



Simultaneous quantification of multiple classes of phenolic compounds in blood plasma by liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

A method using high performance liquid chromatography–electrospray tandem mass spectrometry (LC–ESI–MS/MS) in positive ion mode was developed for the simultaneous analysis of 30 phenolic compounds, including four estrogens, bisphenol A (BPA), 10 hydroxylated polybrominated diphenyl ethers (OH–PBDEs) and 15 bromophenols (BRPs), in blood plasma. In the present method, derivatization with dansyl chloride was employed, and all the derivatized target compounds were well resolved on a 100 mm Xbridge C18 column with acetonitrile and 0.1% formic acid as the mobile phases. Purification procedures, such as liquid–liquid extraction and silica–gel chromatography, were applied to reduce matrix effects in the sample extract and remove excess derivatizing reagents, thus permitting selective and sensitive detection of the target phenolic compounds. The limit of quantification for all analytes, with a signal-to-noise ratio of ~10, was 2–30 pg/g (plasma weight) except for 6-OH-BDE-137 (30 pg/g) and 3-BRP (60 pg/g). The method was validated for recoveries (68–100%), accuracy (84–110%) and precision (3.7–11%) using charcoal-stripped bovine blood plasma spiked with all target compounds (500 and 5000 pg/mL). Finally, the method was applied to analyze six blood plasma samples from frogs and cormorants, where two natural estrogens, one BPA, one OH–PBDE and four BRPs were detected. The greatest total concentrations of estrogens coincided with the least total concentrations of other phenolic compounds for both species. The proposed method based on derivatization followed by LC–MS/MS provides a novel method to simultaneously monitor multiple groups of phenolic compounds in blood plasma.

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1. Introduction

The presence of endocrine-disrupting chemicals in the environment is generating worldwide concern, and most attention has been focussed on the estrogenic potential of these chemicals [1–3]. Many chemicals have been reported to possess estrogenic properties [4,5] and a variety of reproductive disorders in wildlife and humans have been tentatively attributed to these estrogenic pollutants [6,7]. Among the estrogenic compounds, phenolic chemicals, such as natural and synthetic estrogens, bisphenol A, and nonylphenols, have attracted great concerns and have been investigated in a wide variety of biological and environmental matrices [4,5,8].

Besides these well-known estrogenic compounds, some OH–PBDEs and brominated phenols (BRPs) have recently been reported to act as ligands for estrogen receptors [9]. These brominated compounds, primarily originating from natural products and/or man-made flame-retardants [10], have been detected in biological matrices, such as blood of humans and wildlife [11]. Thus, there is a concern about the widespread environmental occurrence of phenolic compounds with potential estrogenic activities, which may impact hormone balance or otherwise disrupt endocrine functions [12]. To assess the effects of these phenolic compounds on the endocrine system, there is a need to develop sensitive methods to measure a broad range of phenolic compounds together with natural estrogens in environmental samples.

Estrogens and BPA have previously been quantified by use of liquid chromatography (LC) with a variety of detection systems, such as fluorescence [13] or mass spectrometry [14–17], some methods based on derivatization followed by gas chromatography coupled to mass spectrometry (GC/MS) have also been used [18]. Recent

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LC-ESI-MS/MS quantification of phenolic compounds belonging to different groups was developed.

2.1. Chemicals and reagents

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Table 1
Analytes, surrogate standards, formulae and selected reaction monitoring conditions.

Analyte	Formula	Transition monitored (<i>m/z</i>)	Collision energy (V)
E1	C ₁₈ H ₂₂ O ₂	504 → 171 ^a	46
		504 → 156	68
d ₄ -E1	C ₁₈ H ₁₈ D ₄ O ₂	508 → 171	46
αE2	C ₁₈ H ₂₄ O ₂	506 → 171 ^a	48
		506 → 156	72
βE2	C ₁₈ H ₂₄ O ₂	506 → 171 ^a	48
		506 → 156	72
d ₄ -βE2	C ₁₈ H ₂₀ D ₄ O ₂	510 → 171	48
EE	C ₂₀ H ₂₄ O ₂	530 → 171 ^a	48
		530 → 156	80
BPA	C ₁₅ H ₁₆ O ₂	695 → 171 ^a	60
		695 → 156	110
d ₁₆ -BPA	C ₁₅ D ₁₆ O ₂	710 → 171	60
2'-OH-6'-Cl-BDE-7	C ₁₂ H ₇ Br ₂ ClO ₂	612 → 171 ^a	48
		614 → 171	48
6'-OH-BDE-17	C ₁₂ H ₇ Br ₃ O ₂	656 → 171 ^a	48
		658 → 171	48
3-OH-BDE-47	C ₁₂ H ₆ Br ₄ O ₂	734 → 171 ^a	48
5-OH-BDE-47		736 → 171	48
6-OH-BDE-47			
4'-OH-BDE-49			
2'-OH-6'-Cl-BDE-68	C ₁₂ H ₅ Br ₄ ClO ₂	770 → 171 ^a	48
		772 → 171	48
6-OH-BDE-90	C ₁₂ H ₅ Br ₅ O ₂	814 → 171 ^a	48
2-OH-BDE-123		816 → 171	48
6-OH-BDE-137	C ₁₂ H ₄ Br ₆ O ₂	894 → 171 ^a	48
		896 → 171	48
3-BRP	C ₆ H ₅ BrO	406 → 171	33
2/4-BRP		408 → 171 ^a	33
4- ¹³ C-BRP	¹³ C ₆ H ₅ BrO	414 → 171	33
2,3-diBRP	C ₆ H ₄ Br ₂ O	484 → 171	38
2,5-diBRP		486 → 171 ^a	38
2,6-diBRP			
3,4-diBRP			
3,5-diBRP			
2,4-diBRP			
2,4- ¹³ C-diBRP	¹³ C ₆ H ₄ Br ₂ O	492 → 171	38
2,3,4-triBRP	C ₆ H ₃ Br ₃ O	562 → 171	43
2,3,5/2,4,5-triBRP		564 → 171 ^a	43
2,3,6-triBRP			
2,4,6-triBRP			
3,4,5-triBRP			
2,4,6- ¹³ C-triBRP	¹³ C ₆ H ₃ Br ₃ O	570 → 171	43

^a MRM transition used for quantification

Table 2

Comparison of instrumental limit of quantifications (LOQs, ng/mL) of analytes by different analytical methods.

Analyte	LC–MS/MS (derivatized with dansyl chloride) ^a	LC–MS/MS (non-derivatized)	GC–HRMS (derivatized with MCF) ^e
Estrone (E1)	0.1	0.1 ^b	
α-Estradiol (E2)	0.04		
β-Estradiol (E2)	0.05	0.25 ^b	
Ethinyl estradiol (EE2)	0.04	0.5 ^b	
Bisphenol A (BPA)	0.1	18 ^c	
2'-OH-6'-Cl-BDE-7	0.1		0.5
6'-OH-BDE-17	0.1	0.8 ^d	0.5
3-OH-BDE-47	0.1	1.1 ^d	1
5-OH-BDE-47	0.08	0.8 ^d	1
6-OH-BDE-47	0.07	0.7 ^d	1
4'-OH-BDE-49	0.04	0.6 ^d	1
2'-OH-6'-Cl-BDE-68	0.3		1
6-OH-BDE-90	0.2		2
2-OH-BDE-123	0.2		2
6-OH-BDE-137	0.5		2
3-BRP	1.0		
2/4-BRP	0.2		
2,3-diBRP	0.06		0.5
2,5-diBRP	0.05		0.5
2,6-diBRP	0.2		0.5
3,4-diBRP	0.04		0.5
3,5-diBRP	0.03		0.5
2,4-diBRP	0.05		0.5
2,4,6-triBRP	0.3		1
2,3,5/2,4,5-triBRP	0.04		1
2,3,4-triBRP	0.12		1
2,3,6-triBRP	0.5		1
3,4,5-triBRP	0.12		1

^a This study.

^b Ref. [16].

^c Ref. [14].

^d Ref. [23].

^e Ref. [10].

triBRP) were obtained from Wellington Laboratories Inc. (Guelph, ON, Canada). Dichloromethane (DCM), n-hexane, methyl *tert*-butyl ether (MTBE), acetone, acetonitrile and methanol were pesticide residue grade obtained from OmniSolv (EM Science, Lawrence, KS, USA). Silica gel (60–100 mesh size), formic acid, hydrochloric acid (37%, A.C.S. reagent), 2-propanol and dansyl chloride were obtained from Sigma–Aldrich.

2.2. Blood plasma collection

Blood plasma of African clawed frogs (*Xenopus laevis*) and double-crested cormorants (*Phalacrocorax auritus*) was analyzed to confirm the performance of the method. The frog blood samples were collected in 2002 from regions in the vicinity of Potchefstroom, South Africa, where corn (*Zea mays*) was the primary cultivated crop [27]. Blood plasma of double-crested cormorants was collected from nestlings in 1991 in the north American Great Lakes in Michigan, USA. The collection details for these samples have been published previously [28]. Blood plasma was prepared by centrifugation of the heparinized blood samples within 12 h of collection and samples were frozen at –20 °C until analysis.

2.3. Preparation of stock and working standards

Stock solutions of estrogens, BPA, OH-PBDEs and BRPs were prepared at 1000, 1000, 100 and 5 µg/mL using acetonitrile, respectively, and stock solutions of isotope-labeled estrogens, BPA, and BRPs were prepared at 1000, 100 and 5 µg/mL, respectively. Working standard solutions were prepared at 50 ng/mL for each

compound by dilution of the stock solutions with acetonitrile. To prepare the calibration standards, five different amounts of target compounds (0.5, 1, 5, 10 and 20 ng/mL) and working surrogate standard solution (10 ng/mL of each surrogate) were added to four 4 mL amber glass tubes.

After drying under a stream of nitrogen, the residues were dissolved in 100 µL of aqueous sodium bicarbonate (100 mmol/L, pH adjusted to 10.5 with NaOH) and 100 µL of dansyl chloride (1 mg/mL in acetone), vortex-mixed for 1 min and incubated at 60 °C for 5 min. Then 1 mL of 18 MΩ water and 2 × 3 mL of hexane were added. After cooling to room temperature the organic fraction was dried under a stream of nitrogen and 100 µL of acetonitrile: water (60:40) was added before LC–MS/MS analysis.

2.4. Sample preparation

Aliquots (0.3–1 mL) of blood plasma samples were transferred into amber tubes and spiked with combined working surrogates. After the addition of 2 mL pure water, 50 µL hydrochloric acid (HCl, 37%) and 3 mL of 3-propanol, the samples were extracted two times with 5 mL hexane/MTBE (1:1; v/v). Extracts were washed four times with 4 mL 18 MΩ water to remove residual acid, and the organic extracts were concentrated and dried under nitrogen. The dried residues were dissolved in 200 µL of aqueous sodium bicarbonate (100 mmol/L, pH adjusted to 10.5 with sodium hydroxide) and 200 µL of dansyl chloride (1 mg/mL in acetone) was added, the sample was vortex-mixed for 1 min and incubated at 60 °C for 5 min. Next, 1 mL of 18 MΩ water and 3 × 3 mL of hexane were added, and the organic solvent layer was removed and was subjected to a silica-gel column (60–100 mesh size) for fractionation. The silica-gel column was wet packed with 4 g silica gel and 4 g sodium sulfate. After application of the extract, the column was rinsed with 15 mL hexane/DCM (1:1, v/v), and then eluted with 20 mL DCM and 30 mL of DCM/acetone (9:1; v/v). The first fraction contained OH-PBDEs and BRPs, and the second fraction contained estrogens and BPA. The two fractions were evaporated to dryness and reconstituted with 50 µL of acetonitrile:water (60:40) before LC–MS/MS analysis.

2.5. LC–MS/MS analysis

Analyses were conducted using an Agilent 1200 series HPLC system (Santa Clara, CA, USA) connected to an API 3000 triple-quadrupole MS/MS system (PE Sciex, Concord, ON, Canada). Both the LC and mass spectrometer were controlled by AB Sciex Analyst 1.4.1 software (Applied Bioscience, Foster City, CA, USA). For chromatographic separation, two chromatographic columns were tested: a Betasil C18 (100 mm × 2.1 mm, 5 µm particle size) from Thermo (Waltham, MA, USA) and an XBrige C18 column (100 mm × 2.1 mm, 3.5 µm particle size) from Waters (Milford, MA, USA). Separations were conducted at room temperature. The sample volume injected onto the column was 20 µL. The mobile phases consisting of acetonitrile (Solvent A) and 0.1% formic acid in water (Solvent B) was used with a gradient elution of A:B = 60:40 (0–1 min) to 95:5 (1–15 min) and 95:5 (15–22 min) at a flow rate of 0.25 mL/min.

The analytes were detected using a turbo ion spray ion source operated in the positive ion multiple-reaction monitoring (MRM) mode. All of the source and instrument parameters were optimized by infusing purified dansyl derivatives of analytes into the mass spectrometer. Both Q1 and Q3 were operated under unit mass resolution (0.7 Da at full width half-maximum). Nitrogen was used as the curtain (setting 8), nebulizer gas, (setting 12), and collision gas (setting 10). The ion spray voltage was set at 3750 V, and the temperature of the turbo ion spray interface was maintained at 475 °C. The formulae and the chosen MRM transitions with optimized colli-

sion energy for all analyzed compounds are summarized in Table 1. A dwell time of 50 ms was used for each of the MRM transitions. The declustering potential, focusing potential, entrance potential, and collision cell exit potential were optimized at 60–75, 220–250, 5–9, and 10–12 V, respectively, for the selected MRM transition of the analytes and isotope-labeled standards.

3. Results and discussion

3.1. Optimization of LC–MS/MS conditions

Dansyl chloride is highly reactive with phenolic hydroxyl groups, which provides a new method to derivatize phenolic compounds, including estrogens, BPA, OH-PBDEs, and BRPs prior to LC–MS analysis [19,20]. As expected, ionization and fragmentation of the isolated dansyl derivatives in electrospray tandem mass spectrometry resulted in protonated molecular ions $[MH]^+$ or $[MH]^+-2$ of their dansyl derivatives, and produced the same major product ions at m/z 171 and 156 (Table 1). The ion m/z 171 originates from a cleavage of a C–S bond in the dansyl portion of the molecule and the ion m/z 156 is produced by loss of the methyl group from the m/z 171. For estrogens and BPA, $[MH]^+ \rightarrow 171$ and

$[MH]^+ \rightarrow 156$ were used as the transitions, but for OH-PBDEs and BRPs, the most intense MRM transition took place at $[MH]^+ \rightarrow 171$ and $[MH]^+-2 \rightarrow 171$ due to the presence of the bromine isotopes in these compounds.

Dansyl derivatives of estrogens have been reported to exhibit greater sensitivities compared with direct LC–MS analysis of estrogens [19,29–31]. In our studies, similar improvements in sensitivities were observed for BPA and OH-PBDEs. The limits of quantification (LOQs) were calculated on the basis of the signal-to-noise ratio of 10:1. The instrumental LOQs of dansyl derivatives of BPA and OH-PBDEs were 8–180 times less than those of non-derivatization methods (Table 2). For OH-PBDEs and BRPs, GC–MS is the method most often applied in various sample matrices, and prior chemical derivatizations (e.g. diazomethane or methyl chloroformate (MCF)) is usually required. The LOQs of the new method are lower than those for a GC–HRMS-based method also developed in our laboratory [10] (Table 2). Furthermore, the reaction of dansyl chloride with phenolic compounds is rapid, being completed in 5 min, which is much less than the time required for other derivatization reagents used for the GC–MS analysis (0.5–1 h). The greater sensitivities, rapid and safe reaction to form dansyl derivatives of target phenolic compounds observed in this study suggest that

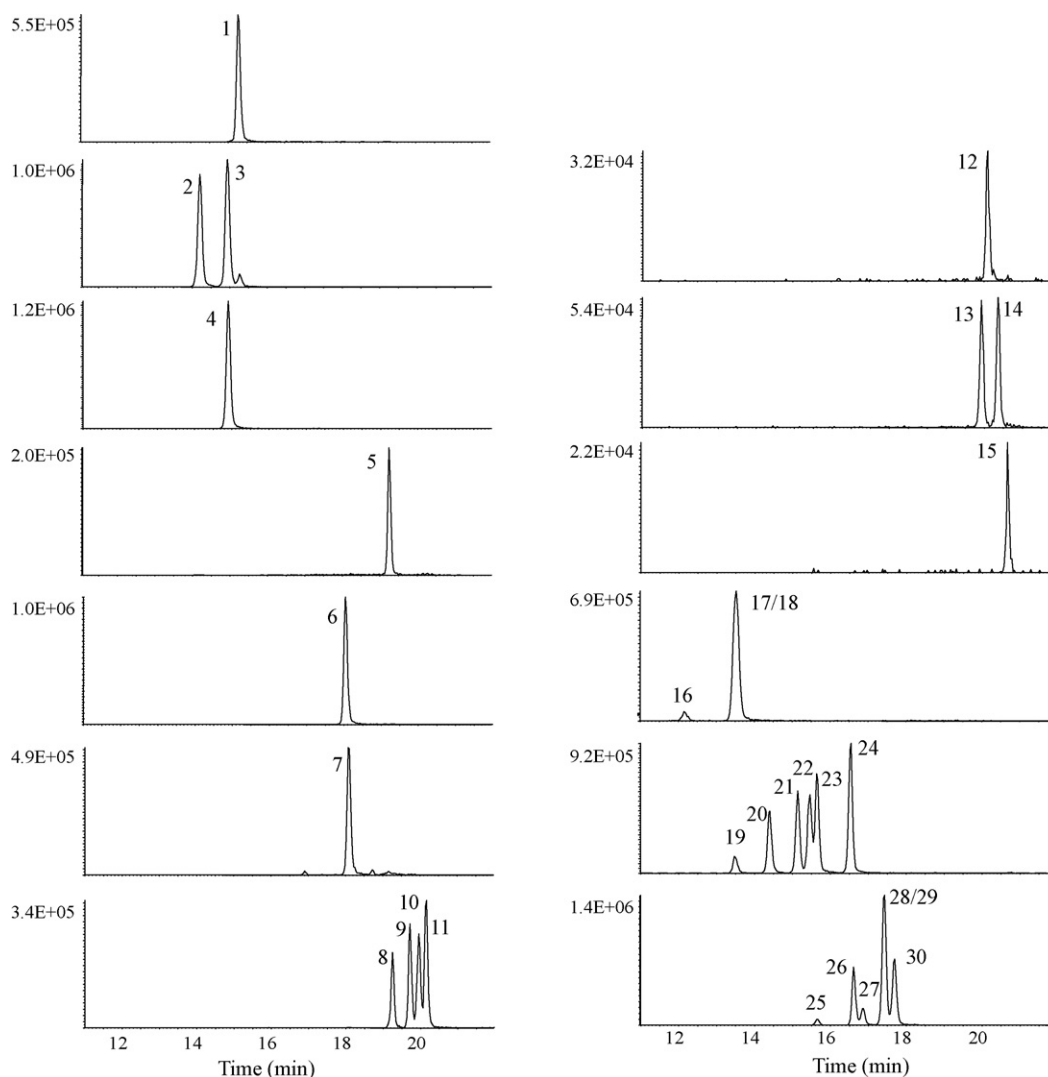


Fig. 1. LC–MS/MS MRM chromatographic profiles of 30 analytes in a standard mixture of 10 ng/mL. E1 (1), β E2 (2), α E2 (3), EE (4), BPA (5), 2'-OH-6'-Cl-BDE-7 (6), 6'-OH-BDE-17 (7), 3-OH-BDE-47 (8), 5-OH-BDE-47 (9), 6-OH-BDE-47 (10), 4'-OH-BDE-49 (11), 2'-OH-6'-Cl-BDE-68 (12), 6-OH-BDE-90 (13), 2-OH-BDE-123 (14), 6-OH-BDE-137 (15), 3-BRP (16), 2/4-BRP (17/18), 2,6-diBRP (19), 2,3-diBRP (20), 2,5-diBRP (21), 2,4-diBRP (22), 3,4-diBRP (23), 3,5-diBRP (24), 2,3,6-triBRP (25), 2,3,4-triBRP (26), 2,4,6-triBRP (27), 2,3,5/2,4,5-triBRP (28/29), 3,4,5-triBRP (30), analyzed on a 100 mm $\times$ 2.1 mm, i.d.; Waters XBridge column.

derivatization with dansyl chloride may be a promising method for the trace analysis of phenolic compounds by LC–MS/MS.

Chromatographic separation has an important role in this method, especially for congeners like OH-tetraBDE, diBRP and triBRP clusters. Thus, two columns were evaluated. Co-elution of OH-tetraBDE clusters was observed on the Thermo Scientific Betasil C18 column regardless of the gradient elution or mobile phase used. The Waters XBridge C18 column provided the best resolution with shorter retention times, although the separation can be further optimized to resolve some di- and tri- BRPs (e.g. 2-BRP and 4-BRP, 2,3,5-triBRP and 2,4,5-triBRP). Characteristic transitions for a standard mixture of four estrogens, one BPA, 10 OH-PBDEs, and 15 BRPs on the Waters XBridge C18 column are given in Fig. 1. Acceptable resolution of the adjacent chromatographic peaks for OH-tetraBDEs was achieved when a binary mixture of acetonitrile and 0.1% formic acid was used. The negative ESI–LC/MS/MS method for directly measuring eight OH-PBDEs has been previously suggested [24]. However, separation of OH-tetraBDEs can only be achieved by use of a ternary mixture of 5 mM ammonium acetate, acetonitrile and methanol as the mobile phases. Here we found that after derivatization with dansyl chloride, the target compounds can be easily separated with a binary mobile phase.

3.2. Optimization of extraction conditions

Sample preparation was designed to quantitatively extract estrogens and other phenolic compounds while minimizing interferences present in environmental samples before injection. In the extraction mixtures, 2-propanol was used to eliminate the

formation of emulsions that appeared during the extractions. Since phenolic compounds are not efficiently extracted into neutral organic solvents when they are still in an ionized form, HCl was used to acidify the solution. Addition of HCl also hydrolyzed conjugated estrogens so that they could be extracted quantitatively and total estrogen concentrations could be determined [32,33].

Estrogens have previously been quantified by LC–MS/MS analysis of crude extracts of human serum after derivatization with dansyl chloride [29–31]. However, for more complicated matrices such as blood plasma and other tissue samples, the derivatized solutions were still not clean enough for direct injection into the detection system. Also the relatively great concentrations of non-volatile carbonate buffer and dansyl chloride used in the sample extracts was found to accumulate in the detector causing a significant loss of sensitivity. Therefore, frequent cleaning of the system was required to maintain analytical performance when directly injecting the derivatization solutions into the instrument. In this study, it was found that all the derivatives of estrogens, BPA, OH-PBDEs and BRPs were extracted by hexane from water-diluted derivatization solutions. This step not only helped clean the samples but also facilitated the analyses of complex environmental samples.

To further clean the extracted samples, a silica-gel column was used for purification after derivatization. The brominated compounds (OH-PBDEs and BRPs) can be separated from estrogens and BPA by use of several eluents. No matrix-induced ion suppression was observed even when the two fractions were combined. Comparison of sample preparation using the silica-gel column and direct injection of derivatized solutions was conducted by analyz-

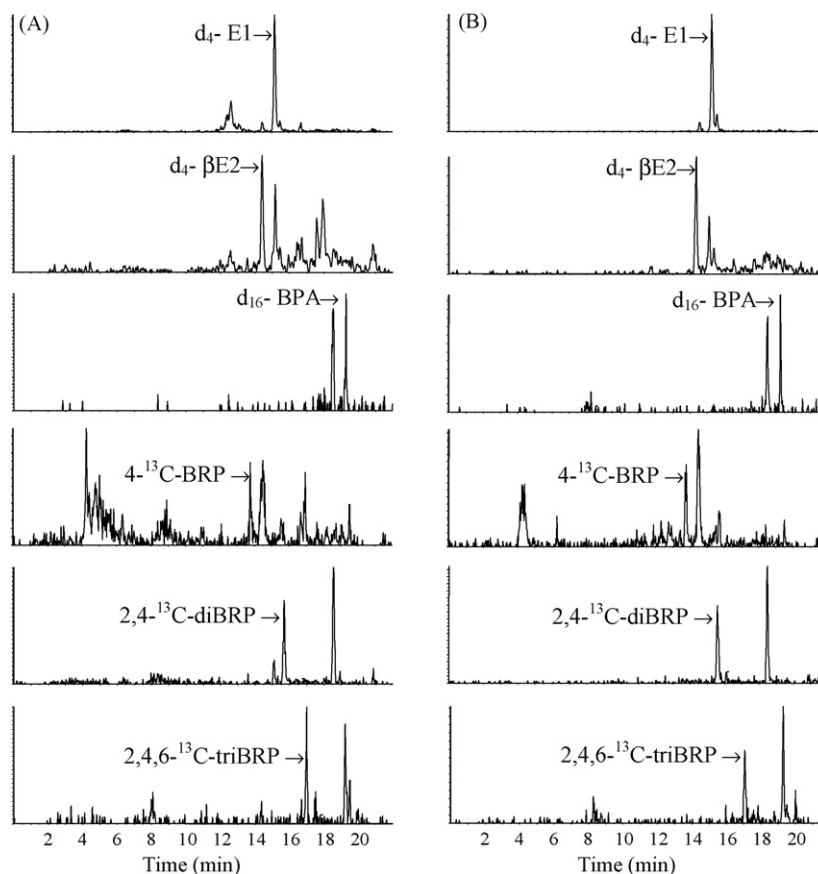


Fig. 2. LC–MS/MS MRM chromatographic profiles of 25 pg/mL of surrogate standards spiked in 1 mL of an identical charcoal-stripped bovine blood plasma sample with (B) and without silica-gel purification (A).

ing extracts of charcoal-stripped bovine blood plasma spiked with 25 pg/mL of surrogate standards (Fig. 2). The signal-to-noise ratios for surrogate standards with silica-gel purification were improved by the removal of several isobaric interferences.

3.3. Performance of the method

To correct for potential losses of analytes during extraction or sample preparation, and to compensate for variations in instrument response between injections isotopically labeled surrogate standards were used. d_4 -E1 was used as the surrogate standard for E1; d_4 - β E2 for α E2, β E2 and EE2. $4-^{13}\text{C}$ -BRP for 2-BRP, 3-BRP and 4-BRP; $2,4-^{13}\text{C}$ -diBRP for 2,6-diBRP, 2,5-diBRP, 2,4-diBRP, 2,3-diBRP, 3,5-diBRP and 3,4-diBRP; and $2,4,6-^{13}\text{C}$ -triBRP for 2,4,6-triBRP, 2,3,5-triBRP, 2,4,5-triBRP, 2,3,4-triBRP and 2,3,6-triBRP, on the basis of similarities in structure and retention time. d_{16} -BPA was used as the surrogate for BPA and all OH-PBDEs, since d_{16} -BPA has been used as the surrogate for eight OH-PBDEs by a previous study [24]. The calibration curve for each of the analytes was developed by plotting peak area ratios of the analyte derivative to its surrogate derivative versus the analyte concentrations and fitting these data with the linear regression. The linear calibration range studied was 0.5–1000 ng/mL with coefficients of determination greater than 0.99. Quantitation of analytes in the blood plasma was carried out using the AB Sciex Analyst 1.4.1 software (Applied Bioscience). Quantitative results for each of the analytes were calculated using the peak area ratios of the analyte derivative to its surrogate derivative in blood plasma samples and the corresponding calibration curve.

Charcoal-stripped bovine blood plasma was employed for preparation of quality control (QC) samples. The QC samples were

prepared at two concentrations: 500 or 5000 pg/mL of each target compounds. The mean ($n=4$) absolute recoveries for each of the target analytes ranged from 68% to 100%. The accuracy of this method was assessed as the recovery percent of the amount of target compounds known to be added, and was based on the mean of four samples analyzed in one batch. The intra-batch precision was based on four samples analyzed in one batch, and inter-batch precision was based on the means for four samples in each of four batches. As shown in Table 3, the accuracy for the two spiked levels of QC samples was 86–105% and 84–110%, respectively. The intra-batch precision was 6.8–11%, and 3.7–10% for the two concentrations, respectively. The inter-batch precision was 8.0–16%, and 5.1–12% for the two concentrations, respectively.

The extent of ESI enhancement/suppression (E/S) in LC-ESI/MS/MS detection was evaluated by spiking extracts of charcoal-stripped bovine blood plasma. At 500 and 5000 pg/mL, the percentage ESI E/S of the peak areas of all analytes in the samples were compared with the peak areas of each target analyte in the standard solution at the same concentration. In the charcoal-stripped bovine blood plasma, the percentage ESI E/S varied from 88% to 102% and there was no significant difference between greater and lesser concentrations spiked.

The method limit of quantification, with a signal-to noise ratio of $\sim 10:1$, was determined by extraction of 1 mL of charcoal-stripped bovine blood plasma and injection of 20 μL . The LOQs were 2–4 pg/g for estrogens, 5 pg/g for BPA, 3–30 pg/g for OH-PBDEs and 3–60 pg/g for BRPs (Table 3). The LOQ of estrogens observed in this study were less than those reported by using LC-MS/MS directly (30–60 pg/g using 2 mL of bovine blood serum) [17]. Similar sensitivity improvement with derivatization of dansyl chloride was also reported for estrogens in previous studies [19]. The present study was the first

Table 3

Accuracy, intra-batch precision and inter-batch precision of LC-MS/MS measurement of analytes and method limit of quantification (LOQs, pg/g) in 1 mL of blood plasma.

Analyte	500 pg/mL in blood plasma			5000 pg/mL in blood plasma			LOQ
	Accuracy (%) ^a	Intra-batch precision (%) ^b	Inter-batch precision (%) ^c	Accuracy (%) ^a	Intra-batch precision (%) ^b	Inter-batch precision (%) ^c	
Estrone (E1)	95	8.6	9.4	96	5.6	8.0	4
α -Estradiol (E2)	98	7.4	10	94	6.9	8.2	2
β -Estradiol (E2)	101	9.8	12	99	5.3	6.6	3
Ethinyl estradiol (EE2)	94	10	9.9	102	4.2	8.0	2
Bisphenol A (BPA)	97	5.8	11	105	4.8	8.1	5
2'-OH-6'-Cl-BDE-7	105	8.1	12	102	6.9	7.3	6
6'-OH-BDE-17	100	10	13	98	3.8	7.5	6
3-OH-BDE-47	97	7.8	14	89	5.9	6.8	6
5-OH-BDE-47	95	9.9	12	95	7.4	8.0	5
6-OH-BDE-47	91	8.7	9.1	91	4.6	6.5	5
4'-OH-BDE-49	89	11	13	88	5.9	6.3	3
2'-OH-6'-Cl-BDE-68	92	10	10	96	7.2	7.9	15
6-OH-BDE-90	93	7.1	8.4	100	3.7	9.4	12
2-OH-BDE-123	87	6.9	8.2	92	10	12	13
6-OH-BDE-137	96	6.8	8.0	87	8.0	8.9	30
3-BRP	89	10	12	86	6.7	5.1	60
2/4-BRP	95	7.0	8.5	93	5.8	7.8	6
2,3-diBRP	92	10	15	88	9.0	11	4
2,5-diBRP	89	9.4	14	96	5.3	11	4
2,6-diBRP	94	10	12	89	7.6	7.6	12
3,4-diBRP	86	7.8	10	91	7.2	8.9	4
3,5-diBRP	90	7.9	9.9	84	5.9	8.7	3
2,4-diBRP	89	11	16	86	7.0	10	4
2,3,4-triBRP	101	8.1	14	91	4.6	9.8	8
2,3,5/2,4,5-triBRP	105	8.9	14	110	6.2	8.7	4
2,3,6-triBRP	87	10	16	92	8.1	5.8	30
2,4,6-triBRP	88	9.8	12	86	6.9	9.0	10
3,4,5-triBRP	97	7.8	13	87	7.4	11	10

^a Accuracy was measured as the percent of the amount of target compounds known to be added to a plasma sample that was recovered. Based on the mean of four samples extracted, derivatized and analyzed together in one batch.

^b Intra-batch precision (coefficient of variation) was measured by the percent RSD. Based on four samples extracted, derivatized and analyzed together in one batch.

^c Inter-batch precision (coefficient of variation) was measured by the percent RSD. Based on four samples extracted, derivatized and analyzed in each of four batches.

Table 4Concentrations (pg/g) of analytes detected in stocked blood plasma from three wild frogs and three wild cormorants^a.

Analyte	Frog 1	Frog 2	Frog 3	Cormorant 1	Cormorant 2	Cormorant 3
Bisphenol A (BPA)	670	3400	N.D.	N.D.	N.D.	N.D.
4'-OH-BDE-49	N.D.	N.D.	N.D.	140	76	72
2/4-BRP	N.D.	N.D.	N.D.	280	63	50
2,4,6-triBRP	90	550	800	280	120	360
2,3,4-triBRP	N.D.	N.D.	N.D.	N.D.	N.D.	120
Sum of phenolic pollutants	760	3950	800	700	259	602
Estrone (E1)	220	1300	N.D.	35	28	13
β -Estradiol (E2)	1400	N.D.	7400	N.D.	44	N.D.
Sum of estrogens	1620	1300	7400	35	72	13

^a Concentration is expressed as the mean of replicate aliquots extracted, derivatized, and analyzed together in one batch. ND: analyte was not detected.

application of dansyl derivatization to BPA analysis, and as with estrogens, the LOQ was found to be less than those reported for direct LC–MS (300 pg/g using 1 mL of blood plasma) [15]. The LOQs reported in two separate studies that used derivatization followed by GC–ECNI–MS were 0.5–2 pg/g for OH-PBDEs and BRPs when 8.9 g human blood plasma was used [11] and 40–150 pg/g for OH-PBDEs when 1 mL glaucous gull blood plasma was used [22]. Thus, in the present study, by using the same amount of blood plasma, the LOQs for OH-PBDEs and BRPs were less than those observed when derivatization followed by GC–MS were used.

Blanks of pure water (1 mL) were analyzed to determine the background contributed during the entire procedure. Only 2/4-BRP, 2,4-diBRP, 2,4,6-triBRP and BPA were detected in the blanks and the blank values were all less than 10% of the average concentrations in blood plasma. No carryover was observed between runs.

To check for the stability of the derivatives, the dansyl derivatives were dissolved in acetonitrile and acetonitrile–0.1% formic acid in water (60:40, v/v) (10 ng/mL), and then stored at room temperature for 7 days. The concentrations of derivatives in the solvents were quantified in the 7-day period and the typical relative standard deviation (RSD) was lower than 12% by day-by-day replicate determinations, suggesting that there was no significant decrease of the concentrations of the dansyl derivatives in either solution over a 7-day period.

3.4. Application of the method to blood plasma

Once the LC and MS conditions necessary to resolve and quantify the 30 analytes in spiked blood plasma samples had been established, characterized and validated, it was applied to three samples of blood plasma from wild frogs and three samples of blood plasma from wild cormorants, collected in 2001 and 1991, respectively. Samples were fortified with isotopically labeled standards before analysis. The recoveries of the surrogate standards were between 72% and 101% and the measured results were corrected by the surrogate recoveries. Concentrations of BPA in frog blood plasma ranged from less than the LOQ to 3400 pg/g, while no BPA was detected in cormorants (Table 4). One OH-BDE (4'-OH-tetraBDE-49) and three BRPs (2/4-diBRP, 2,4,6-triBRP and 2,3,4-triBRP) were detected in cormorants, but only one BRP (2,4,6-triBRP) was detected in frog blood plasma. This could be due to the different natural histories and sampling locations of these animals: cormorants mainly feed on fishes which contain greater concentrations of natural phenolic compounds [10], while frogs were collected from a corn-cultivating region. BPA has been used as an inert ingredient in pesticides, and frogs may readily accumulate this man-made pollutant in environment [34]. Furthermore, the greatest concentrations of the sum of estrogens coincided with the least concentrations of phenolic compounds for both species. This may be due to that the synthetic estrogenic phenolic compounds influencing the production or metabolism of endogenous estrogens in these animals.

4. Conclusions

An LC–MS/MS method with greater sensitivity and better separation efficiency was established for simultaneously analyzing four estrogens, BPA, 10 OH-PBDEs, and 15 BRPs in various blood plasma samples by purification on a silica-gel chromatography after dansyl chloride derivatization. Using LC–MS/MS with dansyl chloride as the derivatizing agent, 30 analytes belonging to four classes of phenolic compounds could be analyzed within 22 min after sample pretreatment. After extraction and derivatization, the sample purification procedure improved the sensitivity for analyzing these phenolic compounds in blood plasma samples by removing several isobaric interferences and reducing the background noise, and eliminating contamination of the LC–MS/MS system with derivatization residuals. This method has acceptable accuracy, intra- and inter-batch precision and is thus adequate for the rapid reassessment of archived samples without cumbersome procedures. Use of the method based on derivatization with dansyl chloride, followed by LC–MS/MS, will be useful for the simultaneous monitoring of endogenous estrogens and estrogenic phenolic compounds in biological matrices, such as blood serum and plasma.

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