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Analysis of estrogenic compounds in environmental and biological samples by liquid chromatography–tandem mass spectrometry with stable isotope-coded ionization-enhancing reagent

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ABSTRACT

A sensitive and reliable stable isotope labeling technology was developed for the determination of estrogenic compounds in environmental and biological samples based on the derivatization of estrogenic compounds with 10-methyl-acridone-2-sulfonyl chloride (d_0-MASC) and its deuterated counterpart d_3-MASC. The labeling reaction of MASC with estrogenic compounds is simple and robust and can be carried out under mild conditions within 5 min. Internal standard-based quantification was achieved by this labeling strategy without the need of using expensive internal standard analogy to every analyte of interest. Meanwhile, the sensitivity obtained by liquid chromatography–tandem mass spectrometry (LC–MS/MS) was enhanced by 2–3 orders of magnitude compared to the underivatized counterparts. Application of the stable isotope labeling technology in relative and absolute quantification of estrogenic compounds in complicated samples indicated that the labeling strategy was effective in overcoming matrix effects. The proposed method was successfully applied to the analysis estrogenic compounds in different environmental and biological samples with high sensitivity and accuracy.

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1. Introduction

Mass spectrometry (MS) is highly popular because of its high sensitivity and specificity compared to other analytical techniques. The hyphenation of MS to liquid chromatography (LC–MS) is especially popular due to its fast and sensitive characterization and quantification. Nowadays, LC–MS plays a growing important role in many fields [1–9]. However, endogenous matrix components may coelute with the analytes of interest and thus affect the reproducibility and accuracy of the method. In extreme cases, matrix components can cause ionization suppression to such an extent that the analytes are rendered undetectable by MS [10].

Great efforts have been made to reduce matrix effects [11,12]. Solid phase extraction (SPE) is one of the often used methods to minimize matrix effects. However, SPE methods are only moderately successful in overcoming matrix effect because it is difficult to remove matrix components possessing similar property to the analytes. These components are likely to coelute with the analytes in LC–MS and continue to cause ionization suppression [10]. Another often used strategy is to use stable isotope labeled (SIL) internal standard [13]. Matrix effects can be reduced to a minimal level since matrix effects observed for the SIL internal standard are generally similar to those observed for the analytes. However, only a limited number of SIL internal standards are commercially available, and it is expensive and not practical to synthesize SIL internal standard to every analyte of interest, especially when there are a variety of target compounds. Recently, a new strategy employing stable isotope labeling overcomes some of the drawbacks mentioned above and becomes popular in many fields [13–18]. Instead of synthesizing an isotope analogy of the analyte of interest, stable isotope labeling method uses a chemical reaction to introduce an isotope tag to the analyte in one sample and another mass-difference isotope tag to the same analyte in another comparative sample (or standard), followed by mixing the two labeled samples for mass spectrometric analysis. The isotopic pairs of the labeled analytes coelute within a single run and have identical retention times. Since they are electrosprayed from identical solution conditions, the matrix effects and ionization efficiencies are expected to be the same.

Estrogenic compounds have gained increasing environmental and social concerns in recent years because of their endocrine-disrupting property and other serious side effects on human health [19–23]. Various approaches have been developed for the

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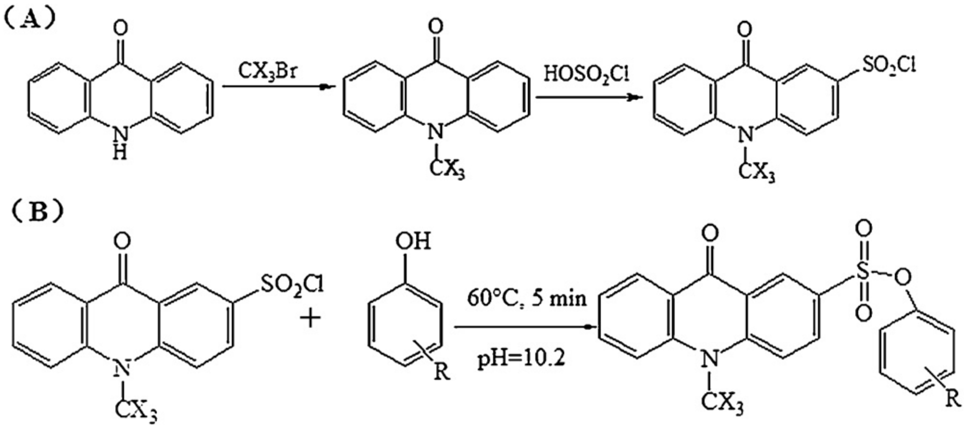

Fig. 1. Schemes of synthesis and derivatization. (A) Synthesis routes of d_0- and d_3-MASC. (B) Reaction scheme of MASC with estrogenic compounds. X = H or D.

determination of estrogenic compounds in these samples, such as liquid chromatography (LC) [24,25], LC–MS [26,27] and gas chromatography–mass spectrometry (GC–MS) [23,28]. However, many studies indicated that the application of these methods was often hampered by the low sensitivity or severe matrix effect [29–31].

In this work, we report a stable isotope labeling technology for improved quantification of estrogenic compounds in environmental and biological samples. Differential isotope labeling of estrogenic compounds with isotope-coded MASC provided isotopic variants which coeluted on a reversed-phase column. Matrix effects and run-to-run ionization differences which were often encountered in direct LC–MS analysis were therefore greatly reduced by the application of deuterated internal standard generated through derivatization. Meanwhile, the ionization efficiency of estrogenic compounds was greatly enhanced through the introduction of a readily ionizable MASC moiety into the analyte. The established method can be well applied to the relative and absolute quantification of unknown substances, such as phenolic- and amino-containing components, which can also react with MASC.

2. Experimental

2.1. Reagents and chemicals

Analytical standards of 4-octylphenol (OP), 4-nonylphenol (NP), bisphenol A (BPA), diethylstilbestrol (DES), estrone (E1), 17α-ethynylestradiol (EE2), 17β-estradiol (E2) and estriol (E3) were all obtained from Dr. Ehrenstorfer (Ausburg, Germany) with purity of higher than 99%. Methanol, dichloromethane, ethyl acetate, n-hexane and acetonitrile were of HPLC grade and purchased from Sigma–Aldrich (USA). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were of HPLC grade or at least of analytical grade. ODS C18 cartridges (500 mg, 6 mL) were obtained from Chrome Expert (CA, USA).

Individual stock solutions of 100 mg/L for all compounds were prepared in HPLC-grade acetonitrile and stored at 4 °C in the dark. Standard solutions containing all compounds were mixed and diluted with acetonitrile, and working solutions of all compounds and calibration concentrations were prepared by appropriate dilution of the stock solutions on the day of analysis.

d_0-MASC or d_3-MASC were synthesized in authors' laboratory as described in the synthesis section and Fig. 1A. The derivatizing reagent solution (1.0×10^{-3} mol/L) was prepared by dissolving 3.1 mg MASC in 10 mL of anhydrous acetonitrile. When not in use, all reagent solutions were stored at 4 °C in a refrigerator.

2.2. Synthesis of d_0-MASC and d_3-MASC

The preparation of stable isotope labeling reagents was carried out by a two-step procedure similar to the previously described method in our laboratory [32]. In brief, to a 200-mL flask, potassium hydroxide (2.6 g) and DMSO (30 mL) were mixed at room temperature for 10 min. Then acridone (5.8 g) in 20 mL of DMSO solution was added and stirred at room temperature for 40 min. A solution of $^1H_3$-bromomethane or $^2H_3$-bromomethane (13 mL) in 5.5 mL of DMSO solution was then added dropwise within 10 min. The contents were kept at room temperature for 24 h with vigorous stirring. The reaction mixture was poured into 100 mL of water with vigorous stirring for 10 min. The precipitated solid was recovered by filtration, washed with water, and dried with P_2O_5 under vacuum for 24 h. The crude products were recrystallized three times from acetonitrile to afford a yellow crystal (10-methyl acridone), yield 5.4 g (86%). The final product of 10-methyl-acridone-2-sulfonyl chloride (MASC) was synthesized by the reaction of chlorosulfonic acid with 10-methyl acridone. The synthesis procedure was exactly the same as that described before [32]. The synthesis procedures of light d_0-MASC and heavy d_3-MASC are depicted in Fig. 1A. The previously reported 10-ethyl-acridone-2-sulfonyl chloride (EASC) was not applied in this method because the synthesis of its deuterated counterpart would be more expensive.

2.3. Sample extraction

To avoid the contaminations from sample analysis process, glass syringes and glass vessels were employed throughout the experiments to avoid the introduction of NP or BPA. Syringes and vessels were all rinsed sequentially with tap water, high-purity water and methanol prior to sample addition.

2.3.1. Wastewater samples

Wastewater samples were collected from three different sites of a small river to which domestic sewage was discharged. Samples were extracted according to the method described in our previous work [33]. Wastewater samples were filtered through 0.45 μm pore size cellulose filters to remove fine particles. They were adjusted to pH 3.0 with 6 M HCl solution. Then 200 mL of water samples were passed through the ODS C18 SPE cartridges previously conditioned with 5 mL of ethyl acetate, 5 mL of methanol, and 5 mL of water. After washing with 10 mL of methanol–water (1/9, v/v), the cartridges were dried under vacuum for 10 min, then the analytes were eluted with 5.0 mL of mixed solvent of n-hexane/dichloromethane (9/1, v/v) and 7.0 mL of ethyl acetate. The eluted solutions were

Table 1
The multiple reaction monitoring (MRM) parameters (Agilent 6460).

Segment	Time (min)	Compound	Precursor ion		Product ion				Fragmentor (V)	CE (V)
			Light	Heavy	Light		Heavy			
1	3.0–3.6	E3	560.3	563.3	209.2	224.1	212.2	227.1	220	45
2	3.6–4.9	E2	544.3	547.3	208.2	224.3	211.2	227.3	190	40
		EE2	568.3	571.3	208.2	224.1	211.2	227.1	190	35
		E1	542.3	545.3	208.2	224.1	211.2	227.1	200	35
3	4.9–5.9	BPA	771.1	777.1	208.3	224.1	211.3	227.1	200	45
		trans-DES	811.3	817.3	208.3	267.1	211.3	267.1	235	55
		cis-DES	811.3	817.3	208.3	267.1	211.3	267.1	235	55
4	5.9–6.9	OP	478.3	481.3	209.1	224.2	212.1	227.2	200	35
		NP	492.3	495.3	209.1	224.1	212.1	227.1	190	35

evaporated to near 1 mL under a gentle stream of nitrogen gas at 40 °C. The residues were transferred into 3-mL vials and evaporated to dryness for derivatization.

2.3.2. *Sediment samples*

River sediment samples were collected from three stations of Xiaoqing He in Jining city. They were dried at ambient temperature, ground and passed through a 2 mm sieve prior to analysis. Sediment sample (2.0 g) was extracted twice by ultrasonication with methanol as extraction solvent. The supernatants of the two times were united and evaporated to dryness in a rotary vacuum evaporator at 45 °C. The residue was redissolved in 10 mL of methanol–water (1:9, v/v) and purified by ODS C18 cartridges under the same procedure as described above for wastewater sample. The eluted solution was evaporated to near 1 mL under a gentle stream of nitrogen gas at 40 °C. The residue was then transferred into a 3-mL vial and further evaporated to dryness for derivatization.

2.3.3. *Biological samples*

Shrimp and pork meat samples were purchased from a local market in Qufu city. They were homogenized by a high-speed blender. Estrogenic compounds in meat samples were extracted by ethyl acetate with the same procedure described above for sediment samples. The eluent of the SPE procedure was dried and reconstituted in 3 mL of acetonitrile. It was then stored at −18 °C for 30 min to remove the lipid. The acetonitrile phase was transferred into a 3-mL vial and further evaporated to dryness for derivatization.

2.4. *Derivatization of estrogenic compounds*

The derivatization of estrogenic compounds with MASC proceeded in basic condition (see Fig. 1B). To a solution containing an appropriate amount of standard or dried sample in a 3-mL vial, 80 μL $NaHCO_3$ buffer (pH 10), 200 μL acetonitrile and 50 μL d_0-MASC or d_3-MASC acetonitrile solution were added. The vial was sealed and vortexed for 1 min. Then it was allowed to react at 60 °C for 5 min in a water bath. After the reaction was completed, the mixture was cooled to room temperature. A 20 μL 50% acetic acid solution was added to adjust pH to lower than 7.0. The derivatized sample solution was then diluted to 500 μL with water–acetonitrile (1:1, v/v).

2.5. *HPLC–MS/MS analysis*

HPLC–MS/MS analysis was carried out on an Agilent 1290 series HPLC system coupled with an Agilent 6460 Triple Quadrupole MS/MS system (Agilent, USA) equipped with an Agilent Jet Stream electrospray ionization source (ESI source). HPLC separation was achieved using a SB C18 column (2.1 mm × 50 mm, 1.8 μm i.d., Agilent, USA). Eluent A was 0.1% formic acid in 5% acetonitrile and B was 0.1% formic acid in acetonitrile. The flow rate was 0.3 mL/min and the column temperature was kept at 30 °C. The elution conditions were as follows: 20–90% B from 0 to 5 min and then held for 2 min. The injection volume was 2 μL. The mass spectrometer was operated in a positive ion mode for the monitoring of $[M+H]^+$. The optimal ESI source conditions were: capillary voltage +4.0 kV; nebulizer 40 psi; dry gas 11.0 L/min; dry temperature 300 °C; Sheath gas temperature 280 °C; Sheath gas flow 10 L/min. The multiple reaction monitoring (MRM) parameters of the target compounds are listed in Table 1. Agilent 1100 Series LC/MSD-Trap-SL liquid chromatograph–mass spectrometry (Agilent, USA) was used to analysis unknown compounds from complex matrix.

2.6. *Matrix effect evaluation*

The matrix effects caused by wastewater, sediments and biological samples were compared between direct LC–MS/MS and stable isotope labeling LC–MS/MS method. After the SPE purification procedure, sample solutions were divided into two groups, each, except for a blank sample, was spiked at a concentration level of 10 μg/L with three replicates. The standard solutions were added after extraction procedure to avoid the possible error occurred during the sample pretreatment procedure. Thus the determined error could be solely attributed to the MS determination process. One group was derivatized by d_0-MASC and then analyzed by external standard method. The other group was first derivatized by d_0-MASC and then mixed with the d_3-MASC labeled standards. The mixed solution was analyzed by stable isotope labeling method. After subtracting the blank value, the mean values of three replicate experiments were used for the evaluation of matrix effects.

2.7. *Method validation*

Procedural blanks and sample blanks were analyzed before the analysis of real sample. The analytical method was then validated by linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision. Calibration curves were first constructed for d_0-MASC and d_3-MASC derivatives by plotting peak areas versus concentrations (0.5–200 ng/mL), then the theoretic peak area ratios of d_0-/d_3-MASC derivatives were compared with the experimental peak area ratios to obtain the linear regression equations for the quantification of stable isotope labeling method. LODs and LOQs for all target compounds were calculated at a signal-to-noise (S/N) ratio of 3 and 10, respectively. Recoveries were carried out by spiking blank samples with three different concentrations of standard solutions. Intra-day precision was determined by analyzing samples spiked at the same three levels of standards with six replicates, and inter-day precision was determined by running samples with spiked standards at the same levels with three replicates on three different days over a period of one week.

3. Results and discussion

3.1. Extraction of estrogenic compounds from different samples

Quick and accurate determination of estrogenic compounds in water, sediment and biological samples is of great importance since they have great influence on our daily life. Good sample extraction method is the basis of accurate quantification. Many extraction methods which were reported to be excellent have been tried in this study [34–36], but the recoveries were not satisfying in our study, especially for OP and NP. According to the method described in our previous work [33,37] and a three years experience in sediment and biological analysis, we proposed three feasible methods for the extraction of estrogenic compounds from different samples. The recoveries of the three extraction methods for all target compounds were higher than 75% and can be applied in the daily analysis of the estrogenic compounds from environmental and biological samples.

3.2. Derivatization of estrogenic compounds

Derivatization of estrogenic compounds with MASC could be achieved at 60 °C within 5 min. There is no increase in response with increased derivatization time. Maximum derivatization yields were obtained by using 0.1 M sodium bicarbonate buffer (pH 10) as basic catalyst. To obtain solely disubstituted derivatives of BPA and DES, a large excess of labeling reagent should be used. Constant peak intensities were achieved with the addition of a seven-fold molar reagent excess to total molar analytes. Further increasing the excess of reagent beyond this level had no significant effect on the yields. For the convenience of operation, 1.0×10^{-3} mol/L MASC was applied in derivatization. This concentration was sufficient enough for daily analysis since the contents of estrogenic compounds in most samples were far below this level.

Peak intensities of disubstituted compounds were usually higher than those of mono-substituted compounds [33]. However, peak intensities of BPA and DES turned out to be lower than those of mono-substituted derivatives such as E1 in this study. The MS1 scan spectrum of the target compounds indicated that BPA and DES were rich in $[M+Na]^+$ adducts, while the mono-substituted compounds such as E1 were dominated by $[M+H]^+$ adducts. Since $[M+H]^+$ ions were monitored in this study, thus the monitored peak intensities of BPA and DES were reduced. Although $[M+Na]^+$ ions were more abundant than $[M+H]^+$ ions for BPA and DES, they were not used as precursor ions because it was difficult to obtain regular product ions from them. Double charged ions of BPA and DES were not observed in the MS1 scan spectrum. It should be pointed out that DES is a mixture of *trans*-DES and *cis*-DES. The amount of *cis*-DES increased at room temperature owing to the *trans* to *cis* conversion. A linear response was observed for the sum of the *trans* and *cis* isomers. As a result, the sum peak areas of *trans*- and *cis*-DES were applied for quantification in this study.

3.3. Stability of MASC and derivatives

Acetonitrile solution of MASC could be stored at room temperature (25 °C) for one week without obvious decrease in derivatization yields for estrogenic compounds compared to those newly prepared MASC solution. When placed at 4 °C, it could be stable for one month with peak area deviations of less than 5% for the derivatized analytes. The stabilities of the corresponding derivatives were also investigated. Standard solution of 10 μg/L, water, sediment and meat samples spiked at 10 μg/L were derivatized by d_0-MASC and d_3-MASC, respectively. The derivatized solutions were then neutralized to pH < 7.0. These solutions were repeatedly analyzed by LC–MS/MS after being placed at room temperature for 0, 4, 8, 12, 24, 48, 72 and 96 h, respectively. The corresponding derivatives were stable with peak area deviations (RSDs) of less than 4.6%.

3.4. Mass enhancement

All eight estrogenic compounds contain, at least, one or two phenolic hydroxyl functional groups. The phenolic hydroxyl group usually exhibits relatively low ionization efficiency in negative ion mode. Therefore, these compounds typically have below-average sensitivity in electrospray ionization mass spectrometry analysis, compared to ionizable organic compounds such as amine or carboxylic acids [29,30,38–40]. For example, the ionization ratio of EE2 is less than 0.001% [41]. A number of derivatization procedures have been applied to enhance the ionization efficiency of estrogenic compounds in LC–MS analysis [32,40,42]. Derivatizing reagents containing a sulfuryl chloride group have often been used for the derivatization of estrogens. For example, Xu et al. applied pyridine-3-sulfonyl to the analysis of steroidal estrogens with product ions rich in analyte-specific fragment ions [40], while Anari et al. applied dansyl chloride to the analysis of EE2 with high sensitivity and specificity [41]. In our previous study we have compared the mass enhancement effects between EASC and dansyl chloride, and the results indicated that EASC was superior to dansyl chloride in mass enhancement [32].

To further demonstrate the signal enhancement effect of MASC, estrogenic compounds at a concentration of 500 μg/L were directly analyzed by negative ion mode LC–MS/MS. To obtain better ionization efficiency, the SB C18 column was operated at its highest pH value of 8 with 0.01% ammonia added in the mobile phase. At the same time, a standard solution was derivatized with MASC and analyzed by positive ion mode LC–MS/MS (final concentration: 10 μg/L). The signal intensities of OP, NP, E1, E2, E3, EE2, BPA and DES were enhanced by 240, 790, 500, 4700, 4000, 2850, 1000 and 250-fold, respectively. It should be pointed out that the sensitivity of negative mode LC–MS/MS would be enhanced if the analysis could be operated at higher pH value, and the signal enhancement factors may decrease somewhat.

In addition, derivatization obviously increased the hydrophobicity and *m*/*z* values of the analytes. In this case, the MASC derivatives were shifted out of the low mass region that typically exhibits significant background noise from solvent clusters and common contaminants during LC–MS analysis [14,43]. At the same time, the increased retention time resulted in more efficient ionization owing to the high acetonitrile content in mobile phase [17].

3.5. Relative quantification

Relative quantification is extremely important for complex sample analysis, especially when ionization efficiencies of analytes show tremendous differences between two single runs or the standard solutions are not available. Relative quantification of estrogenic compounds in two comparative samples can be done by light labeling one sample and heavy labeling the other sample, followed by mixing the two labeled samples and injecting the mixture into the LC–MS for analysis. To investigate the capability of our method for accurate relative quantification, d_0- and d_3-labeled standards were mixed with the ratios of 1:20, 1:10, 1:5, 1:1, 5:1, 10:1 and 20:1 and then injected into the LC–MS/MS for analysis. All analyses were done in triplicate. Average peak area ratios of the d_0- and d_3-labeled ions were used for relative quantification of estrogenic compounds. The results indicated that the experimental data and the theoretical ratios were in good linearity with correlation coefficients of >0.9923, while the relative standard deviations (RSDs) of the triplicate analysis were lower than 4.8%.

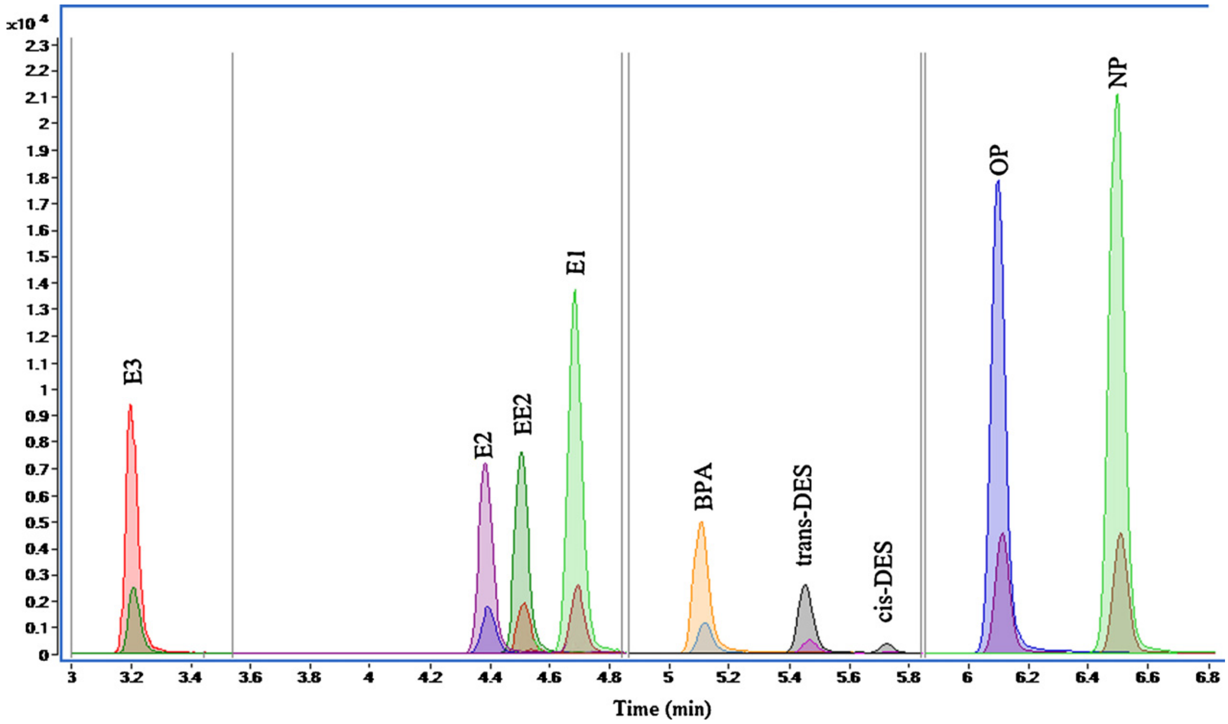

Fig. 2. Extracted ion chromatograms of d_0-MASC (the lower chromatogram) and d_3-MASC (the upper chromatogram) labeled estrogenic compounds in spiked water sample.

To demonstrate the feasibility of relative quantification by the established labeling technology, a water sample was divided into two equal fractions and spiked at a concentration ratio of 4:1. The low concentration fraction was labeled with d_0-MASC and the other fraction was labeled with d_3-MASC under the same condition. Subsequently, the two fractions were well mixed and analyzed by LC–MS/MS. As can be seen from Fig. 2, d_3-MASC derivatives coeluted with their d_0-MASC counterparts in RPLC. The ratios of integrated peak areas for d_3- and d_0-labeled ion pairs were in the range of 3.6–4.2, in good agreement with the expected value of 4.

3.6. Absolute quantification

Accurate absolute quantification is the goal of many researchers and test organizations. It is possible to determine the absolute concentration of the target compounds as long as the analyte standards are available. However, the accuracy of the results was often discounted since the sample and standard showed different behaviors in MS detecting process. Stable isotope labeling method provides a good solution to this problem. Stable isotopic tags were introduced into the standards by the labeling reaction, while the mass-difference isotopic tags were introduced into the samples to be analyzed. Standards and samples were then mixed and analyzed by LC–MS/MS. Since standards and sample derivatives eluted at the same time, matrix effects and ionization process for the light-labeled analytes were expected to be identical with the heavy-labeled standards.

As an example of absolute quantification, sediment sample solutions were light labeled with d_0-MASC. The estrogenic compound standards of known concentration were heavy labeled with d_3-MASC at the same time. The two labeled samples were well mixed and then analyzed by LC–MS/MS (see Fig. S1 in Supplementary Data). The obtained peak abundance ratio of d_0-/d_3-MASC labeled NP was calculated to be 0.83. Since the concentration of d_3-MASC labeled NP was already known (5 μg/L), the absolute concentration of NP can be readily calculated to be 3.9 μg/L. Similarly, OP and BPA were detected in the sediment sample with concentrations of 1.8 and 1.7 μg/L, respectively.

Both d_0-MASC and d_3-MASC derivatives of the target compounds showed good linearity in the concentration range of 0.5–200 μg/L with correlation coefficients of higher than 0.996. The linear regression equations obtained by comparing theoretic concentration ratios of d_0-/d_3-MASC derivatives with the experimental concentration ratios, method LODs and LOQs are shown in Table 2. Recoveries were determined by spiking 5.0, 10.0 and 20.0 ng/L of standards in wastewater samples, and 1.0, 5.0 and 10.0 μg/kg of standards in sediment and shrimp samples. The spiked samples were treated according to the procedure described in Section 2 and light labeled by d_0-MASC, while the standards were heavy labeled by d_3-MASC. Then the spiked samples and standards were mixed and determined by LC–MS/MS. The method was confirmed to be free of procedural interferences of the target compounds, and BPA was found in some sample blanks. Based on the formula of (measured value − endogenous value)/added value × 100, the recoveries were between 75% and 95% for all eight estrogenic compounds with relative standard deviations of less than 5.8%. The results were listed in Table 3. The intra-day precisions for the tested samples were in the range of 3.2–6.0%, while the inter-day precisions were between 5.9% and 8.6%.

3.7. Evaluation of matrix effect

Matrix effect is a well known problem that greatly influence the accuracy of mass spectrometry [11,12]. Stable isotope labeling method provides a good strategy to overcome matrix effects. The differential isotope labeled samples and standards coeluted and were therefore detected by MS detector at the same time. Thus, matrix effects or instrumental variations observed for the standards and analytes were expected to be the same. In this study, matrix effects caused by wastewater, sediment and biological samples were compared between direct LC–MS/MS and stable isotope labeling LC–MS/MS method according to the method described in Section 2, and the results were shown in Tables 4 and 5.

Table 2
Method linearity, dynamical range, LOD and LOQ.

Analyte	Linearity	R^2	Dynamical ratio range	LOD[b]			LOQ[b]		
				Water	Sediment	Meat	Water	Sediment	Meat
OP	$Y = 0.9661X$[a]	0.9996	20:1–1:20	0.70	0.10	0.09	2.1	0.30	0.25
NP	$Y = 1.0687X$	0.9952	20:1–1:20	0.60	0.08	0.09	2.0	0.25	0.30
BPA	$Y = 1.0287X$	0.9952	20:1–1:20	1.0	0.15	0.15	3.0	0.45	0.45
DES	$Y = 0.8524X$	0.9978	20:1–1:20	1.5	0.17	0.17	4.5	0.50	0.50
E2	$Y = 0.9523X$	0.9952	20:1–1:20	0.80	0.09	0.09	2.5	0.30	0.30
EE2	$Y = 0.9406X$	0.9965	20:1–1:20	0.75	0.09	0.10	2.5	0.25	0.30
E1	$Y = 0.8786X$	0.9972	20:1–1:20	0.50	0.06	0.05	1.5	0.20	0.15
E3	$Y = 1.0214X$	0.9923	20:1–1:20	0.70	0.07	0.08	2.0	0.20	0.25

[a] X, theoretic concentration ratio; Y, experimental mass spectrometric peak intensity ratio.
[b] The unit of water sample was ng/L, and the units for sediment and meat sample were μg/kg.

Table 3
Recoveries of estrogenic compounds in wastewater, sediment and shrimp samples ($n = 3$).

Sample	Spiked level	Recovery (%)							
		OP	NP	BPA	DES	E1	EE2	E2	E3
Wastewater (ng/L)	5	84.3 ± 4.1[a]	80.2 ± 3.9	94.4 ± 3.9	89.5 ± 4.2	93.2 ± 3.8	94.6 ± 4.0	90.8 ± 3.6	93.6 ± 4.2
	10	86.0 ± 4.5	82.4 ± 4.2	93.7 ± 4.8	91.4 ± 5.1	94.8 ± 2.5	92.5 ± 3.4	88.6 ± 3.3	95.2 ± 3.1
	20	87.4 ± 3.2	83.5 ± 4.6	95.1 ± 3.1	92.0 ± 3.8	95.0 ± 3.6	93.0 ± 2.6	89.5 ± 2.7	93.3 ± 2.8
Sediment (μg/kg)	1	81.6 ± 3.9	78.6 ± 3.2	91.2 ± 2.9	86.7 ± 5.2	91.6 ± 4.2	92.3 ± 3.4	87.9 ± 3.4	91.4 ± 4.7
	5	83.4 ± 4.1	80.5 ± 3.5	93.4 ± 3.8	88.3 ± 3.4	91.0 ± 2.4	91.7 ± 4.5	86.5 ± 3.0	92.4 ± 4.1
	10	83.5 ± 3.1	81.4 ± 3.8	92.5 ± 4.3	87.5 ± 4.4	93.5 ± 3.7	93.2 ± 3.7	88.2 ± 3.6	93.5 ± 3.7
Shrimp (μg/kg)	1	77.5 ± 3.8	75.3 ± 3.5	89.6 ± 4.3	85.3 ± 4.5	90.5 ± 3.1	92.5 ± 4.3	86.6 ± 4.0	90.5 ± 3.9
	5	78.6 ± 3.5	76.1 ± 3.9	90.5 ± 3.4	87.1 ± 3.9	91.4 ± 3.6	91.2 ± 3.6	87.3 ± 3.5	91.3 ± 4.3
	10	80.2 ± 3.1	75.8 ± 4.2	91.6 ± 4.4	87.8 ± 4.3	90.8 ± 4.1	92.3 ± 3.5	88.5 ± 2.8	90.4 ± 3.8

[a] Data are expressed as mean recovery (%) ± SD.

Table 4
Comparison of the accuracy of direct LC–MS/MS and stable isotope labeling LC–MS/MS methods in wastewater sample analysis.

Analyte	Add amount[a] (μg/L)	LC–MS/MS		Stable isotope labeling LC–MS/MS	
		Determined amount[b]	RSD (%)	Determined amount[b]	RSD (%)
OP	10	5.0	9.8	9.5	3.6
NP	10	4.5	8.7	9.2	3.4
BPA	10	7.5	5.0	10.3	4.5
DES	10	8	5.2	8.9	5.0
E2	10	7.2	4.8	9.4	3.0
EE2	10	7.4	5.7	9.2	4.2
E1	10	7.0	4.5	9.0	3.8
E3	10	9.2	5.6	10	2.8

[a] Standards were added after sample extraction procedure.
[b] Results are shown by μg/L.

As can be seen from the results of wastewater sample listed in Table 4, the determined values of direct LC–MS/MS method showed, to some extent, an obvious deviation to that of the real value. The low determined values of OP and NP may be caused by the ionization competition from the matrix in wastewater sample or the contaminations introduced from other sources. Contrarily, when the stable isotope labeling technique was used, little matrix effects were observed. The improved accuracy may be

Table 5
Comparison of the accuracy of direct LC–MS/MS and stable isotope labeling LC–MS/MS methods in sediment and biological sample analysis.

Analyte	Add amount[a] (μg/L)	LC–MS/MS				Stable isotope labeling LC–MS/MS			
		Determined amount[b]		RSD (%)		Determined amount[b]		RSD (%)	
		Sediment	Meat	Sediment	Meat	Sediment	Meat	Sediment	Meat
OP	10	5.1	5.8	10.2	8.7	9.0	9.2	4.0	4.6
NP	10	6.2	5.5	7.6	9.2	9.6	9.5	3.6	3.7
BPA	10	7.2	7.5	4.5	4.2	10.5	9.8	4.2	4.1
DES	10	7.0	7.3	4.8	5.6	8.8	9.1	5.2	4.8
E2	10	7.7	7.8	5.3	5.4	9.5	9.4	3.8	3.4
EE2	10	7.5	7.0	6.2	5.3	9.2	9.5	4.9	4.6
E1	10	8.5	8.2	4.2	4.9	8.9	9.3	4.6	3.5
E3	10	9.4	8.5	6.6	5.8	9.8	10	3.2	2.9

[a] Standards were added after sample extraction procedure.
[b] Results are shown by μg/L.

Table 6
The concentration of estrogenic compounds in different samples.

Sample		Estrogenic compound							
		OP	NP	BPA	DES	E1	EE2	E2	E3
Water (ng/L)	1	4.0	5.8	21	–	6.5	–[a]	3.7	–
	2	3.2	3.7	15	–	7.4	–	5.2	–
	3	5.3	4.6	12	–	7.2	–	4.8	–
Sediment (μg/kg)	1	1.0	2.7	1.6	–	–	–	–	–
	2	0.90	2.0	0.85	–	–	–	–	–
	3	2.1	3.6	2.5	–	–	–	–	–
Shrimp (μg/kg)	1	–	–	–	–	–	–	–	–
	2	–	–	1.2	–	–	–	–	–
	3	–	–	4.0	–	–	–	–	–

[a] –: not detected.

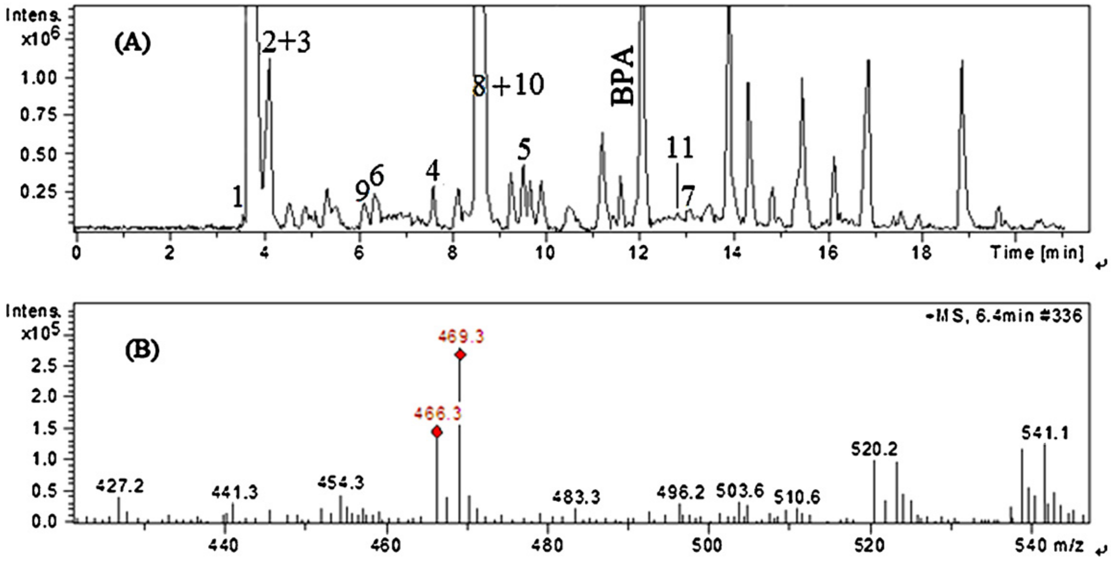

Fig. 3. (A) Base peak ion chromatogram of mixed shrimp and meat sample. 1–11: Peaks containing ion-pairs with a mass difference of 3 amu or 6 amu. (B) An expanded mass spectrum showing an ion pair with a mass difference of 3 amu from the chromatogram A.

attributed to the deuterated internal standard generated through derivatization.

Sediment or meat samples usually contain more complex matrices than water samples. To remove the lipid matrix in meat samples, a further purification procedure (freezing–lipid filtration) was carried out. However, severe matrix effects were still observed (see Table 5). Some results obtained by direct LC–MS/MS method obviously deviated from their real values, especially for OP and NP. On the contrary, the values obtained by stable isotope labeling method were in good accordance with their real values (88–105%). Besides the improvement in accuracy, the method RSDs were also improved. Therefore, it can be concluded that stable isotope labeling technique is an efficient approach for the analysis of estrogenic compounds in complex samples.

Besides the minimization of matrix effects, another important improvement of stable isotope labeling technique was the reduction of the run-to-run error. During direct LC–MS analysis, different injection orders between the standard and real samples often resulted in several-fold differences for the obtained results. Furthermore, a minor alteration of MS or LC parameters between two runs can result in an obvious change in signals [13]. These problems were not observed when stable isotope labeling technique was used. This was due to the fact that the sample and standard were determined in a single run.

3.8. *Applications*

The utility of the method was examined in absolute quantification of estrogenic compounds in wastewater, sediment and shrimp samples. These samples were purified by the procedures described in Section 2.3 and then derivatized by d_0-MASC, while standard solutions were derivatized by d_3-MASC at the same time under the same conditions. Samples and standards were mixed and analyzed by LC–MS/MS. As shown in Table 6, BPA was found in all three kinds of samples. OP and NP were found in both wastewater and sediment samples. Endogenous estrogenic compounds of E1 and E2 were found only in wastewater samples. DES, EE_2 and E_3 were not detected in all three kinds of samples.

The usefulness of the proposed method in relative quantification was examined in the analysis of unknown compounds in shrimp and pork meat samples by Agilent 1100 Series Ion Trap LC–MS. Two samples were, respectively, purified according to the method described in Section 2. The purified shrimp sample was labeled by d_0-MASC, while the pork meat sample was labeled by d_3-MASC. Then the two differentially labeled samples were well mixed and analyzed by LC–MS (see Fig. 3A). Besides BPA, 11 unknown ion-pairs with a mass difference of 3 amu or 6 amu were monitored (Table S1 in Supplementary Data). As an example, Fig. 3B shows an unknown ion-pair with a mass difference of 3 amu. The peak abundance ratio of the two ions (469.3/466.3) is calculated to be 1.35. Thus, the concentration of the unknown compound in pork meat sample was about 1.35-fold of that in shrimp sample. The calculated molecular weight of the unknown compound was 194.2. Since only phenolic- or amino-containing compounds could react with MASC, the unknown compound was speculated to be 4-hydroxybenzoic acid isobutyl ester, an often used preservative in food. 4-Hydroxybenzoic acid isobutyl ester standard and the mixed sample were then analyzed by LC–MS/MS. The retention time and product ions (208.1 and 392.0) were in good accordance. Therefore, the compound could be identified as 4-hydroxybenzoic acid isobutyl ester. The structures of the other compounds need to be further identified in our laboratory.

4. Conclusions

In this work, a new sensitive stable isotope labeling method was developed for the analysis of estrogenic compounds in environmental and biological samples. One of the features of the proposed method is that the stable isotope labeling reagents can be readily synthesized and the labeling process is fast and robust. A second feature is the success of this labeling strategy in solving the problems of lacking of internal standards and severe matrix effects. Matrix effects and ionization differences which were often encountered in direct MS analysis were overcome by the coeluting of the analyte and the standard. A third feature is the significant signal enhancement effect through the introduction the MASC moiety with high proton affinity to analyte molecules. A fourth feature is the utility of the proposed method in complex sample analysis. Detailed sample pretreatment procedure and matrix effect were studied for wastewater, sediment and biological samples, respectively. The results indicated that analytical procedure described in this paper was suitable for the analysis of estrogenic compounds in different samples. The proposed method was successfully applied to the relative and absolute quantification of estrogenic compounds in different environmental and biological samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.01.045.

References

[1] G.L. Bosco, TrAC, Trends Anal. Chem. 29 (2010) 781.
[2] N. Deshmukh, I. Hussain, J. Barker, A. Petroczi, D.P. Naughton, Steroids 75 (2010) 710.
[3] B. Kammerer, R. Kahlich, M. Ufer, S. Laufer, C.H. Gleiter, Anal. Biochem. 339 (2005) 297.
[4] J.R. Kesting, J.Q. Huang, D. Sørensen, J. Pharm. Biomed. Anal. 51 (2010) 705.
[5] C.T. Kim, E.S. Hwang, H.J. Lee, Food Chem. 101 (2007) 401.
[6] D.T. Modhave, T. Handa, R.P. Shah, S. Singh, J. Pharm. Biomed. Anal. 56 (2011) 538.
[7] O. Nunez, E. Moyano, M.T. Galceran, TrAC, Trends Anal. Chem. 24 (2005) 683.
[8] M. Rauh, J. Steroid Biochem. Mol. Biol. 121 (2010) 520.
[9] M. Vogeser, F. Kirchhoff, Clin. Biochem. 44 (2011) 4.
[10] E.T. Gangl, M. Annan, N. Spooner, P. Vouros, Anal. Chem. 73 (2001) 5635.
[11] P.J. Taylor, Clin. Biochem. 38 (2005) 328.
[12] J.C. Van De Steene, K.A. Mortier, W.E. Lambert, J. Chromatogr. A 1123 (2006) 71.
[13] K. Guo, C. Ji, L. Li, Anal. Chem. 79 (2007) 8631.
[14] K. Guo, L. Li, Anal. Chem. 81 (2009) 3919.
[15] X. Huang, F.E. Regnier, Anal. Chem. 80 (2008) 107.
[16] S.M. Lamos, M.R. Shortreed, B.L. Frey, P.J. Belshaw, L.M. Smith, Anal. Chem. 79 (2007) 5143.
[17] N. Abello, P.P. Geurink, M. Toorn, A.J.M. Oosterhout, J. Lugtenburg, G.A. Marel, H.A.M. Kerstjens, D.S. Postma, H.S. Overkleeft, R. Bischoff, Anal. Chem. 80 (2008) 9171.
[18] S.H. Kirsch, J.P. Knapp, W. Herrmann, R. Obeid, J. Chromatogr. B 878 (2010) 68.
[19] M.J. Alda, D. Barceló, Fresenius J. Anal. Chem. 371 (2001) 437.
[20] A. Ballesteros-Gómez, S. Rubio, D. Pérez-Bendito, J. Chromatogr. A 1216 (2009) 449.
[21] R. Carabias-Martinez, E. Rodriguez-Gonzalo, P. Revilla-Ruiz, J. Chromatogr. A 1056 (2004) 131.
[22] R. Jeannot, H. Sabik, E. Sauvard, T. Dagnac, K. Dohrendorf, J. Chromatogr. A 974 (2002) 143.
[23] R. Liu, J. Zhou, A. Wilding, J. Chromatogr. A 1022 (2004) 179.
[24] L. Wang, Y.Q. Cai, B. He, C.G. Yuan, D.Z. Shen, J. Shao, G.B. Jiang, Talanta 70 (2006) 47.
[25] S. Wang, W. Huang, G. Fang, J. He, Y. Zhang, Anal. Chim. Acta 606 (2008) 194.
[26] K. Mitani, M. Fujioka, H. Kataoka, J. Chromatogr. A 1081 (2005) 218.
[27] A. Nieto, F. Borrull, E. Pocurull, R.M. Marcé, J. Chromatogr. A 1213 (2008) 224.
[28] Y. Zhou, J. Zhou, Y. Xu, J. Zha, M. Ma, Z. Wang, Environ. Monit. Assess. 158 (2009) 35.
[29] Y. Iwasaki, Y. Nakano, K. Mochizuki, M. Nomoto, Y. Takahashi, R. Ito, K. Saito, H. Nakazawa, J. Chromatogr. B 879 (2011) 1159.
[30] A. Salvador, C. Moretton, A. Piram, R. Faure, J. Chromatogr. A 1145 (2007) 102.
[31] R.L. Gomes, E. Avcioglu, M.D. Scrimshaw, J.N. Lester, TrAC, Trends Anal. Chem. 23 (2004) 737.
[32] J. You, H. Zhao, Z. Sun, Y. Suo, G. Chen, Chromatographia 70 (2009) 45.
[33] S. Zhang, J. You, Z. Sun, C. Song, S. Ning, C. Zhao, Y. Suo, Microchem. J. 103 (2012) 90.
[34] J. Wang, H. Pan, Z. Liu, F. Ge, J. Chromatogr. A 1216 (2009) 2499.
[35] O. Ballesteros, A. Zafra, A. Navalón, J.L. Vílchez, J. Chromatogr. A 1121 (2006) 154.
[36] Z.L. Zhang, A. Hibberd, J.L. Zhou, Anal. Chim. Acta 577 (2006) 52.
[37] X. Zhang, Y. Xu, X. Gong, H. Xing, S. Zhang, X. Liu, J. Fish. Sci. China 16 (2009) 791.
[38] W. Li, Y. Li, A. Li, S. Zhou, W. Naidong, J. Chromatogr. B 825 (2005) 223.
[39] X.M. Zhuang, M. Yuan, Z.W. Zhang, X.Y. Wang, Z.Q. Zhang, J.X. Ruan, J. Chromatogr. B 876 (2008) 76.
[40] L. Xu, D.C. Spink, Anal. Biochem. 375 (2008) 105.
[41] M.R. Anari, R. Bakhtiar, B. Zhu, S. Huskey, R.B. Franklin, D.C. Evans, Anal. Chem. 74 (2002) 4136.
[42] W.C. Yang, F.E. Regnier, D. Sliva, J. Adamec, J. Chromatogr. B 870 (2008) 233.
[43] B.O. Keller, J. Sui, A.B. Young, R.M. Whittal, Anal. Chim. Acta 627 (2008) 71.