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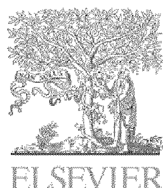

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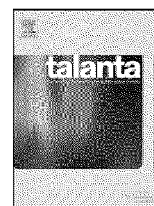

Automated on-line column-switching HPLC–MS/MS method for measuring environmental phenols and parabens in serum

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ABSTRACT

We developed a method using on-line solid phase extraction (SPE) coupled to high performance liquid chromatography–isotope dilution tandem mass spectrometry (HPLC–MS/MS) to measure the serum concentrations of seven environmental phenols and five parabens: bisphenol A; *ortho*-phenylphenol; 2,4-dichlorophenol; 2,5-dichlorophenol; 2,4,5-trichlorophenol; benzophenone-3; triclosan; and methyl-, ethyl-, propyl-, butyl-, and benzyl-parabens. The phenols and parabens present in serum were retained and concentrated on a C18 reversed-phase size-exclusion SPE column, back-eluted from the SPE column while the eluate was diluted through a mixing Tee (analyte peak focusing), separated using a pair of monolithic HPLC columns, and detected by isotope dilution-MS/MS. Sample preparation did not require protein precipitation, only dilution of the serum with 0.1 M formic acid. This method, which combines an on-line SPE with analyte peak focusing feature and the selective atmospheric pressure photoionization MS detection, resulted in limits of detection ranging from 0.1 to 0.5 ng/mL for most of the analytes. The high throughput and adequate sensitivity with yet a relative low serum volume used (100 μL) confirm that analytically it is possible to measure simultaneously these phenols and parabens with the precision and accuracy at sub-parts-per-billion levels required for biomonitoring. However, important additional factors, including validated sample collecting, handling, and storing protocols, as well as toxicokinetic data, are required if these measures are used for exposure assessment.

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1. Introduction

Humans are exposed to environmental phenols and parabens through industrial pollution, pesticide use, food consumption, and use of personal care and consumer products. Bisphenol A (BPA) is used to manufacture polycarbonate plastic, which can be found in water and infant bottles, and epoxy resins, which can be used in protective coatings on food containers and in dental composites and sealants [1]. Some chlorophenols have been used in the wood preservation industry, as intermediates in the production of pesticides, and as disinfectants or fungicides for industrial and indoor home use [2]. Other phenols, including the sunscreen agent 2-hydroxy-4-methoxybenzophenone (benzophenone-3, BP-3) and the antimicrobial agent 2,4,4′-trichloro-2′-hydroxydiphenyl ether (triclosan), are used extensively in personal care and consumer products [3]. Parabens, esters of *p*-hydroxybenzoic acid, are widely used as antimicrobial preservatives in cosmetics, pharmaceuticals, and in food and beverage processing [4].

The results from human and animal studies have demonstrated that after exposure and absorption, several environmental phenols, such as BPA, BP-3, and triclosan, are mainly metabolized by glucuronidation or sulfatation to facilitate their urinary excretion [5–8]. Therefore, these conjugated and free species can be used as valid biomarkers for exposure assessment in humans [9–11]. Animal studies show that parabens, after being absorbed, are mainly hydrolyzed to *p*-hydroxybenzoic acid, which can be excreted in the urine also as glycine, glucuronide and sulfate conjugates [4]. However, measuring *p*-hydroxybenzoic acid and its conjugates may not be the best approach for assessing human exposure to parabens because *p*-hydroxybenzoic acid is a non-specific biomarker and different parabens can possess rather different estrogenic bioactivities. It has been suggested that the unchanged precursor parabens and their conjugates could be valid biomarkers to assess human exposure to these compounds [12].

Because of their extensive use, human exposure to some phenols and parabens is widespread in the general US population as demonstrated by the high frequency of detection of these compounds in urine [9–13]. Although some of these phenols are toxic in animals, their potential toxic effects in humans are, for the most part, largely unknown. Urinary concentrations of these phenols can be used to

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estimate the prevalence of exposure to these compounds. However, the presence of phenols and parabens in urine does not indicate that these compounds are detrimental to human health [13]. To answer this question, information on the concentration of the compounds available to interact at the target organ(s) is needed. Assuming that the free form of these compounds is the pharmacologically active species, the concentrations of these free species in blood would be helpful for risk assessment.

Reports exist on the quantification of environmental phenols and parabens in serum using analytical techniques. Gas chromatography–mass spectrometry (GC–MS) had been used to measure alkyl phenols, BPA, and triclosan in human cord blood, plasma, and serum [14–16]. However, GC methods usually require a relatively large amount of sample (1 mL), extensive sample cleanup, and a derivatization step due to the relatively low volatility of these phenols. More recently, high-performance liquid chromatography (HPLC)–tandem mass spectrometry (MS/MS) was used for measuring the concentrations of some of these environmental phenols, such as BPA, in plasma [17] and serum [18]. We report the development and validation of a new on-line SPE–HPLC–MS/MS method to measure simultaneously the serum concentrations of seven environmental phenols and five parabens: BPA; *ortho*-phenylphenol (*O*-PP); 2,4-dichlorophenol (2,4-DCP); 2,5-dichlorophenol (2,5-DCP); 2,4,5-trichlorophenol (2,4,5-TCP); BP-3; triclosan; and methyl-, ethyl-, propyl-, butyl-, and benzyl-parabens.

2. Experimental

2.1. Analytical standards and reagents

Methanol (MeOH) and water, purchased from Caledon (Ontario, Canada) were analytical or HPLC grade. Formic acid (98%) was purchased from EM Science (Gibbstown, NJ, USA). BPA; *O*-PP; 2,4-DCP; 2,5-DCP; 2,4,5-TCP; triclosan; methyl-, ethyl-, propyl-, butyl-, and benzyl-parabens; 4-methylumbelliferyl glucuronide; 4-methylumbelliferyl sulfate; ammonium acetate (>98%); β-glucuronidase/sulfatase (*Helix pomatia*, H1) were purchased from Sigma–Aldrich Laboratories, Inc. (St. Louis, MO, USA). BP-3 (Eusolex 4360) was provided by EMD Chemicals, Inc. (Hawthorne, NY, USA). $^{13}C_{12}$-BPA; $^{13}C_6$-OPP; $^{13}C_6$-2,4-DCP; $^{13}C_6$-2,5-DCP; $^{13}C_6$-2,4,5-TCP; $^{13}C_4$-4-methylumbelliferone were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). D_3,^{13}C-BP-3 was obtained from Los Alamos National Laboratory (Los Alamos, NM, USA). $^{13}C_6$-triclosan was purchased from Wellington laboratories, Inc. (Ontario, Canada). D_4-methyl paraben was purchased from CDN Isotopes (Quebec, Canada) and D_4-ethyl-, D_4-propyl-, D_4-butyl-parabens were purchased from CanSyn Chem Corp. (Toronto, Canada). 15 commercial human serum samples for method validation were purchased from Interstate Blood Bank, Inc. (Memphis, TN, USA).

2.2. Preparation of standards and quality control materials

The initial stock solutions of analytical standards and stable isotope-labeled internal standards were prepared by dissolving measured amounts of the analytes of interest in MeOH. 10 working standard spiking solutions that contained all 12 compounds were generated by serial dilution of the initial stock with MeOH to a final concentration such that a 100-μL spike in 100 μL serum would cover a concentration range from 0.1 to 100 ng/mL for all of the analytes except triclosan (1–1000 ng/mL). The stable isotope-labeled internal standard working solution was prepared by diluting the internal standard stock solutions in MeOH, so that a 50-μL aliquot in 100 μL serum resulted in a concentration level of 50 ng/mL. All standard stock solutions and spiking solutions were dispensed into glass vials and stored at −70 °C until used.

Quality control (QC) materials were prepared from calf serum (Gibco, Grand Island, NY, USA). The serum was mixed uniformly and divided into two aliquots for QC low (QCL) and QC high (QCH) concentration pools. The QCL and the QCH pools were enriched with different levels of native target compounds. Initially we added the standard solutions directly into the serum. However, because of the precipitation caused by MeOH, getting homogeneous QC pools was difficult. Therefore, we modified the spiking procedure by first mixing the standard solutions with 3 mL of 0.1 M formic acid then adding serum onto the mixture. We observed no substantial precipitation during the QC preparation using the new procedure and obtained homogeneous QC pools. These pools were mixed thoroughly, sonicated for 15 min, and dispensed in aliquots of 1.5 mL in silanized glass vials (to minimize adsorption of some of the compounds, such as methyl paraben, to the glass). All QC materials were stored at −70 °C until used.

A mixture of $^{13}C_4$-4-methylumbelliferone, 4-methylumbelliferyl sulfate, and 4-methylumbelliferyl glucuronide was prepared in H_2O and stored at 4 °C until use. 50 μL of this mixture was added to each sample and used as a deconjugation standard to quantify the extent of the enzymatic reaction. After incubation, 4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide were deconjugated to free 4-methylumbelliferone, and the 4-methylumbelliferone/$^{13}C_4$-4-methylumbelliferone peak area ratio was monitored to check the extent of the deconjugation. The enzyme solution was prepared daily for each run by dissolving 0.04 g of β-glucuronidase/sulfatase (463,000 U/g solid) in 10 mL of 1 M ammonium acetate buffer (pH 5.0).

2.3. Sample preparation

To measure both the concentration of free and total species, each unknown sample was prepared in two different ways: one sample was processed without enzyme treatment; the other was treated with β-glucuronidase/sulfatase. Serum was thawed, sonicated, vortex mixed, and divided into aliquots. For a preparation without enzyme treatment to estimate the concentrations of free species, 50 μL of internal standard solution and 100 μL of serum were added into 850 μL of 0.1 M formic acid in 1.5 mL conical bottom autosampler vial. To determine the concentrations of the free plus conjugated species (total) of the compounds, 50 μL of internal standard, 50 μL of 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate/$^{13}C_4$-4-methylumbelliferone mixed standard (0.5 μg/mL), and 50 μL of enzyme solution were added to 100 μL of serum in an autosampler vial. After gentle mixing, the sample was incubated at 37 °C for 4 h. After incubation, 750 μL of 0.1 M formic acid was added to the sample. Since some precipitation was observed during incubation, before placing the samples on the HPLC autosampler for the on-line SPE–HPLC–MS/MS analysis, all samples were vortex mixed and centrifuged at 812 × g for 15 min. The autosampler injector needle was programmed to withdraw the sample 4.5 mm above the bottom of the autosampler vial, so that the precipitate would not be withdrawn into the HPLC system. We prepared analytical standards, QCs, and serum blanks using the same procedure as described above but replaced the serum by the same volume of standard stock solution, QC serum, or calf serum (for blanks).

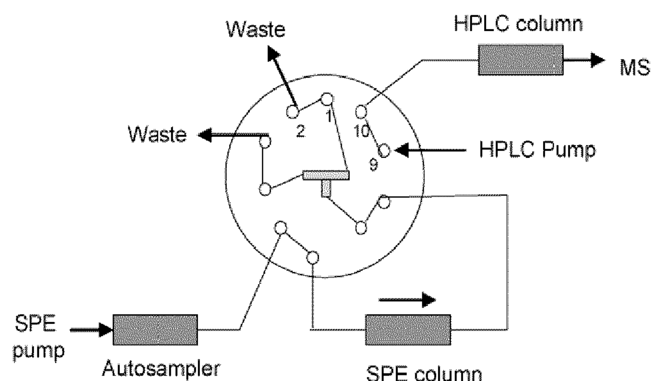
2.4. On-line SPE–HPLC–MS/MS

The on-line SPE–HPLC–MS/MS system was built from several Agilent 1100 modules (Agilent Technologies, Wilmington, DE, USA) coupled with an API 4000 Q Trap^{TM} mass spectrometer (Applied

Biosystems, Foster City, CA, USA) equipped with an atmospheric pressure photoionization (APPI) interface. The on-line SPE–HPLC system consisted of two binary pumps with degassers, an autosampler with a 900-μL injection loop, a high pressure mixing Tee, and one column compartment with a 10-port switching valve. The mass spectrometer and Agilent modules were programmed and controlled using the Analyst 1.4.1 software (Applied Biosystems), and the on-line SPE–HPLC–MS/MS acquisition method was built in 'LC sync' mode (i.e., acquisition was only triggered after the sample injection was completed). The SPE column was a LiChrosphere RP-18 ADS (25 mm × 4 mm, 25 μm particle size, 60 Å pore size, Merck KGaA, Germany), and the HPLC columns were two Chromolith™ Performance RP-18 (100 mm × 4.6 mm; Merck KGaA, Germany) in tandem.

The on-line SPE–HPLC–MS/MS system used in this study was modified from the one used for measuring urinary concentrations of phenols [19]. We simplified the original set up so that the autosampler valve did not require custom configuration to perform peak focusing (Fig. 1), and advanced programming for the autosampler was not required. The procedure for extracting the environmental phenols and parabens from the serum involved three periods (Table 1). The solvent gradient programs of SPE pump and HPLC pump, and the time schedules of the 10-port switching valve are also listed in Table 1. During the first period (0–3 min), with the 10-port valve at positions 1–2, 700 μL of the sample injected was loaded onto the SPE column by the SPE pump with 20% MeOH:80% H_2O at a flow rate of 1 mL/min. During the second period (3–5 min), the 10-port valve was switched to its alternative positions (1–10), and the analytes retained on SPE column were back-eluted by the HPLC pump, with 50% MeOH:50% H_2O at a flow rate of 0.5 mL/min. At the same time, the SPE eluate was diluted through a mixing tee with 100% H_2O at a flow rate of 0.25 mL/min provided by the SPE pump. During the third period, the 10-port valve was switched to its original positions (1–2), and the analytes were transferred to the HPLC column by the HPLC pump using a slow gradient program at a flow of 0.75 mL/min (Table 1). Regeneration of the SPE column by the SPE pump with 100% MeOH and SPE column equilibration with 20% MeOH:80% H_2O were also performed during this third period.

(A): Position 1-2 (0-3 min and 5-21 min)



(B): Position 1-10 (3-5 min)

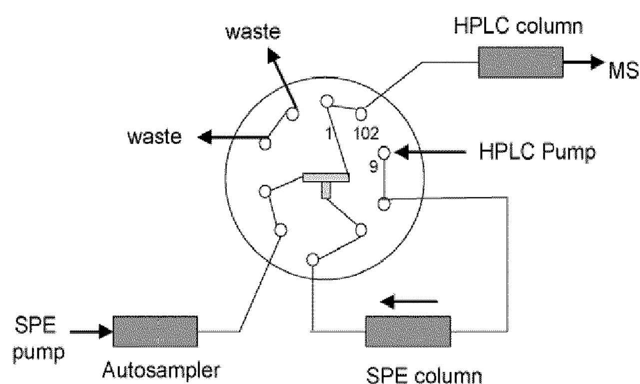


Fig. 1. On-line SPE–HPLC–MS/MS set-up.

2.5. Mass spectrometry

The mass spectrometer equipped with an APPI interface was used in negative ion mode. The analytes in the sample were ionized by photoionization induced by a continuous beam of ultraviolet radiation in the presence of a dopant (toluene) within the source house. The dopant was provided at a flow rate of 75 μL/min by an isocratic pump controlled with the Analyst 1.4.1 software. The APPI settings were curtain gas (N_2) flow: 20 arbitrary units (au); collision gas (N_2) flow: 9 au; nebulizer gas (N_2) flow: 60 au; lamp gas (N_2) flow: 20 au; nebulizing gas temperature: 500 °C; and ion transfer voltage: −800 V. Q1 and Q3 were set at unit resolution. Ionization parameters and collision cell parameters were optimized separately for each analyte. The negative fragment ions used for quantification and the retention time for the analytes are listed in Table 2.

3. Results and discussion

3.1. Pretreatment of serum samples

SPE has gained popularity in the past 20 years as a useful technique for extracting a wide range of analytes from serum and other biological and environmental samples [20,21]. When SPE is used in a stand-alone fashion, the process often involves time-consuming vaporization and reconstitution steps [18,22,23]. By contrast, when SPE is directly coupled to a HPLC–MS system (i.e., online-SPE), these steps are no longer necessary [24–26]. Furthermore, when using

Table 1
Gradient programs of SPE pump and HPLC pump, and the time schedule of the 10-port switching valve

	Period 1: sample loading and SPE washing	Period 2: analytes transfer and peak focusing	Period 3: HPLC separation and SPE regeneration and equilibration
Time (min)	0–3	3–5	5–21
10-Port valve position	1–2	1–10	1–2
SPE gradient A: H_2O; B: MeOH	20% B (1.0 mL/min)	100% A (0.25 mL/min)	5–10 min: 100% B 10–21 min: 20% B (1.0 mL/min)
HPLC gradient A: H_2O; B: MeOH	50% B (0.5 mL/min)	50% B (0.5 mL/min)	5–10 min: 50% B–65% B 10–17 min: 65% B–100% B 17–20 min: 100% B 20–21 min: 50% B (0.75 mL/min)

Table 2
Analyte retention time (RT), and precursor ion → product ion transitions monitored for quantitation (and confirmation) of native compounds and corresponding isotope-labeled internal standards

Analyte	RT (min)	Precursor ion → product ion (*m*/*z*)	
		Native analyte	Internal standard
Bisphenol A	15.4	227 → 133 (212)	239 → 139
Triclosan	19.3	252 → 216	264 → 228
Benzophenone-3	18.2	227 → 183 (211)	231 → 183
Ortho-phenylphenol	16.3	169 → 115 (141)	175 → 121
2,4-Dichlorophenol	16.2	161 → 125 (163 → 125)	167 → 131
2,5-Dichlorophenol	15.9	161 → 125 (163 → 125)	167 → 131
2,4,5-Trichlorophenol	18.0	195 → 159 (197 → 161)	201 → 165
Methyl paraben	11.5	151 → 92 (136)	155 → 96
Ethyl paraben	13.3	165 → 92 (137)	169 → 96
Propyl paraben	15.2	179 → 92 (136)	183 → 96
Butyl paraben	16.6	193 → 92 (136)	197 → 96
Benzyl paraben	16.6	227 → 92 (136)	197 → 96[a]

[a] D_4-butyl paraben is used as the internal standard of benzyl paraben.

SPE for serum samples, most approaches require an additional sample pre-treatment step, such as protein precipitation, to prevent proteins from clogging the SPE column [27]. In this study, we used an on-line SPE–HPLC–MS/MS approach to measure the serum concentrations of seven environmental phenols and five parabens. The sample pre-treatment step was simple and only involved dilution of the serum sample with 0.1 M formic acid, followed by centrifugation. Of interest, the addition of formic acid both eliminated the need of a protein precipitation step and improved the retention of some compounds, especially 2,4,5-TCP, on the SPE column. Formic acid has been shown to effectively suppress the interaction of polyfluoroalkyl compounds with serum macromolecules (e.g., proteins) and facilitate binding of these compounds to the SPE sorbent [26].

3.2. Peak focusing of the analytes

Our on-line SPE approach not only simplified the sample extraction procedure but also was amenable to provide the peak focusing feature. One of the most common limitations of on-line SPE is compromised HPLC resolution [28]. In our case, elution of the phenols of interest from the SPE column required 0.5 mL/min 50% MeOH:50% water for 2 min. However, a starting HPLC gradient with 50% MeOH content would broaden the chromatographic peaks for the most polar phenols, such as methyl paraben (Fig. 2). However, using a 10-port switching valve and a mixing Tee, we were able to dilute the HPLC flow (0.5 mL/min 50% MeOH) with 100% H_2O (0.25 mL/min) provided by the SPE pump. As a result, the HPLC resolution was improved greatly as illustrated by the decrease in the peak width of the methyl paraben signal at half height from 0.45 to 0.25 min (Fig. 2).

3.3. MS detection

Previously, we used atmospheric pressure chemical ionization (APCI) for measuring the concentrations in urine of 9 phenols [19]. Specifically, due to the dissociation of triclosan into dichlorophenol in Q_0 under the harsh APCI conditions, we used the *m*/*z* 161 → 125 and 163 → 125 dichlorophenol transitions at the retention time of triclosan for quantification and confirmation of triclosan, respectively [19]. Although the *m*/*z* 161 → 125 transition was more sensitive than the *m*/*z* 287 → 142 (triclosan molecular ion → product ion) transition in the urine matrix [19], the sensitivity of the *m*/*z* 161 → 125 transition was poor in serum due to the more severe ionization suppression from the matrix. Therefore, we chose to use a novel ionization technique, atmospheric

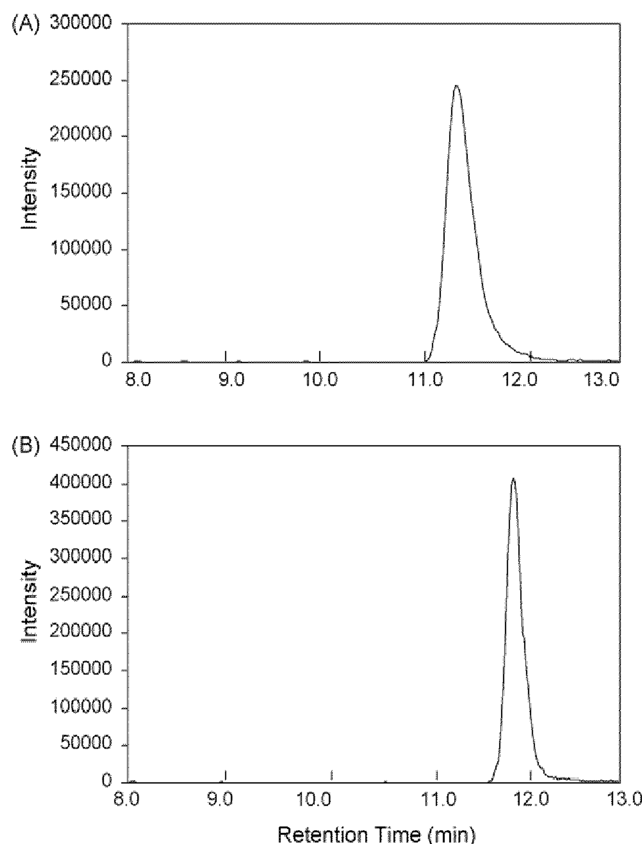


Fig. 2. The extracted ion chromatograms (MRM) of methyl paraben: (A) without peak focusing, HPLC-0.75 mL/min 50% MeOH; (B) with peak focusing, HPLC flow (0.5 mL/min 50% MeOH) was diluted with 100% H_2O (0.25 mL/min) from 3 to 5 min.

pressure photoionization (APPI), instead of APCI. The full scan mass spectrum of triclosan by APPI gave three ions at *m*/*z* 287 $[M-H]^-$, *m*/*z* 252 $[(M-H)-Cl]^-$, and *m*/*z* 161 (the dichlorophenol ion from the break down of triclosan in Q_0). The product ion scan of *m*/*z* 252 gave an ion at *m*/*z* 216. More importantly, the ion transition *m*/*z* 252 → 216 was about 10 times more sensitive than *m*/*z* 287 → 142 and 161 → 125. Therefore, for triclosan, we monitored *m*/*z* 252 → 216 (quantitation ion) and 287 → 142 (confirmation ion). The use of APPI also improved the ionization of *O*-PP and BP-3. For the rest of the analytes, ionization was comparable regardless of the technique used.

3.4. Method validation and quality control

Calf serum spiked with standard and isotope-labeled standard solutions was analyzed repeatedly to determine the limit of detection (LOD), accuracy, and precision of the method. The LOD was calculated as $3S_0$, where S_0 is the standard deviation as the concentration approaches zero [29]. S_0 was determined from five repeated measurements of low-level standards prepared in calf serum. The calculated LODs ranged from 0.1 to 0.5 ng/mL, except for triclosan (1.1 ng/mL) (Table 3). These values reflect the good sensitivity of the method, especially considering the relatively low sample volume (100 μL) used and the simplicity of the sample preparation procedure. Typical chromatograms for a reagent blank and a low concentration standard are shown in Fig. 3. The method accuracy was assessed by five replicate analyses of calf serum spiked at four different concentrations and was expressed as the percentage of expected levels (Table 3). The intra-day variability, reflected in the method accuracy, ranged from 82 to 113% for all of the analytes at the four spiking levels (Table 3). We determined the method precision from 40 repeated measurements of QCL and QCH materials

Table 3
Solid-phase extraction (SPE) recoveries, spiked standard concentration recoveries, and limits of detection (LOD)

Analyte	SPE recovery (%)	(Standard concentration) (ng/mL) spiked recovery (%)				LOD (ng/mL)
Bisphenol A	81	(0.5) 113	(1) 115	(5) 115	(10) 108	0.3
Triclosan	25	(5) 100	(10) 110	(25) 111	(50) 100	1.1
Benzophenone-3	96	(1) 87	(5) 106	(10) 106	(25) 101	0.5
Ortho-phenylphenol	76	(0.5) 113	(1) 112	(5) 101	(10) 101	0.1
2,4-Dichlorophenol	88	(0.5) 101	(1) 86	(5) 103	(10) 110	0.1
2,5-Dichlorophenol	82	(1) 101	(5) 105	(10) 111	(50) 106	0.4
2,4,5-Trichlorophenol	101	(2.5) 108	(5) 105	(7.5) 108	(10) 113	1.0
Methyl paraben	83	(0.5) 108	(1) 106	(5) 107	(10) 105	0.1
Ethyl paraben	90	(0.5) 100	(1) 99	(5) 107	(10) 106	0.1
Propyl paraben	89	(0.5) 82	(1) 101	(5) 105	(10) 105	0.2
Butyl paraben	89	(0.5) 103	(1) 99	(5) 105	(10) 106	0.2
Benzyl paraben	88	(0.5) 95	(1) 92	(5) 101	(10) 102	0.1

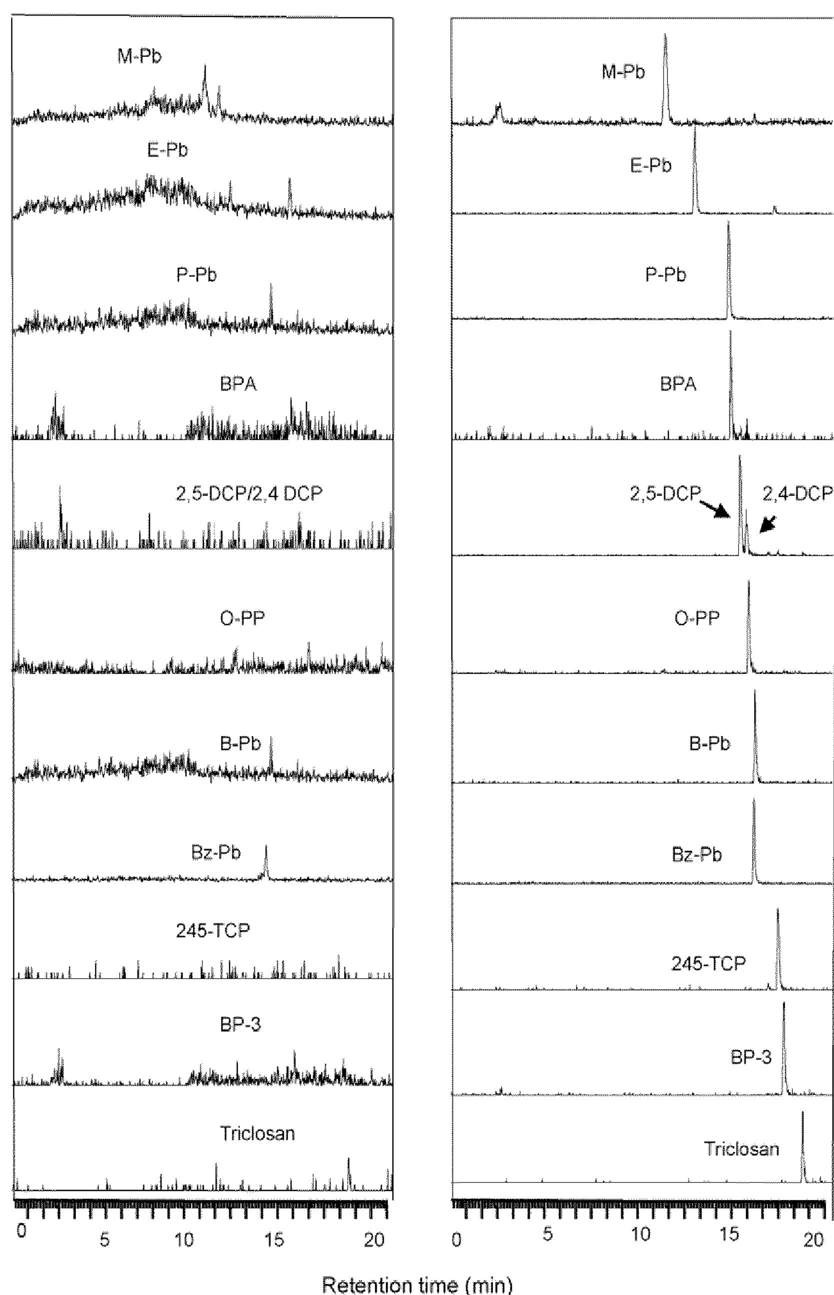


Fig. 3. Typical HPLC–MS/MS extracted ion chromatograms for a serum blank spiked with a low concentration (0.5–1 ng/mL in serum) calibration standard (right) and a serum blank (left). The calculated concentrations of the serum blank were <LOD for all analytes. The *y*-axis scales of the serum blank were magnified 5–10× times compared to those used for displaying the chromatograms of the spiked serum.

Table 4
Precision of concentration measurements in spiked quality control (QC) samples

Analyte	QC low		QC high	
	Mean (ng/mL)	R.S.D. (%)	Mean (ng/mL)	R.S.D. (%)
Bisphenol A	5.6	9.3	9.5	6.2
Triclosan	4.4	21.5	7.9	17.0
Benzophenone-3	5.7	7.7	9.5	8.7
Ortho-phenylphenol	5.2	8.0	8.8	5.2
2,4-Dichlorophenol	4.1	8.8	6.9	6.0
2,5-Dichlorophenol	5.9	6.8	10.2	8.5
2,4,5-Trichlorophenol	5.6	6.6	9.3	6.0
Methyl paraben	6.7	11.5	13.0	5.0
Ethyl paraben	5.6	7.4	9.5	7.0
Propyl paraben	5.4	7.0	8.9	6.0
Butyl paraben	4.8	9.5	7.0	10.5
Benzyl paraben	5.3	6.8	8.4	7.5

over a period of 2 weeks (Table 4). The R.S.D., which reflect the intra- and inter-day variability of the method, ranged from 5.0 to 21.5%.

Calibration curves were obtained from the standards spiked in water and calf serum. Because the slopes of the calibration curves from water and calf serum were very similar (e.g., for BPA: 0.0204 (water) and 0.0214 (serum), for methyl paraben: 0.0264 (water) and 0.0265 (serum), and for triclosan: 0.00225 (water), and 0.00231 (serum)), only the calibration curve obtained from water was used for quantification. Calibration curves in water showed adequate linearity, $\leq$100 ng/mL for all of the analytes except for triclosan ($\leq$1000 ng/mL) with correlation coefficients greater than 0.99. Inter-day variation of the calibration curve slopes, measured as the R.S.D., was <10%.

The SPE recoveries of the analytes from serum were calculated on the basis of the following experiment: first, 100 μL of serum mixed with a known amount of native analyte standards and 0.1 M formic acid was injected on the SPE column. Right after the native compounds were backflushed from the SPE column and before HPLC separation, 50 μL of internal standard solution was injected into the HPLC gradient flow (using a second Agilent 1100 autosampler). Although native compounds and isotope-labeled standards were injected separately, they all eluted from the HPLC column and were detected by MS/MS at about the same time. A response factor (RF_a) for each analyte was calculated from this experiment as the ratio of peak areas of native compound to its corresponding labeled analog. Second, 100 μL of serum spiked with the same amount of native and internal standards was injected on the SPE column, and 50 μL of HPLC solvent (50% MeOH) was injected into the HPLC flow. Response factor (RF_b) was calculated as before. The two experiments differed in that the first (RF_a), internal standards did not go through the SPE cleanup but the second (RF_b) did. The SPE recovery was calculated from RF_a/RF_b because the internal standard amount used for both experiments was the same and matrix effects were equivalent.

Good SPE recoveries (71–101%) were obtained for all of the analytes except for triclosan (25%) (Table 3). Triclosan was the last compound eluted from the HPLC column. Because the SPE and HPLC columns contain similar sorbents, triclosan was expected to retain strongly on the SPE column; 2 min of backflushing the column with 50% of MeOH may not have been enough time to transfer triclosan completely onto the HPLC column. Even after we extended the backflush time from 2 to 5 min with 50% MeOH, we observed only a slight increase in the SPE recovery of triclosan. However, when we backflushed the SPE column with a higher organic content (70% MeOH) solvent for 3 min, the SPE recovery of triclosan increased to ~80%. Unfortunately, backflushing the SPE column with this higher organic content eluate compromised the separation of the two dichlorophenol isomers. Even with peak focusing before HPLC, the baseline separation of these two isomers could not be accomplished. Nevertheless, although the SPE recovery for triclosan is low, the sensitivity (LOD = 1.1 ng/mL) and accuracy (spiked recoveries are between 100 and 110% at four spiking levels) are still acceptable (Table 3) and may be attributed to the use of the isotope labeled triclosan as internal standard.

3.5. *Quantification of phenols and parabens in commercial serum samples*

We tested the usefulness of this method by analyzing 15 commercially available serum samples collected between 1998 and 2003 from 4 male and 11 female donors. No other demographic information or information regarding potential exposure to the analytes of interest from the donors was available. The mean, median, and range of the concentrations of free and total (free + conjugated) species, and the mean % of the conjugated species of selected analytes are listed in Table 5. Because analytical standards of the conjugated (glucuronidated and sulfated) phenols and parabens were not commercially available, we determined the concentration of these conjugates by subtracting the total concentrations from the respective concentrations of free species. The mean % of the conjugated species of each analyte, calculated as

Table 5
Frequency of detection, mean and median concentrations of free, and total (free plus conjugated) species, range of concentrations, and the mean % of the conjugated species of selected environmental phenols in 15 serum samples[a]

Compound	Frequency of detection (%)	Mean (ng/mL)	Median (ng/mL)	Range (ng/mL)	Mean conjugate (%)
Methyl paraben free	60	1.3	0.2	<LOD-9.8	90
Methyl paraben total	100	42.4	10.9	0.4-301	
Ethyl paraben free	0	<LOD	<LOD	<LOD	100
Ethyl paraben total	53	0.6	0.2	<LOD-5.4	
Propyl paraben free	47	0.4	<LOD	<LOD-2.3	87
Propyl paraben total	80	8.0	1.4	<LOD-67.4	
Triclosan free	0	<LOD	<LOD	<LOD	100
Triclosan total	67	9.3	0.8	<LOD-13.7	
2,5-DCP free	0	<LOD	<LOD	<LOD	100
2,5-DCP total	67	19.5	1.7	<LOD-152	
O-PP free	0	<LOD	<LOD	<LOD	100
O-PP total	22	0.3	0.2	<LOD-0.9	

[a] The limits of detection (LODs) were 0.1 ng/mL (O-PP, and methyl-, ethyl-parabens), 0.2 ng/mL (propyl paraben), 0.4 ng/mL (2,5-DCP), and 1.1 ng/mL (triclosan). Concentrations <LOD were imputed a value of LOD divided by the square root of 2 for the statistical calculations. The calculated mean conjugate % only included the samples with detectable total species concentrations.

the ratio of concentrations of conjugated and total species, only included samples with total concentration values above the LOD.

In the current study, of the 12 analytes examined, 6 were detected infrequently (<10% of samples analyzed). For example, BPA was detected in only one of the 15 commercial samples tested, and the free and total serum concentrations of BPA in this sample were similar (1.5 ng/mL). In one study, the concentrations of BPA in all 19 human blood samples analyzed were <LOD (0.5 ng/mL) [17], but BPA has been detected in blood or serum in several other studies [30–33] using various analytical detection methods, including enzyme-linked immunosorbent assay [34,35] which may overestimate BPA concentrations [18,31,36]. The BPA results presented here must be interpreted with caution because the frequency of detection of BPA was low (6.7%) and we had no information on the procedures for collection, processing, and storage of the samples analyzed, which may be of critical importance to rule out the potential for contamination when measuring concentrations of BPA in the sub-part-per-billion levels in blood or serum [30,37].

By contrast, 6 analytes, namely methyl-, ethyl-, and propyl-parabens, triclosan, 2,5-DCP, and *O*-PP, were detected frequently in the 15 serum samples analyzed. For these compounds, the mean % of the conjugated species ranged from 87 to 100% (Table 5) suggesting that the conjugated species rather than the free forms of these phenols predominated in serum. This finding was in agreement with the data collected from urine, in which the conjugated species were also found to be dominant [12,38].

4. Conclusions

We developed a sensitive, selective, and precise automated on-line SPE–HPLC–MS/MS method with peak focusing for the simultaneous measurement of seven environmental phenols and five parabens in serum. The method required a small amount of serum (0.1 mL) and minimum sample pretreatment without protein precipitation. This method is rugged, labor and cost effective, and allows for the analysis of large number of samples for epidemiological studies. However, based on a small number of commercial sera analyzed, the analytical sensitivity may not be high enough to allow for the quantitative determination of the analytes detected at the lowest concentrations (e.g., BPA). Because we only tested 15 samples, our findings should be replicated in future studies. More importantly, although analytically it is possible to measure several of these phenols and parabens simultaneously with the precision and accuracy at sub-parts-per-billion levels required for biomonitoring purposes, important additional considerations, such as toxicokinetic data, as well as adequate and validated collection protocols, handling and storage of the samples, including data on the temporal stability of the analytes in serum, are needed to demonstrate the utility of these measures for exposure and risk assessment purposes.

Disclaimer

The use of trade names is for identification only and does not constitute endorsement by the US Department of Health and Human Services or the Centers for Disease Control and Prevention. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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