JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Dynamic Microwave-Assisted Extraction Coupled with Salting-Out Liquid–Liquid Extraction for Determination of Steroid Hormones in Fish Tissues

Hui Wang, Xiuqing Zhou, Yiqun Zhang, Haiyan Chen, Guijie Li, Yang Xu, Qi Zhao, Weitao Song, Haiyan Jin, and Lan Ding*

College of Chemistry, Jilin University, 2699 Qianjin Street, Changchun 130012, China

ABSTRACT: In this work, a simple and fast sample pretreatment method was proposed for determination of steroid hormones in fish tissues by coupling dynamic microwave-assisted extraction with salting-out liquid–liquid extraction. The steroid hormones were successively extracted with acetonitrile and water under the action of microwave energy. Subsequently, the extract was separated into an acetonitrile phase and an aqueous phase with ammonium acetate. The acetonitrile phase containing the target analytes was concentrated and determined by LC–MS/MS. The limits of detection for the steroid hormones were in the range of 0.03–0.15 ng g^{-1}. This method was successfully applied to analyze seven kinds of fish tissues, and the recoveries of the steroid hormones for the spiked samples were in the range of 75.3 ± 4.9% to 95.4 ± 6.2%. Compared with the traditional method, the proposed method could reduce the consumption of the organic solvent, shorten the sample preparation time, and increase the sample throughput.

KEYWORDS: *dynamic microwave-assisted extraction, salting-out liquid–liquid extraction, steroid hormones, fish tissue, liquid chromatography–tandem mass spectrometry*

1. INTRODUCTION

Steroid hormones have been widely employed in farming and aquaculture as growth promoters to increase the weight of animals and improve efficiency of feed conversion. However, the use of steroid hormones in animal feeding has been prohibited in many countries including European Union[1] and China [2] due to their toxic effects on public health.

Numerous methods have been developed for determination of steroid hormones in edible tissues such as immunoassay,[3,4] gas chromatography–mass spectrometry (GC–MS),[5,6] and liquid chromatography–MS (LC–MS).[7−10] So far, the most common assay used for monitoring steroid hormones in biological samples is immunoassay which is simple and fast. However, the reliability of the obtained results has been questioned for its lack of specificity.[11] GC–MS is recognized as a highly sensitive and suitable technique for analysis of steroid hormones, but it requires derivatization to reduce the polarity and thermal instability of steroid hormones.[12−17] LC–MS/MS offers a simplified and specific alternative to GC–MS, which could dispense with the need for derivatization.[18−20]

A sample pretreatment procedure including extraction and cleanup plays an essential and crucial role in the whole analytical procedure. The extraction process provides suitable recoveries for analytes, and the cleanup step removes some of the coextracted compounds. In the established methods, steroid hormones were extracted with organic solvent under sonication, homogenization, and microwave irradiation, followed by centrifugation, and then the extract was cleaned up by solid-phase extraction (SPE).[21−23] In recent years, lipophilic sorbent materials such as neutral alumina, neutral silica gel, and Florisil have been employed to remove lipid from biological matrices.[24−26]

Over the past decade, pressurized microwave-assisted extraction (PMAE) has been extensively used for the extraction of steroid hormones in biological and environmental samples.[27−29] The sample preparation time and solvent consumption could be reduced by PMAE; however, some thermolabile steroid hormones would decompose under high-temperature conditions (above 70 or 110 °C).[28,29] In addition, when the extraction was accomplished, the vessels must be cooled to room temperature to avoid the loss of volatile analytes, and the extract should be filtered or centrifuged, for which the overall extraction time was increased.[30] To overcome these weaknesses, dynamic MAE (DMAE) has been developed, which can continuously supply an extraction vessel with fresh extraction solvent, and analytes could be transferred out of the extraction vessel as soon as they were extracted.[31−37] It was especially important to avoid the degradation or contamination of the analytes. Moreover, the extract could be filtered online, and DMAE could be coupled with other sample pretreatment techniques.

Salting-out liquid–liquid extraction (SLLE) is a classic homogeneous liquid–liquid extraction method. An appropriate salting-out agent is added into the sample solution to induce phase separation of organic solvent from the bulk aqueous phase.[38] Compared with conventional LLE, substantial volumes of organic solvents and vigorous mechanical shaking are not required in the SLLE method to obtain higher extraction efficiency. SLLE has been demonstrated to be useful for sample

Received: April 23, 2012
Revised: September 28, 2012
Accepted: September 28, 2012
Published: September 28, 2012

cleanup and analyte enrichment, which was widely applied in HPLC and LC−MS analysis.[39−42]

This paper describes a rapid analytical method based on DMAE−SLLE combined with LC−MS/MS for the determination of nine steroid hormones in fish tissues. The approach combined benefits of DMAE and SLLE technology, and 15 samples could be simultaneously treated. Ammonium acetate, a mass spectrometry friendly salt, was used as the salting-out reagent in SLLE. The main experimental parameters were studied and optimized. The proposed method was employed to analyze seven fish tissues, and the obtained results were compared with those provided by the conventional method.

2. MATERIALS AND METHODS

2.1. Materials and Reagents. Standards of corticosterone (purity >95.0%), estrone (purity >99.0%), estriol (purity >97.0%), medroxyprogesterone (purity >99.0%), progesterone (purity >99.0%), 17-α-estradiol (purity >99.0%), 17-α-hydroxyprogesterone (purity >97.0%), testosterone (purity >99.5%), and 19-nortestosterone (purity >99.5%) were provided from Sigma (St. Louis, MO, USA). The structures of the steroid hormones are shown in Figure 1. Chromatographic grade

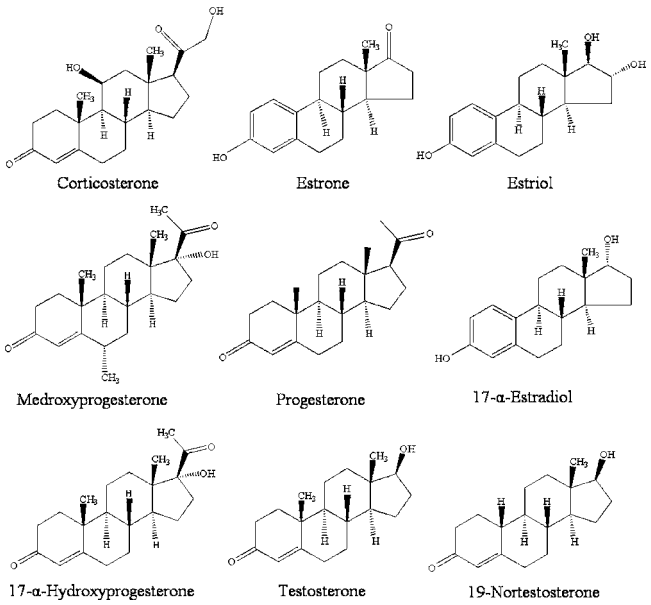

Figure 1. Chemical structures of nine steroid hormones.

acetonitrile was obtained from Fisher (Pittsburgh, PA, USA). Purified water was obtained from a Millipore purification system (Billerica, MA, USA) operating at a resistivity of 18.2 MΩ cm^{-1}. Analytical grade methanol, acetonitrile, ethanol, acetic acid, ammonia−water, ammonium sulfate, sodium chloride, ammonium acetate, sodium sulfate, and magnesium sulfate were purchased from Beijing Chemical (Beijing, China). Analytical grade neutral alumina was obtained from Sinopharm (Shanghai, China). Membrane filters (0.45 μm, nylon) were provided from Millipore (Bedford, MA, USA).

Individual steroid stock solutions (100 μg mL^{-1}) were prepared in methanol and stored at −18 °C. A mixed stock solution (10 μg mL^{-1}) of nine steroid hormones was prepared by diluting the individual steroid stock solutions with methanol and stored at 4 °C in a dark glass bottle. The mixed solution should be replaced every two weeks to prevent the decomposition of the steroid hormones.

Seven frozen fish samples belonging to crustacea (shrimp), cephalopoda (squid), and pisces (cod, hairtail, whitebait, yellow croaker, and mackerel) were purchased from local supermarkets. All fish originated from the Bohai Sea, China. The skin and bones of the fish were removed, and then the tissue was minced and kept at −18 °C prior to analysis. Four samples showed the absence of the steroid hormones, so we randomly selected a sample (whitebait) for recovery studies and named it sample 1.

Spiked fish tissue samples were prepared by adding different volumes of the steroid hormone standard solution into sample 1. The mixture was equilibrated by stirring for 15 min and placed in the dark for more than 24 h until methanol was completely volatilized. The spiked samples were kept at −18 °C until analysis.

2.2. Apparatus. The DMAE−SLLE system was illustrated in Figure 2, which was modified based on our previous work.[37] The system consisted of a vacuum pump (HPD-25, Beijing, China), a vacuum SPE manifold (Waters, Milford, MA, USA), and a flat countertop microwave oven (Panasonic, Shanghai, China). The extraction vessels were polyethylene tubes (85.0 mm long, 15.0 mm i.d.). The upper ends and the bottoms of the vessels were connected with a solvent container and the ports of the vacuum SPE manifold by tubes (T1 and T2) (polyethylene, 500.0 mm long, 0.5 mm i.d.), respectively. Fifteen glass test tubes (100.0 mm long, 14.0 mm i.d.) with a plastic plug were used as collection tubes.

2.3. Extraction Procedures. *2.3.1. Dynamic Microwave-Assisted Extraction Coupled with Salting-out Liquid−liquid Extraction.* Fish tissue was accurately weighted (3.00 g) and thoroughly mixed with neutral alumina (4.0 g) which was used as a degreaser and dispersant. The mixture was placed between two absorbent cotton plugs in the extraction vessel and then carefully compressed. Fifteen extraction vessels were loaded by the same method and systematically arranged in the microwave oven and then connected with the solvent container and the vacuum SPE manifold by T1 and T2.

Subsequently, the vacuum pump was activated, and 5 mL of acetonitrile and about 5 mL of water were successively passed through the extraction vessels at the flow rate of 1.0 mL min^{-1}. When the extraction vessel was properly filled with acetonitrile, microwave heating was started with the power of 800 W, and the extract was directly transferred into the collection tube. When the volume of the extract in the collection tube was 9 mL, the rate control valve was stopped. Once the extraction of 15 samples was completed, the vacuum pump and the microwave irradiation were stopped.

Finally, 2.0 g of ammonium acetate was added to the collection tube, and then the extract was separated into the acetonitrile phase and aqueous phase after 6 min. The acetonitrile phase (about 1.2 mL) was transferred to a conical vial with a syringe and evaporated to dryness at 40 °C under a stream of N_2. The residue was reconstituted with 0.3 mL of acetonitrile and filtered through a 0.45 μm membrane. An aliquot of 20 μL was injected into the LC−MS/MS system for analysis.

2.3.2. Traditional Liquid Extraction. Traditional liquid extraction of steroid hormones from fish tissues was performed according to the Chinese national standard.[43] Fish tissue (5.00 g) was placed in a 50 mL centrifuge tube, and then 3 mL of sodium carbonate solution (0.1 g mL^{-1}) and 25 mL of methyl *tert*-butyl ether were added into it. The mixture was homogenized for 30 s and shaken for 10 min and subsequently centrifuged at 6000 rpm for 10 min. The supernatant was added into a 100 mL heart-shaped flask. The other 25 mL of methyl *tert*-butyl ether was added into the centrifuge tube, and the previous treatment was repeated. The supernatants were combined and evaporated to dryness on a rotary evaporator at 40 °C. The residue was reconstituted with 2 mL of acetonitrile aqueous (50%, v:v). Subsequently, the extract was frozen for 30 min and centrifuged at 17 000*g* for 5 min. Then the solution was filtered through a 0.22 μm membrane, and a 20 μL aliquot was injected into the LC−MS/MS system for analysis.

2.4. LC−MS/MS Analysis. Liquid chromatography was performed on an Agilent Series 1100 HPLC system (Palo Alto, CA, USA) equipped with a degasser, a quaternary pump, an autosampler, and a heated column compartment. Nine steroid hormones were separated by a Zorbax Eclipse C_{18} column (150 mm × 4.6 mm i.d., 3.5 μm, Agilent, USA). A gradient elution solvent containing acetonitrile as mobile phase A and water as mobile phase B was applied. The mobile-phase gradient profile was as follows: t_0, A = 40%; t_{5min}, A = 40%; t_{15min}, A = 85%; t_{16min}, A = 40%. The flow rate was 0.8 mL min^{-1}. The eluate from the HPLC column was split and then introduced into the

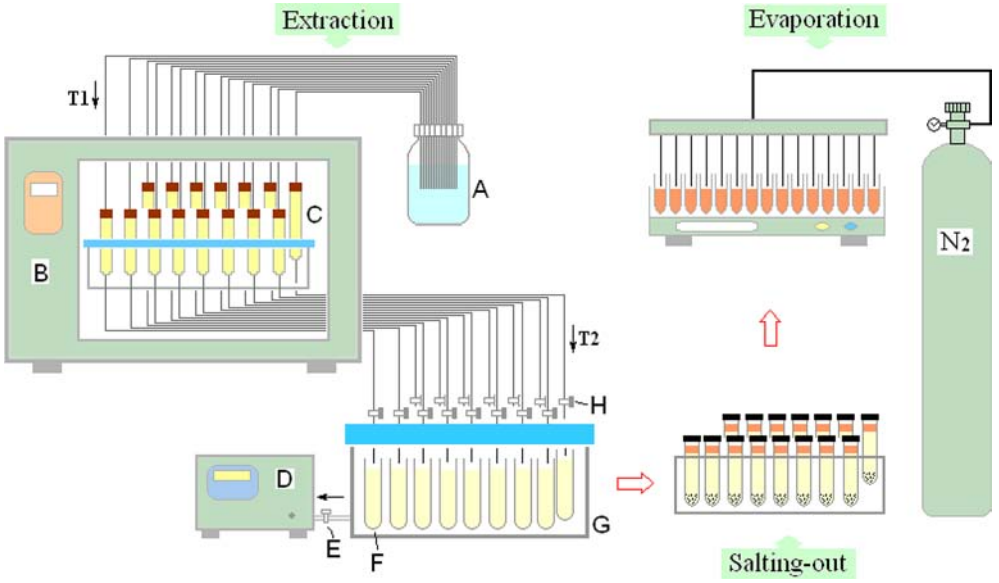

Figure 2. Sketch of the DMAE−SLLE setup. A, solvent (acetonitrile or water) container; B, microwave oven; C, extraction vessel; D, vacuum pump; E, vacuum valve; F, collection tube; G, vacuum SPE manifold; H, rate control valve.

Table 1. Optimum Conditions for LC−MS/MS Analysis of Steroid Hormones

analyte	retention time (min)	precursor ion (m/z)	product ions (m/z)	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
estriol	2.6	287.2 $[M - H]^-$	171.0[a]	−50	−10	−42	−40	−4
			159.1	−50	−10	−40	−40	−6
corticosterone	4.7	345.3 $[M - H]^-$	123.1[a]	−50	−10	−42	−40	−4
			241.2	−55	−8	−40	−38	−4
17-α-estradiol	9.2	271.2 $[M - H]^-$	145.0[a]	−50	−8	−40	−42	−4
			239.1	−50	−10	−40	−40	−6
estrone	10.6	269.3 $[M - H]^-$	145.0[a]	−50	−8	−42	−40	−4
			159.1	−50	−10	−42	−40	−4
17-α-hydroxyprogesterone	10.9	329.4 $[M - H]^-$	285.2[a]	−45	−10	−40	−38	−4
			301.3	−50	−10	−40	−40	−4
medroxyprogesterone	12.8	343.4 $[M - H]^-$	299.2[a]	−50	−10	−42	−40	−4
			315.2	−50	−10	−44	−42	−4
19-nortestosterone	5.5	275.3 $[M + H]^+$	109.1[a]	55	10	32	40	4
			145.2	50	10	40	42	4
testosterone	6.1	289.3 $[M + H]^+$	97.0[a]	50	10	36	40	4
			109.1	55	10	32	40	6
progesterone	9.7	315.3 $[M + H]^+$	97.1[a]	55	10	32	40	4
			109.1	55	10	40	40	4

[a]The product ion used for quantification.

Table 2. Calibration and Statistical Validation Parameters

		solvent standard		matrix-matched standard			
steroid hormones	linearity range (ng g^{-1})	calibration equation	correlation coefficient (r^2)	calibration equation	correlation coefficient (r^2)	LOD (ng g^{-1})	LOQ (ng g^{-1})
estriol	0.5−100	$y = 1213x + 1126$	0.9990	$y = 1342x + 1403$	0.9979	0.13	0.44
corticosterone	0.5−100	$y = 1422x + 1275$	0.9981	$y = 1676x + 1724$	0.9957	0.15	0.47
17-α-estradiol	0.5−100	$y = 1299x + 1313$	0.9988	$y = 1407x + 1583$	0.9974	0.12	0.39
estrone	0.2−100	$y = 2044x + 3122$	0.9984	$y = 2118x + 3371$	0.9988	0.04	0.14
17-α-hydroxyprogesterone	0.2−100	$y = 2229x + 2671$	0.9991	$y = 2290x + 2892$	0.9958	0.03	0.11
medroxyprogesterone	0.2−100	$y = 1961x + 2870$	0.9983	$y = 2084x + 3026$	0.9983	0.05	0.17
19-nortestosterone	0.2−100	$y = 2395x + 3268$	0.9982	$y = 2643x + 3265$	0.9979	0.07	0.23
testosterone	0.2−100	$y = 1897x + 2673$	0.9997	$y = 2044x + 2789$	0.9997	0.05	0.16
progesterone	0.2−100	$y = 2059x + 2084$	0.9993	$y = 2251x + 2227$	0.9993	0.06	0.20

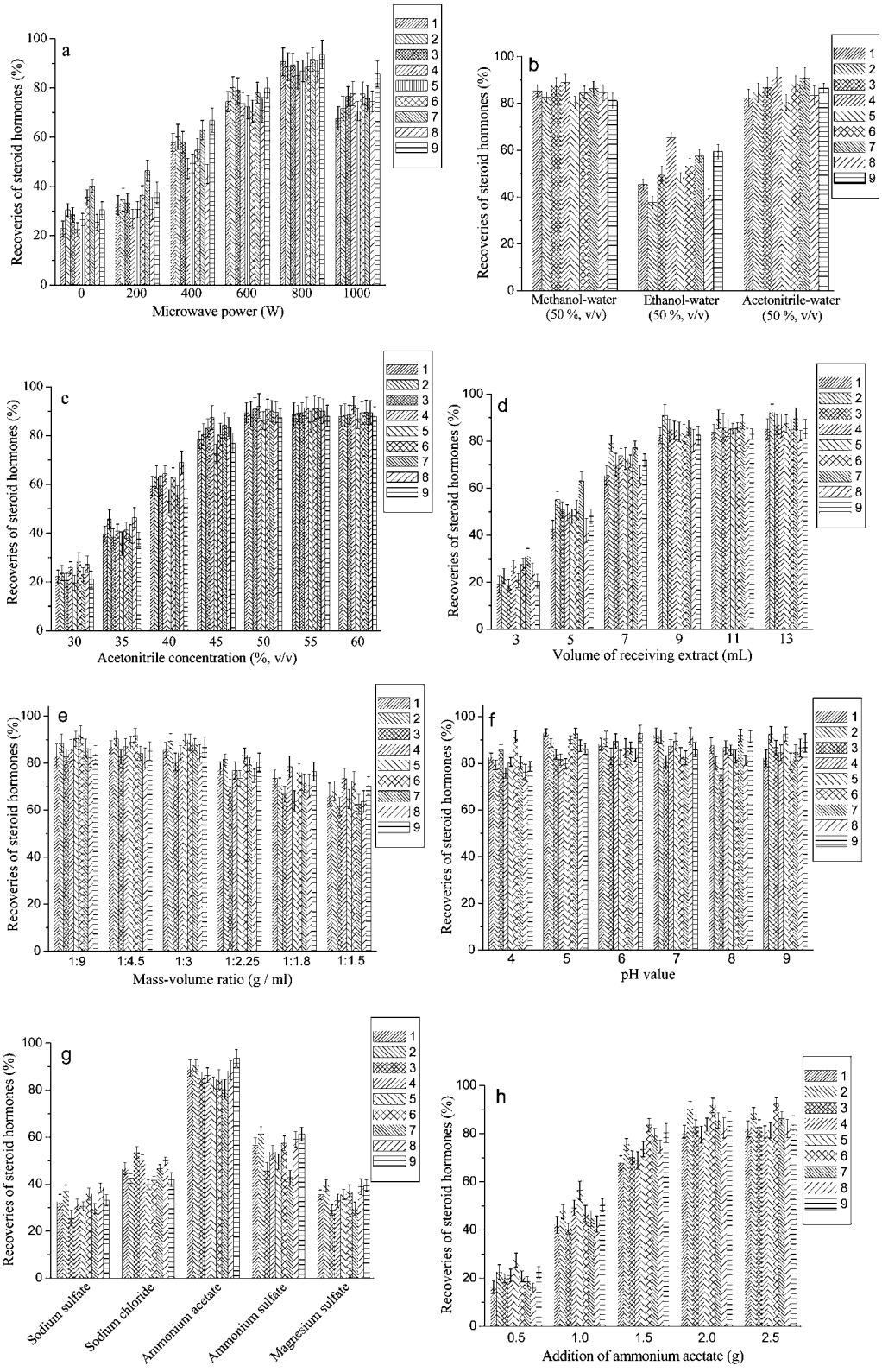

Figure 3. Optimization of DMAE−SLLE conditions on the recoveries of the steroid hormones (n = 3): (a) microwave power; (b) kinds of extraction solvent; (c) acetonitrile concentration; (d) volume of receiving extract; (e) mass−volume ratio; (f) pH value; (g) kinds of salt; and (h) addition of ammonium acetate. Steroid: 1, estriol; 2, corticosterone; 3, 19-nortestosterone; 4, testosterone; 5, 17-α-estradiol; 6, progesterone; 7, estrone; 8, 17-α-hydroxyprogesterone; 9, medroxyprogesterone. The time of microwave irradiation was about 9.5 min.

MS detector at the flow rate of 0.25 mL min^{-1}. The injection volume was 20 μL.

A Q-Trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) interfaced with the HPLC system was used for the measurement of the steroid hormones. Optimization of the ion source and MS/MS settings was performed by the automatic optimization function of the MS software (Analyst 1.4.1, Applied Biosystems) assisted by manual optimization. The ESI−MS/MS

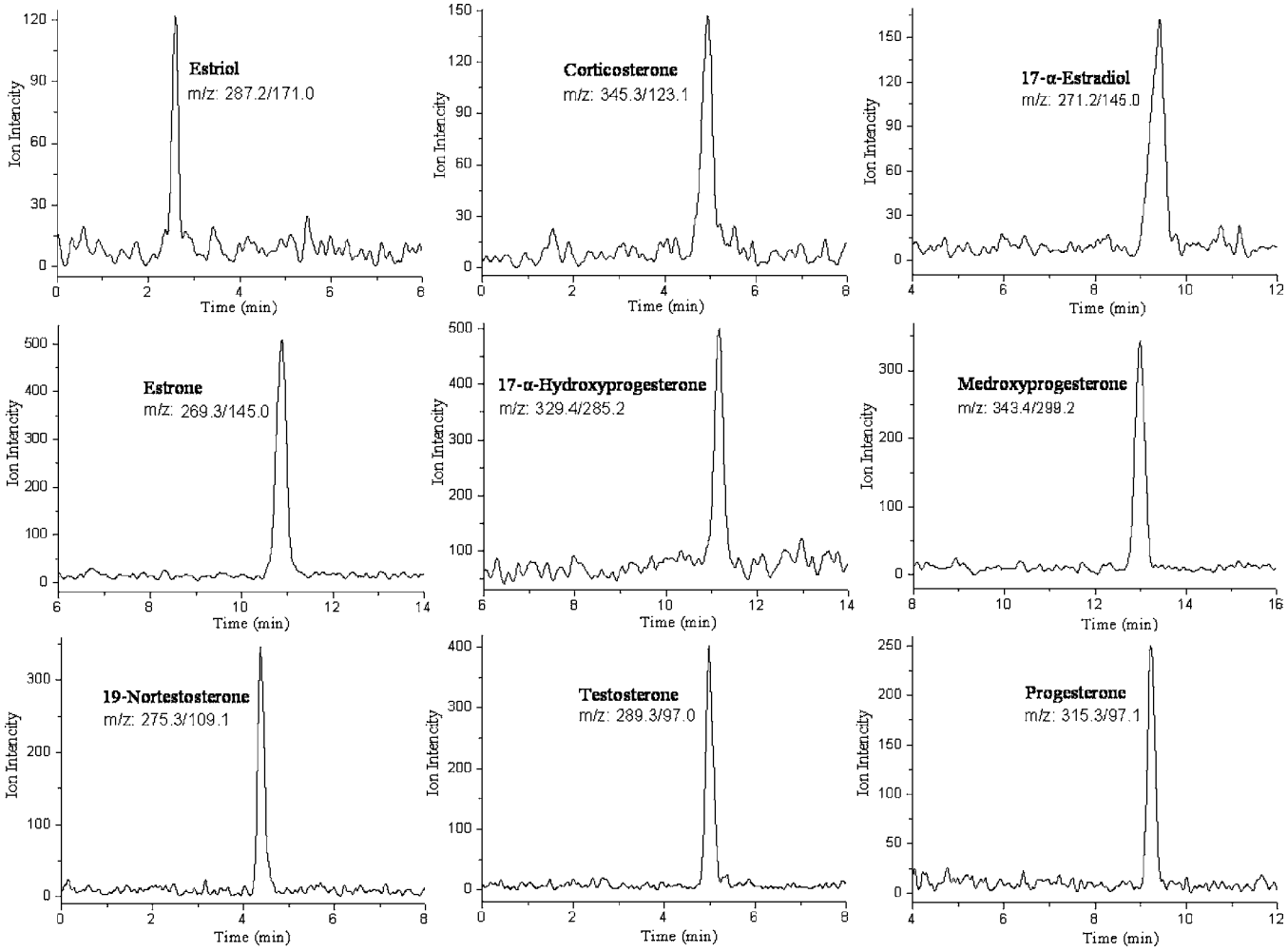

Figure 4. LC−MS/MS chromatograms of blank fish tissue extract spiked with 0.5 ng g^{-1} of steroid hormones.

detection was performed in the positive mode, and the source-dependent parameters were as follows: curtain gas, N_2 (30 psi); collision gas, N_2 (high); gas 1, N_2 (30 psi); gas 2, N_2 (50 psi); ion spray voltage, 5200 V; temperature, 400 °C. The base peaks selected for quantitation of six steroid hormones corresponded to the deprotonated molecule $[M - H]^-$, whereas the progesterone, testosterone, and 19-nortestosterone are detected as protonated molecules $[M + H]^+$. The data acquisition was performed in the multiple reaction monitoring (MRM) mode which records the transitions between the precursor ion and the two most abundant product ions for each target analyte. MRM transitions as well as the corresponding declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell entrance potential (CEP), and collision cell exit potential (CXP) are shown in Table 1. All transitions were recorded in one single retention time window with a dwell time of 100 ms.

3. RESULTS AND DISCUSSION

3.1. Calibration. Calibration curves were constructed at six concentration levels by appropriately diluted standards with solvent and blank fish tissue extract. Both solvent and matrix-matched standard calibration curves showed good linearity in the studied range (0.5−100 ng g^{-1} for estriol, corticosterone, and 17-α-estradiol and 0.2−100 ng g^{-1} for the rest of the steroid hormones) with the correlation coefficients in the ranges of 0.9981−0.9997 and 0.9957−0.9997 for solvent and matrices, respectively (Table 2).

3.2. Evaluation of the Homogeneity of the Spiked Fish Tissue Sample. Before the optimization study of the extraction process, the homogeneity of the spiked samples was assessed. Six spiked fish tissue samples (3 g) were extracted by the standard method described in Section 2.3.2. Average recoveries were 82.7, 78.2, 80.3, 88.6, 90.1, 92.4, 84.1, 85.0, and 89.9% for estriol, corticosterone, 17-α-estradiol, estrone, 17-α-hydroxyprogesterone, medroxyprogesterone, 19-nortestosterone, testosterone, and progesterone, respectively, and relative standard deviations (RSDs) were in the range of 5.2−7.6%. The results show that the spiked fish tissue samples were considered to be homogeneous when sample amounts were equal to or larger than 3 g.

3.3. Optimization of DMAE Conditions. *3.3.1. Effect of Microwave Power.* The effect of microwave power on the recoveries of the steroid hormones in the spiked sample was studied by varying the power between 0 and 1000 W. Figure 3a shows that the recoveries of the steroid hormones increased with the change of the microwave power from 0 to 800 W and then decreased from 800 to 1000 W which might be caused by the partial decomposition of steroid hormones under high temperature.[44] Thus, 800 W was selected as the best microwave power.

3.3.2. Effect of Extraction Solvent. Aqueous solutions of methanol (50%, v/v), ethanol (50%, v/v), and acetonitrile (50%, v/v) were evaluated as the extraction solvent (Figure

Table 3. Comparisons of the Proposed Method with Other Methods Used in the Literature

sample	sample preparation	volume of organic solvent (mL)	detection	LOD	LOQ	recovery (%)	precision (RSD, %)	ref
animal muscle tissues	homogenization (1 min) → enzymolysis (3−4 h) → ultrasonic extraction (5 min) → centrifugation (8 min) → SPE cleanup (10 min) → evaporation → reconstitution	73	LC−MS/MS	0.06−0.22 $\mu g\ kg^{-1}$	0.12−0.54 $\mu g\ kg^{-1}$	64−77	7.1−20.3	21
food	homogenization → centrifugation (10 min) → liquid−liquid extraction → SPE cleanup	310	GC−MS	0.01−0.3 $\mu g\ kg^{-1}$		no report		22
sediment	pressurized microwave-assisted extraction (15 min) → cooling (60 min) → rinses → evaporation → SPE cleanup → derivatization	48	GC−MS/MS	0.05−0.14 $\mu g\ kg^{-1}$	0.17−0.47 $\mu g\ kg^{-1}$	86−102	1.22−20.0	27
urine	pressurized microwave-assisted extraction (60 min) → evaporation → derivatization	10	GC−MS	1−70 ng mL^{-1}		no report		28
	pressurized microwave-assisted extraction (60 min) → evaporation		LC−MS/MS	3−100 ng mL^{-1}				
sediments	pressurized microwave-assisted extraction (15 min) → cooling → rinses → centrifugation (5 min) → evaporation → SPE cleanup → evaporation → derivatization	60	GC−MS	0.2−1.0 $\mu g\ kg^{-1}$	0.5−3.4 $\mu g\ kg^{-1}$	61.5−133	<24.3	29
fish and clams	homogenization → SPE cleanup → concentration → centrifugation (5 min) → concentration	20	UHPLC−MS/MS	0.049−19.8 $\mu g\ kg^{-1}$	0.12−21.7 $\mu g\ kg^{-1}$	30.0−111	<15	48
sludge and sediments	ultrasonic extraction (20 min) → centrifugation (5 min) → evaporation → reconstitution → silica gel cleanup → SPE → HPLC cleanup → evaporation → derivatization	13	GC−MS/MS	no report	2−4 $\mu g\ kg^{-1}$	73−121	3−51	49
fish	dynamic microwave-assisted extraction (9 min) → salting-out liquid−liquid extraction (6 min) → evaporation → reconstitution	5	LC−MS/MS	0.03−0.15 $\mu g\ kg^{-1}$	0.11−0.47 $\mu g\ kg^{-1}$	78.9−94.3	5.9−7.4	proposed method

3b). The results demonstrated that methanol aqueous and acetonitrile aqueous provided the higher recoveries of the steroid hormones than ethanol aqueous. The possible reason is that methanol and acetonitrile have a relatively higher dielectric constant than ethanol (the dielectric constants of methanol, acetonitrile, and ethanol were 33, 38, and 25, respectively), which means that, compared with ethanol, methanol and acetonitrile could be polarized more easily with an electric field.[45] Moreover, when adequate salt was added, the acetonitrile aqueous could be separated faster than methanol aqueous into two distinct phases. In addition, acetonitrile could induce protein precipitation and release steroid hormones from the fish matrix.[12] So acetonitrile aqueous was chosen as the extraction solvent.

Subsequently, the effect of acetonitrile concentration in the extraction solvent was investigated. The experiments were performed with 12 mL of acetonitrile aqueous at various concentrations (30−60%, v/v), and the results were shown in Figure 3c. The recoveries of the steroid hormones increased with increasing acetonitrile concentrations from 30 to 50% and then remained relatively constant when the acetonitrile concentration was higher than 50%.

Lastly, the volume of receiving extract was investigated. It can be seen from Figure 3d that 9 mL of the receiving volume offers the satisfying recoveries of the steroid hormones (above 80%). When receiving volume was above 9 mL, the increase of the volume did not result in any noticeable improvement on the recoveries of the steroid hormones. It is worth noting that a great deal of tiny bubbles was generated when the acetonitrile aqueous (50%, v/v) was passed through the fish tissue sample. To solve this problem, acetonitrile and water were successively passed through the extraction vessel. The results demonstrated that the recoveries of the steroid hormones were increased (from 72.8 ± 5.4%−86.1 ± 4.9% to 79.0 ± 4.6%−93.4 ± 5.8%), and the bubble was avoided. Thus, 5 mL of acetonitrile and about 5 mL of water were used as the extraction solvent, and the volume of the receiving extract was 9 mL.

3.3.3. Effect of Mass−Volume Ratio. The effect of mass−volume ratio on the recoveries of the steroid hormones in the spiked sample was studied by varying the ratio between 1:9 and 1:1.5 g/mL. Figure 3e shows that the recoveries of the steroid hormones did not significantly change when the mass−volume ratio was changed from 1:9 to 1:3 g/mL. From 1:3 to 1:1.5 g/mL, the recoveries of the steroid hormones significantly decreased. Hence, the mass−volume ratio of 1:3 g/mL was chosen.

3.3.4. Effect of Extraction Solution Flow Rate. The effect of the extraction solution flow rate from 0.5 to 2.5 mL min^{-1} on the recoveries of the steroid hormones was investigated. The results indicated that the flow rate in the range of 1.0−2.0 mL min^{-1} had no significant effect on the recoveries of the steroid hormones. The recoveries were decreased when the flow rate was higher than 2.0 mL min^{-1}. On the contrary, the overall extraction time was prolonged when the flow rate was lower than 1.0 mL min^{-1}. In this study, approximately 1.0 mL min^{-1} of the extraction solution flow rate was chosen.

3.4. Optimization of SLLE Conditions. The optimization of SLLE conditions was carried out in 10 mL of fish tissue extract spiked with the steroid hormones at the concentration of 0.5 ng mL^{-1}. The effects of pH were examined in the range of 4−9 on the recoveries of the steroid hormones. Figure 3f shows that the recoveries of the steroid hormones were not affected significantly by the pH of the extract in the selected

range. The pH of the obtained extract was 6, and pH adjustment was not required.

Series of salts (ammonium sulfate, sodium chloride, ammonium acetate, sodium sulfate, and magnesium sulfate) were screened as possible salting-out reagents. It was found that all salts could induce phase separation, but ammonium acetate was the most suitable one because it was a mass spectrometry friendly salt and could provide the highest recoveries (80.4 ± 4.1%−93.7 ± 3.6%) (Figure 3g). Additionally, the presence of ammonium acetate minimizes the formation of the sodium adduct and thus enhances the MS response.[42] Therefore, ammonium acetate was selected as the salting-out reagent in this work.

Subsequently, different amounts of ammonium acetate (0.5−2.5 g) were added into the extract. Figure 3h shows that the recoveries of the steroid hormones were improved as the amounts of salt addition increased from 0.5 to 2.0 g and were approximately constant in the range of 2.0−2.5 g. So 2.0 g of ammonium acetate was added into the extract.

As a result of compromising, the best DMAE−SLLE conditions were as follows: microwave power, 800 W; extraction solvent, acetonitrile and water; receiving extract volume, 9 mL; mass−volume ratio, 1:3 g/mL; extraction solvent flow rate, 1.0 mL min^{-1}; ammonium acetate addition, 2.0 g.

3.5. Validation of the Method. Numerous studies have revealed that the MS response signal of the analytes in a complex matrix can be greatly affected by competitive effects due to the presence of matrix components.[46,47] Matrix effects can affect the ionization efficiency of analytes, leading to suppression or enhancement of signal depending on the analyte/matrix combination.

To evaluate the matrix effects, calibration curves were compared between solvent and blank fish tissue extract in a concentration range of 0.5−100 ng g^{-1} for estriol, corticosterone, and 17-α-estradiol and 0.2−100 ng g^{-1} for the rest of the steroid hormones (Table 2). The results showed that the slopes of calibration curves obtained for matrix-matched standard were higher than those obtained for solvent standard, which indicated the signal enhancement of the steroid hormones. In this work, the matrix-based calibration curves were used for the reliable quantification. Limits of detection (LODs) and limits of quantification (LOQs) were estimated as the analyte concentration, producing a signal/noise ratio of 3 and 10, respectively. The details of the calibration curves, correlation coefficients, LODs, and LOQs of the proposed method were shown in Table 2. LC−MS/MS chromatograms of spiked fish tissue extract (0.5 ng g^{-1}) are shown in Figure 4.

The precision of the method was evaluated by measuring intra- and interday RSDs. Intraday precision was performed by analyzing spiked sample 1 five times in one day at three different fortified concentrations of 0.5, 5, and 50 ng g^{-1}. The mean values of RSDs (%) were in the range of 3.3−5.0%. The interday precision was performed over five days by analyzing spiked sample 1 at three different fortified concentrations of 0.5, 5, and 50 ng g^{-1}. The mean values of RSDs (%) were in the range of 3.5−5.6%. In addition, microwave energy distribution for 15 samples was evaluated. Fifteen spiked samples (5 ng g^{-1}) were simultaneously treated by the proposed method. The recoveries of the steroid hormones for 15 spiked samples were in the range of 78.9−94.3%, and the RSDs were in the range of 5.9−7.4%.

The accuracy of the method was evaluated by the analysis of seven fish tissues spiked with the steroid hormone standards at the levels of 0.5 and 5 ng g^{-1}, respectively. The spiked samples were equilibrated by stirring for 15 min and placed in the dark for more than 24 h. The recoveries of the steroid hormones obtained by the proposed method were in the range of 75.3 ± 4.9%−92.1 ± 6.6% and 82.0 ± 5.1%−95.4 ± 6.2% for the two concentrations, respectively.

3.6. Application of the Proposed Method. To demonstrate the applicability of the proposed method, several fish samples collected from local supermarkets in Changchun (China) were analyzed. Testosterone was detected with the concentration of 0.45 ± 0.03 ng g^{-1} in the yellow croaker. 17-α-Estradiol and 19-nortestosterone were detected with the contents of 0.39 ± 0.03 and 0.30 ± 0.01 ng g^{-1} in the shrimp. Corticosterone, 17-α-estradiol, and 17-α-hydroxyprogesterone were found in the hairtail at levels of 0.57 ± 0.03, 0.44 ± 0.02, and 0.26 ± 0.01 ng g^{-1}, respectively. The result obtained by the proposed method was compared with that obtained by the standard method which was described in Section 2.3.2, and the obtained results by the two methods were similar.

The proposed method was compared with the methods used in the literature for analyzing steroid hormones (Table 3). It is obvious that the proposed method reduces the consumption of the organic solvent, shortens the sample preparation time, and increases the sample throughput. The entire sample pretreatment procedures including extraction, salting-out, and evaporation can be accomplished within 20 min, which is suitable for the analysis of trace steroid hormones in fish tissues.

In conclusion, the proposed method of DMAE−SLLE coupled with LC−MS/MS could be employed to analyze nine steroid hormones in fish tissues. The method combined advantages of the DMAE and SLLE technique, and the total pretreatment time of 15 samples was about 20 min. DMAE−SLLE was an effective technique in reducing the sample preparation time and solvent consumption. In addition, extraction, cleanup, and enrichment were completed in one step, and the sample pretreatment process became simple. Ammonium acetate was used as the salting-out reagent, which is suitable for LC−MS/MS analysis. The proposed method was successfully applied to the analysis of seven fish tissues. It is envisioned that the DMAE−SLLE will become an alternative sample preparation approach for the analysis of trace organic compounds in fish tissues.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86-431-85168399. Fax: +86-431-85112355. E-mail: dinglan@jlu.edu.cn.

Funding

This work was supported by the Development Program of the Ministry of Science and Technology of Jilin Province, China (grant number 20120327), and the Natural Science Foundation of China (Grant No.: 20875037).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Commission of the European Communities, *Council Directive 96/22/EC, Off. J. Eur. Commun. Legis. L125*, 1996; 3.

(2) The Ministry of Agriculture, PR China, *Regulation No. 193*, 2002.

(3) Scippo, M. L.; G., P.; Degand, G.; Brose, F.; Maghuin-Rogister, G. Control of the illegal administration of natural steroid hormones in

urine and tissues of veal calves and in plasma of bulls. *Anal. Chim. Acta* **1993**, *275*, 57−74.
(4) Peteghem, C. H. v.; Look, L. J. v. A comparison of chemiluminescence immunoassay and radioimmunoassay for the detection of nortestosterone residues in meat samples. *Anal. Chim. Acta* **1988**, *205*, 223−228.
(5) Courant, F.; Antignac, J.-P.; Laille, J.; Monteau, F.; Andre, F.; Bizec, B. L. Exposure assessment of prepubertal children to steroid endocrine disruptors. 2. determination of steroid hormones in milk, egg, and meat samples. *J. Agric. Food Chem.* **2008**, *56*, 3176−3184.
(6) Impens, S.; Wasch, K. D.; Cornelis, M.; Brabander, H. F. D. Analysis on residues of estrogens, gestagens and androgens in kidney fat and meat with gas chromatography−tandem mass spectrometry. *J. Chromatogr. A* **2002**, *970*, 235−247.
(7) Farke, C.; Rattenberger, E.; Roiger, S. U.; Meyer, H. H. D. Bovine colostrum: determination of naturally occurring steroid hormones by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *J. Agric. Food Chem.* **2011**, *59*, 1423−1427.
(8) Malekinejad, H.; Scherpenisse, P.; Bergwerff, A. A. Naturally occurring estrogens in processed milk and in raw milk (from gestated cows). *J. Agric. Food Chem.* **2006**, *54*, 9785−9791.
(9) Shao, B.; Zhao, R.; Meng, J.; Xue, Y.; Wu, G.; Hu, J.; Tu, X. Simultaneous determination of residual hormonal chemicals in meat, kidney, liver tissues and milk by liquid chromatography−tandem mass spectrometry. *Anal. Chim. Acta* **2005**, *548*, 41−50.
(10) Taamalli, A.; Arráez-Román, D.; Ibañez, E.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Optimization of microwave-assisted extraction for the characterization of olive leaf phenolic compounds by using HPLC-ESI-TOF-MS/IT-MS^2. *J. Agric. Food Chem.* **2012**, *60*, 791−798.
(11) Taieb, J.; Benattar, C.; Birr, A. S.; Lindenbaum, A. Limitations of steroid determination by direct immunoassay. *Clin. Chem.* **2002**, *48*, 583−585.
(12) Daeseleire, E.; Vandeputte, R.; Peteghem, C. V. Validation of multi-residue methods for the detection of anabolic steroids by GC-MS in muscle tissues and urine samples from cattle. *Analyst* **1998**, *123*, 2595−2598.
(13) Stolker, A. A. M.; Linders, S. H. M. A.; Ginkel, L. A. v.; Brinkman, U. A. T. Application of the revised EU criteria for the confirmation of anabolic steroids in meat using GC-MS. *Anal. Bioanal. Chem.* **2004**, *378*, 1313−1321.
(14) Blokland, M. H.; Rossum, H. J. v.; Herbold, H. A.; Sterk, S. S.; Stephany, R. W.; Ginkel, L. A. v. Metabolism of methyltestosterone, norethandrolone and methylboldenone in a heifer. *Anal. Chim. Acta* **2005**, *529*, 317−323.
(15) Fuh, M.-R.; Huang, S.-Y.; Lin, T.-Y. Determination of residual anabolic steroid in meat by gas chromatography−ion trap−mass spectrometer. *Talanta* **2004**, *64*, 408−414.
(16) Rambaud, L.; Monteau, F.; Deceuninck, Y.; Bichon, E.; André, F.; Bizec, B. L. Development and validation of a multi-residue method for the detection of a wide range of hormonal anabolic compounds in hair using gas chromatography−tandem mass spectrometry. *Anal. Chim. Acta* **2007**, *586*, 93−104.
(17) Seo, J.; Kim, H.-Y.; Chung, B. C.; Hong, J. Simultaneous determination of anabolic steroids and synthetic hormones in meat by freezing-lipid filtration, solid-phase extraction and gas chromatography−mass spectrometry. *J. Chromatogr. A* **2005**, *1067*, 303−309.
(18) Guan, F.; Soma, L. R.; Luo, Y.; Uboh, C. E.; Peterman, S. Collision-induced dissociation pathways of anabolic steroids by electrospray ionization tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 477−489.
(19) Stolker, A. A. M.; Zuidema, T.; Nielen, M. W. F. Residue analysis of veterinary drugs and growth-promoting agents. *Trends Anal. Chem.* **2007**, *26*, 967−979.
(20) Kaklamanos, G.; Theodoridis, G.; Dabalis, T. Determination of anabolic steroids in muscle tissue by liquid chromatography−tandem mass spectrometry. *J. Chromatogr. A* **2009**, *1216*, 8072−8079.
(21) Xu, C. L.; Chu, X. G.; Peng, C. F.; Jin, Z. Y.; Wang, L. Y. Development of a faster determination of 10 anabolic steroids residues in animal muscle tissues by liquid chromatography tandem mass spectrometry. *J. Pharm. Biomed.* **2006**, *41*, 616−621.
(22) Hartmann, S.; Lacorn, M.; Steinhart, H. Natural occurrence of steroid hormones in food. *Food Chem.* **1998**, *62*, 7−20.
(23) Dévier, M.-H.; Labadie, P.; Togola, A.; Budzinski, H. Simple methodology coupling microwave-assisted extraction to SPE/GC/MS for the analysis of natural steroids in biological tissues: Application to the monitoring of endogenous steroids in marine mussels Mytilus sp. *Anal. Chim. Acta* **2010**, *657*, 28−35.
(24) Capriotti, A. L.; Cavaliere, C.; Giansanti, P.; Gubbiotti, R.; Samperi, R.; Laganà, A. Recent developments in matrix solid-phase dispersion extraction. *J. Chromatogr. A* **2010**, *1217*, 2521−2532.
(25) Canosa, P.; Rodríguez, I.; Rubí, E.; Ramil, M.; Cela, R. Simplified sample preparation method for triclosan and methyltriclosan determination in biota and foodstuff samples. *J. Chromatogr. A* **2008**, *1188*, 132−139.
(26) Cheng, J.; Liu, M.; Yu, Y.; Wangb, X.; Zhang, H.; Ding, L.; Jin, H. Determination of pyrethroids in porcine tissues by matrix solid-phase dispersion extraction and high-performance liquid chromatography. *Meat Sci.* **2009**, *82*, 407−412.
(27) Hibberd, A.; Maskaoui, K.; Zhang, Z.; Zhou, J. L. An improved method for the simultaneous analysis of phenolic and steroidal estrogens in water and sediment. *Talanta* **2009**, *77*, 1315−1321.
(28) Mazzarino, M.; Riggi, S.; Torre, X. d. l.; Botrè, F. Speeding up the process urine sample pre-treatment: Some perspectives on the use of microwave assisted extraction in the anti-doping field. *Talanta* **2010**, *81*, 1264−1272.
(29) Liu, R.; Zhou, J. L.; Wilding, A. Microwave-assisted extraction followed by gas chromatography−mass spectrometry for the determination of endocrine disrupting chemicals in river sediments. *J. Chromatogr. A* **2004**, *1038*, 19−26.
(30) Camel, V. Microwave-assisted solvent extraction of environmental samples. *Trends Anal. Chem.* **2000**, *19*, 229−248.
(31) Wang, H.; Zhao, Q.; Song, W.; Xu, Y.; Zhang, X.; Zeng, Q.; Chen, H.; Ding, L.; Ren, N. High-throughput dynamic microwave-assisted extraction on-line coupled with solid-phase extraction for analysis of nicotine in mushroom. *Talanta* **2011**, *85*, 743−748.
(32) Wang, H.; Chen, L.; Xu, Y.; Zeng, Q.; Zhang, X.; Zhao, Q.; Ding, L. Dynamic microwave-assisted extraction coupled on-line with clean-up for determination of caffeine in tea. *LWT–Food Sci. Technol.* **2011**, *44*, 1490−1495.
(33) Ericsson, M.; Colmsjö, A. Dynamic Microwave-Assisted Extraction Coupled On-Line with Solid-Phase Extraction and Large-Volume Injection Gas Chromatography: Determination of Organophosphate Esters in Air Samples. *Anal. Chem.* **2003**, *75*, 1713−1719.
(34) Luque-García, J. L.; Castro, M. D. L. d. Extraction of polychlorinated biphenyls from soils by automated focused microwave-assisted Soxhlet extraction. *J. Chromatogr. A* **2003**, *998*, 21−29.
(35) Chen, L.; Zeng, Q.; Du, X.; Sun, X.; Zhang, X.; Xu, Y.; Yu, A.; Zhang, H.; Ding, L. Determination of melamine in animal feed based on liquid chromatography tandem mass spectrometry analysis and dynamic microwave-assisted extraction coupled on-line with strong cation-exchange resin clean-up. *Anal. Bioanal. Chem.* **2009**, *395*, 1533−1542.
(36) Wang, H.; Xu, Y.; Song, W.; Zhao, Q.; Zhang, X.; Zeng, Q.; Chen, H.; Ding, L.; Ren, N. Automatic sample preparation of sulfonamide antibiotic residues in chicken breast muscle by using dynamic microwave-assisted extraction coupled with solid-phase extraction. *J. Sep. Sci.* **2011**, *34*, 2489−2497.
(37) Wang, H.; Li, G.; Zhang, Y.; Chen, H.; Zhao, Q.; Song, W.; Xu, Y.; Jin, H.; Ding, L. Determination of triazine herbicides in cereals using dynamic microwave-assisted extraction with solidification of floating organic drop followed by high-performance liquid chromatography. *J. Chromatogr. A* **2012**, *1233*, 36−43.
(38) Wang, M.; Cai, Z.; Xu, L. Coupling of acetonitrile deproteinization and salting-out extraction with acetonitrile stacking in chiral capillary electrophoresis for the determination of warfarin enantiomers. *J. Chromatogr. A* **2011**, *1218*, 4045−4051.

(39) Buratti, M.; Pellegrino, O.; Valla, C.; Rubino, F. M.; Verduci, C.; Colomb, A. Polyurethane foam chips combined with liquid chromatography in the determination of unmetabolized polycyclic aromatic hydrocarbons excreted in human urine. *Biomed. Chromatogr.* **2006**, *20*, 971−978.
(40) Yoshida, M.; Akane, A.; Nishikawa, M.; Watabiki, T.; Tsuchihashi, H. Extraction of thiamylal in serum using hydrophilic acetonitrile with subzero-temperature and salting-out methods. *Anal. Chem.* **2004**, *76*, 4672−4675.
(41) Zhang, J.; Wu, H.; Kim, E.; El-Shourbagy, T. A. Salting-out assisted liquid/liquid extraction with acetonitrile: a new high throughput sample preparation technique for good laboratory practice bioanalysis using liquid chromatography−mass spectrometry. *Biomed. Chromatogr.* **2009**, *23*, 419−425.
(42) Wu, H.; Zhang, J.; Norem, K.; El-Shourbagy, T. A. Simultaneous determination of a hydrophobic drug candidate and its metabolite in human plasma with salting-out assisted liquid/liquid extraction using a mass spectrometry friendly salt. *J. Pharm. Biomed.* **2008**, *48*, 1243−1248.
(43) The Ministry of Agriculture, PR China, *Regulation No. 1031*, 2008.
(44) Esmelindro, M. C.; Toniazzo, G.; Lopes, D.; Oliveira, D.; Dariva, C. Effects of processing conditions on the chemical distribution of mate tea leaves extracts obtained from CO_2 extraction at high pressures. *J. Food Eng.* **2005**, *70*, 588−592.
(45) Larhed, M.; Hallberg, A. Microwave-assisted high-speed chemistry: a new technique in drug discovery. *Drug Discovery Today* **2001**, *6*, 406−416.
(46) Chen, L.; Zeng, Q.; Wang, H.; Su, R.; Xu, Y.; Zhang, X.; Yu, A.; Zhang, H.; Ding, L. On-line coupling of dynamic microwave-assisted extraction to solid-phase extraction for the determination of sulfonamide antibiotics in soil. *Anal. Chim. Acta* **2009**, *648*, 200−206.
(47) Kebarle, P.; Tang, L. From ions in solution to ions in the gas phase-the mechanism of electrospray mass spectrometry. *Anal. Chem.* **1993**, *65*, 972A−986A.
(48) Chen, W.-L.; Wang, G.-S.; Gwo, J.-C.; Chen, C.-Y. Ultra-high performance liquid chromatography/tandem mass spectrometry determination of feminizing chemicals in river water, sediment and tissue pretreated using disk-type solid-phase extraction and matrix solid-phase dispersion. *Talanta* **2012**, *89*, 237−245.
(49) Ternes, T. A.; Andersen, H.; Gilberg, D.; Bonerz, M. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. *Anal. Chem.* **2002**, *74*, 3498−3504.