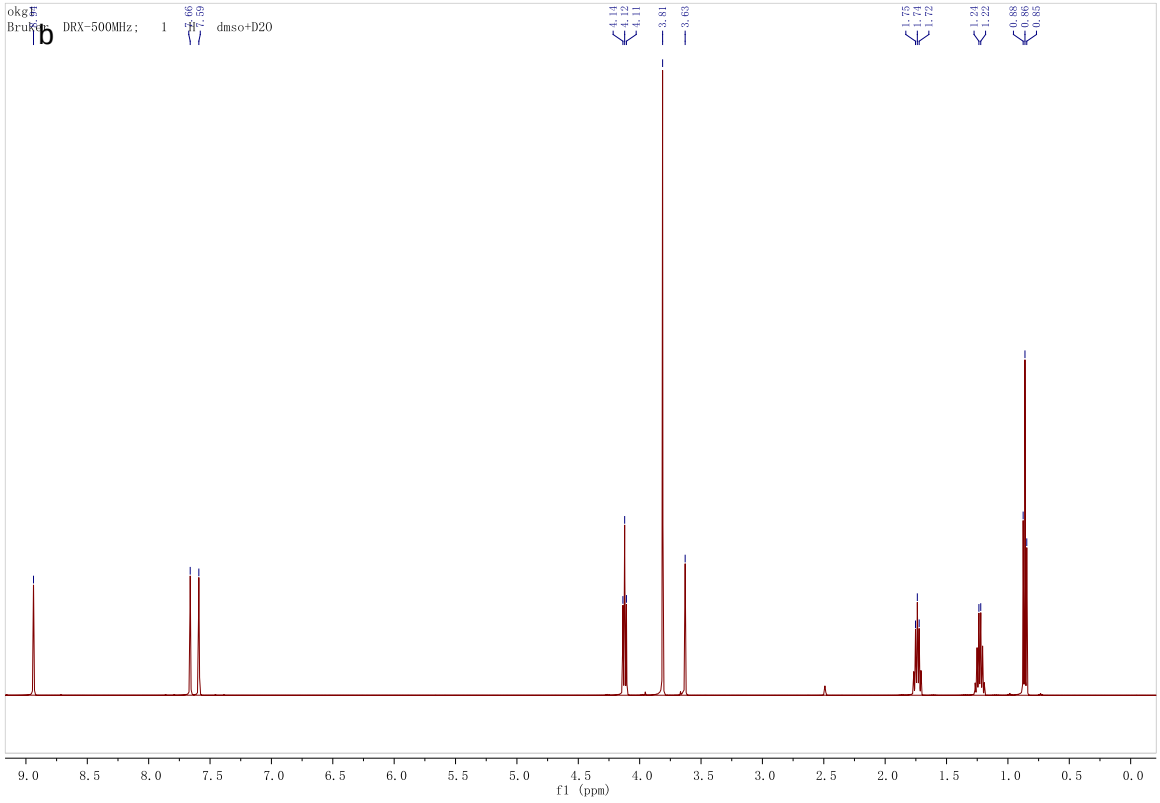
Fig. 6: HPLC–DAD chromatograms: (a) sample milk spiked with estrogens by SS-VA-HF-LPME (100 ng mL^{-1}) and (b) sample milk spiked with estrogens by SS-BVMME (100 ng mL^{-1}).

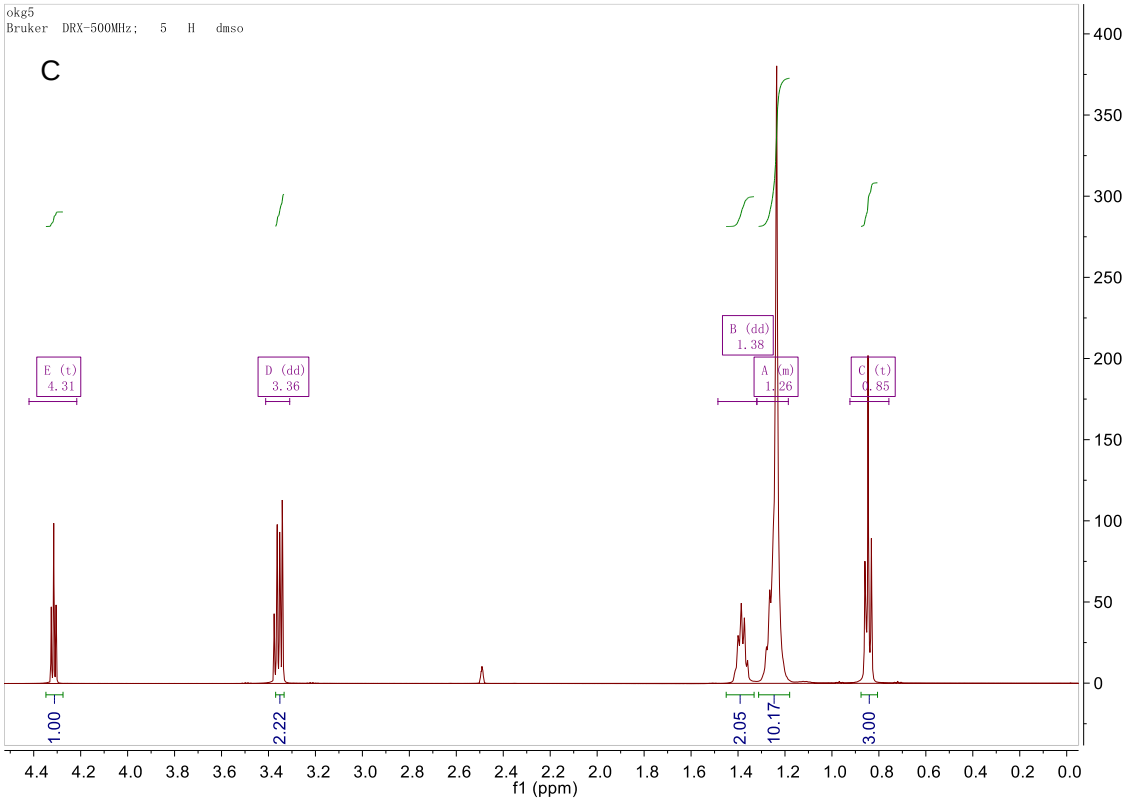
HPLC–DAD conditions—gradient program: 0–4.5 min, 35:65; 6.0–20 min, 55:45; acetonitrile:water, v/v; flow rate: 1.0 mL min^{-1}; temperature: 25 ℃; injecton volume: 20 μL.

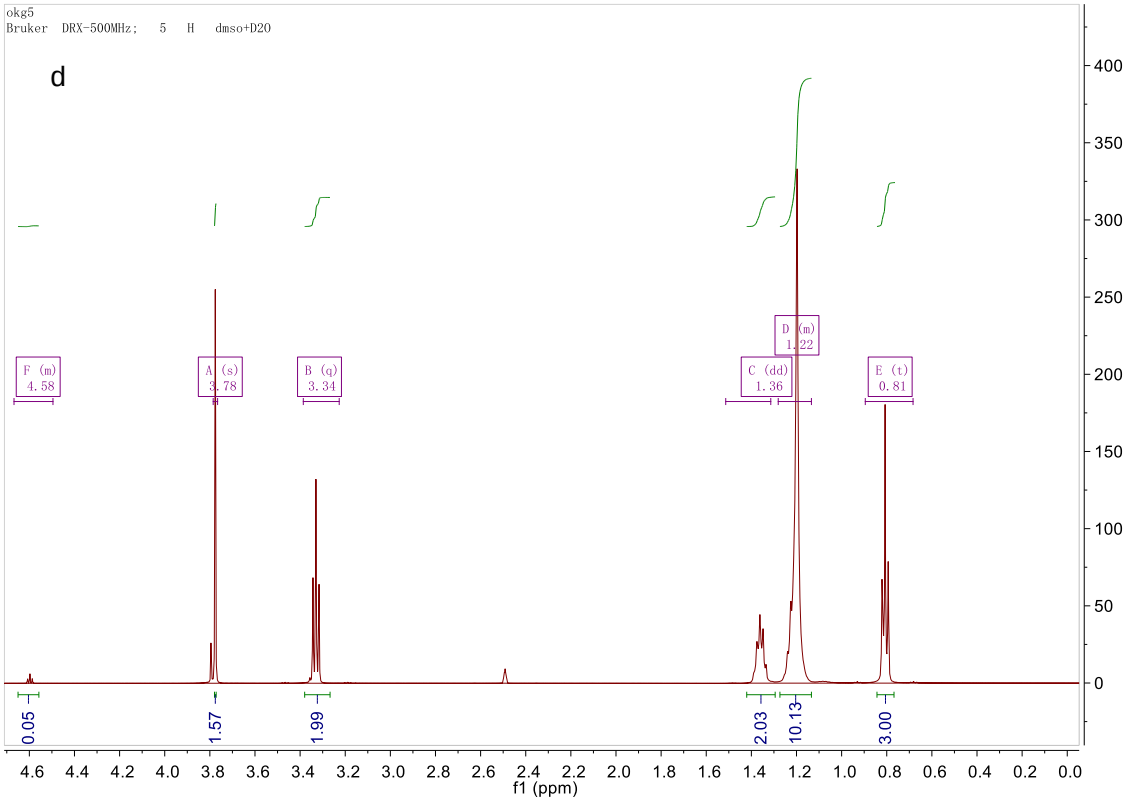
In this study, hydrogen bond was found to be the major driving force of supramolecular solvents self-assembly, contributing to extraction of estrogens from milk samples. ^{1}H and ^{2}H-MR spectroscopy was applied to detect possible intramolecular or intermolecular hydrogen bond in the extraction solution. In Fig. 7(c) and (d), chemical shift changes from 4.3 to 4.6 after D_2O exchange, indicating that the hydrogen of the hydroxyl group of n-octanol is active and likely to form hydrogen bond. No change was found in the chemical shift and integral of sample 1 in spite of D_2O exchange, which shows that there is no active hydrogen (see (Fig. 7(a) and (b)). Sample 3 is the supramolecular solvent, no chemical shift of H was found after D_2O exchange, which indicates that there is no active H replacement (see (Fig. 7 (e) and (f)). All Figs above show the possibility of formation of sTable hydrogen bonds between alcoholic hydroxyl of n-octanol and fluorine of ionic liquid $[BMIM]BF_4$ (sample 3). The chemical shift of 4.1 of sample 4 shows that it is likely to form unsTable hydrogen bonds before D_2O exchange, indicating interactions between hydroxyl groups of estrogens and fluorine of ionic liquid $[BMIM]BF_4$ (see Fig. 7 (g) and (h)).

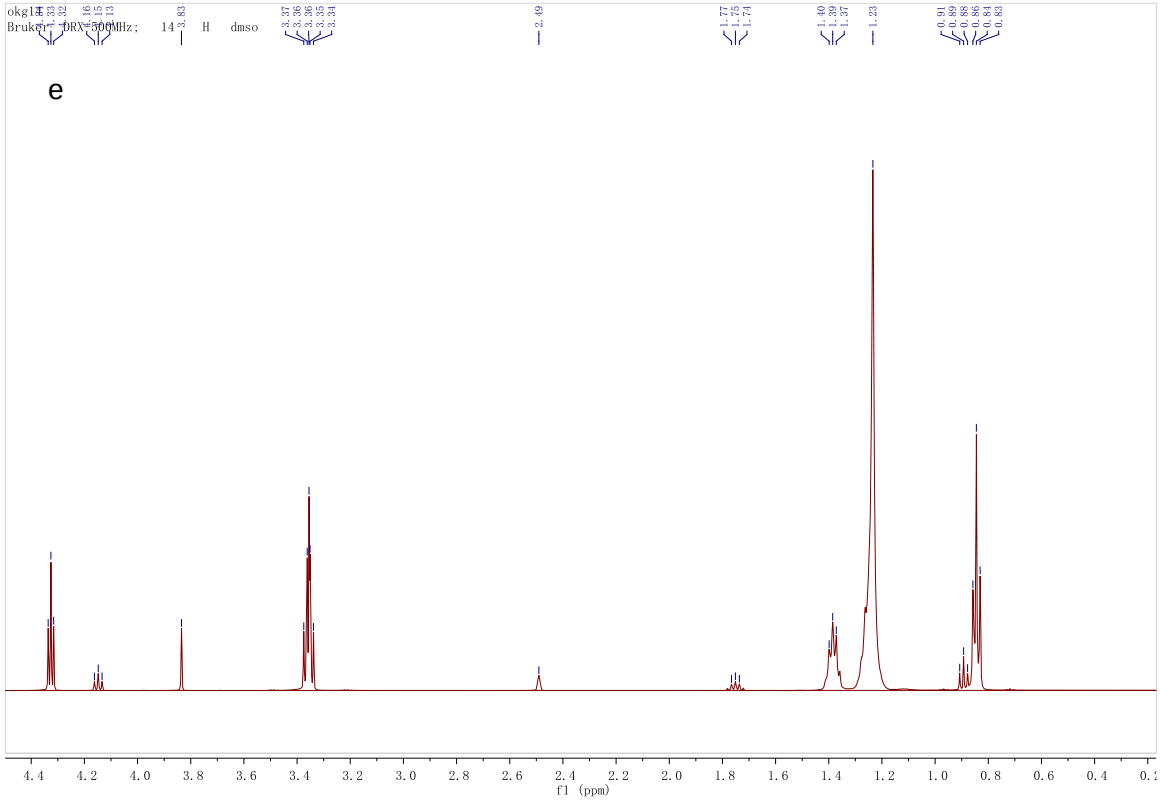
Zhao et al. [39] confirmed formation of hydrogen bonds between boron tetrafluoride anion and hydrogen of imidazole in $[BMIM]BF_4$ using infrared spectroscopy. So, infrared spectroscopy was applied to detect possible hydrogen bond in our study (Fig. 8). In Fig. 8(a) and (c), stretching vibration peak of hydroxyl group of n-octanol changed from 3310 to 3325 cm^{-1} with an increase in intensity. Simultaneously, stretching vibration peak of 2953 changed to 2960 cm^{-1} with an obvious increase in peak intensity (Fig. 8 (a), (b) and (c)). All of the above changes in wave number and intensity indicated the possibility of formation of intermolecular hydrogen bond between alcoholic hydroxyl of n-octanol and fluorine of boron tetrafluoride anion. Those characteristics are identical with the results of ^{1}H and ^{2}H-MR spectroscopy. Both the hydrogen bonds and interactions contribute to the extraction of estrogens.


a
Bruker DRX-500MHz; 1 dmso
4.16
4.14
4.13
3.83
3.36
1.77
1.75
1.74
1.25
1.24
0.90
0.89
0.87
f1 (ppm)


b
DRX-500MHz; 1 dmso+D2O
7.66
7.59
4.14
4.12
4.11
3.81
3.63
1.75
1.74
1.72
1.24
1.22
0.88
0.86
0.85
f1 (ppm)


okg5
Bruker DRX-500MHz; 5 H dmso
C
E (t) 4.31
D (dd) 3.36
B (dd) 1.38
A (m) 1.26
C (t) 0.85
1.00
2.22
2.05
10.17
3.00
f1 (ppm)


okg5
Bruker DRX-500MHz; 5 H dmso+D2O
d
F (m) 4.58
A (s) 3.78
B (q) 3.34
D (m) 1.22
C (dd) 1.36
E (t) 0.81
0.05
1.57
1.99
2.03
10.13
3.00
f1 (ppm)


e
f1 (ppm)
f
f1 (ppm)

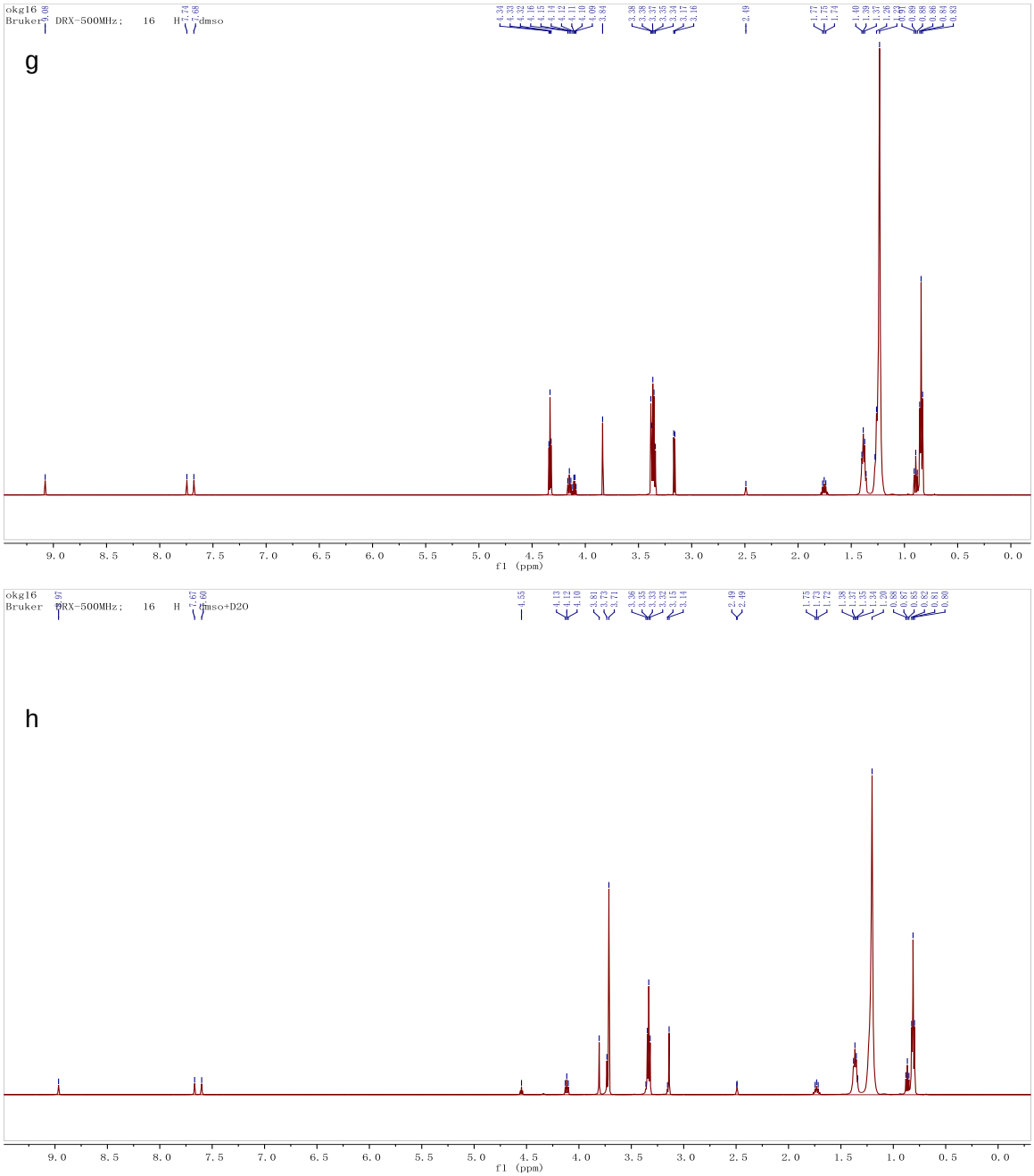
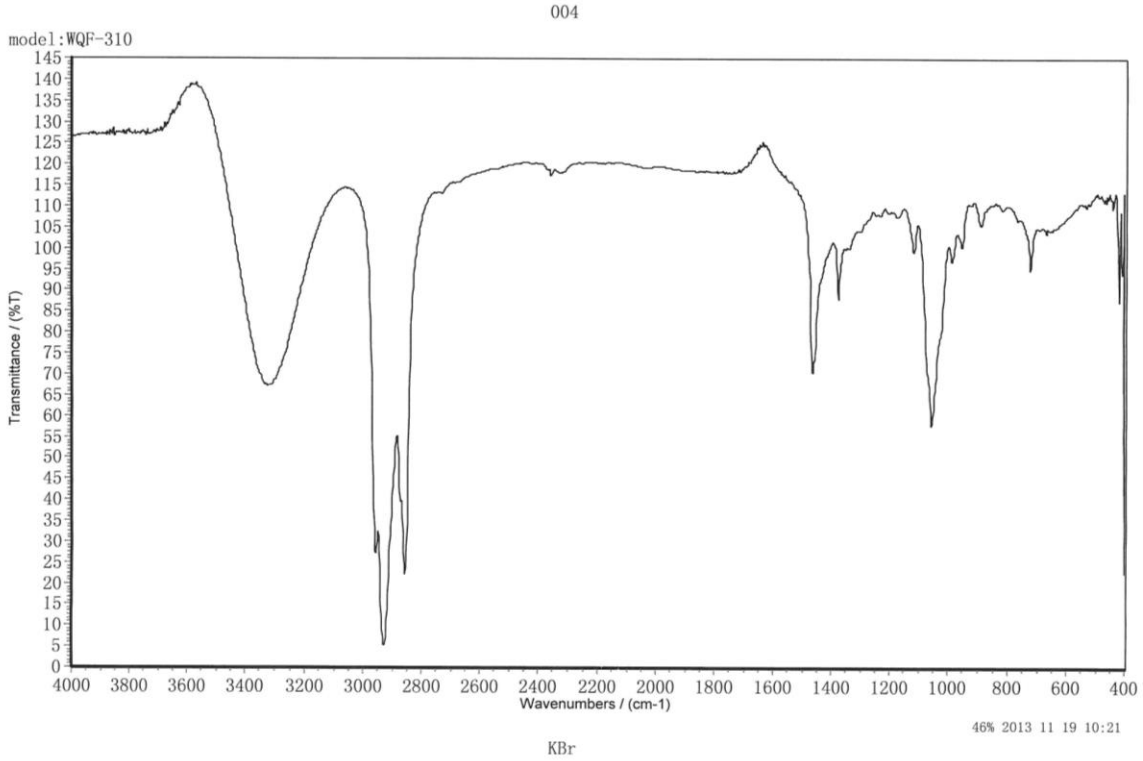


Fig**. 7:** (a) Sample 1: ionic liquids (1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4), solvent: DMSO. (b) Sample 1: ionic liquids (1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4), solvent: DMSO + D_2O. (c) Sample 2: n-octanol, solvent: DMSO. (d) Sample 2: n-octanol, solvent: DMSO + D_2O (e) Sample 3: aggregates of n-octanol and [BMIM]BF_4, solvent: DMSO. (f) Sample 3: aggregates of n-octanol and [BMIM]BF_4, solvent: DMSO + D_2O. (g) Sample 4: aggregates of n-octanol, [BMIM]BF_4 and mixed standard (17β-estradiol (17β-E2), estrone (E1), and diethylstilbestrol (DES)) after extraction, solvent: DMSO. (h) Sample 4: aggregates of n-octanol, [BMIM]BF_4 and mixed standard (17β-estradiol (17β-E2), estrone (E1), and diethylstilbestrol (DES)) after extraction, solvent: DMSO + D_2O.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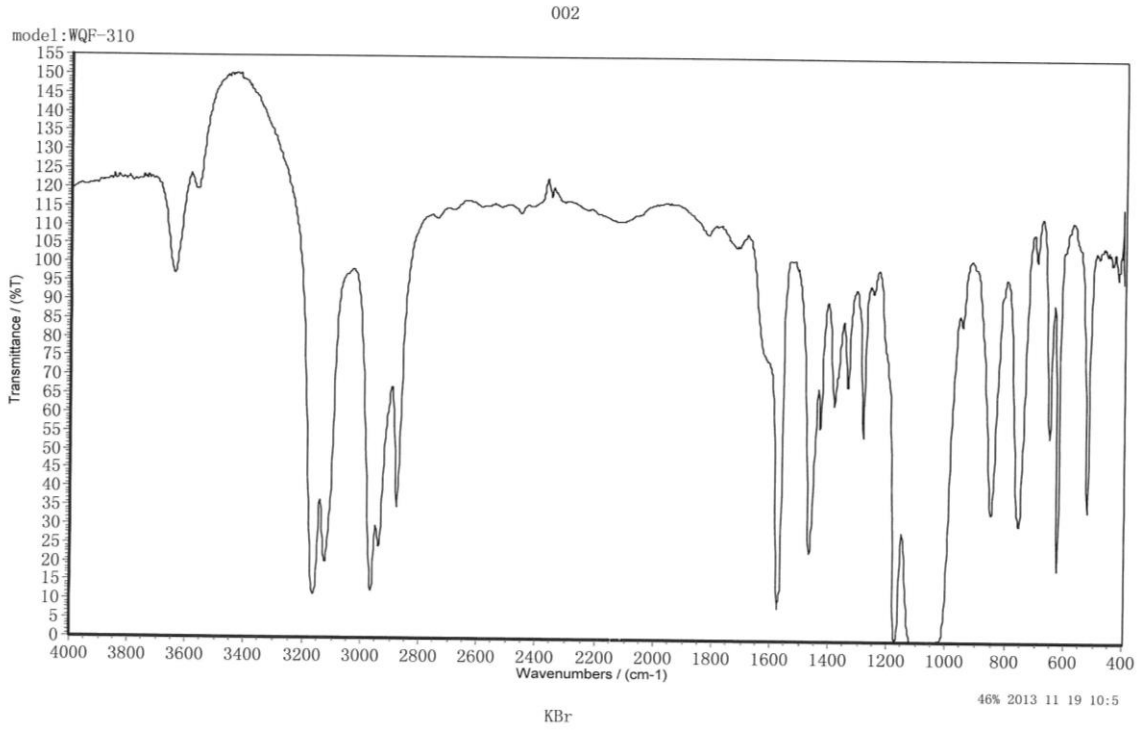
Transmittance / (%T)
Wavenumbers / (cm-1)
46% 2013 11 19 10:21
KBr

(a)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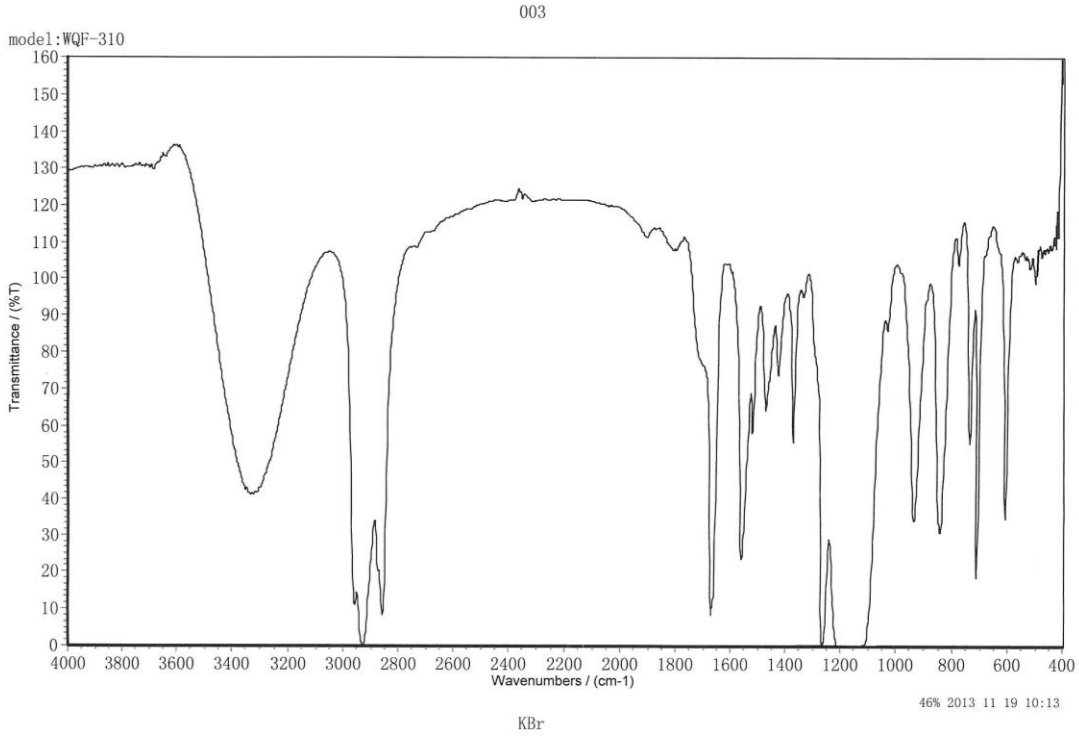
model:WQF-310
Transmittance / (%T)
Wavenumbers / (cm-1)
46% 2013 11 19 10:5
KBr

(b)



(c)

Fig. 8: (a) n-octanol (b) ionic liquids (1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4) (c) aggregates of n-octanol and [BMIM]BF_4.

Conclusions

In this report, supramolecular solvent-based vortex-assisted hollow fibre liquid-phase microextraction has been successfully used to quantify trace amounts of estrogens, 17β-E2, E1, and DES, in milk samples. The novel supramolecular solvent was composed of ionic liquids 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4), n-octanol and water. Hydrogen bond was found to be the major driving force of supramolecular solvents self-assembly, contributing to extraction of estrogens from milk samples. The regulation of supramolecular self-assembly process for the extraction is successful, which was confirmed by ^{1}H, ^{2}H-MR and infrared spectroscopy. The problem of prolonged extraction time of traditional HF-LPME procedure was solved by using vortex-mixing instead of stirring. Coupled with high performance liquid chromatography diode-array detector, the method has been proven to be rapid, simple, and reliable for estrogens assay in milk samples.

Acknowledgments

This study was funded by Science and Technology Project of Hebei Education Department (ZC2023180), Hebei Province, China.

References

1. Z. Yan, Y.H. Li, J. Hua, H.N. Tang, D.Q. Zou, M.S. Liu and Y.L. Yang, Determination of estrogens in human urine by high-performance liquid chromatography/diode array detection with ultrasound-assisted cloud-point extraction, *Anal. Biochem.*, **421**, 378(2012).
2. H. Noppe, B.L. Bizec, K. Verheyden and H.F.D. Brabander, Novel analytical methods for the determination of steroid hormones in edible matrices, *Anal. Chim. Acta.*, **611**, 1(2008).
3. E.R. Prossnitz and M. Barton. Estrogen biology: new insights into GPER function and clinical opportunities. *Mol Cell Endocrinol.*, **389**, 71(2014).
4. P. Monteleone, M. Luisi, B. Colurcio, E. Casarosa, P. Monteleone and R. Ioime, Plasma levels of neuroactive steroids are increased in untreated women with anorexia nervosa or bulimia nervosa, *Psychosom Med.*, **63**, 62 (2001).

5. I. Jochmanov, Z. Lazurov, M. Rudnay, I. Bačová, M. Mareková and I Lazúrová. Environmental estrogen bisphenol A and autoimmunity. *Lupus.*, 24, 392 **(2015).**
6. Y.L. Zhao, M.B. Jessica; B.S. Michael and X.F. Li, Liquid chromatography tandem mass spectrometry determination of free and conjugated estrogens in breast cancer patients before and after exemestane treatment, *Anal. Chim. Acta.*, **806**, 172 (2014).
7. S. Du, H.G. Gao and R.Y. Wang. Research progress of estrogen detection methods in animal-derived foods. *Chin J Hyg Inspec*t., **26**1, 822(2016).
8. Y.Y. Wen, J.H. Li and J.S. Liu, Dual cloud point extraction coupled with hydrodynamic-electrokinetic two-step injection followed by micellar electrokinetic chromatography for simultaneous determination of trace phenolic estrogens in water samples, *Anal. Bioanalytical Chem.*, **405**, 5843 (2013).
9. M. L. Érika Sousa, A. S.Reyla Dias, E. R. Sousa, N. M. Brito,A.S. Freitas, G. S. Silva, L. K. Silva, D. L. D Lima, V. I. Esteves and G. S. Silva, Determination of three estrogens in environmental water samples using dispersive liquid-liquid microextraction by high-performance liquid chromatography and fluorescence detector, *Water, Air, & Soil Pollution.*, **231**, 480 (2020).
10. F. Guo, Q. Liu and G.B. Qu, Simultaneous determination of five estrogens and four androgens in water samples by online solid-phase extraction coupled with high-performance liquid chromatography-tandem mass spectrometry, *J. Chromatogr A.*, **1281,** 9 (2013).
11. J.W. Tang, J.X. Wang and L.J. Yuan, Trace analysis of estrogens in milk samples by molecularly imprinted solid phase extraction with genistein as a dummy template molecule and high performance liquid chromatography-tandem mass spectrometry, *Steroid*s., **145**, 31(2019).
12. A. Eugenio, A. Eleonora, B. Cristina, V. Sara, G. Giusy, G. Enrico,S. Alberto and V. Marco， Optimization and validation of a GC-MS quantitative method for the determination of an extended estrogenic profile in human urine. Variability intervals in a population of healthy women, *Biomedical chromatography.*, **35**, 4967 (2020).
13. M. Hansen, J.W. Naja and F.K. Nielsen, Magnetic polyimide nanosheet microspheres for trace analysis of estrogens in aqueous samples by magnetic solid-phase extraction-gas chromatography–mass spectrometry, *J. Chromatogr A.*, **1675**, 463184 (2022).
14. X. Xiong, D. Li and Z.F. Du, Magnetic solid-phase extraction modified quick, easy, cheap, effective, rugged and safe method combined with pre-column derivatization and ultra-high performance liquid chromatography-tandem mass spectrometry for determination of estrogens and estrogen mimics in pork and chicken samples. *J Chromatogr A.*, **1622**, 461137 (2020).
15. G.C.K. Gunter, D.A. Caterina, M.A. Sue, A.R. Shirley, A.M. Angela and A.B. Sheila, Phytoestrogen content of beverages, nuts, seeds, and oils. *J. Agric. Food Chem.*, **56**, 7311 (2008).
16. A. Lourdes, S.M. Dolores and R.Soledad, Ribbon-shaped supramolecular solvents: Synthesis, characterization and potential for making greener the microextraction of water organic pollutants, *Talanta.*, 255,124227 (2022).
17. H. Watanabe and H. Tanaka, A non-ionic surfactant as a new solvent for liquid-liquid extraction of zinc(II) with 1-(2-pyridylazo)-2-naphthol, *Talanta.*, **25** , 585589 (1978).
18. A. Ballesteros-Gómez and S. Rubio, Environment-Responsive alkanol-based supramolecular solvents: characterization and potential as restricted access property and mixed-mode extractants, *Anal. Chem.*, **84**, 342 (2012).
19. S. Pedersen-Bjergaard and K.E. Rasmussen, Liquid-phase microextraction with porous hollow fibers, a miniaturized and highly flexible format for liquid-liquid extraction. *J. Chromatogr. A.*, **1184**, 132 (2008).
20. H. Ebrahimzadeh, N. Shekari, Z. Saharkhiz and A.A. Asgharinezhad, Simultaneous determination of chloropheniramine maleate and dextromethorphan hydrobromide in plasma sample by hollow fiber liquid phase microextraction and high performance liquid chromatography with the aid of chemometrics. *Talanta.*, **94**, 77 (2012).
21. Y.L. Wu and B. Hu, Deep eutectic solvent as acceptor phase in three-phase hollow fiber liquid-phase microextraction for determination of pyrethroid insecticides from environmental water samples prior to HPLC, *Biomed chromatogr .* **36**, *(*2022).
22. H. Hansson and U. Nilsson, Assessment of a dynamic hollow-fibre liquid phase microextraction system for human blood plasma samples, *Talanta.*, **77**, 1309 (2009).
23. M. Hadjmohammadi, H. Karimiyan and V. Sharifi, Hollow fibre-based liquid phase

microextraction combined with high-performance liquid chromatography for the analysis of flavonoids in Echinophora platyloba DC. and Mentha piperita, *Food Chem.,* **141**, 731 (2013).

24. A. Rahimi, S. Nojavan and M. Maghsoudi, Analysis of basic drugs in biological samples using dynamic single-interface hollow fiber liquid-phase microextraction combined with fast electromembrane extraction, *Microchem J.,* **157**, 5031 (2020).
25. Du, Ji. Yu and M. Mei, Simultaneous trace determination of acidic non-steroidal anti-inflammatory drugs in purified water, tap water, juice, soda and energy drink by hollow fiber-based liquid-phase microextraction and ultra-high pressure liquid chromatography coupled to tandem mass spectrometry, *Talanta.,* **109**, 177 (2013).
26. E. Ali, Y. Yadollah and S. Shahab, Hollow fiber-based liquid phase microextraction combined with high-performance liquid chromatography for extraction and determination of some antidepressant drugs in biological fluids, *Anal. Chim. Acta.,* **604**, 127 (2007).
27. X. Xu, F.H. Liang, J.Y. Shi, X. Zhao, Z. Liu, L.J. Wu, Ying. Song, H.Q. Zhang and Z.M. Wang, Determination of hormones in milk by hollow fiber-based stirring extraction bar liquid–liquid microextraction gas chromatography mass spectrometry, *Anal. Chim. Acta.*, **790**, 39 (2013).
28. R. Fatemeh, Y. Yadollah, M. Morteza and D. Bahram, Supramolecular solvent-based hollow fiber liquid phase microextraction of benzodiazepines, *Anal. Chim. Acta.,* **804**, 135 (2013).
29. H. Qin, X.Y. Qiu, Jiao. Zhao, M.S. Liu and Y.L. Yang, Supramolecular solvent-based vortex-mixed microextraction:determination of glucocorticoids in water samples, *J. Chromatogr. A.,* **1311**, 11 (2013).
30. X.Q. Liu and H.R. Dong, total Magnolol in Magnolia solvent flotation separation and enrichment research, *Chi. J. Chin. Mate. Medi.*, **33**, 1217 (2008).
31. E. Tahmasebi, Y. Yamini and A. Saleh, Extraction of trace amounts of pioglitazone as an anti-diabetic drug with hollow fiber liquid phase microextraction and determination by high-performance liquid chromatography-ultraviolet detection in biological fluids, *J. Chromatogr. A.,* **877**, 1923 (2009).
32. R.B. Socas, R.M. Asensio, B.J. Hernandez and D.M.A. Rodriguez, Hollow-fiber liquid-phase microextraction for the determination of natural and synthetic estrogens in milk samples, *J. Chromatogr. A.,* **1313**, 175 (2013).
33. B.B. Chen, Y.L. Huang, Man. He and Bin. Hu, Hollow fiber liquid-liquid-liquid microextraction combined with high performance liquid chromatography-ultraviolet detection for the determination of various environmental estrogens in environmental and biological samples, *J. Chromatogr. A.,* **1305**, 17 (2013) .
34. Y. Zou, Y.H. Li, H. Jin, D.Q. Zou, M.S. Liu and Y.L. Yang, Ultrasound-Assisted Surfactant-Enhanced Emulsification Microextraction Combined with HPLC for the Determination of Estrogens in Water, *J. Braz. Chem. Soc.* **23**, 694 (2012).
35. Q.S. Zhong, Y.F. Hu, Y.L. Hu and G.K. Li, Dynamic liquid–liquid–solid microextraction based on molecularly imprinted polymer filaments on-line coupling to high performance liquid chromatography for direct analysis of estrogens in complex samples, *J. Chromatogr. A.,* **1241**, 13 (2012).
36. Y. Zou, Y.H Li, H. Jin, H.N. Tang, D.Q. Zou, M.S. Liu and Y.L. Yang, Determination of estrogens in human urine by high-performance liquid chromatography/diode array detection with ultrasound-assisted cloud-point extraction, *Anal. Biochem.*, **421**, 378 (2012).
37. M.R. Hadjmohammadi, Ghoreishi and S. Saboora, Determination of estrogens in water samples using dispersive liquid liquid microextraction and high performance liquid chromatography, *Acta Chim. Slov.,* **58**, 765 (2011).
38. H. Wan, D.Y. Huang, Y. Cai and G.F. Guan, Study on extraction of phenolic compounds using [omim] BF_4 ionic liquid, *J. Chem. Eng. Chin. Univ.*, **22**, 1 (2008).
39. J.Y. Zhao and A.F. Ding, Quantum chemical study of group tetrafluoroborate ionic liquids, *J. Nanjing. Xiao. Zhua. Univ.* **6**, 58 (2009).