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Authors

Wang, Huili
Gao, Ming
Wang, Mei
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Integration of phase separation with ultrasound-assisted salt-induced liquid–liquid microextraction for analyzing the fluoroquinones in human body fluids by liquid chromatography

Huili Wang[a], Ming Gao[b,1], Mei Wang[a], Rongbo Zhang[a], Wenwei Wang[a], Randy A. Dahlgren[c], Xuedong Wang[a,*]

[a] College of Life Sciences, Wenzhou Medical University, Wenzhou 325035, China
[b] College of Environmental Sciences and Public Health, Wenzhou Medical University, Wenzhou 325035, China
[c] Department of Land, Air and Water Resources, University of California, Davis, CA 95616, USA

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ABSTRACT

Herein, we developed a novel integrated device to perform phase separation based on ultrasound-assisted salt-induced liquid–liquid microextraction for determination of five fluoroquinones (FQs) in human body fluids. The integrated device consisted of three simple HDPE components used to separate the extraction solvent from the aqueous phase prior to retrieving the extractant. A series of extraction parameters were optimized using the response surface method based on central composite design. Optimal conditions consisted of 945 μL acetone extraction solvent, pH 2.1, 4.1 min stir time, 5.9 g Na_2SO_4, and 4.0 min centrifugation. Under optimized conditions, the limits of detection (at S/N = 3) were 0.12–0.66 μg L^{-1}, the linear range was 0.5–500 μg L^{-1} and recoveries were 92.6–110.9% for the five FQs extracted from plasma and urine. The proposed method has several advantages, such as easy construction from inexpensive materials, high extraction efficiency, short extraction time, and compatibility with HPLC analysis. Thus, this method shows excellent prospects for sample pretreatment and analysis of FQs in human body fluids.

1. Introduction

The widespread application of fluoroquinolones (FQs) in human and food-producing animals has increased human health concerns because of induced pathogen resistance and possible allergic hypersensitivities in humans [1–6]. Previous research has demonstrated that low amounts of FQs were often found in plasma and their main excretion pathway in urine [5,6]. Because of the possible resistance of human pathogens to antibiotics, there is currently concern about low level exposure to these compounds [7]. Therefore, sensitive and selective analytical methods for the determination of FQs in plasma and urine or other biological fluids are urgently required. To date, many methods have been developed for the determination of FQs in many matrices, such as spectroscopy [8], capillary electrophoresis [9], spectrofluorometry [10–12], potentiometric titration [13] and high performance liquid chromatography (HPLC) [14,15] coupled with mass spectrometry (MS) [16]. Because of interference from complex matrices in biological fluids, these analytical methods often require extensive sample preparation. Accordingly, there is considerable interest in developing a cost-effective, efficient and reliable extraction and quantification method for FQs in complex matrices.

Abbreviations: PS-USLM, phase separation based on ultrasound-assisted, salt-induced, liquid–liquid microextraction; FQs, fluoroquinones; HPLC-FLD, high performance liquid chromatography equipped with fluorescence detector; PPCPs, pharmaceuticals and personal care products; HPLC, high performance liquid chromatography; MS, mass spectrometry; SPE, solid-phase extraction; LLE, liquid–liquid extraction; LPME, liquid-phase microextraction; DLLME, dispersive liquid-liquid microextraction; SALLME, salting-out assisted liquid–liquid microextraction; RSM, response surface method; CCD, central composite design; LOD, limits of detection; ER, extraction recovery; FLE, fleroxacin; OFL, ofloxacin; NOR, norfloxacin; CIP, ciprofloxacin; ENR, enrofloxacin; $MgSO_4$, magnesium sulfate; Na_2SO_4, sodium sulfate; CH_3COONa, sodium acetate; $(NH_4)_2SO_4$, ammonium sulfate; CH_3COONH_4, ammonium acetate; HDPE, high-density polyethylene; LDR, linear dynamic range; RSDs, relative standard deviations; RR, relative recovery; LLE-HPLC, liquid-liquid extraction combined with high performance liquid chromatography; SPE-HPLC, solid phase extraction combined with high performance liquid chromatography; SOMC-HPLC, second-order multivariate calibration combined with high performance liquid chromatography; SSPC-HPLC, single-step precipitation cleanup method combined with high performance liquid chromatography; UFLC, ultra-fast liquid chromatography; HF-LPME-HPLC, hollow fiber-based liquid phase microextraction combined with high performance liquid chromatography.

* Corresponding author. Tel.: +86 577 86689733; fax: +86 577 86699122.
E-mail addresses: gaom625@126.com (M. Gao), zjuwxd@163.com (X. Wang).
[1] Co-first author: Ming Gao.

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Salting-out assisted liquid–liquid microextraction (SALLME) is based on phase separation of water-miscible organic solvents from the aqueous phase at high salt concentration [17]. In the SALLME procedure, the collection and measurement of microliter volumes of the separated organic phase are difficult because the wide diameter glass tube makes the thin layer of extractant difficult to retrieve and requires relatively long extraction times. A few studies have introduced extraction devices or vessels to classical dispersive liquid–liquid microextraction (DLLME) that allow for the use of lower density organic extraction solvents, either by using a narrow-necked glass tube [18], or by using a glass vial [19]. Hashemi et al. [20] introduced a home-made, narrow-necked glass tube for the effective collection of extractant, and inserted it into a centrifuge tube for centrifugation after extraction. Zhang et al. [21] designed a special flask equipped with two narrow open necks with one having a capillary tip to facilitate the DLLME process. However, these glass-based devices are fragile and require special design, therefore their cost is relatively high and their commercial availability is limited [22].

Recently, a cheap, flexible and disposable polyethylene Pasteur pipette was introduced as an extraction device for low-density solvent-based DLLME [23,24]. Wang et al. [19] developed a new device, which consisted of a dropper and a sample vial, to perform extraction, separation and concentration of trace pesticides from solvents. The bulb end of the cut polyethylene dropper was inserted into the neck of the sample vial and the tip end of the polyethylene dropper was cut to an appropriate length [22]. The plastic pipette afforded advantages of low cost, use of easily available materials and ease of operation. However, the major drawback of this device is that the extracted organic phase was difficult to completely retrieve because the organic and aqueous phases were not separated prior to collection of the extractant. Thus, repartitioning of extractant into the aqueous phase can occur over the relatively long retrieval time, which will result in a low extraction recovery.

To overcome the above-mentioned limitations of current methods, this study developed and optimized a novel integrated device and methodology for extracting and isolating FQs for HPLC quantification using phase separation based on ultrasound assisted, salt-induced, liquid–liquid microextraction (PS-USLM). The proposed PS-USLM method was optimized for major operational factors (stirring time, pH, salt type and volume, solvent type and volume, and centrifugation time) using a response surface method (RSM) based on central composite design (CCD). The optimized method was compared with other commonly used LPME methods to evaluate its advantages and feasibility for determining trace levels of FQs in plasma and urine samples. To the best of our knowledge, this integrated device, designed to completely and rapidly separate the organic and aqueous phases prior to collection of the extractant, is the first reported use of this approach for determination of FQs in biological fluids.

2. Experimental

2.1. Reagents and materials

Analytical standards for fleroxacin (FLE), ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP) and enrofloxacin (ENR) were purchased from J&K Chemical, China and used without further purification. HPLC-grade ethanol, methanol, ethyl acetate, acetonitrile and acetone were sourced from Merck (www.merck.com.cn). Salts (magnesium sulfate ($MgSO_4$), sodium sulfate (Na_2SO4), sodium acetate (CH_3COONa), ammonium sulfate ($(NH_4)_2SO_4$) and ammonium acetate (CH_3COONH_4)) with purities ≥99% were obtained from Aladdin Industrial Co. Ltd.

Stock standard solutions (1000 μg mL^{-1}) for each FQ were prepared by dissolving each compound in methanol and stored at 4 °C. Stock solutions were diluted with methanol to prepare a secondary mixed stock solution of 10 μg mL^{-1}. Mixtures of standard working solutions for extraction at different concentrations were prepared daily by dilution with Milli-Q ultrapure water (Millipore, Bedford, USA).

2.2. Preparation of plasma and urine samples

Drug-free whole blood plasma and urine samples from male and female volunteers were collected from healthy individuals at Wenzhou Medical University, Wenzhou, China. The plasma samples were taken intravenously in the presence of EDTA-2Na as an anticoagulant and were centrifuged at 12,000 rpm for 10 min at 4 °C (the partial impurities in the blood settled at the bottom of the centrifuge tube). Then, the above treated blood samples or the collected urine samples were transferred into individual 50 mL polypropylene centrifuge tubes immediately after filtration using a 0.45 μm membrane filter and stored at −20 °C until analysis or validation of the analytical method. Before use, the samples were thawed at ambient temperature. Ethical approval for this study was obtained from the Ethics Committee at Wenzhou Medical University.

2.3. Instrumentation

FQs were analyzed with an Agilent 1260 HPLC equipped with a fluorescence detector (FLD). A Zorbax Eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm particle size) was used and injections were performed manually using a 20.0-μL sample loop. The operating conditions were as follows: mobile phase, methanol–acetonitrile–water (14:7:79, v/v; water consisting of 3.4 mL orthophosphoric acid and 6.0 mL triethylamine per liter); flow rate, 0.8 mL min^{-1}; column temperature, 40 ± 1 °C; and excitation and emission wavelengths of 290 and 455 nm, respectively. Solutions were heated and ultrasonicated using a model KS-600EI ultrasonic washing unit from Ningbo Kesheng Ultrasonic Equipment Factory, China. Centrifugation used a model TDL-50C centrifuge from Anting Instrument Factory, China and TGL-20M centrifuge from Xiangli Instrument Factory, China.

2.4. PS-USLM procedure

A schematic of the integrated PS-USLM procedure is shown in Fig. 1. This novel integrated device consists of three components: (1) a high-density polyethylene (HDPE) centrifuge tube with 12.0-cm height, 1.6-cm external diameter and 1.4-cm internal diameter (Fig. 1A); (2) an inverted cut HDPE dropper with 1.0-cm height and 1.4-cm external diameter joined to a 3.0-cm length of capillary tube (Fig. 1I); and (3) a "V" HDPE capillary tube with a 10.0-cm total length and 0.5 cm internal diameter (Fig. 1I). The inverted cut disposable HDPE dropper was inserted into the centrifuge tube, and the "V" tube was easily attached/detached from the inverted HDPE dropper (Fig. 1F and G). Advantages of this integrated device are simple design, low cost, practicability, HDPE material is not easily broken, repeatable application and ease of operation.

In operation, the sample solution was first added to the centrifuge tube followed by the extraction solvent, which was water-miscible and lower density than water. After centrifugation, the sedimented proteins and other interfering compounds were discarded (Fig. 1A). Then, an appropriate amount of salt was added to the remaining solution (Fig. 1B). After salting-out, the extraction solvent will float on the top of the sample solution following ultrasound treatment and centrifugation (Fig. 1C and D; extraction solvent, Fig. 1D-1; sample solution, Fig. 1D-2; undissolved salt, Fig. 1D-3)). The inverted HDPE dropper was then placed into

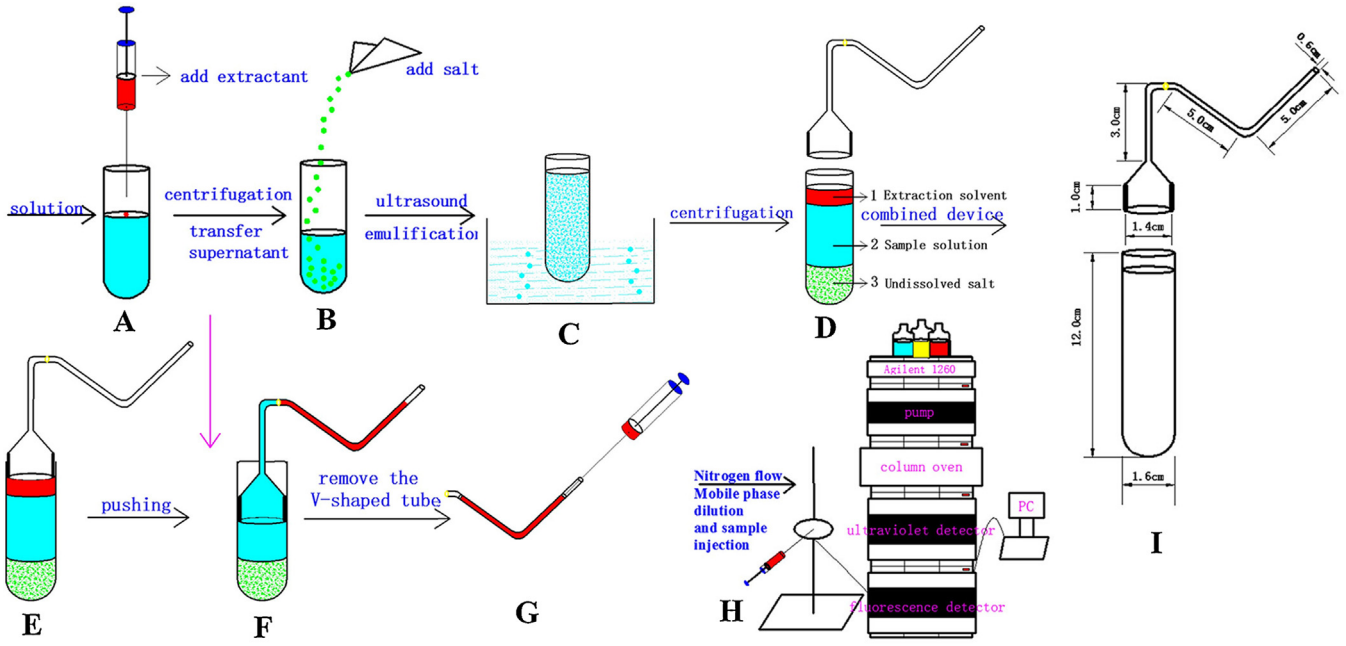

Fig. 1. The integrated device and schematic procedures for the PS-USLM method. *Note*: Each step in the PS-USLM procedure is described in the text.

the sample solution and the extractant was extruded through the tip of the dropper (Fig. 1E and F). When the extractant was fully transferred into the "V" tube, the "V" tube was detached and the extractant was collected with a microsyringe (Fig. 1G). The extractant was then dried using a gentle nitrogen flow, dissolved with 50 μL of mobile phase and quantified by HPLC-FLD (Fig. 1H).

For pretreatment, plasma and urine samples were thawed at ambient temperature after −20 °C storage. One milliliter of plasma or urine was placed into triplicate 15 mL centrifuge tubes. Ultrapure water (9 mL) was added to each sample followed by acidification to pH 1.0–3.0 with sulfuric acid. The water-miscible organic solvent (560–1240 μL) was slowly introduced into the aqueous phase with a 1000-μL micropipette. The emulsion was centrifuged at 4000 rpm for 2 min resulting in sedimentation of protein impurities. An appropriate amount of salt (3.8–7.2 g) was added followed by ultrasonic extraction for 1–7 min at 25 °C and centrifugation at 4000 rpm for 5 min. The ultrasonic extraction was performed at 40 °C and 1200 W. The ultrasonic time was referred to as extraction time, one of the main factors in the PS-USLM procedure. Finally, the extraction solvent was isolated as the top layer of the sample solution and recovered using the inverted dropper as described above.

2.5. Experimental design

Four main factors, extraction solvent volume (A), pH (B), extraction time (C) and weight of salt (D), were chosen based on previous studies [25–27] and preliminary experiments. For each variable, high and low set points were selected to construct an orthogonal design (Table 1). CCD was used to optimize values for each factor based on ER. The CCD included 22 treatments in five levels ($-\alpha$, −1, 0, +1, $+\alpha$) for four factors, and consisting of two blocks (Table 2). It contained an imbedded half-fraction factorial design ($N_f = 2^{f-1}$) with a set of center points (N_0) that was augmented with a group of "star points" ($N_\alpha = 2f$) that allow for estimation of curvature [18], where "f" indicates the number of experimental factors. As a result, the 22 treatments included 8 half-fraction factorial design points, 8 "star points" and 6 center points. The average ER was considered as the "experimental response" to evaluate method performance [18]. A quadratic polynomial model Eq. (1) was used to predict the response of dependent variables for the ERs of FQs:

$$Y = \mathrm{b0} + \sum_{i=1}^{4} bixi + \sum_{ij=1(i\neq j)}^{6} bijxixj + \sum_{i=1}^{4} biix_i^2 \quad (1)$$

where Y is the dependent variable, x_i the independent variable, b_0 the intercept, b_i the coefficient of linear effect, b_{ij} the coefficient of interaction effect, and b_{ii} is the coefficient of the squared effect [28]. The software package Design-Expert 8.0.5 (Minneapolis, USA) was employed to analyze the data and experimental design. Analysis of variance (ANOVA) was used to evaluate the model and to obtain response surfaces for factor optimization.

2.6. Method performance

ER is an indicator of absolute recovery, which takes into account the matrix effect and is calculated by Eq. (2):

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

Table 1
Factors, symbols and levels for the CCD.

Factor	Symbol	Level				
		$-\alpha$ (low)	−1	0	1	$+\alpha$ (high)
Extraction solvent volume (μL)	A	560	700	900	1100	1240
pH	B	1	1.5	2	2.5	3
Extraction time (min)	C	1	2	4	6	7
Weight of salt (g)	D	3.8	4.5	5.5	6.5	7.2

Table 2
Design matrix and responses for the CCD.

Run	Block	*A*: Extraction solvent volume (μL)	*B*: pH	*C*: Extraction time (min)	*D*: Weight of salt (g)	Recovery (%)
1	1	900	2	4	5.5	80.34
2	1	700	2.5	2	6.5	59.14
3	1	700	1.5	6	4.5	68.28
4	1	900	2	4	5.5	97.76
5	1	900	2	4	5.5	91.18
6	1	1100	2.5	6	4.5	67.83
7	1	1100	1.5	2	6.5	63.18
8	1	1100	1.5	6	6.5	77.25
9	1	1100	2.5	2	4.5	55.48
10	1	900	2	4	5.5	88.57
11	1	700	1.5	2	4.5	55.84
12	1	700	2.5	6	6.5	72.32
13	2	900	2	4	7.2	85.81
14	2	900	2	1	5.5	50.01
15	2	900	2	4	5.5	91.93
16	2	900	3	4	5.5	82.28
17	2	1240	2	4	5.5	83.99
18	2	900	2	7	5.5	82.21
19	2	900	2	4	5.5	85.01
20	2	900	2	4	3.8	42.16
21	2	560	2	4	5.5	41.14
22	2	900	1	4	5.5	46.64

where C_{sed} is concentration of the analyte in the sedimented phase; C_0 is the initial concentration of analyte in the aqueous phase; and V_{sed} and V_{aq} are the volumes of sedimented and aqueous phases, respectively [18]. In comparison, relative recovery (RR) deducts the matrix effect, and is used to appraise the analytical performance of the optimized method after fortification of standard samples in matrices. RR is computed by Eq. (3):

$$RR = \frac{C_{found} - C_{real}}{C_{added}} \quad (3)$$

where C_{found}, C_{real}, and C_{added} are the concentration of analyte in the final solution after PS-USLM procedures in plasma or urine samples, the concentration of analyte in the unfortified samples, and the concentration of a known amount of standard spiked into the samples, respectively.

Under optimal conditions, the precision study was carried out in six parallel experiments by determining the intra- and inter-day RSDs (relative standard deviations) at three fortified levels (5, 10 and 20 μg L^{-1}). The intra-day experiments were performed in one day (6 recovery experiments at 12 h, each experiment at 2 h interval). The inter-day experiments were carried out over 6 continuous days (one recovery experiment for each day). The mean recovery and RSD were computed using recovery data for the 6 replicates.

3. Results and discussion

3.1. Selection of extraction solvent and salt type

In the PS-USLM method, the selection of an appropriate extraction solvent is based on several basic requirements, such as lower density than water, miscibility with the aqueous phase, ease of phase separation in high salt concentrations, good chromatographic behavior, and high extraction efficiency for target analytes. According to these considerations, tetrahydrofuran, ethanol, methanol, acetonitrile and acetone were examined for their "salting-out" phenomena and extraction efficiencies for FQs (Fig. 2). When the sample solution and extraction solvent volumes were 10.0 and 0.9 mL, respectively, the salting-out effect was examined for five salts ($MgSO_4$, Na_2SO_4, CH_3COONH_4, CH_3COONa and $(NH_4)_2SO_4$) in the range of 3.8–7.2 g. The methanol–water mixture did not show any phase separation even when the mixture was saturated with salts. Additionally, ethanol showed indistinct phase separation even after centrifugation. In contrast,

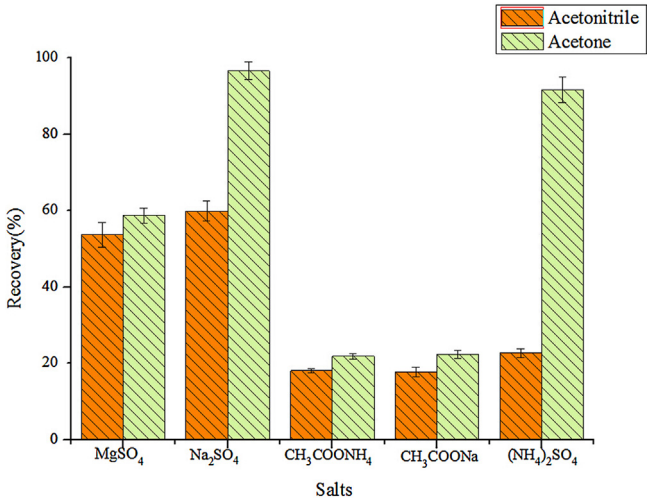

Fig. 2. Effect of extraction solvent and salt on the average extraction recovery of five FQs. Experimental conditions: pH 2.1, extraction time, 4.1 min and extraction temperature, 25 °C

water–tetrahydrofuran, water–acetonitrile and water–acetone mixtures gave a clear separation in the presence of all four salts under the conditions of 10.0 mL of water and 0.9 mL of solvent. Under the above conditions, the volumes of organic solvent-rich phase/water-rich phase after separation were 0.6/10.9 mL for both water–acetonitrile and water–acetone. Although tetrahydrofuran produced a salting-out phenomenon and a dissolved-out organic phase, a severe overlapping phenomena occurred between the peaks of analytes, and poor chromatographic peak shape was observed, which could not be used for accurate quantification. The highest ER was observed in water/acetone/Na_2SO_4 treatment (96.63 ± 2.27%), followed by water/acetone/$(NH_4)_2SO_4$ (91.62 ± 3.31%) (Fig. 2). As a result, acetone and Na_2SO_4 were chosen for subsequent experiments.

3.2. Optimization of the extraction temperature

The effect of temperature on extraction efficiency was investigated by setting a series of temperatures (10–65 °C at a 5 °C interval) during the ultrasonic extraction step. There was no ER of targets at

Table 3
Analysis of variance (ANOVA) for the CCD.

Source	Sum of squares	Mean		*F*-value	*p*-value	Significant
		df	Square		Prob > *F*	
Block	86.38	1	86.38			
Model	5914.1	14	422.44	9.04	0.0063	Significant
A-Extraction solvent volume	918.06	1	918.06	19.64	0.0044	
B-pH	635.1	1	635.1	13.59	0.0103	
C-Extraction time	805.45	1	805.45	17.24	0.006	
D-Weight of salt	952.66	1	952.66	20.39	0.004	
AB	321	1	321	6.87	0.0395	
AC	0.08	1	0.08	1.71E−03	0.9683	
AD	410.67	1	410.67	8.79	0.0251	
BC	0.12	1	0.12	2.57E−03	0.9612	
BD	450.2	1	450.2	9.63	0.021	
CD	0.76	1	0.76	0.016	0.9029	
A^2	843.67	1	843.67	18.05	0.0054	
B^2	655.02	1	655.02	14.02	0.0096	
C^2	632.03	1	632.03	13.52	0.0104	
D^2	733.84	1	733.84	15.7	0.0074	
Residual	280.4	6	46.73			
Lack of fit	100.64	2	50.32	1.12	0.411	Not significant
Pure error	179.76	4	44.94			
Cor total	6280.88	21				

10 °C, which could be explained by the rapid crystallization of anhydrous Na_2SO_4 at low temperatures, which inhibited the salting-out phenomenon. The ERs increased from 81.3% to 95.2% with increasing exaction temperatures from 10 to 25 °C. However, with a further increase of temperature from 25 to 50 °C, the ER remained nearly constant (~94.2%), but it was reduced from 55 to 65 °C. Thus, the ambient temperature (25 °C) was selected in this investigation.

3.3. Optimization of the PS-USLM procedures using CCD

The optimization experiments were randomized in order to minimize the effects of uncontrolled factors. As it was not possible to complete each experiment during a single work day, they were divided into two blocks and carried out over two sequential days to remove any variations caused by changes occurring over the course of the experiments [18]. Each factor was evaluated at 5 levels ($-\alpha$, −1, 0, 1, $+\alpha$) with the central level set at 900 μL solvent volume, pH 2, 4 min extraction time and 5.5 g of salt (Table 1). The experimental design matrix, which is composed of the number and order of the experiments, levels of factors in each experiment and the extraction recovery, is summarized in Table 2.

ANOVA was used to evaluate the significance of the model equation and related terms (Table 3). The model was highly significant and the "probe > *F*" value for the "lack of fit component" was 0.4110, which indicates that the other factors in this experiment had a small influence and the model represents the data well. The significant model, with a "probe > *F*" value 0.0063, indicated that the equation was a good fit for representing the relationship between ER and the four main factors. Based on the significant effects for "probe > *F*" values <0.0500, it was concluded that *A*, *B*, *C*, *D*, *AB*, *AD*, *BD*, A^2, B^2, C^2 and D^2 all showed significant effects. A second-order polynomial provided the strongest statistical fit and was considered as the best response surface model to fit the experimental data [18]. As can be seen in Eq. (3), there were four main effects (*A*, *B*, *C* and *D*), three two-factor interaction effects (*AB*, *AD* and *BD*), and four curvature effects (A^2, B^2, C^2 and D^2):

$$Y = b_0 + b_1A + b_2B + b_3C + b_4D + b_5AB + b_6AC + b_7AD + b_8BC + b_9BD + b_{10}CD + b_{11}A^2 + b_{12}B^2 + b_{13}C^2 + b_{14}D^2$$

with $b_0 = 131.63$; $b_1 = -0.08$; $b_2 = -119.31$; $b_3 = 18.53$; $b_4 = -4.86$; $b_5 = 0.098$; $b_6 = 2.50 \times 10^{-4}$; $b_7 = 0.051$; $b_8 = -0.12$; $b_9 = 23.17$; $b_{10} = 0.15$; $b_{11} = -1.82 \times 10^{-4}$; $b_{12} = -19.45$; $b_{13} = -1.92$; and $b_{14} = -6.80$.

Here, *Y* is the ER, b_0 the intercept and b_1 to b_{14} are parameter coefficients. The relationship between the related effect and the response is indicated by "+" or "−" for each coefficient. A "+" means the coefficient and the extraction recovery have a positive relationship, while a "−" indicates a negative relationship. The absolute value of the coefficients indicates the strength of the relationship between the coefficient and the extraction recovery (*Y*).

The goodness of fit for the polynomial model was expressed by the coefficient of determination (R^2, adjusted-R^2). The R^2 (0.9547) is a measure of the amount of variance around the average explained by the model. The adjusted-R^2 (0.8491) is the R^2 adjusted for the number of terms in the model, and it decreases as the number of terms in the model increases and those additional terms do not add value to the model [25]. The high R^2 values indicated that we can use the model to analyze and optimize the effects of extraction conditions on ER. As can be seen from Fig. 3a, most of the data points were scattered near the regression line, suggesting a good correlation between predicted and actual responses and a good fit for the quadratic model. In addition, the residual plots were randomly scattered (Fig. 3b) indicating that the variance of the experimental measurements was constant for all values of *Y*.

In order to obtain more details of the experimental factors on the ER, 3D response surfaces and contour lines were plotted. These plots represent the relationship between the response and levels of two factors simultaneously, while holding the other factors fixed at their central levels [26]. The 3D response surfaces and contour lines shown in Fig. 4 represents the relationship between ER and the four experimental factors (extraction solvent volume, pH, extraction time and weight of salt). For example, Fig. 4a describes the 3D response surface and contour line for the effect of extraction solvent volume and pH on ER under fixed conditions of 4-min extraction time and 5.5 g salt. The ERs of FQs increased with increasing extraction solvent volume from 560 to 945 μL and pH from 1.0 to 2.1. However, with a further increase in extraction solvent volume from 945 to 1240 μL and pH from 2.1 to 3.0, the ERs of FQs declined. Fig. 4b depicts the 3D response surface and contour line for the effect of extraction solvent volume and the weight of salt on ER when the pH and exaction time were set at 2.0 and 4.0 min, respectively. The maximum ER was observed at 945 μL of extraction solvent and 5.9 g salt. With further increases in extraction solvent volume (945–1240 μL) and salt weight (5.9–7.2 g), the ERs decreased sharply. Fig. 4c demonstrates the 3D response surface and contour line for the effect of pH and salt weight on the ERs when the extraction volume and extraction time were set at 900 μL and 4.0 min, respectively. When the pH increased from 1 to

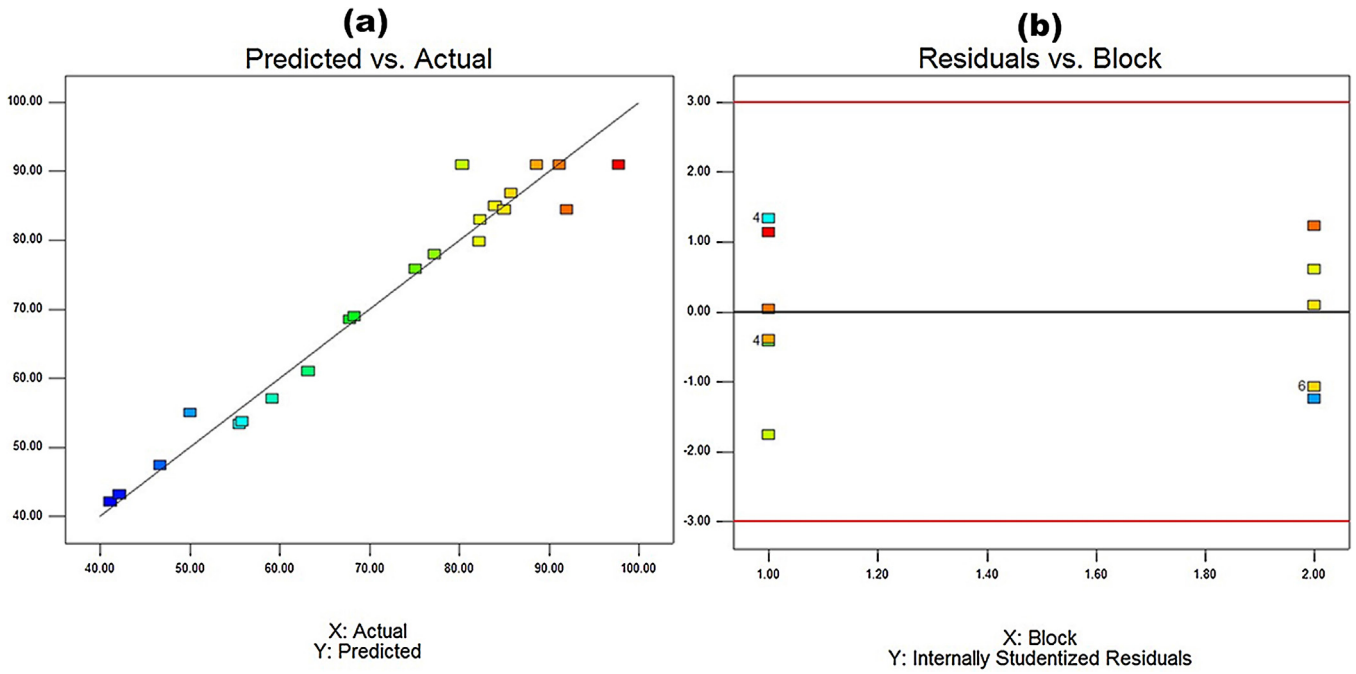

Fig. 3. (a) The predicted response vs. the observed response and (b) a plot of the internally studentized residuals vs. the predicted response.

2.1 and the weight of salt increased from 3.8 to 5.9 g, the ER reached its maximum, and then quickly declined with further increases of pH (2.1–3.0) and salt weight (5.9–7.2 g). After rigorous analysis of the interaction factors in Fig. 4, the optimal set points for the four parameters were determined to be 945 μL extraction solvent, pH 2.1, 4.1 min extraction time, 5.9 g salt and 25 °C, and the recovery (~98%) was obviously higher than for the other combinations, for example, 900 μL extraction solvent, pH 2.0, 4.0 min extraction time, 5.5 g salt and 25 °C yielded a recovery of ~90%.

3.4. Method evaluation

Under the optimized experimental conditions, the performance of PS-USLM was evaluated for linear range, LOD, precision

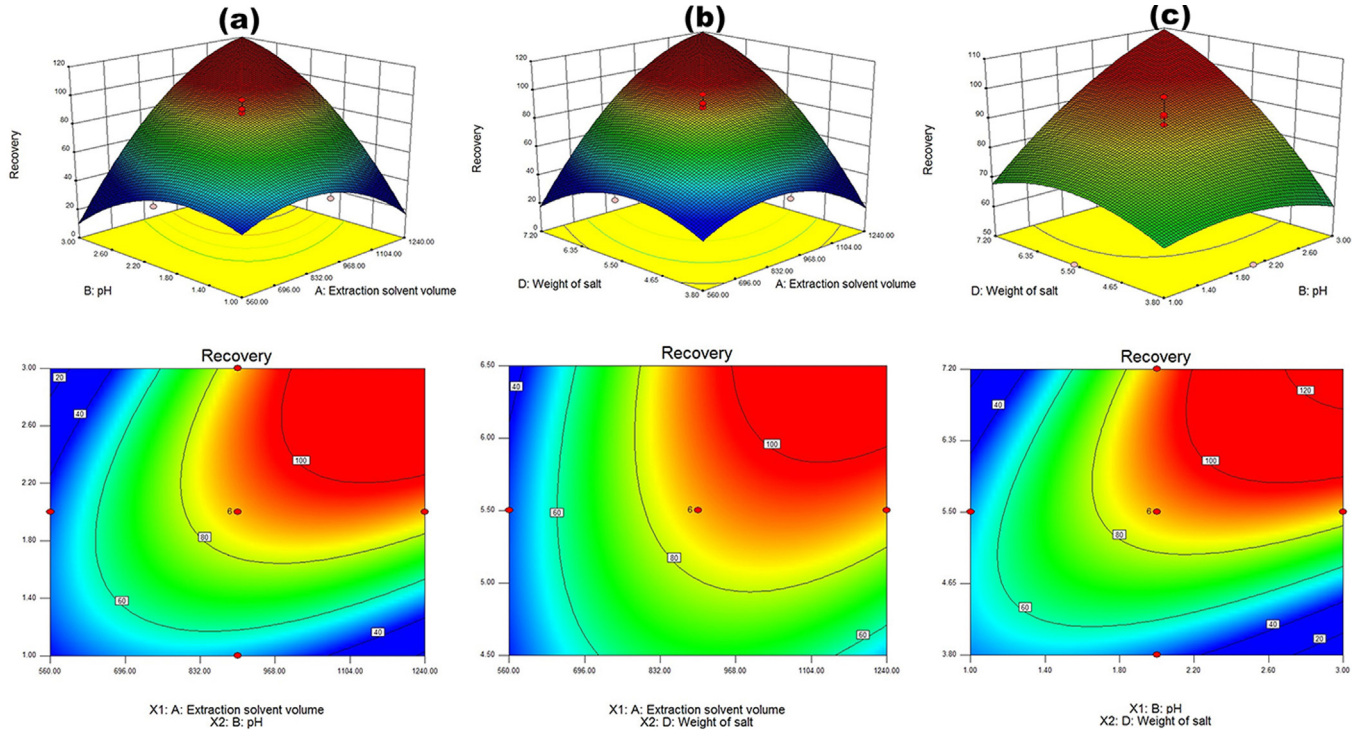

Fig. 4. (a) 3D response surface and contour plots for the extraction solvent volume and pH at constant extraction time (4 min) and salt weight (5.5 g) on the average extraction recovery, (b) 3D response surface and contour plots for the extraction solvent volume and salt weight at constant pH (2.0) and extraction time (4.0 min) on the average extraction recovery and (c) 3D response surface and contour plots for pH and salt weight at constant extraction volume (900 μL) and extraction time (4.0 min) on the average extraction recovery.

Table 4
The analytical performance of the PS-USLM-HPLC method.

Sample	Analytes	Regression equations	Determination coefficients (R^2)	Linear range ($\mu g\,L^{-1}$)	LOD ($\mu g\,L^{-1}$)
Plasma	FLE	$y = 0.2947x - 0.0679$	0.9996	0.5–500	0.1315
	OFL	$y = 0.0387x - 0.0396$	0.9997	3–500	0.6592
	NOR	$y = 0.0938x - 0.0116$	0.9998	2–500	0.5186
	CIP	$y = 0.4242x - 0.3748$	0.9994	1–500	0.1901
	ENR	$y = 0.8465x + 0.0367$	0.9995	0.5–500	0.1191
Urine	FLE	$y = 0.2795x + 0.1491$	0.9992	0.5–500	0.1534
	OFL	$y = 0.0368x - 0.0055$	0.9994	3–500	0.6747
	NOR	$y = 0.0938x - 0.0062$	0.9996	2–500	0.5894
	CIP	$y = 0.3949x + 0.1307$	0.9993	1–500	0.2060
	ENR	$y = 0.8581x - 0.0269$	0.9998	0.5–500	0.1465

and ER (Table 4). The coefficients of determination (R^2) for the five FQs were in the range of 0.9991–0.9998. The LODs at a signal/noise ratio of 3 for plasma and urine samples were in the range 0.13–0.15 $\mu g\,L^{-1}$ for FLE; 0.66–0.67 $\mu g\,L^{-1}$ for OFL; 0.52–0.59 $\mu g\,L^{-1}$ for NOR; 0.19–0.21 $\mu g\,L^{-1}$ for CIP and 0.12–0.15 $\mu g\,L^{-1}$ for ENR. The linear dynamic range (LDR) was 0.5–500 $\mu g\,L^{-1}$ for FLE, 3–500 $\mu g\,L^{-1}$ for OFL, 2–500 $\mu g\,L^{-1}$ for NOR, 1–500 $\mu g\,L^{-1}$ for CIP and 0.5–500 $\mu g\,L^{-1}$ for ENR. The precision study was carried out in six parallel experiments by determining the intra- and inter-day RSDs (relative standard deviations) at three fortified FQ concentrations (high = 20 $\mu g\,L^{-1}$, medium = 10 $\mu g\,L^{-1}$ and low = 5 $\mu g\,L^{-1}$). The RSDs varied between 0.89% and 5.02% for intra-day analysis, and ranged from 1.86% to 6.52% for inter-day analysis.

3.5. Analysis of plasma and urine samples

The PS-USLM method was applied for the determination of five FQs in plasma and urine samples. Fig. 5 illustrates a typical FQ chromatogram for plasma and urine samples at a fortification level of 10 $\mu g\,L^{-1}$ using the optimized PS-USLM method. The results showed that the concentrations of FLE, OFL, NOR and CIP were all below their respective detectable level in the blank plasma and urine samples. For the three spiked levels (5, 10 and 20 $\mu g\,L^{-1}$), the RRs for the five FQs were in the range of 92.6–108.8% for plasma and 92.8–110.9% for urine (Table 5). In summary, the optimized PS-USLM method can be effectively used to analyze trace levels of FQs in plasma and urine samples with high precision and accuracy.

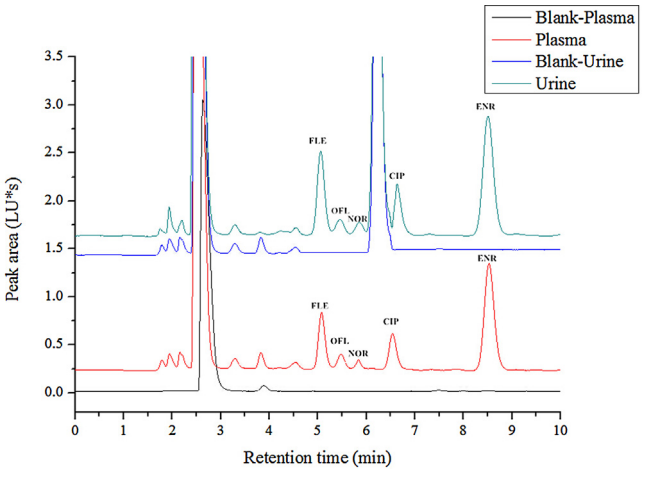

Fig. 5. Chromatogram of analytes obtained by the proposed PS-USLM-HPLC method under optimized conditions. *Note*: (1) FLE; (2) OFL; (3) NOR; (4) CIP; (5) ENR. Experimental conditions: (a) plasma and urine samples were fortified with FQs at 10 $\mu g\,L^{-1}$; (b) extraction solvent volume = 945 μL, pH 2.1, extraction time = 4.1 min, Na_2SO_4 = 2.5 g, and extraction temperature = 25 °C.

3.6. Merits of the integrated extraction device

In traditional microextraction procedure, the extraction solvent (acetone in this investigation) and aqueous phase are not separated prior to retrieving of the extraction solvent. The retrieving of extraction solvent requires about 3 min, in which 5–10% of the extraction solvent will be resolved into the aqueous solution, leading to a similar percentage of recovery loss. The above analysis was proved by our preliminary experiment, and the recovery was about 80% if we did not use the integrated extraction device. Wang et al. [19] developed a new device, which consisted of a cut polyethylene dropper and a sample vial, to perform extraction, separation and concentration of trace pesticides from solvents. However, the new device cannot separate extraction solvent and aqueous phase prior to retrieving the extractant, and the mean recovery is 79.4%, which is obvious less than that (92.6%) obtained in this study. These results showed that our proposed integrated extraction device can help to eliminate repartition of extractant into aqueous phase during collection, decrease organic-collected time and improve extraction recovery.

3.7. Comparison of PS-USLM with other pretreatment methods

The PS-USLM method developed and optimized in this study was compared with other previously reported methods, such as liquid–liquid extraction combined with HPLC (LLE-HPLC) [27], solid-phase extraction combined with HPLC (SPE-HPLC) [29], second-order multivariate calibration combined with HPLC (SOMC-HPLC) [30], single-step precipitation cleanup method combined with HPLC (SSPC-HPLC) [31], direct ultra-fast liquid chromatography (UFLC) [32], and hollow fiber-based liquid phase microextraction combined with HPLC (HF-LPME-HPLC) [33]. The results were compared with reference to sample preparation time, LOD, RR, extraction solvent and injection method. The sample preparation time (~11.1 min) for PS-USLM-HPLC was much shorter than those of SPE-HPLC (38 min), SSPC-HPLC (21 min), direct UFLC (33 min) and HF-LPME-HPLC (5.5 h), while it was comparable with those of direct LLE-HPLC (12 min) (Table 6) [27]. The LODs (0.12–0.67 $\mu g\,L^{-1}$) for our PS-USLM-HPLC method were lower than those of LLE-HPLC, SPE-HPLC, SOMC-HPLC, SSPC-HPLC, UFLC and HF-LPME-HPLC. The RRs of PS-USLM-HPLC (92.6–110.9%) were higher or comparable with the other referenced methods (100.3–103.5%). Less volume of extraction solvent (acetone, <1 mL) was required in our PS-USLM method when compared with the other methods. Similar acidic conditions (pH 2.1) to the PS-USLM procedure were also required in the other methods (SPE-, SOME-, and SSPC-HPLC-FLD; pH 3.0–3.5). Additionally, the PS-USLM-HPLC method gave higher precision with RRs very close to 100%. The higher precision can be explained by low repartitioning of extractant into the aqueous phase during collection as a result of complete

Table 5
The relative recoveries of FQs by the PS-USLM-HPLC method in plasma and urine samples.

FQs	Plasma (n = 6, mean ± SD)				Urine (n = 6, mean ± SD)			
	Blank	Added ($\mu g\,L^{-1}$)	Found ($\mu g\,L^{-1}$)	RR (%)	Blank	Added ($\mu g\,L^{-1}$)	Found ($\mu g\,L^{-1}$)	RR (%)
FLE	ND	5	4.63 ± 0.58	92.6	ND	5	5.28 ± 0.49	105.6
	ND	10	9.31 ± 0.66	93.1	ND	10	9.79 ± 0.72	97.9
	ND	20	21.09 ± 0.72	105.4	ND	20	19.41 ± 0.69	97.0
OFL	ND	5	5.06 ± 0.22	101.2	ND	5	20.28 ± 0.35	101.4
	ND	10	10.64 ± 0.76	106.4	ND	10	10.19 ± 0.32	101.9
	ND	20	18.96 ± 0.49	94.8	ND	20	19.65 ± 0.58	98.2
NOR	ND	5	5.54 ± 0.21	108.8	ND	5	5.12 ± 0.38	102.4
	ND	10	9.26 ± 0.34	92.6	ND	10	10.13 ± 0.44	101.3
	ND	20	21.02 ± 0.63	105.1	ND	20	20.17 ± 0.63	100.8
CIP	ND	5	4.76 ± 0.39	95.2	ND	5	4.84 ± 0.29	92.8
	ND	10	9.52 ± 0.57	95.3	ND	10	10.03 ± 0.59	100.3
	ND	20	19.22 ± 0.44	96.1	ND	20	19.72 ± 0.61	98.6
ENR	ND	5	5.25 ± 0.42	105.0	ND	5	4.86 ± 0.33	97.2
	ND	10	10.22 ± 0.67	102.2	ND	10	11.09 ± 0.59	110.9
	ND	20	20.47 ± 0.58	102.4	ND	20	20.18 ± 0.68	100.9

Note: RR indicates the relative recovery.

Table 6
Comparison of the PS-USLM-HPLC method with others for determination of FQs in biological fluid samples.

Methods	Matrices	FQs	Extraction time (min)	LOD ($\mu g\,L^{-1}$)	RR (%)	References	Extraction solvent	pH	Injection
Direct LLE-HPLC-FLD	Plasma	LEV, CIP	12	12.5–50	70.3–122.6	[32]	Methanol		Direct injection
SPE-HPLC-FLD	Serum	ENO, OFL, ENR, NOR	38	9.8–300		[34]	Tetrahydro-furan	3.0	Direct injection
	Urine	ENO, OFL, ENR, NOR	38	9.8–300		[34]			
SOMC-HPLC-FLD	Urine	PIP, MAR, CIP, ENR, OFL, NOR, LOM, DAN		2–8		[35]		3.5	Direct injection
SSPC-HPLC-FLD	Porcine plasma	ENR, CIP, DAN	21	1–8	86.5–106	[36]	Phosphate buffer-acetonitrile (82:18)	3.0	Direct injection
UFLC-FLD	Rat plasma	SIN	33	2	100.3–103.5	[37]	Acetonitrile		Direct injection
HF-LPME-HPLC-FLD	Bovine urine	MRB, NRF, CRP, DNF, ENR, GTF, GRP, FLM	5.5 h			[38]	1-Octanol	7	Direct injection
PS-USLM/HPLC-FLD	Plasma	FLE, OFL, NOR, CIP, ENR	11.1	0.12–0.66	92.6–108.8	This work	Acetone	2.1	Nitrogen-flow dry
	Urine	FLE, OFL, NOR, CIP, ENR	11.1	0.15–0.67	92.8–110.9	This work			Dissolved injection

Note: (1) Direct LLE-HPLC-FLD indicates liquid–liquid extraction combined with high performance liquid chromatography equipped with FLD detector. (2) SPE-HPLC indicates solid-phase extraction combined with high performance liquid chromatography equipped with FLD detector. (3) SOMC-HPLC-FLD indicates second-order multivariate calibration combined with high performance liquid chromatography equipped with FLD detector. (4) SSPC-HPLC-FLD indicates single-step precipitation cleanup method combined with high performance liquid chromatography equipped with FLD detector. (5) UFLC-FLD indicates ultra fast liquid chromatography combined equipped with FLD detector. (6) HF-LPME-HPLC-FLD indicates hollow fiber-based liquid phase microextraction combined with high performance liquid chromatography equipped with FLD detector. (7) PS-USLM/HPLC-FLD indicates phase separation based ultrasound-assisted salt-induced liquid–liquid microextraction combined with high performance liquid chromatography equipped with FLD detector.

separation of extractant from the aqueous solution prior to collection.

4. Conclusion

The novel integrated device can be used to separate the extraction solvent from the aqueous phase prior to retrieving the extractant, and it reduces repartitioning of extractant into the aqueous phase during collection, decreases organic phase-collection time and improves extraction efficiency. The proposed PS-USLM-HPLC method has several advantages: high extraction efficiency, easy construction with inexpensive HDPE materials, laboratory accessibility, short extraction time, and compatible for subsequent HPLC analysis. Using the response surface method based on central composite design, the optimal conditions consisted of 945 μL acetone extraction solvent, pH 2.1, 4.1 min stir time, 5.9 g Na_2SO_4, and 4.0 min centrifugation. At the three spiked levels (5, 10 and 20 $\mu g\,L^{-1}$), the RRs for the five FQs were in the range of 92.6–108.8% for plasma and 92.8–110.9% for urine. The low LODs (0.12–0.67 $\mu g\,L^{-1}$) were also acquired in plasma and urine samples. The RSDs of the proposed method varied between 0.89% and 5.02% for intra-day analysis, and ranged from 1.86% to 6.52% for inter-day

analysis. The PS-USLM method has excellent prospects for sample pretreatment and quantification of trace levels of FQs in biological samples.

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