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Determination of conjugated estrogens in human urine using carrier-mediated hollow-fiber liquid phase microextraction and LC-MS/MS

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ABSTRACT

The extraction conditions of the conjugated estrogens, including estrone-3-glucuronide (E1-3G), 17β-estradiol-3-glucuronide (E2-3G), and 17α-ethynylestradiol-3-glucuronide (EE2-3G), were optimized using carrier-mediated hollow-fiber liquid phase microextraction (HF-LPME) and then applied in the analysis of human urine samples. The optimal extraction conditions were as follows: organic phase composed of 20% (w/w) tri-octylamine (TOA) in 1-octanol; 2 M NaCl as the acceptor phase; 800 rpm of agitation; and 50 min of extraction time. The extraction efficiency of carrier-mediated LPME was 11 times higher than that of the three-phase LPME. Estrogens and their conjugates in urine samples from pregnant and non-pregnant women were extracted and analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS). The results showed that carrier-mediated HF-LPME had good recoveries and linearity in the calibration curve. Estrogens in pregnant and non-pregnant women were mainly excreted in a conjugated form (94.85 ± 4%), whereas free estrogen was detected at a ratio of 5.15%. Both free and conjugated form of E1 were the most dominant species in all forms, which accounted for 72.7% of all the measured estrogens. Consequently, the HF-LPME in carrier-mediated mode was effectively applied to extract the estrogen conjugates in only 10 ml of human urine samples, which is the most significant advantage.

Keywords: Estrogens; Conjugated estrogens; Carrier-mediated HF-LPME; Human urine

1. Introduction

A concern that endocrine disruptors (EDs) are environmental pollutants, has been increasing since the 1980s [1]. This class of EDs includes a broad range of chemicals, including natural estrogens and androgens, phytoestrogens, synthetic estrogens and androgens, and various industrial chemicals [2]. Among these, natural estrogens, such as 17β-estradiol (E2), estrone (E1), and the synthetic estrogen 17α-ethinylestradiol (EE2) have been identified as the most potent estrogenic compounds [3].

Humans are known to be a major source of natural estrogens in the environment [4]. However, estrogens

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excreted from humans mainly exist in conjugated form, especially glucuronide [5]. Conjugation to sulfate or glucuronide is a major metabolization pathway (phase II) in the liver for the excretion of natural estrogens. Because sulfate and glucuronide belong to the hydrophilic functional group, the conjugated estrogens (log K_{ow} = 1.14 − 1.5) are easily excreted through urine [6]. Specifically, the conjugation of estrogens occurs at C_3 or C_{17} position, but only C_3 position-conjugated estrogens are biologically inactive, because the estrogen receptor reacts only with the phenolic group at the C_3 position. For this reason, the most conjugated estrogens have not been regarded as EDs. However, the conjugated moiety can be easily cleaved in the environment when excreted, resulting in the reactivation of estrogenicity [7]. Reactivation caused by deconjugation depends on the acid–base properties of the environmental matrix and bacterial processes. Moreover, several studies have demonstrated that deconjugation occurred during the STP processes [8–11]. In particular, estrogen glucuronates were more susceptible to deconjugation than estrogen sulfates in domestic wastewater [9,11]. D'ascenzo et al. [9] found that large quantities of β-glucronidase released by fecal bacteria (*Escherichia coli*) might be a major contributor to the deconjugation of estrogen glucuronates, whereas arylsulfatase activity was insufficient to deconjugate estrogen sulfates [9]. Therefore, conjugated estrogens should be considered as potential EDs, and thus need to be monitored for the quantification of whole estrogens in the samples.

Recently, hollow fiber-liquid phase microextraction (HF-LPME), an alternative sample clean-up technique used to determine various environmental pollutants [12] has been successfully applied in biological samples [13–16]. In the HF-LPME system, analytes are extracted from aqueous samples through a thin layer of organic solvent immobilized within the porous membrane and into the acceptor solution inside the lumen of HF. Because HF-LPME samples require only a small volume, they are suitable for biological fluids, such as blood, serum, and urine. Moreover, HF-LPME is an effective sample purification method that provides a high degree of selectivity by eliminating the carry-over from the matrix. Compared to the conventional LLE and SPE for the sample clean-up, LPME showed fewest interferences in urine samples [14].

LPME has been commonly applied to hydrophobic analytes, which are easily extracted from an aqueous sample and placed in an organic solvent. It was not suitable for hydrophilic analytes such as conjugated estrogens. However, Ho et al. [17] demonstrated the possibility of carrier-mediated transport of hydrophilic drugs from biological fluid in order to enhance the extraction efficiency. Carrier-mediated HF-LPME is an effective extraction technique and has been discussed in several studies [17–20]. In the present study, HF-LPME, which has many strong points for use in the extraction of analytes from biological samples, was developed to extract the conjugated estrogens from human urine. In our previous study, free-form estrogens (E1, E2, and EE2) were effectively extracted by three-phase LPME [21]. However, estrogens are mainly discharged through urine in the form of glucuronide conjugates, and thus the quantified estrogens in a real biological sample can be underestimated by the three-phase LPME method. In our knowledge, little previous research has applied HF-LPME mediated by a carrier to conjugated estrogens. Hence, this study aimed to optimize the carrier-mediated HF-LPME with liquid chromatography tandem mass spectrometry (LC-MS/MS), which was applied to determine estrogen conjugates (E1-3G, E2-3G, and EE2-3G). The optimization parameters, including carrier type, carrier concentration, counter-ion concentration, extraction time, and agitation rate were evaluated. In addition, the optimized extraction conditions of carrier-mediated HF-LPME were then applied to human urine samples.

2. Materials and methods

2.1. Chemicals and reagents

Standard estrogens, including estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), estrone-3-glucuronide (E1-3G), 17β-estradiol-3-glucuronide (E2-3G), and 17α-ethynylestradiol-3-glucuronide (EE2-3G), were purchased from Sigma-Aldrich (St. Louis, MO, USA). As an internal standard, stable isotopes of estrone-16, 16-d_2 (E1-d_2) were obtained from C/D/N Isotopes Inc. (Pointe Claire, Quebec, Canada). 1-octanol and sodium carbonate (Na_2CO_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and tri-octylamine (TOA) and Aliquot-336 (N-methyl-N,N-dioctyloctan-1-ammonium chloride), which were used as carriers, were obtained from Sigma-Aldrich. HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Biological sample collection

Pregnancy urine samples were collected from 10 healthy pregnant women (aged 23–38) in the fourth to ninth months of pregnancy. Non-pregnancy urine samples were collected from seven healthy women (aged 24–29), except during menstruation. All urine samples were collected in the early morning and stored at −20°C until the analysis. The stored samples were analyzed within two weeks.

2.3. Carrier-mediated hollow fiber-liquid phase microextraction (HF-LPME) for conjugated estrogens

The technical setup for the LPME was based on a U-shaped fiber, using two microsyringes (Fig. 1). A 10 μl microsyringe, which was used to inject an acceptor phase, was purchased from Agilent Technologies (Palo Alto, CA, USA). This syringe has a cone-shaped needle tip that fits the hollow fiber. Accurel Q3/2 polypropylene hollow-fiber (600 μm inner diameter, 200 μm wall thickness, 0.2 μm wall pore size) was purchased from Membrana (Wuppertal, Germany). The hollow fiber was cut to a length of 4.5 cm to suit the capacity of the acceptor phase (10 μl). A 10 μl microsyringe was prefilled with 10 μl of 2 M NaCl solution as the acceptor phase. The needle tip of the syringe was inserted into the hollow fiber, which was immersed in the organic phase for 10 s for impregnation. The organic phase was 1-octanol containing 20% (v/v) trioctylamine (TOA), which is used as a carrier. A syringe with an impregnated fiber was placed into a sample vial containing a 10 ml aliquot of the sample solution. The 10 μl acceptor phase was then introduced into the fiber, and the extraction was carried out at room temperature. The following procedure was identical to HF-LPME for free estrogen. After extraction, 10 μl of the acceptor phase was drawn into the syringe, and the hollow fiber was then removed. The acceptor phase was moved into the insertion vial, followed by the injection of 10 μl of internal standard (E1-d_2, 50 ng ml^{-1}) and adjusted to 100 μl by adding methanol. A 20 μl aliquot of the total volume was introduced for quantification.

An optimization experiment for carrier-mediated LPME was performed according to the following extraction parameters: (1) carrier type; (2) carrier concentration; (3) NaCl concentration in the acceptor phase; (4) extraction time; (5) agitation. For the carrier type, the intensity of two carriers including Aliquat-336 and TOA was compared. Next, 0, 5, 10, 20, and 30% of the carrier content were tested. The effect of counter-ion concentration in the acceptor phase was tested at 0, 0.5, 1, 2, and 3 M. Finally, the extraction time and degree of agitation were conducted in ranges of 10–50 min and 0–800 rpm, respectively. The extraction of free estrogens was conducted according to the procedure used in our previous study [21].

2.4. Quantification of target compounds

Quantification of the target compounds was conducted using Alliance 2695 (separation module) liquid chromatography and Quattro micro API Mass-mass spectroscopy (Waters, USA), using a Sunfire C18 column (3.5 μm particle size, 2.1 × 150 mm, Waters, USA). The system consists of a pair of LC pumps, a degasser, an autosampler, and a detector. The gradient program was carried out following the method in Tso and Aga [22]. The mobile phase solvents were 5 mM ammonium hydroxide/methanol (96/4, v/v) (A) and 5 mM ammonium hydroxide/methanol/

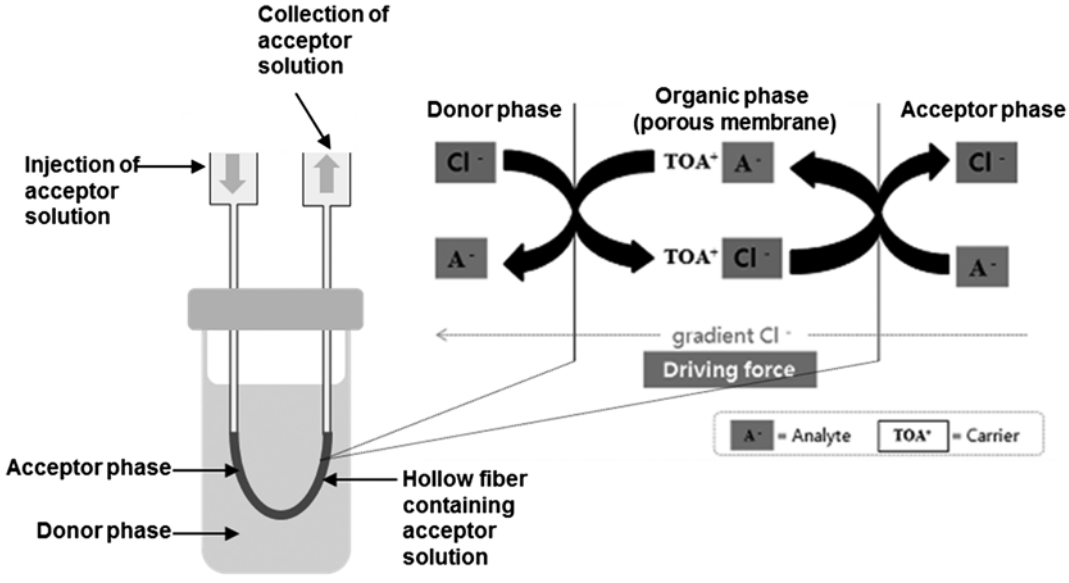


Fig. 1. Technical setup for carrier-mediated HF-LPME and its mechanism.

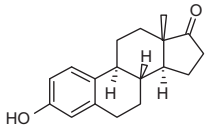
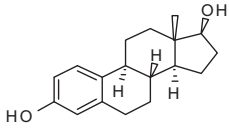
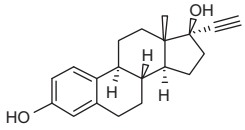
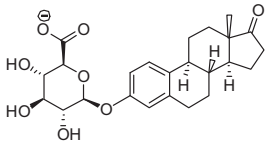
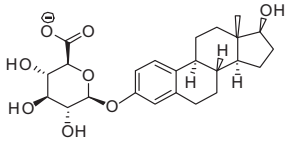
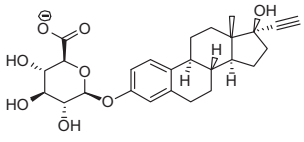
acetonitrile (10/10/80, v/v/v) (B) at a flow rate of 0.2 ml min^{-1}. The mobile phase gradient, starting with 0% B for 2 min, reached 100% B within 12 min and then held constant for 3 min, before returning to 0% B within 1 min. The total run time per sample was 18 min, including the post run of 7 min. The analytes were detected in negative electron spray ionization (ESI) mode using multiple reaction monitoring (MRM) and selected ion monitoring mode. The drying gas flow was 11 L min^{-1}, and the nebulizer gas pressure was 22 psi. The capillary voltage was 3.5 kV. The fragmentor and MRM parameters were adjusted for each analyte to achieve the optimum signal intensity (Table 3). An injection volume of 20 μL was used for analysis. The curve of quantity vs. the relative response of analytes to the internal standard exhibited good linearity of the target compounds (r^2 = 0.9931–0.9996).

3. Results and discussion

3.1. Selection of carrier-mediated HF-LPME mode for conjugated estrogens and the extraction mechanism

Estrogen glucuronide conjugates, including estrone-3-glucuronide (E1-3G), 17β-estradiol-3-glucuronide (E2-3G), and ethynylestradiol-3-glucuronide (EE2-3G), are more hydrophilic than free estrogens (Table 1). Therefore, a comparative study of several HF-LPME techniques was conducted to determine an appropriate method for the extraction of the conjugated estrogens: (a) three-phase LPME, (b) carrier-mediated LPME, and (c) simultaneous LPME, including pH adjustment and carrier mediation (Table 2). In the three-phase LPME for estrogen conjugates, the pH of the donor phase was adjusted to a lower value than the pK_a of E2-3G for the de-protonation of the target compound. Octanol was used as the organic phase because it has a higher

Table 1
Physicochemical properties of target compounds

Target compounds	Acronym	Structure	MW	pK_a	log K_{ow}
Estrone	E1		270.37	10.3–10.8[a]	3.1–3.4[a]
17β-estradiol	E2		272.38	10.5–10.7[a]	3.1–4.0[a]
17α-ethynylestradiol	EE2		296.40	10.4–10.7[b]	3.67–4.15[b]
Estrone-3-glucuronide	E1-3G		446.49	2.80[c]	1.144[c]
Estradiol-3-glucuronide	E2-3G		448.50	NA	NA
Ethynylestradiol-3-glucuronide	EE2-3G		471.51	NA	NA

[a]Ref. [27].
[b]Ref. [28].
[c]Ref. [29].

Table 2
Operating condition of HF-LPME mode for free and conjugated estrogens

Target chemical	LPME mode	Donor phase	Organic phase	Acceptor phase	
Free estrogens	Three-phase	pH 7	Toluene	pH 11 (0.5 M NaOH)	
Conjugated estrogens	Three-phase	pH 1	Octanol	pH 11 (0.5 M NaOH)	(a)
	Carrier-mediated	pH 6	Octanol + TOA	NaCl	(b)
	Simultaneous (pH adjustment + carrier)	pH 6	Octanol + TOA	NaCl + 0.5 M NaOH (pH 11)	(c)

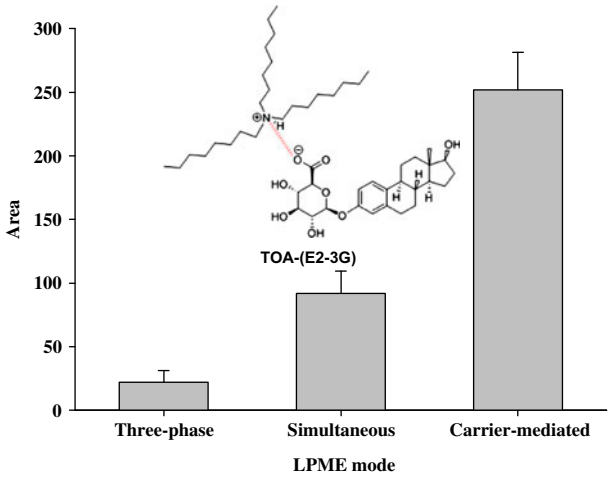


Fig. 2. The comparison of extraction efficiency of conjugated estrogen using different LPME modes. The optimization test was conducted using E2-3G. Structure of hydrophobic ion-complex of 17β-estradiol-3-glucuronide (E2-3G) and carrier (tri-octylamine, TOA).

polarity of conjugates. The results showed that carrier-mediated LPME methods had higher extraction efficiency than other methods, at 3 and 11 times higher than simultaneous and three-phase LPME, respectively (Fig. 2).

Glucuronide includes many hydroxyl groups, which makes the compound hydrophilic, resulting in an increased water solubility of the conjugated form. Therefore, water-soluble estrogen conjugates are difficult to extract into an organic phase using only a pH adjustment. The results indicated that carriers (TOA) significantly increased the extraction efficiency of the hydrophilic estrogen conjugates. On the other hand, simultaneous LPME showed an efficiency only three times greater than that of the three-phase mode. Based on these results, carrier-mediated HF-LPME was selected as the extraction mode of the conjugated estrogens and used in the further optimization study.

The pK_a of estrogen glucuronide conjugates was low (Table 1) because of the carboxyl group (–COOH) in the glucuronide moiety (pK_a = 3.68) [23]. In the neutral pH range (6–7) of the sample solution, glucuronide conjugates were protonated and had a negative charge, whereas TOA (pK_a = 8.3) in the organic phase was deprotonated and had a positive charge. During the extraction, the charged analytes were diffused into the fiber and meet the opposite charge of the carrier (TOA) in the organic phase. Both analytes and carrier combined to form a hydrophobic ion-complex by electrostatic attraction (Fig. 2). This complex was easily dissolved into the organic phase before meeting the counter-ion (Cl^-) in the acceptor phase at the contact surface. Because chloride ions have a higher affinity with amine (TOA) than analyte (COO^-), a hydrophobic ion-complex is easily dissociated. The dissociated analyte, which has a negative charge, was diffused and collected into the acceptor phase. Concurrently, the TOA^+–Cl^- complex in the organic phase was dissociated at the surface of the donor phase, and chloride ions were diffused into the donor phase (Fig. 1). The driving force of this mechanism was the mass transfer of the counter ions from the acceptor to the donor phase, which was caused by the chloride concentration gradient.

3.2. Optimization of extraction parameters for carrier-mediated HF-LPME

The extraction parameters for carrier-mediated HF-LPME were optimized based on the relative peak area obtained from the LC-MS/MS analysis. The carrier type, carrier concentration, counter-ion concentration, extraction time, and agitation rate were selected as the parameters that could affect the efficiency of the extraction. The optimization was conducted by extracting the represented estrogen conjugate (5 ng ml^{-1} of E2-3G) from the standard spiked water solution. First, the effect of carrier type on E2-3G was determined by monitoring the relative peak area (Fig. 3(a)). TOA and aliquot-336 were selected as the anionic carrier candidates for extraction. The results showed that the extraction efficiency of TOA was 4.4

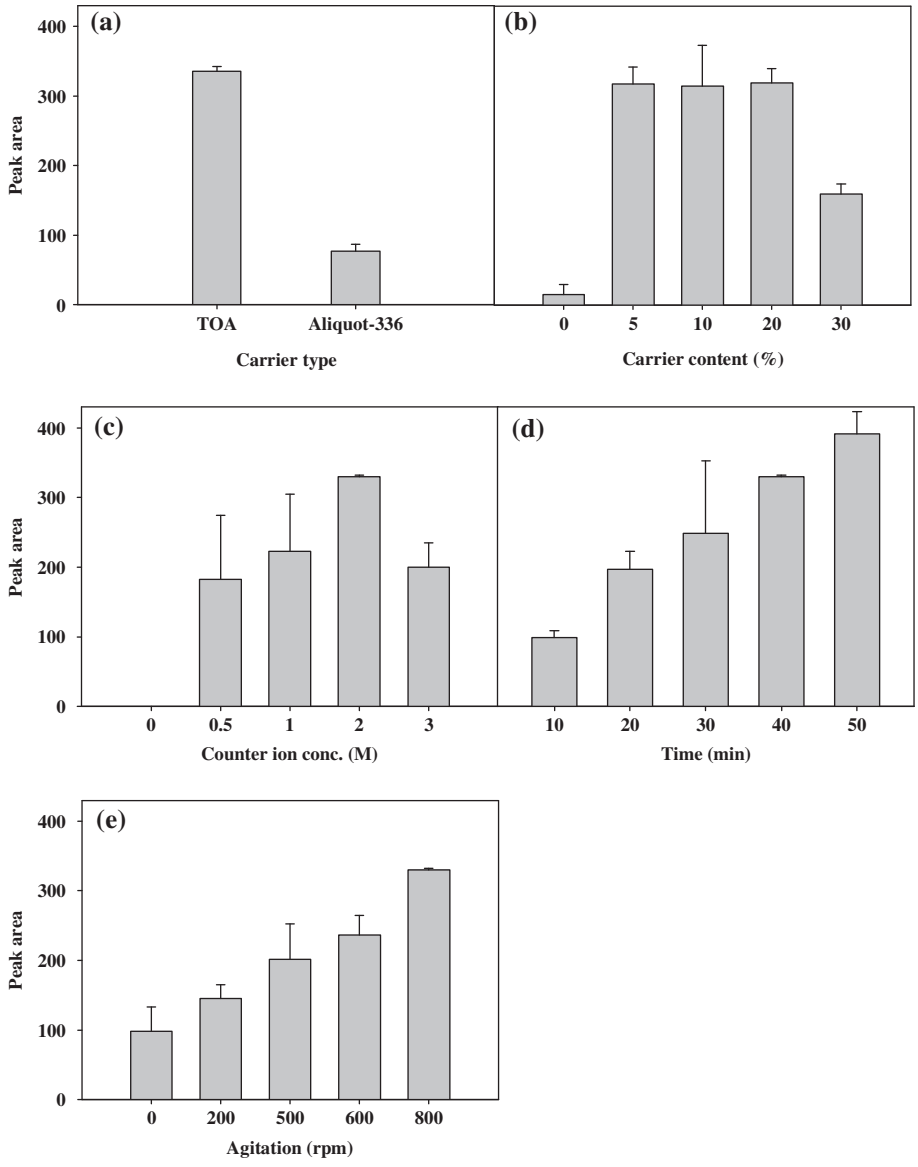


Fig. 3. Optimization of extraction parameters for carrier-mediated HF-LPME. (a) Carrier type, (b) carrier concentration in organic phase, (c) counter-ion concentration in acceptor phase, (d) extraction time, and (e) agitation. The optimization test was conducted using E2-3G.

times higher than that of Aliquot-336. Thus, TOA was selected as the appropriate carrier in this study. Next, the concentration of the optimized carrier was investigated (Fig. 3(b)). Because of its long alkyl chain, TOA dissolves easily in organic solvent. In this study, 1-octanol was selected as the organic solvent used in the impregnation of the pore of the hollow fiber. In particular, octanol was an appropriate organic solvent for HF-LPME because of its good compatibility with the fiber and its immiscibility in the aqueous phase. TOA was added to 1-octanol at concentrations of 0, 5, 10, 20, and 30%. The results showed that the extraction efficiency of E2-3G was highly increased in 5% and significantly decreased in 30% of the TOA content. E2-3G was not affected by TOA content between 5 and 20%. However, 20% TOA was selected as the optimal carrier because another target (EE2-3G) showed the highest efficiency at 20% in a further experiment (data not shown).

The effect of counter ions on the efficiency of the extraction was investigated by adding various concentrations of chloride ions to the acceptor phase, within the range of 0, 0.5, 1, 2, and 3 M (Fig. 3(c)). The counter-ion concentration induced the driving force of

carrier-mediated HF-LPME. The conjugated estrogen was not extracted without the driving force caused by the chloride ions. The extraction efficiency was increased as the Cl^- concentration increased from 0 to 2 M and then decreased. Accordingly, a 2 M counter-ion concentration was selected as the optimized condition. In the selection of the appropriate extraction time, several extraction times were compared across the range of 10, 20, 30, 40, and 50 min (Fig. 3(d)). Because the equilibrium of the mass-transfer in LPME is a time-dependent process, the extraction efficiency increased when the extraction time was increased from 10 to 50 min. However, it was not practical to extend the extraction time longer than 50 min, in terms of temporal efficiency. Therefore, 50 min was selected as the optimal extraction time. Agitation of the sample solution accelerated the efficiency of the extraction because the stirring rate allowed the solvent to be in continuous contact with the aqueous sample. Different degrees of agitation, ranging from 0 to 800 rpm, were investigated to determine the optimal agitation speed (Fig. 3(e)). The extraction efficiency gradually increased as the agitation speed increased. Thus, 800 rpm was selected as the optimal stirring condition. The optimized carrier-mediated HF-LPME conditions are summarized as follows: TOA as the carrier, 20% carrier in 1-octanol, Cl^- concentration of 2 M, extraction time of 50 min, and a stirring rate of 800 rpm.

3.3. Method validation and performance evaluation of carrier-mediated HF-LPME

Extracted free-form estrogens (E1, E2, EE2) and conjugated forms (E1-3G, E2-3G) were analyzed simultaneously using LC-MS/MS, following the method used in Tso and Aga [22]. Although as an internal standard, both E2 and E1-d_2 have the same m/z value (271 > 145), they were confirmed by different retention times. Generally, a pretreatment method with a recovery of 80–120% is required for an accurate chemical quantitative analysis. The LPME-applied calibration curve was drawn by using the peak area extracted from 0.1, 0.5, 1, 2.5, and 5 ng ml^{-1} estrogen mixtures in de-ionized water. Theoretically, the analyte in the sample solution was enriched 100-fold (10, 50, 100, 250, and 500 ng ml^{-1}), when the recovery value was 100%. All LPME-applied calibration curves exhibited good linearity (r^2) (Table 3), showing high r^2 values of 0.9981 (E1), 0.9988 (E2), and 0.9987 (EE2), 0.9974 (E1-3G), 0.9990 (E2-3G), and 0.9996 (EE2-3G).

The matrix effect of HF-LPME was demonstrated by comparing the distilled water (DW) and urine samples. The recovery of each estrogen species in the DW-applied and urine-applied calibration is shown in Table 3. All estrogen species exhibited good recovery in DW, at 95.0% (E1), 97.7% (E2), 96.9% (EE2), 93.2% (E1-3G), 12.8% (E2-3G), and 91.1% (EE2-3G). However, recovery from the urine samples was lower than DW (Table 3). This may have been caused by many impurities in human urine, such as urea and creatinine, as well as other ions, such as chloride, potassium, ammonium, sulfates, and phosphates [24], the matrix effect of which might have affected the efficiency of extracting the estrogens. The recovery of the conjugated estrogen in the urine samples was lower than the DW-spiked recovery, at 33.2, 46.2, and 43.8% for E1-3G, E2-3G, and EE2-3G, respectively. Free estrogens also showed decreased recovery values of 37.7, 43.4, and 66.6% for E1, E2, and EE2, respectively.

Table 3
MRM parameters for estrogens' analysis and analytical data by LC-MS/MS

						LPME calibration					
Compounds	MRM ions	RT (min)	CP (kV)[a]	CV (V)[b]	CE (V)[c]	Linearity (r^2)	Recovery in urine (%)	Recovery in distilled water (%)	Linear range (μg L^{-1})	Precision (RSD)	LOD (μg L^{-1})
E1-3G	445 > 269	11.50	3.5	35	25	0.9974	33.2 ± 15.2	93.2 ± 24.1	10–500	5.2	0.24
E2-3G	447 > 271	11.24	3.5	35	27	0.9970	46.2 ± 3.7	112.8 ± 36.8	10–500	6.3	0.38
EE2-3G	471 > 295	11.54	3.5	35	24	0.9996	43.8 ± 8.4	91.1 ± 17.3	10–500	9.0	0.31
E1	269 > 145	17.19	3.5	25	35	0.9981	37.7 ± 3.7	95.0 ± 10.7	10–1,000	8.7	0.37
E2	271 > 145	16.53	3.5	50	42	0.9988	43.4 ± 4.5	97.7 ± 16.7	10–1,000	5.8	0.11
EE2	295 > 145	16.86	3.5	50	35	0.9987	66.6 ± 9.5	96.9 ± 20.5	10–1,000	9.4	0.07
E1-d_2 (ISTD)	271 > 145	17.16	3.5	50	42						

[a]CP = capillary pressure.
[b]CV = cone voltage.
[c]CE = collision energy.

Table 4
Comparison of MDL and recovery of conjugated estrogen extracted using SPE and LPME

Compounds	SPE		LPME	
	MDL (ng ml^{-1})	Recovery (%)	MDL (ng ml^{-1})	Recovery (%)
E1-3G	0.052	106.9	0.073	93.2
E2-3G	0.039	73.3	0.115	112.8
EE2-3G	0.045	101.5	0.094	91.1

The linear range of LPME-applied calibration is shown in Table 3. The linear range of conjugated estrogens (E1-3G, E2-3G, and EE2-3G) was 10–500 ng ml^{-1}, which was shorter than that of free estrogen (10–1,000 ng ml^{-1}), which was because of the fiber capacity of LPME. The extraction efficiency of the conjugated estrogen was facilitated by carrier-mediated LPME. The precision of HF-LPME was characterized by a relatively standard deviation (RSD (%) = standard deviation (SD)/mean × 100 (%)). The RSD of all estrogen species was less than 10%, which demonstrated good precision. The repeatability of HF-LPME method was confirmed for free and conjugated estrogens with RSDs within 5.2–9.4%, which are acceptable values for the analysis of biological samples [14].

The application of LPME to conjugated estrogens was validated by comparing the method detection limit (MDL) with that of SPE (Table 4). SPE was performed according to the procedure described by Kuuranne et al. [14]. A conjugated estrogen mixture in 10 ml of DW sample was extracted by 10 ml of methanol: water (1:1, v/v). The HF-LPME method exhibited good recovery, and the MDL value was similar to that of SPE, despite the much lower amount of organic solvent (10 μl) than SPE (10 ml) and the one-step extraction procedure did not include a drying process. During the drying process of SPE, some conjugated estrogens might be broken into free form because of elevated temperature and a long drying time. This was demonstrated by an 18% increase of E2 extracted in SPE, which was not detected by the HF-LPME procedure. These results confirmed that HF-LPME is an appropriate and convenient sample pretreatment method for the conjugated estrogens.

3.4. Application of HF-LPME in human urine samples

Using the optimized HF-LPME parameters, free and conjugated estrogens were extracted from real human urine samples of non-pregnant ($n = 7$) and pregnant women ($n = 10$). The pregnant women were in different stages of pregnancy: 4 ($n = 1$), 6 ($n = 1$), 7 ($n = 2$), 8 ($n = 2$), and 9 months ($n = 4$). The estrogens analyzed in the urine samples were compensated by the recovery ratio. The concentrations of estrogens in the urine samples from the non-pregnant and pregnant woman are compared in Fig. 4.

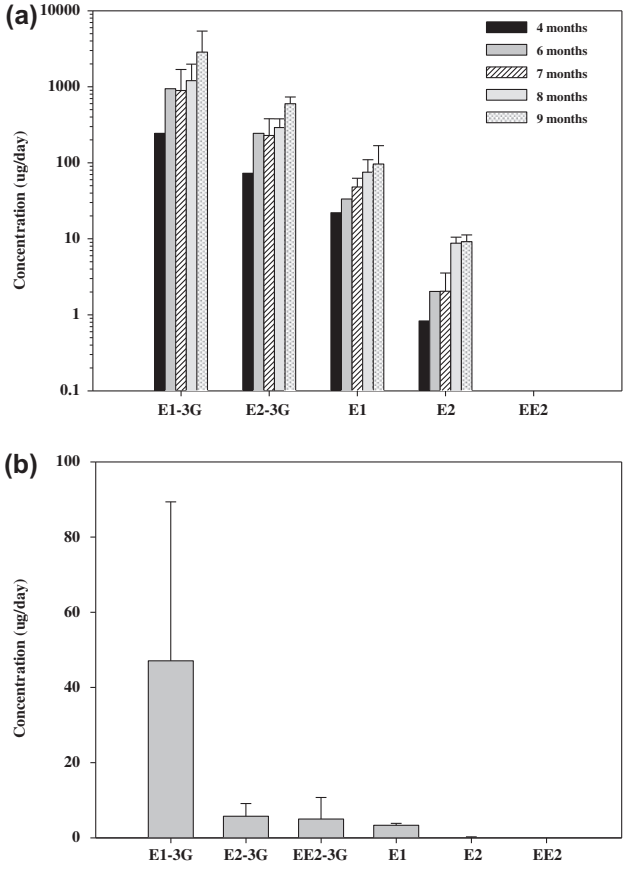


Fig. 4. Detected amount of estrogens (μg d^{-1}) in advanced pregnancy samples (a) and non-pregnancy samples (b).

In non-pregnancy urine, the daily excreted amount of the conjugated estrogens was 47.08, 5.74, and 5.02 μg d^{-1} for E1-3G, E2-3G, and EE2-3G, respectively, assuming a daily urine volume of 1.0 L. These levels were in good agreement with the previous study, especially the level of E2-3G (4.95 μg d^{-1}) [9]. The average data from pregnancy urine samples showed a much higher value of estrogens and their conjugates than non-pregnancy urine, at 1,228.36 and

286.98 μg d^{-1} for E1-3G and E2-3G, respectively. In the case of late pregnancy urines (9 months), four samples excreted 2,858.36 (E1-3G) and 597.95 (E2-3G) μg d^{-1} on average. Similar estrogen values were detected in the previous studies [25,26]. Adessi et al. [25] also observed 5,240 μg d^{-1} for E1 and 980 μg d^{-1} for E2 in late pregnancy urine after enzymatic hydrolysis, which might be the total estrogen level, including free and conjugated forms. In addition, free estrogens were detected at higher values than in non-pregnant women, at 55.09 μg d^{-1} for E1 and 4.57 μg d^{-1} for E2. The oral contraceptive pill EE2 was observed less than the detection limit in all the urine samples of non-pregnant and pregnant woman.

The excretion of estrogens was increased with advancing pregnancy from four to nine months (Fig. 4). Although, there were individual variations, the estrogen levels of both conjugated and free forms tended to increase in the advanced stages of pregnancy. In particular, the concentrations of E1-3G, E2-3G, and E1 showed a significant increase with advancing pregnancy. These results indicate that estrogen levels increased as delivery approached. Estrogens were mainly excreted in a conjugated form (94.85 ± 4%), whereas free estrogen was detected at a ratio of 5.15%. The estrogen levels were much higher in the urine of pregnant women than in the non-pregnant women, showing values that were higher by 5.18–60.72 (E1-3G) and 12.71–104.17 (E2-3G) times. The most dominant species of estrogen in pregnancy urine was E1 (conjugated and free form combined) at a ratio of 72.7 ± 9%. The results indicated that although, E1 has only 23% of the estrogenic potency of E2 [21], it can be a major contributor to estrogen pollution because its concentration in human urine is 4.5-fold higher than that of E2. These results suggest that both free and conjugated forms of E1 are the most significant EDs among the estrogen species.

The results of this study showed that HF-LPME was an appropriate extraction method for conjugate estrogens in urine samples. Biological samples that might have contained a large variety of interfering substances were completely cleared, which was demonstrated by the higher recovery and linearity found by analysis. The most significant advantage is that only 10 ml of the biological sample was sufficient to measure all estrogens using the HF-LPME technique.

4. Conclusion

In the present study, HF-LPME in carrier-mediated mode was effectively applied to extract estrogen conjugates in human urine samples. The extraction efficiency of carrier-mediated LPME for estrogen conjugates was 11 times higher than that of non-carrier HF-LPME. The optimal extraction conditions were the following: the organic phase, composed of 20% (w/w) TOA in 1-octanol; 2 M NaCl as the acceptor phase; agitation at 800 rpm; extraction time at 50 min. The extraction method with the optimized parameters was conducted to demonstrate the feasibility of HF-LPME in human urine samples. In the monitoring study, 94.8% of the total amount of estrogens in urine existed in the conjugated form. E1 was the most dominant species in all subjects (E1 > E2 > EE2), in which free and conjugated forms accounted for 72.7% of all estrogens. Based on these results, the HF-LPME is a good sample pretreatment technique and could be applied to several substances and matrices.

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