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Gao, Ming
Xu, Youqu
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A phase separation method for analyses of fluoroquinones in meats based on ultrasound-assisted salt-induced liquid–liquid microextraction and a new integrated device

Huili Wang [a], Ming Gao [b,1], Youqu Xu [b], Wenwei Wang [b], Lian Zheng [b], Randy A. Dahlgren [c], Xuedong Wang [b,*]

[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 in meats by HPLC analysis. The novel integrated device consisted of three simple HDPE (high density polyethylene) parts that were used to separate the solvent from the aqueous solution prior to retrieving the extractant. The extraction parameters were optimized using the response surface method based on central composite design: 589 μL of acetone solvent, pH 2.1, 4.1 min extraction time and 3.5 g of Na_2SO_4. The limits of detection were 0.056–0.64 μg kg^{-1} and recoveries were 87.2–110.6% for the five fluoroquinones in muscle tissue from fish, chicken, pork and beef. This method is easily constructed from inexpensive materials, extraction efficiency is high, and the approach is compatible with HPLC analysis. Thus, it has excellent prospects for sample pre-treatment and analysis of fluoroquinones in meat samples.

1. Introduction

Fluoroquinolones (FQs) are widely used as antibacterial agents in human and veterinary medicines due to their broad spectrum activity against both Gram-positive and Gram-negative bacteria through inhibition of DNA gyrase (Gao et al., 2011). Fleroxacin (FLE), ofloxacin (OFL), norfloxacin (NOR) and ciprofloxacin (CIP) are third-generation FQs used in treating human and animal diseases, while enrofloxacin (ENR) is used only for treating animal diseases. With the overuse of these FQs in animal husbandry and aquaculture, they are widely detected in all kinds of matrices, especially in meat such as fish (Huet et al., 2008; Huet, Charlier, Weigel, Godefroy, & Delahaut, 2009), chicken (He, Lv, Yu, & Feng, 2010; Lee, Kim, & Kim, 2013; Tian et al., 2014), pork (Li et al., 2009; Lee et al., 2013) and beef (Lee et al., 2013; Sheng et al., 2009). Because of the complex matrix interferences in meat, the previously reported analytical methods often require extensive sample preparation (Vazquez, Vazquez, Galera, & Garcia, 2012; Ebrahimpour, Yamini, & Moradi, 2012). Accordingly, there is considerable interest in developing a cost-effective, efficient and reliable extraction method for the analysis of complex samples prior to FQ quantification.

In recent years, some novel liquid-phase microextraction (LPME) techniques have been developed such as dispersive liquid–liquid microextraction (DLLME), ionic liquid-based homogeneous liquid–liquid microextraction (IL-HLLME) (Gao et al., 2011) and ion pair-based surfactant-assisted microextraction (IP-SAME) (Ebrahimpour et al., 2012). However, a major drawback for the use of non-polar, water-immiscible, organic solvents in all types of LPME is their low dielectric constant, making extraction of polar solutes relatively poor (Gupta, Archana, & Verma, 2009). More polar solvents, such as acetonitrile and ethanol, which provide solubility for polar to non-polar compounds, are frequently water-miscible and thus can't be used in conventional LPME. Salting-out is a process of electrolyte addition to an aqueous phase in order to increase the distribution ratio of a particular solute. The term also connotes reduction of mutual miscibility of two liquids by addition of electrolytes. Weak intermolecular forces, e.g., hydrogen bonds, between organic molecules or non-electrolytes and water are easily disrupted by the hydration of electrolytes. Salting-out assisted liquid–liquid microextraction (SALLME) is based on phase separation of water-miscible organic solvents from the aqueous solutions at high salt concentrations (Tsai et al., 2009). It uses water-miscible organic solvents that, generally, have low toxicity and small amounts of salt that cause little environmental pollution. Additionally, this method has the advantages of simplicity and sensitivity and uses less solvents, and the product is compatible for subsequent

* Corresponding author. Tel.: +86 577 86689733; fax: +86 577 86699122.
E-mail address: zjuwxd@163.com (X. Wang).
[1] Co-first author.

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analysis by HPLC (Myasein, Kim, Zhang, Wu, & Tawakol, 2009; Cai et al., 2007). In SALLME, a glass centrifuge tube is often used as the extraction device. However, collection and measurement of microliter volumes of organic phase are difficult because the thin layer of extract is difficult to retrieve from the wide diameter glass tube increasing extraction time. To solve the above-mentioned problem, a few approaches have been reported for introducing extraction apparatuses into the microextraction process that allows for the use of low-density solvent, either by using a narrow-necked tube (Ye, Zhou, & Wang, 2007) or by using a sample vial (Cheng, Matsadiq, Liu, Zhou, & Chen, 2011). Two narrow open necks were specially designed to be equipped in a round-bottom flask, among which one had a capillary tip making the collection step more convenient (Zhang, Shi, Yu, & Feng, 2011). Chen et al. utilized a plunger plug to push the upper layer solvent into a capillary tip, which made the final collection step rapid (Chen, Liu, Lin, Ponnusamy, & Jen, 2013). However, all of the glass apparatuses used to collect the low-density extractant have some prominent drawbacks. For example, the narrow-necked glass tube is easily broken, requires special design and is costly. Consequently, there is limited commercial availability for these specially designed glass tip tubes (Wang, Cheng, Zhou, Wang, & Cheng, 2013).

In recent years, many researchers used a polyethylene plastic tube as an extraction device for extracting low-density solvents in the microextraction procedure (Hu, Wu, & Feng, 2010; Guo & Lee, 2011). A polyethylene dropper and a sample vial were integrated to conduct microextraction of organic pollutants in a single step (Cheng et al., 2011). The plastic tube has advantages of low cost, use of easily available materials, ease of operation and avoidance of carryover problems (Wang et al., 2013). However, the major drawback of this device is that the organic phase was difficult to completely retrieve because the organic phase and aqueous solution were not separated prior to the collection of the extractant. The repartitioning of the extractant into the aqueous phase may occur over a long retrieval time, which will possibly result in low extraction efficiency.

To overcome the above-mentioned limitations of current methods, this study developed and optimized a novel integrated device and methodology for extraction of FQs by means of a phase separation method based on ultrasound-assisted, salt-induced, liquid–liquid microextraction (PS-USLM). The proposed PS-USLM method was optimized for major operational factors (extraction time, pH, salt kind and volume, solvent kind 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 fish, chicken, pork and beef. 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 meat.

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 Corporation (Shanghai, China) and used when received. The chemical structures and molecular weight of the five FQs are shown in Supplementary Fig. 1, and they have a common 4-oxo-1,4-dihydroquinoline skeleton, where the pharmacophore unit consists of a pyridine ring with a carboxyl group, a piperazinyl group and a fluorine atom placed at positions 3, 6 and 7 (Gajda, Posyniak, Zmudzki, Gbylik, & Bladek, 2012). HPLC-grade ethanol, methanol, ethyl acetate, acetonitrile and acetone were sourced from Merck Corporation (Shanghai, China). Salts (magnesium sulfate ($MgSO_4$), sodium sulfate (Na_2SO_4), ammonium sulfate ($(NH_4)_2SO_4$) and ammonium acetate (CH_3COONH_4)) with purities ≥99% were obtained from Aladdin Industrial Co. Ltd. (Shanghai, China).

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 by dilution with Milli-Q ultrapure water (Millipore, Bedford, USA).

Fish, chicken, pork and beef muscles (the meat of fish body on both sides, chicken breast tenderloin, pork fillet and beef sirloin) were purchased from local markets in Wenzhou, China. In order to increase the representativeness of the meat samples, we purchased three batches of meat samples on July 14th, 20th and 25th, 2014 in the three local markets. These samples were ground and stored at −20 °C until analysis within one week.

2.2. Preparation of meat samples

Fortified samples of fish, chicken, pork and beef were prepared by adding the appropriate volumes of the mixed standards to ground muscle tissues. Prior to sample treatment and analysis, all samples were stirred and allowed to stand in the dark for 30 min at ambient temperature to permit full interaction between the antibiotics and muscle tissue. All samples were prepared in triplicate.

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 (15:5:80, 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 stirred with a model HJ-6A magnetic heater–stirrer with an 8 mm × 4 mm stir bar (Jiangsu Jintan Medical Instrument Factory (Jintan, China)). Centrifugation used a model TDL-50C centrifuge from Anting Instrument Factory (Shanghai, 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 parts: (1) a high-density polyethylene (HDPE) centrifuge tube (8 cm × 1.6 cm external diameter, 1.4 cm internal diameter, Fig. 1-A); (2) an inverted cut HDPE dropper (1 cm × 1.4 cm external diameter joined to a 3 cm length of capillary tube); and (3) a "V" HDPE capillary tube (10 cm × 0.5 cm internal diameter). 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. 1-G and H).

In the operation, the sample solution was first added to the centrifuge tube followed by the *n*-hexane and extraction solvent, which was water-miscible and had density lower than that of water. After centrifugation, the sedimented proteins, floating fat and other interfering compounds were discarded (Fig. 1-A and B). After that, an appropriate amount of salt was added to the remaining solution (Fig. 1-C). After the salting-out process, the extraction solvent floated on the top of the sample solution following ultrasound and centrifugation (Fig. 1-D–E) (extraction solvent, Fig. 1-E-1; Sample solution, Fig. 1-E-2; Undissolved salt, Fig. 1-E-3). The inverted HDPE dropper was then placed into the sample solution and the extractant was extruded through the tip of the dropper (Fig. 1-F–G). When the extractant was fully transferred into the "V" tube, the "V" tube was detached and the extractant was collected with a microsyringe (Fig. 1-H). The extractant was then dried

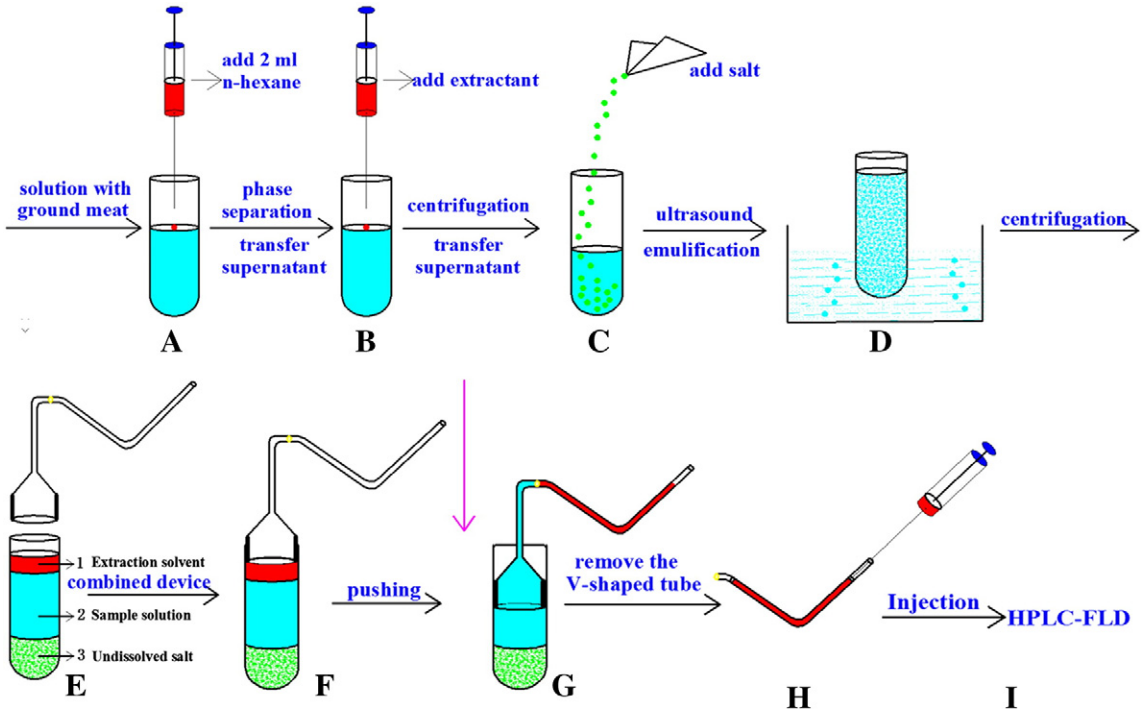

Fig. 1. The integrated device and schematic procedures of the PS-USLM method. *Note*: Each step in the PS-USLM procedure is described in the text.

using a gentle nitrogen flow, dissolved with 50 μL of mobile phase and quantified by HPLC-FLD analysis (Fig. 1-I).

For the pre-treatment of meat samples, 2 g of ground fish, chicken, pork and beef samples was placed in 10 mL centrifuge tubes. Each sample was added using ultrapure water to obtain a final volume of 5 mL, and followed by acidification to pH 1.0–3.0 with sulfuric acid. Two milliliters of *n*-hexane was slowly introduced into the sample solution. After 2 min of vortex, the emulsion was centrifuged at 5000 rpm for 2 min, and the sedimented fat impurities were removed (Fig. 2-b). Then, the water-miscible organic solvent (430–770 μL) was slowly introduced into the sample solution with a 1000-μL micropipette. The emulsion was centrifuged at 5000 rpm for 2 min, and subsequently the sedimented protein impurities were removed (Fig. 2-e). An appropriate amount of salt (2.0–5.0 g) was added, followed by ultrasonic extraction for 1.5–6.5 min at 25 °C and centrifugation at 5000 rpm for 3 min. Finally, the extraction solvent was isolated in the top layer of the sample solution and recovered using the inverted dropper as described above.

2.5. Non-PS-USLM procedure

For the pre-treatment of the meat samples, 2 g of ground fish, chicken, pork and beef samples was placed in 10 mL centrifuge tubes. Each sample was added using acetone to obtain a final volume of 2 mL, and after 2 min of vortex, the emulsion was centrifuged at 5000 rpm for 2 min, the acetone was collected with a microsyringe. The acetone was then dried using a gentle nitrogen flow, dissolved with 50 μL of mobile phase and quantified by HPLC-FLD analysis.

2.6. Experimental design

To decrease the influence of uncontrolled factors, a randomized experimental design was employed. Because there were too many treatments to complete in a working day, we divided these treatments into two blocks and completed each batch of experiments in two sequential days (Sereshti, Izadmanesh, & Samadi, 2011). According to our preliminary experiments, the most sensitive parameters for the PS-USLM procedure were solvent volume (A), pH (B), extraction time (C) and weight of salt (D). An orthogonal design was used to set high and low points for each of the analyzed variables (Supplementary Table 1). Extraction recovery (ER) was recognized as the response parameter to assess/

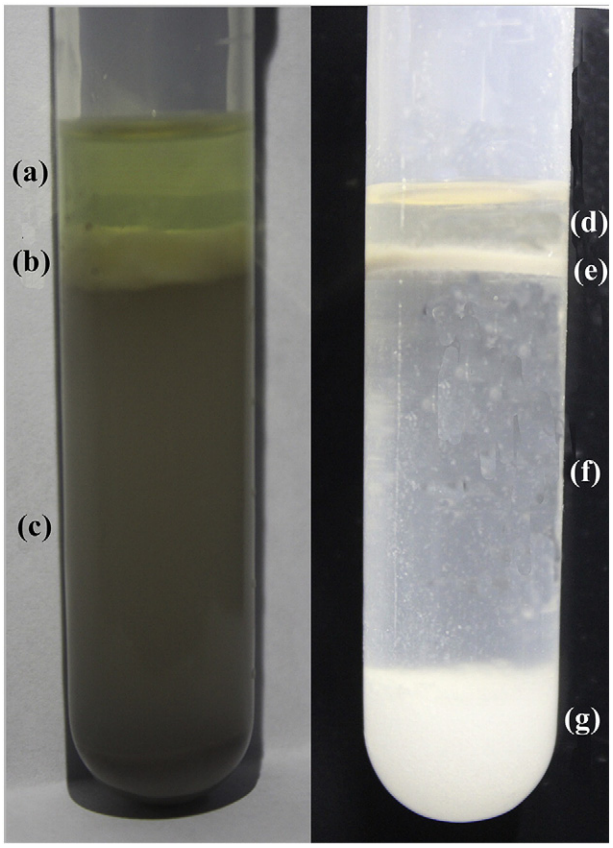

Fig. 2. The profiles of the meat samples after PS-USLM procedures. *Note*: (a) *n*-hexane; (b) fat; (c) sample solution with ground meat; (d) extraction solvent; (e) protein; (f) sample solution and (g) undissolved salt.

optimize each factor. To determine the best response conditions and decrease the number of treatments, a small CCD, one of the most common response surface designs, was employed. According to CCD, 22 treatments were adopted and graded into five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) for the four variables (Supplementary Table 2). Several superimposed designs were constructed to comprise a half-fraction factorial design ($N_f = 2^{f-1}$). A set of center points (N_0) were augmented with a group of "star points" ($N_\alpha = 2f$). The "f" parameter was the number of optimized factors (equal to 4 in this investigation). Twenty-two treatments, based on CCD, were composed of 8 half-factorial design points, 8 "star points" and 6 center points, respectively. The mean extraction recovery (ER) was used to assess analytical performance; calculation methods followed Sereshti and coworkers (Sereshti, Heravi, & Samadi, 2012). A quadratic polynomial model equation was used to predict the response of dependent variables for the ERs of FQs:

$$Y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{ij=1(i\neq j)}^{6} b_{ij} x_i x_j + \sum_{i=1}^{4} b_{ii} x_i^2$$

where Y is the dependent variable, b_0 is the intercept, b_i is the coefficient of linear effect, x_i is the independent variable, b_{ij} is the coefficient of interaction effect, and b_{ii} is the coefficient of the squared effect (Mohammadi et al., 2013). The software Design-Expert 8.0.5 (Minneapolis, USA) was employed to design the experimental scheme, and to draw the 3D response surface and contour plot. Analysis of variance (ANOVA) was applied for appraising the goodness-of-fit for the model and for obtaining the maximum ERs of FQs.

3. Results and discussion

3.1. Selection of solvent and salt

In the PS-USLM procedures, an appropriate solvent must meet 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. After considering these requirements, ethyl acetate, ethanol, methanol, acetonitrile and acetone were examined for their "salting-out" phenomena and extraction efficiencies for FQs (Fig. 3). Using a 5 mL sample and a 0.6 mL solvent, we examined the salting-out effect of 3.5 g of four salts ($MgSO_4$, Na_2SO_4, CH_3COONH_4 and $(NH_4)_2SO_4$). The methanol–water mixture did not show any phase separation even when the mixture was saturated with salts. Additionally, ethyl acetate and ethanol showed indistinct phase separation even after centrifugation. In contrast, the water–acetonitrile and water–acetone mixtures gave a clear separation in the presence of all four salts under the above conditions. Similarly, the volume of organic solvent-rich phase/water-rich phase after separation was 0.5/5.6 mL for water–acetonitrile and water–acetone. The highest ER was observed in water/acetone/Na_2SO_4 (93.7 ± 2.8%), followed by water/acetone/$(NH_4)_2SO_4$ (80.8 ± 2.4%) and water/acetonitrile/$(NH_4)_2SO_4$ (79.5 ± 3.5%) (Fig. 3, n = 6). As a result, acetone and Na_2SO_4 were chosen in subsequent experiments.

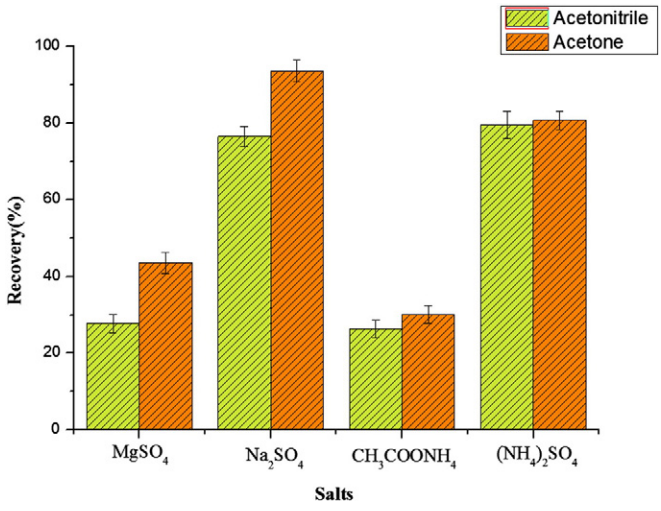

Fig. 3. Effect of extraction solvent and salt on the average extraction recovery of the five FQs. Experimental conditions: pH of 2.1, extraction time of 4.1 min and extraction temperature of 25 °C.

3.2. Optimization of the extraction temperature

A series of temperatures, from 15 to 65 °C at an interval of 10 °C, was set in the process of ultrasonic extraction to analyze the effects of temperature. As shown in Supplementary Fig. 2, the ERs (n = 6) increased from 80.3% to 96.0% with increasing extraction temperatures from 15 to 25 °C. However, with a further increase of temperature from 25 to 45 °C, the ER remained nearly constant (~94%), followed by a reduction from 55 to 65 °C. The above phenomenon may be explained by increasing temperatures resulting in the FQs becoming more and more unstable with possible degradation at higher temperatures. Thus, the ambient temperature (25 °C) was selected in this investigation.

3.3. Optimization of the PS-USLM procedure using CCD

The experimental design matrix consisting of the experiments (number and order), factors and extraction recoveries is summarized in Supplementary Table 2. The significance of the model equation and related terms was evaluated using ANOVA (Supplementary Table 3). The resulting model was highly significant and the "probe > F" value for the "lack-of-fit component" was 0.4039 indicating that other factors had a small amount of interference and the model accurately describes the data. The model equation showed a strong fit between ER and the four main factors with a "probe > F" value less than 0.0001. The goodness of fit for the polynomial model (R^2) was 0.9969 and represents the amount of variance explained by the model. Further, the adjusted-R^2 value was 0.9998; this value adjusts for the number of terms in the model and accounts for possible over-parameterization of the model (Sereshti et al., 2012). The high R^2 values support the efficacy of the model for analyzing and optimizing the effects of the various factors on ER. As seen in Supplementary Fig. 3a, the majority of the data points lie close to the regression line indicating a strong correlation between predicted and observed data. Residual plots demonstrated random scatter (Supplementary Fig. 3b) indicating similar variance of experimental measurements across the range of Y values.

To further probe the effects of the various experimental factors for optimization of extraction recovery, 3D response surfaces and contour lines were generated. These resulting plots evaluate the relationship between ER and the levels of two experimental factors simultaneously, while fixing other experimental factors at their central levels (Sereshti et al., 2011). Fig. 4 shows the relationship between ER and the four experimental factors of solvent volume, pH, extraction time and mass of salt. Specifically, Fig. 4A describes the 3D response surface and contour line for the effect of extraction solvent volume and pH on ER when extraction time (4.0 min) and salt mass (3.5 g Na_2SO_4) were held constant. The ERs of FQs increased with increasing extraction solvent volume from 430 to 589 μL and with increasing pH from 1.0 to 2.1. However, with a further increase in extraction solvent volume from 589 to 770 μL and pH from 2.1 to 3.0, the ERs of FQs declined. Similarly, Fig. 4B depicts the relationship for the effect of extraction solvent volume and salt mass on ER when the pH (2.0) and extraction time (4.0 min) were fixed. The maximum ER was observed at 589 μL of solvent and 3.5 g of Na_2SO_4. With further increases in extraction solvent volume (589–770 μL) and salt mass (3.5–5.0 g), the ERs decreased

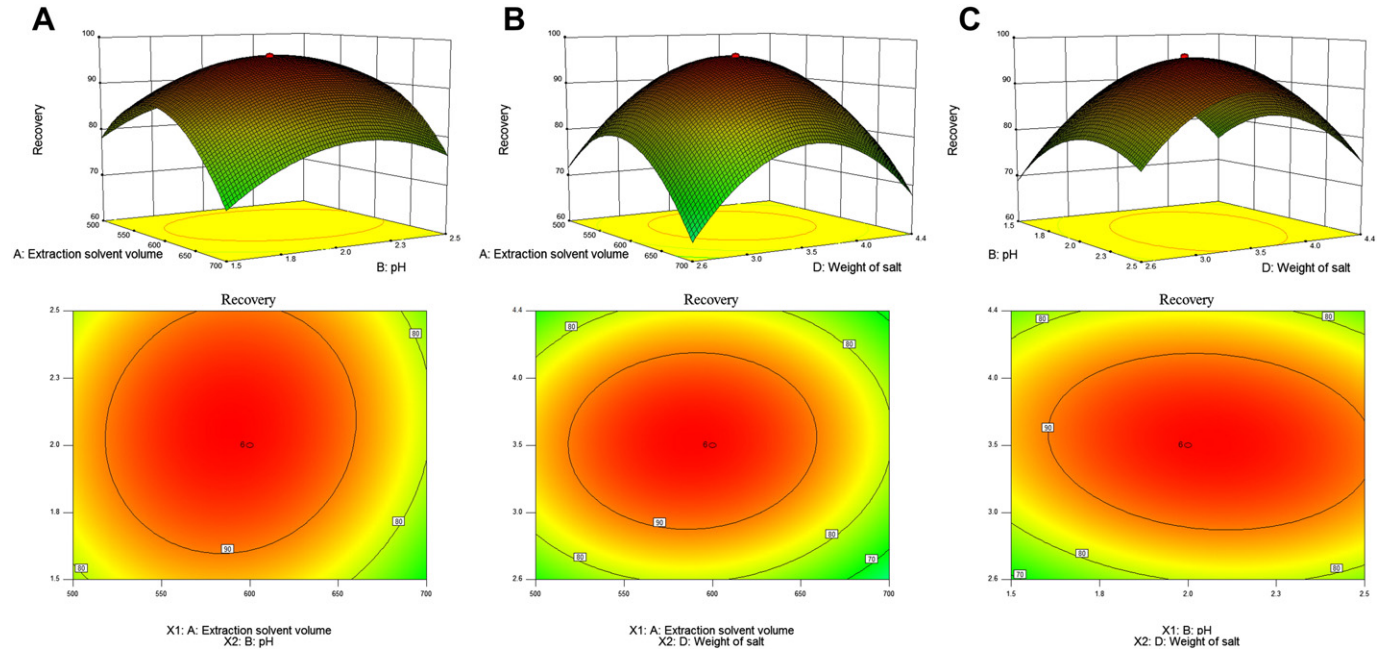

Fig. 4. The response surface and contour plots for the different factors. *Note*: (A) 3D response surface and contour plots for the extraction solvent volume and pH at a constant extraction time of 4.0 min and 3.5 g weight of salt on the average extraction recovery, (B) 3D response surface and contour plots for the extraction solvent volume and the weight of salt at a constant pH of 2.0 and exaction time of 4.0 min on the average extraction recovery, (C) 3D response surface and contour plots for the pH and the weight of salt at constant extraction volume of 600 μL and exaction time of 4.0 min on the average extraction recovery.

sharply. Finally, Fig. 4C demonstrates the effect of pH and salt mass on the ERs when the extraction volume (600 μL) and extraction time (4.0 min) were held constant. When the pH increased from 1.0 to 2.1 and the salt mass increased from 2.0 to 3.5 g, the ERs gradually increased. The maximum ER was observed at approximately a pH of 2.1 and salt mass of 3.5 g. After this rigorous analysis of the interaction among factors, the optimal set points for the four parameters were determined to be: solvent volume = 589 μL, pH = 2.1, extraction time = 4.1 min and mass of salt = 3.5 g (Na_2SO_4).

After the CCD optimization experiment, a comparison was made between PS-USLM and non-PS-USLM treatments to evaluate their performance for extraction of the five FQs from the meat samples (Supplementary Fig. 4). The extraction yield for the five FQs using the non-PS-USLM treatment was very low for OFL and NOR (less than detection limit) (Supplementary Fig. 4a), and the chromatograms for FLE, CIP and ENR were of poor quality preventing accurate quantification of these compounds. Thus, many impurities interfered with the FQ peaks in the chromatogram of the non-PS-USLM treatment limiting its use for detection of low concentrations of FQs in the meat samples In contrast, high extraction efficiencies were obtained with the PS-USLM treatment and chromatographic interferences were not evident in PS-US treated samples (Supplementary Fig. 4).

3.4. Method evaluation

Under the optimal conditions determined in this study, the performance of PS-USLM was evaluated for regression equations, correlation coefficients (R^2), linear range, limits of detection (LODs) and limits of quantity (LOQ) (Table 1). The coefficients of determination (R^2) for linearity of standard curves for the five FQs were in the range of 0.9980–

Table 1
The analytical performance of the PS-USLM–HPLC method.

Sample	Analytes	Regression equations	Correlation coefficients (R^2)	Linear range (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Fish	FLE	y = 0.0775x + 0.0434	0.9992	0.50–250	0.148	0.493
	OFL	y = 0.0270x + 0.0064	0.9992	2.00–500	0.472	1.574
	NOR	y = 0.0142x + 0.0552	0.9992	2.50–500	0.640	2.134
	CIP	y = 0.0552x + 0.0678	0.9988	1.00–250	0.196	0.653
	ENR	y = 0.1489x + 0.6504	0.9992	0.50–250	0.084	0.281
Chicken	FLE	y = 0.0804x + 0.0374	0.9988	0.50–250	0.109	0.362
	OFL	y = 0.0234x + 0.0243	0.9980	1.50–500	0.332	1.107
	NOR	y = 0.0143x + 0.0491	0.9988	2.00–500	0.509	1.697
	CIP	y = 0.0550x + 0.0769	0.9989	1.00–250	0.180	0.599
	ENR	y = 0.1497x + 0.5962	0.9989	0.50–250	0.073	0.242
Pork	FLE	y = 0.0771x + 0.0480	0.9994	0.50–250	0.100	0.333
	OFL	y = 0.0285x + 0.0014	0.9997	1.50–500	0.319	1.063
	NOR	y = 0.0141x + 0.0590	0.9992	2.00–500	0.530	1.767
	CIP	y = 0.0549x + 0.0897	0.9990	1.00–250	0.171	0.570
	ENR	y = 0.1468x + 0.7997	0.9998	0.50–250	0.065	0.217
Beef	FLE	y = 0.0799x + 0.0415	0.9992	0.50–250	0.106	0.355
	OFL	y = 0.0252x + 0.0118	0.9987	1.50–500	0.366	1.220
	NOR	y = 0.0143x + 0.0464	0.9987	1.50–500	0.450	1.502
	CIP	y = 0.0546x + 0.1079	0.9992	1.00–250	0.158	0.527
	ENR	y = 0.1507x + 0.5213	0.9985	0.50–250	0.056	0.187

Table 2
Intra-day and inter-day precision of the five FQs by PS-USLM–HPLC method.

Analytes	Intra-day precision (RSD%, n = 6)			Inter-day precision (RSD%, n = 6)		
	Low	Medium	High	Low	Medium	High
FLE	6.45	5.02	2.26	6.89	5.65	4.02
OFL	5.61	4.64	2.76	5.83	3.96	3.30
NOR	3.79	3.86	2.08	4.85	4.06	3.15
CIP	5.61	4.95	4.13	3.39	2.55	2.19
ENR	4.45	2.55	1.14	5.84	3.91	1.80

0.9998. The limits of detection (LODs at S/N = 3) for the fish, chicken, pork and beef samples were in the range of 0.11–0.15 μg kg^{-1} for FLE; 0.32–0.47 μg kg^{-1} for OFL; 0.45–0.64 μg kg^{-1} for NOR; 0.16–0.20 μg kg^{-1} for CIP and 0.06–0.08 μg kg^{-1} for ENR. The linear dynamic range (LDR) was 0.50–250 μg kg^{-1} for FLE and ENR, 1.50–500 μg kg^{-1} for OFL and NOR, and 1.00–250 μg kg^{-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 fortification levels of FQs. The RSDs varied between 1.1% and 6.5% for intra-day analysis and ranged from 1.8% to 6.9% for inter-day analysis (Table 2).

3.5. Analysis of meat

The PS-USLM method was applied for the determination of five FQs in the fish, chicken, pork and beef samples. Fig. 5 illustrates typical chromatograms for the fish, chicken, pork and beef samples at fortification levels of 10 μg kg^{-1} for the five FQs using the optimized PS-USLM method. The relative recovery (RR) was used to assess the analytical performance of the PS-USLM method as calculated by the method of Wang and coworkers (Wang et al., 2013). We determined FQ concentrations in three batches of blank meat samples (without fortification) and in three batches of fortified meat samples (5, 10 and 20 μg kg^{-1} for low, middle and high fortification levels), collected on July 14th, 20th and 25th, 2014. Considering the data quantity, it was not convenient to list all data in one table. Additionally, we did not calculate the average FQ concentrations for the different batches of meat samples because of FQ differences in different batches of blank samples. As a result, we selected the meat samples, collected in July 14th, 20th and 25th, as the respective blank samples and treatments fortified at 5, 10 and 20 μg kg^{-1} levels, respectively. Such data reflected the representativeness of actual meat samples and the differences between different batches. As listed in Table 3, the concentrations of FLE and OFL in the control samples were all below their respective detectable levels in fish, chicken, pork and beef samples. However, NOR was detected in the blank sample of chicken (0.83 μg kg^{-1}), CIP was detected in chicken and beef (2.01–2.82 μg kg^{-1} in chicken, 0.79–1.24 μg kg^{-1} in beef samples) and ENR was detected in chicken and beef (1.96–3.08 μg kg^{-1} in chicken and 1.31–1.74 μg kg^{-1} in beef samples). For the three fortified levels, the RRs for the five FQs were in the range of 88.2–110.6% for fish, 87.2–107.2% for chicken, 89.4–108.4% for pork and 90.2–102.2% for beef. In total, these results demonstrate that the optimal PS-USLM method can be effectively used to analyze trace levels of FQs in meat with high precision and accuracy.

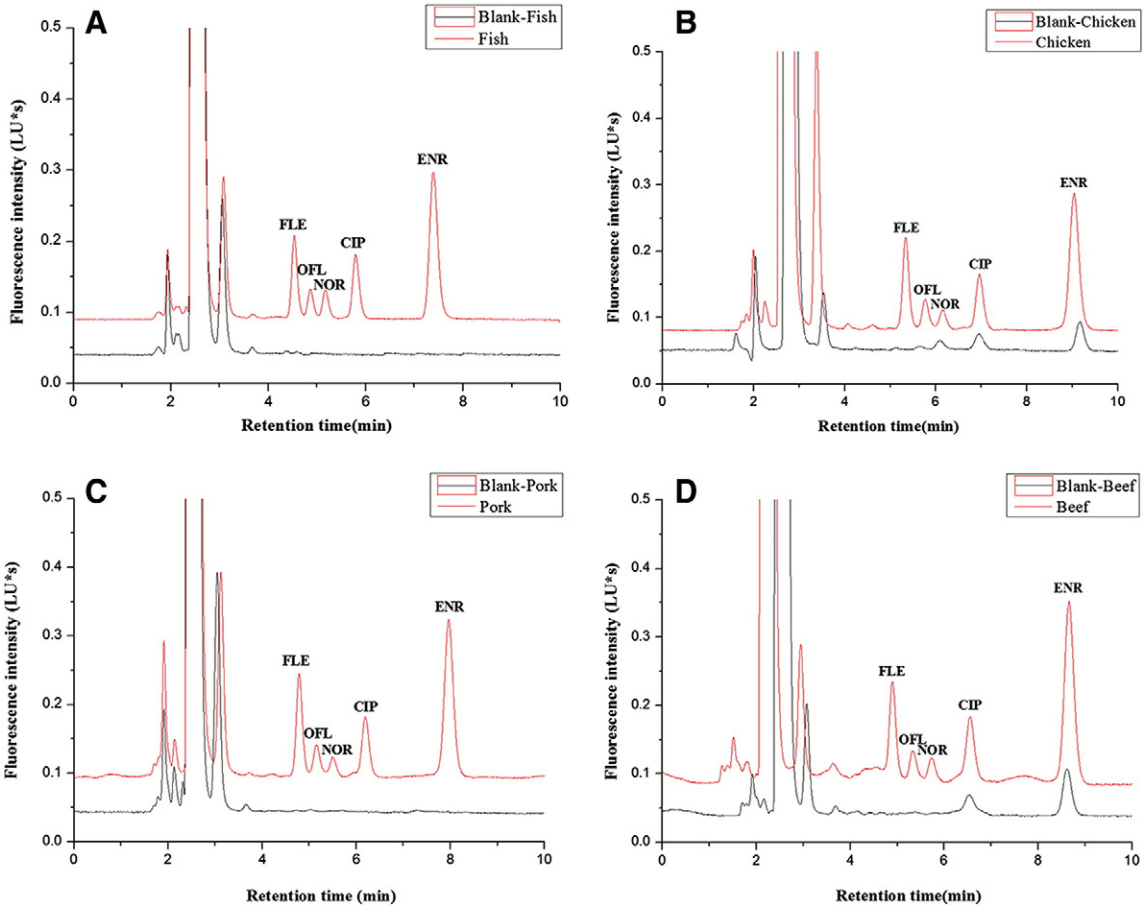

Fig. 5. Chromatograms of analytes obtained by the proposed PS-USLM–HPLC method under optimal conditions. *Note*: Experimental conditions: (1) the meat samples were fortified by FQs at 10 μg L^{-1}; (2) extraction solvent volume of 589 μL, pH of 2.1, extraction time of 4.1 min, weight of Na_2SO_4 of 3.5 g and an extraction temperature of 25 °C.

Table 3
The relative recoveries of FQs by the PS-USLM–HPLC method in the meat samples.

FQs	Fish (n = 6, mean ± SD)				Chicken (n = 6, mean ± SD)				Pork (n = 6, mean ± SD)				Beef (n = 6, mean ± SD)			
	Blank	Added (μg kg^{-1})	Found (μg kg^{-1})	RR (%)	Blank	Added (μg kg^{-1})	Found (μg kg^{-1})	RR (%)	Blank	Added (μg kg^{-1})	Found (μg kg^{-1})	RR (%)	Blank	Added (μg kg^{-1})	Found (μg kg^{-1})	RR (%)
FLE	ND	5	4.41 ± 0.62	88.2	ND	5	5.36 ± 0.45	107.2	ND	5	5.42 ± 0.56	108.4	ND	5	5.11 ± 0.43	102.2
	ND	10	9.69 ± 0.32	96.9	ND	10	9.62 ± 0.58	96.2	ND	10	10.39 ± 0.47	103.9	ND	10	10.05 ± 0.31	100.5
	ND	20	20.88 ± 0.77	104.4	ND	20	19.34 ± 0.71	96.7	ND	20	20.46 ± 0.69	102.3	ND	20	20.24 ± 0.54	101.2
OFL	ND	5	5.22 ± 016	104.4	ND	5	4.87 ± 0.51	97.4	ND	5	4.93 ± 0.26	98.6	ND	5	4.88 ± 0.32	97.6
	ND	10	10.04 ± 0.37	100.4	ND	10	9.39 ± 0.37	93.9	ND	10	9.99 ± 0.31	99.9	ND	10	9.53 ± 0.45	95.3
	ND	20	19.55 ± 0.66	97.8	ND	20	19.26 ± 0.53	96.3	ND	20	20.06 ± 0.24	100.3	ND	20	20.41 ± 0.66	102.0
NOR	ND	5	5.45 ± 0.57	109.1	ND	5	4.36 ± 0.44	87.2	ND	5	4.47 ± 0.39	89.4	ND	5	4.51 ± 0.42	90.2
	ND	10	10.41 ± 0.58	104.1	0.83 ± 0.34	10	10.28 ± 0.56	94.5	ND	10	9.45 ± 0.61	94.5	ND	10	9.31 ± 0.46	93.1
	ND	20	20.98 ± 0.83	104.9	ND	20	18.96 ± 0.67	94.8	ND	20	18.83 ± 0.58	94.2	ND	20	18.69 ± 0.77	93.4
CIP	ND	5	5.53 ± 0.44	110.6	ND	5	4.88 ± 0.31	97.6	ND	5	4.55 ± 0.28	91.0	ND	5	4.54 ± 0.44	90.8
	ND	10	10.38 ± 0.51	103.8	2.82 ± 0.77	10	12.31 ± 0.51	94.9	ND	10	9.62 ± 0.54	96.2	0.79 ± 0.36	10	9.85 ± 0.71	90.6
	ND	20	20.42 ± 0.48	102.1	2.01 ± 0.55	20	22.65 ± 0.58	103.2	ND	20	19.05 ± 0.62	95.2	1.24 ± 0.28	20	19.89 ± 0.38	93.2
ENR	ND	5	5.09 ± 0.43	101.8	ND	5	4.95 ± 0.41	99.0	ND	5	4.81 ± 0.45	96.2	1.31 ± 0.51	5	6.22 ± 0.43	98.2
	ND	10	10.27 ± 0.52	102.7	3.08 ± 0.62	10	12.88 ± 0.57	98.0	ND	10	10.16 ± 0.63	101.6	1.74 ± 0.30	10	11.33 ± 0.29	95.9
	ND	20	18.15 ± 0.66	90.8	1.96 ± 0.51	20	21.04 ± 0.49	95.4	ND	20	19.38 ± 0.57	96.9	ND	20	20.03 ± 0.59	100.2

Note: (1) RR indicates the relative recovery; (2) the blank and added meat samples (5 μg kg^{-1}) were collected on July 14th, 2014; (3) the blank and added meat samples (10 μg kg^{-1}) were collected on July 20th, 2014 and (4) the blank and added meat samples (20 μg kg^{-1}) were collected on July 25th, 2014.

3.6. Merits of the integrated extraction device

In the traditional LPME procedure, the extraction solvent and aqueous phase are not separated prior to retrieving the extraction solvent. According to our preliminary experiment, retrieving the extraction solvent requires about 3 min, and repartitioning of the extraction solvent into the aqueous solution occurs in the retrieval process, leading to a 5–10% loss of recovery. In this investigation, the mean recovery of FQs was about 80% if we did not use the integrated extraction device. Wang et al. integrated a cut plastic tube within a sample vial to carry out the microextraction of pesticides from waters in one step (Wang et al., 2013). Because the new device could not separate the extraction solvent and aqueous phase prior to retrieving the extractant, the mean recovery was 79.4%, which was significantly less (92.6%) than that obtained in this study. In another study, an 8-mL glass tube was specially designed with a self-scaled capillary tip to perform LPME of triclosan in cosmetics. After the extraction, a plunger plug was employed to push the upper layer solvent into a capillary, which resulted in a simple, convenient and rapid collection (Chen et al., 2013). However, the device, developed by Chen and coworkers, can't perform phase separation prior to collection of extractant, and also it is not suitable for centrifugation because of the rubber plunger plug. Therefore, it can only be used for pretreatment of relatively clean liquid matrices, but not for complex biological samples, such as animal meats. In summary, our proposed integrated device can help eliminate repartitioning of extractant into the aqueous phase during collection, decrease the collection time of the

Table 4
Comparison of the PS-USLM–HPLC method with others for determination of FQs in meat samples.

Methods	Matrices	Extraction time (min)	LOD (μg kg^{-1})	RR (%)	References
OBII-SPR	Fish	83	0.30	41–115	Huet et al. (2008)
	Chicken		0.13		
OBII-SPR	Fish	83	0.9^{-}3.7	26–183	Huet et al. (2009)
	Chicken		0.4^{-}1.9		
SPE–HPLC/ESI-MS/MS	Fish	50	0.1	79.7–94.2	Li et al. (2009)
	Pork			75.3–96.3	
PMME–FASS–CE/UV	Chicken	60	2.4–34.0	81.2–100	He et al. (2010)
ID–LC–MS/MS	Chicken	60	0.01–0.1	95	Lee et al. (2013)
	Pork				
	Beef				
LLE–CE–UV	Chicken	145	25–47	81.8–109.9	Tian et al. (2014)
SPE–LC–MS/MS	Pig kidney	2 days	2.17–288	88–115	Toussaint et al. (2005)
ELISA	Chicken	30	1.1	92–105	Sheng et al. (2009)
	Pork		2.1	87–101	
	Beef		1.9	85–101	
PS-USLM/HPLC-FLD	Fish	13.1	0.084–0.64	88.2–110.6	This work
	Chicken		0.073–0.51	87.2–107.2	
	Pork		0.065–0.53	89.4–108.4	
	Beef		0.056–0.45	90.2–102.2	

Note: (1) OBII-SPR indicates optical biosensor inhibition immunoassay based on the surface plasma resonance.
(2) SPE–HPLC/ESI-MS/MS indicates solid phase extraction combined with high-performance liquid chromatography electrospraying ionization tandem mass spectrometry.
(3) PMME–FASS–CE/UV indicates poly monolith microextraction coupled with field-amplified sample stacking and capillary electrophoresis–ultraviolet.
(4) ID–LC–MS/MS indicates isotope dilution–liquid chromatography/tandem mass spectrometry.
(5) Direct LLE–CE/UV indicates liquid–liquid extraction combined with capillary electrophoresis–ultraviolet.
(6) SPE–LC–MS/MS indicates solid phase extraction combined with high-performance liquid chromatography ionization tandem mass spectrometry.
(7) Direct ELISA indicates enzyme-linked immunosorbent assay.

organic phase and improve extraction recovery, and also it is suitable for the pretreatment of complex biological samples.

3.7. Comparison of PS-USLM with other pretreatment methods

The PS-USLM method developed and optimized in this study was compared with other methods from the literature, such as solid-phase extraction (SPE) (Li et al., 2009; Toussaint, Chedin, Vincent, Bordin, & Rodriguez, 2005), polymonolith microextraction (PMME) (He et al., 2010), liquid–liquid extraction (LLE) (Tian et al., 2014) and so on. As summarized in Table 4, the sample preparation time for PS-USLM is much shorter (13.1 min) than those of optical biosensor inhibition immunoassay (OBII) (Huet et al., 2008; Huet et al., 2009) (83 min), SPE (50 min and 2 days), PMME (60 min), isotope dilution (ID) (Lee et al., 2013) (60 min) and LLE (145 min). The LODs for PS-USLM-HPLC-FLD were in the range of 0.056–0.64 μg kg^{-1}, which were comparable with those of MSPE, and lower than those of OBII, SPE, PMME, LLE and enzyme-linked immunosorbent assay (ELISA) (Sheng et al., 2009). The RRs of PS-USLM (87.2–110.6%) were much better than those of other referenced methods (ca. 80–110%), with the exception of ID (95%) and ELISA (85–105%). Additionally, the PS-USLM method gave higher precision with RRs very close to 100%. The higher precision could be explained by low repartitioning of extractant into the aqueous solution during collection as a result of complete separation of extractant from the aqueous phase prior to collection.

4. Conclusion

This study developed a new and simple integrated device for extraction and quantification of five FQs in meat samples. The novel integrated device consisted of three simple HDPE parts that were used to separate the solvent from the aqueous solution prior to retrieving the extractant. This technique reduces repartitioning of extractant into the aqueous phase during collection, decreases organic phase-collection time and improves extraction efficiency. As compared with other methodologies, the PS-USLM method has several advantages, such as high extraction efficiency, easily constructed with inexpensive HDPE materials, laboratory accessibility, short extraction time and compatible for subsequent HPLC analysis. It was successfully applied to determine five FQs with high RRs (87.2–110.6%) and low LODs (0.056–0.64 μg kg^{-1}) in the fish, chicken, pork and beef samples. As a result, the proposed method has excellent prospects for sample pretreatment and quantification of trace levels of FQs in meat.

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