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Automated On-Line Column-Switching HPLC-MS/MS Method with Peak Focusing for the Determination of Nine Environmental Phenols in Urine

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We developed a method using isotope dilution on-line solid-phase extraction (SPE) coupled to high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) for the determination in urine of nine environmental phenolic compounds: Bisphenol A; 4-*tert*-octylphenol; *o*-phenylphenol; 2,4-dichlorophenol; 2,5-dichlorophenol; 2,4,5-trichlorophenol; 2,4,6-trichlorophenol; benzophenone-3 (2-hydroxy-4-metoxybenzophenone); and triclosan (2,4,4′-trichloro-2′-hydroxyphenyl ether). A unique fully automated column-switching system, constructed using 1 autosampler, 2 HPLC pumps, and a 10-port switching valve, was designed to allow for concurrent SPE-HPLC operation with peak focusing. The phenols present in 100 μL of urine were retained and concentrated on a C18 reversed-phase size-exclusion SPE column. Then, the phenols were "back-eluted" from the SPE column and diluted through a mixing Tee before being separated from other urine matrix components using a pair of monolithic HPLC columns. The phenols were detected by negative ion-atmospheric pressure chemical ionization-MS/MS. The efficient preconcentration of the phenols by the SPE column, analyte peak focusing by the dilution, and minimal ion suppression in the LC/MS interface by the buffer-free mobile phases resulted in limits of detection as low as 0.1−0.4 ng/mL for most analytes. The method was validated on spiked pooled urine samples and on urine samples from 30 adults with no known occupational exposure to environmental phenols. The method can be used for quick and accurate analysis of large numbers of samples in epidemiologic studies for assessing the prevalence of human exposure to environmental phenols.

Industrial pollution, pesticide use, food consumption, or the use of personal care products exposes humans and wildlife to a large variety of phenolic compounds. Bisphenol A (BPA) is used in the manufacture of polycarbonate plastic and epoxy resins, which are used in baby bottles, as protective coatings on food containers, and as composites and sealants in dentistry.[1−3] Alkylphenols (APs), such as *tert*-octylphenol, are used in the manufacture of nonionic surfactants used in detergents.[4,5] 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. The manufacture of other chlorinated aromatic compounds can produce chlorophenols as byproducts. Phenols are also used as sunscreen agents (e.g., benzophenone-3 (2-hydroxy-4-metoxybenzophenone), BP-3) or bactericides (e.g., triclosan (2,4,4′-trichloro-2′-hydroxyphenyl ether)) in personal care products. Some of these environmental phenols are considered potentially estrogenic, carcinogenic, or both. BPA and APs can disrupt the endocrine system in laboratory and wildlife animals.[5−8] Although chlorinated phenols are toxic for a wide range of wildlife organisms,[9] the toxic effects of these phenolic compounds in humans are largely unknown. The potential for human exposure to these compounds is high because of their widespread use. Therefore, a growing need exists for their biomonitoring to assess the prevalence of human exposure and whether environmental exposure to any of these environmental phenols may pose potential risks to public health.[10,11]

After exposure and absorption, phenolic compounds are metabolized and eliminated in urine and feces. These compounds or their metabolites can be further metabolized by glucuronidation or sulfatation to increase their water solubility, which facilitates their urinary excretion. Therefore, human exposure to phenolic compounds can be assessed by measuring the conjugated or free phenolic metabolites in urine or feces. For nonoccupational exposure studies, the analytical method must be selective and sensitive enough at the concentration ranges at which these

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(1) Howe, S. R.; Borodinsky, L.; Lyon, R. S. *J. Coat. Technol.* **1998**, *70*, 69−74.
(2) Sajiki, J.; Yonekubo, J. *Chemosphere* **2003**, *51*, 55−62.
(3) Arenholt-Bindslev, D.; Breinholt, V.; Preiss, A.; Schmalz, G. *Clin. Oral Invest.* **1999**, *3*, 120−5.
(4) Montgomery-Brown, J.; Reinhard, M. *Environ. Eng. Sci.* **2003**, *20*, 471−86.
(5) Ying, G. G.; Williams, B.; Kookana, R. *Environ. Int.* **2002**, *28*, 215−26.
(6) Kwack, S. J.; Kwon, O.; Kim, H. S.; Kim, S. S.; Kim, S. H.; Sohn, K. H.; Lee, R. D.; Park, C. H.; Jeung, E. B.; An, B. S.; Park, K. L. *J. Toxicol. Environ. Health, Part A* **2002**, *65*, 419−31.
(7) Laws, S. C.; Carey, S. A.; Ferrell, J. M.; Bodman, G. J.; Cooper, R. L. *Toxicol. Sci.* **2000**, *54*, 154−67.
(8) Lee, H. B.; Peart, T. E. *Water Qual. Res. J. Can.* **2002**, *37*, 681−96.
(9) *Chlorophenols*; Environmental Health Criteria Monographs (EHCs) 93; International Programme on Chemical Safety (IPCS), World Health Organization: Geneva, 1989.
(10) Daston, G. P.; Cook, J. C.; Kavlock, R. J. *Toxicol. Sci.* **2003**, *74*, 245−52.
(11) Rudel, R. A.; Camann, D. E.; Spengler, J. D.; Korn, L. R.; Brody, J. G. *Environ. Sci. Technol.* **2003**, *37*, 4543−53.

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compounds are found. At the urinary levels expected (low nanograms per milliliter), the analytical method of choice is usually gas chromatography (GC) coupled with mass spectrometry (MS) detection. However, GC methods may require a relatively large amount of sample, an extensive sample cleanup, derivatization of the phenols in the extracted urine, and preconcentration and reconstitution of the urine extract.[12−20] In contrast, analysis by high-performance liquid chromatography (HPLC) separation coupled with MS detection has the advantage over GC/MS of not requiring a derivatization step, thus potentially simplifying the sample preparation procedure. However, coeluting matrix components may have an important effect on the ionization of some phenols during HPLC−MS.[21] Therefore, a thorough and selective sample cleanup is very important.

Solid-phase extraction (SPE) has gained popularity in the past 20 years as a useful technique for extracting a wide range of analytes from biological and environmental samples. Specifically, off-line[12,22−25] and on-line SPE[26,27] have been used to measure phenolic compounds in urine. On-line SPE has also been used for the analysis of a variety of environmental phenols in water.[28−32]

Usually automated on-line SPE-HPLC systems are constructed from an autosampler, two HPLC pumps, and one six-port switching valve.[33−35] During on-line SPE-HPLC, the sample is loaded onto a SPE column where the analytes are retained while the unretained matrix components are washed away. Then, the analytes are automatically transferred, usually by reversed flow, from the SPE column to the analytical HPLC column for their chromatographic separation. Because the evaporation and reconstitution steps usually present in off-line SPE are eliminated, on-line SPE methods are less labor intensive.

However, conventional on-line SPE has some limitations.[36] The starting organic content of the HPLC solvent gradient is limited by the minimum organic content needed to elute the analytes from the SPE column. As a result, the analytical separation on the HPLC column, encompassing not only the separation of the analytes from each other but also the separation of the analytes from the residual matrix components that can cause suppression of the ionization of the analytes of interest during MS analysis, may be compromised. On the other hand, if the initial organic content is too low, HPLC signals from the analytes that bind strongly to the SPE column will tail.[35]

We addressed the above limitations by using a unique on-line SPE-HPLC system setup coupled with tandem mass spectrometry (MS/MS) detection. For improved analytical separation, we used peak focusing (i.e., diluting the SPE eluate before HPLC). An analogous on-line SPE-HPLC operation can be achieved by using commercial (but often costly) systems with multiple switching valves and special software.[29,31,32] In the present report, we demonstrate that these on-line SPE functionalities can be achieved by in-house-built systems using common HPLC modules and only one 10-port switching valve. The method has sensitivity and selectivity comparable to off-line SPE-derivatization-GC/MS methods for measuring urinary phenol derivatives.[23−25,37] Furthermore, the method allows for the analytical separation and quantification of dichlorophenol and trichlorophenol isomers.

EXPERIMENTAL SECTION

Materials. Methanol (MeOH) and water, purchased from Tedia (Fairfield, OH) were analytical or HPLC grade. BPA, 4-*tert*-octylphenol (*t*-OP), *o*-phenylphenol (OPP), triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol), 2,4-dichlorophenol (2,4-DCP), 2,5-diclorophenol (2,5-DCP), 2,4,5-trichlorophenol (2,4,5-TCP), 2,4,6-trichlorophenol (2,4,6-TCP), 4-methylumbelliferyl glucuronide, 4-methylumbelliferyl sulfate, and β-glucuronidase/sulfatase (*Helix pomatia*, H1) were purchased from Sigma Aldrich Laboratories, Inc. (St. Louis, MO). Benzophenone-3 (BP-3, Eusolex 4360) was kindly provided by EMD Chemicals Inc. (Hawthorne, NY). $^{13}C_{12}$-BPA, $^{13}C_6$-OPP, $^{13}C_{12}$-triclosan, $^{13}C_6$-2,4-DCP, $^{13}C_6$-2,5-DCP, $^{13}C_6$-2,4,5-TCP, and $^{13}C_6$-2,4,6-TCP were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). D_4-*t*-OP was purchased from Hayashi Pure Chemical Ind., Co. Ltd.

Preparation of Standards and Quality Control Materials. The initial stock solutions were prepared by dissolving measured amounts of the phenols in MeOH. Nine working standard spiking solutions, containing all nine analytes, were generated by serial dilution with MeOH of the initial stock solutions. These standards covered concentration ranges of 0.1−100 (2,4-DCP, 2,4,5-TCP, 2,4,6-TCP, OPP, BPA, *t*-OP), 0.1−200 (BP-3), and 0.1−1000 ng/mL (2,5-DCP and triclosan). The isotope-labeled standard spiking solution was also prepared in MeOH. All standard stock solutions and spiking solution were dispensed into vials and stored at −20 °C until use. The quality control (QC) materials were prepared

(12) Brock, J. W.; Yoshimura, Y.; Barr, J. R.; Maggio, V. L.; Graiser, S. R.; Nakazawa, H.; Needham, L. L. *J. Expos. Anal. Environ. Epidemiol.* **2001**, *11*, 323−8.
(13) Jeannot, R.; Sabik, H.; Sauvard, E.; Dagnac, T.; Dohrendorf, K. *J. Chromatogr., A* **2002**, *974*, 143−59.
(14) Kojima, M.; Tsunoi, S.; Tanaka, M. *J. Chromatogr., A* **2003**, *984*, 237−43.
(15) Lerch, O.; Zinn, P. *J. Chromatogr., A* **2003**, *991*, 77−97.
(16) Louter, A. J. H.; Jones, P. A.; Jorritsma, J. D.; Vreuls, J. J.; Brinkman, U. A. T. *HRC−J. High Resolut. Chromatogr.* **1997**, *20*, 363−8.
(17) Rinken, M. J. *Int. J. Environ. Anal. Chem.* **2002**, *82*, 77−85.
(18) Schonfelder, G.; Wittfoht, W.; Hopp, H.; Talsness, C. E.; Paul, M.; Chahoud, I. *Environ. Health Perspect.* **2002**, *110*, A703−7.
(19) Zafra, A.; del Olmo, M.; Pulgar, R.; Navalon, A.; Vilchez, J. L. *Chromatographia* **2002**, *56*, 213−8.
(20) Rosenfeld, J. M.; Moharir, Y.; Hill, R. *Anal. Chem.* **1991**, *63*, 1536−41.
(21) Reemtsma, T. *J. Chromatogr., A* **2003**, *1000*, 477−501.
(22) Inoue, K.; Kawaguchi, M.; Okada, F.; Takai, N.; Yoshimura, Y.; Horie, M.; Izumi, S.; Makino, T.; Nakazawa, H. *Anal. Chim. Acta* **2003**, *486*, 41−50.
(23) Kawaguchi, M.; Inoue, K.; Yoshimura, M.; Ito, R.; Sakui, N.; Okanouchi, N.; Nakazawa, H. *J. Chromatogr., B* **2004**, *805*, 41−8.
(24) Kuklenyik, Z.; Ekong, J.; Cutchins, C. D.; Needham, L. L.; Calafat, A. M. *Anal. Chem.* **2003**, *75*, 6820−5.
(25) Tsukioka, T.; Brock, J.; Graiser, S.; Nguyen, J.; Nakazawa, H.; Makino, T. *Anal. Sci.* **2003**, *19*, 151−3.
(26) Inoue, K.; Kawaguchi, M.; Okada, F.; Yoshimura, Y.; Nakazawa, H. *Anal. Bioanal. Chem.* **2003**, *375*, 527−33.
(27) Inoue, K.; Kawaguchi, M.; Funakoshi, Y.; Nakazawa, H. *J. Chromatogr., B* **2003**, *798*, 17−23.
(28) Kataoka, H.; Ise, M.; Narimatsu, S. *J. Sep. Sci.* **2002**, *25*, 77−85.
(29) Lopez-Roldan, P.; de Alda, M. J. L.; Barcelo, D. *Anal. Bioanal. Chem.* **2004**, *378*, 599−609.
(30) Watabe, Y.; Kondo, T.; Morita, M.; Tanaka, N.; Haginaka, J.; Hosoya, K. *J. Chromatogr., A* **2004**, *1032*, 45−9.
(31) Asperger, A.; Efer, J.; Koal, T.; Engewald, W. *J. Chromatogr., A* **2002**, *960*, 109−19.
(32) Hennion, M. C. *Analusis* **1998**, *26*, M131−7.
(33) Kato, K.; Shoda, S.; Takahashi, M.; Doi, N.; Yoshimura, Y.; Nakazawa, H. *J. Chromatogr., B* **2003**, *788*, 407−11.
(34) Koch, H. M.; Gonzalez-Reche, L. M.; Angerer, J. *J. Chromatogr., B* **2003**, *784*, 169−82.
(35) Kuklenyik, Z.; Ye, X.; Reich, J. A.; Needham, L. L.; Calafat, A. M. *J. Chromatogr. Sci*. **2004**, *42*, 495−500.
(36) Hennion, M. C. *J. Chromatogr., A* **1999**, *856*, 3−54.
(37) Kawaguchi, M.; Inoue, K.; Sakui, N.; Ito, R.; Izumi, S.; Makino, T.; Okanouchi, N.; Nakazawa, H. *J. Chromatogr., B* **2004**, *799*, 119−25.

Table 1. Concurrent SPE Cleanup and HPLC–MS/MS Analysis Time Line

			SPE of Sample $N + 1$				
period		1	2	3	4	5	6
	start	analyte transfer and dilution	regenerate SPE column	equilibrate SPE column	sample loading	SPE column wash	stop pump 1
time (min)	0	0.1–2	2–5	5–9	9–15.5	15.5–18.8	18.8–21
autosampler valve	6–1	6–1	6–1	6–1	1–2	6–1	1–2
pump 1							
mL/min	0	0.25	1.0	1.0	1.0	1.0	0
MeOH (%)	20	20	100	20	20	20	20
			SPE of Sample N				
		analyte transfer	HPLC separation and MS/MS acquisition				equilibrate pump 2
time (min)	0	0.1–2	2–10	10–17	17–18.5	18.5–18.8	18.8–21
10-port valve	10–1	1–2	10–1	10–1	10–1	10–1	10–1
pump 2							
mL/min	0.5	0.5	0.75	0.75	0.75	0.75	0.75
MeOH (%)	50	50	50–65	65–100	100	100–50	50

from a urine pool obtained from multiple anonymous adult donors. The urine pool was divided into two parts for QC low (QCL) and QC high (QCH) pools. The QCL and the QCH pools were enriched with different levels of native target compounds. All QC materials were stored at −20 °C. The QC pools were characterized to define the mean and the 95 and 99% control limits of phenols concentrations by a minimum of 50 repeated measurements in a 4-week period. QC materials reextracted and analyzed after the initial characterization showed that the phenols remained stable at -20 °C for at least 6 months.

A mix of $^{13}C_4$-4-methylumbelliferone, 4-methylumbelliferyl sulfate, and 4-methylumbelliferyl glucuronide was added to all samples and used as a deconjugation standard. The 4-methylumbelliferone/$^{13}C_4$-4-methylumbelliferone peak area ratio was monitored to check the extent of the deconjugation reaction. The enzyme solution was prepared by dissolving 0.1 g of β-glucuronidase/sulfatase (*H. pomatia*, 463 000 units/g of solid) in 50 mL of 1 M ammonium acetate buffer solution (pH 5.0).

Sample Preparation. All unknown, blank, and QC samples were prepared in conical bottom autosampler vials. A 100-μL aliquot of urine was mixed with 50 μL of internal standard, 50 μL of enzyme/ammonium acetate, and 10 μL of deconjugation standard solutions. Calibration standards were prepared as unknowns by adding 100 μL of native spiking solution instead of urine. After gentle mixing, all samples, including QCs, blanks, and standards, were incubated at 37 °C overnight. After the incubation, the deconjugated urine samples were diluted with 800 μL of 0.1 M formic acid (Tedia, Fairfield, OH) and centrifuged.

On-Line SPE-HPLC-MS/MS. The on-line SPE-HPLC-MS/MS system was constructed from several Agilent 1100 modules (Agilent Technologies, Wilmington, DE) coupled to a triple quadrupole API 4000 mass spectrometer (Applied Biosystems, Foster City, CA). The mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI) 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 a column compartment with a 10-port switching valve. The mass spectrometer and Agilent modules were programmed and controlled using the Analyst 1.4 software (Applied Biosystems). The SPE column was a LiChrosphere RP-18 ADS (25 × 4 mm, 25-μm particle size, 60-Å pore size, Merck

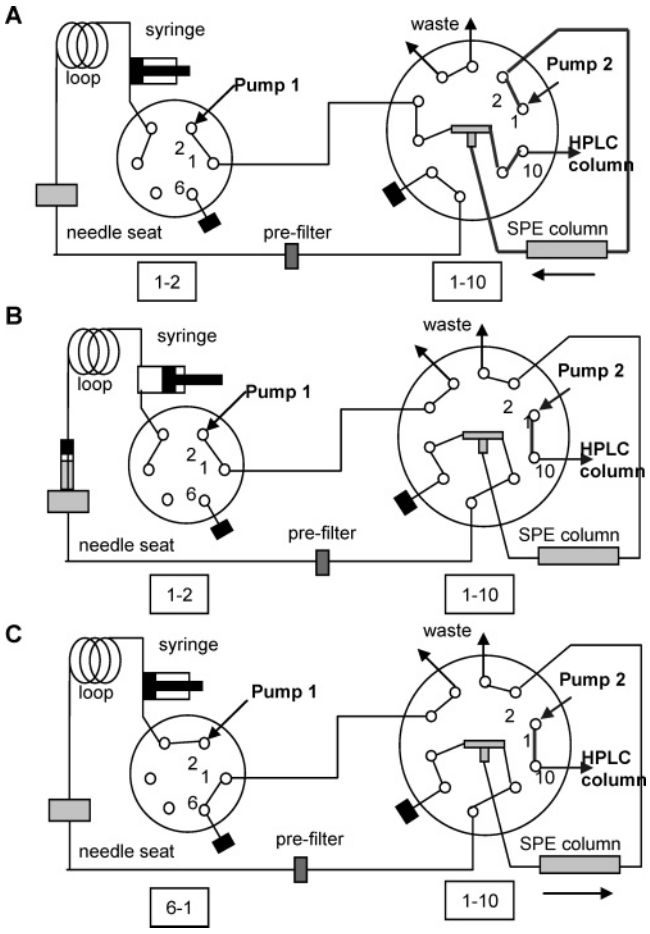

Figure 1. Tubing setup for the autosampler valve, the 6-port valve and the 10-port valve with configurations for three selected periods of Table 1: (A) analyte transfer and dilution (period 1), (B) sample loading (period 5), and (C) SPE column wash (period 6).

Table 2. Analyte Retention Time (RT) and Precursor Ions → Product Ion Transitions Monitored for Quantitation (and Confirmation) of Native Compounds and Corresponding Internal Standards

analyte	RT (min)	precursor ion → product ion (m/z) native analyte	internal standard
Bisphenol A	13.6	227 → 133 (212)	239 → 141
4-*tert*-octylphenol	19.3	205 → 133	209 → 137
benzophenone-3	17.8	227 → 183 (221)	
o-phenylphenol	15.1	169 → 115 (141)	175 → 121
triclosan	19.1	161 → 125 (287 → 142)	167 → 131 (299 → 148)
2,4-dichlorophenol	14.8	161 → 125 (163 → 125)	167 → 131
2,5-dichlorophenol	14.2	161 → 125 (163 → 125)	167 → 131
2,4,5-trichlorophenol	17.4	195 → 159 (197 → 161)	201 → 165
2.4,6-trichlorophenol	16.9	195 → 159 (197 → 161)	201 → 165

KGaA), and the HPLC columns were two Chromolith Performance RP-18 (100 × 4.6 mm; Merck KGaA) in tandem.

The procedure for extracting the phenols from the urine involved concurrent SPE and HPLC−MS/MS cycles (Table 1). The autosampler and pump 1 were used for the SPE cleanup of one sample, and the 10-port switching valve, pump 2, and mass spectrometer were used to acquire data from the previous sample. The HPLC−MS/MS acquisition method was built in "no sync" mode (i.e., all devices were programmed to start at the same time). The different combinations of autosampler valve and switching valve positions, and the timing of the gradient of the two binary pumps divided the concurrent SPE cleanup and HPLC−MS/MS data collection into six periods (Table 1, Figure 1).

First, the analytes from the previously injected sample that had been retained by the SPE column were eluted using 50% MeOH/50% water at 0.5 mL/min provided by pump 2. Through a mixing Tee, the 0.5 mL/min SPE eluate was diluted with 20% MeOH/80% water (0.25 mL/min) provided by pump 1. Then, the analytes were transferred to the HPLC column (Figure 1A, Table 1). At 2 min, the collection of the HPLC−MS/MS data started while the SPE column was regenerated and equilibrated with 100% MeOH (1 mL/min for 3 min) and 20% MeOH/80% water (1 mL/min for 4 min), respectively. The loading of a 1-mL sample (containing 100 μL of urine) was performed by execution of two sequential "500-μL sample draw" and "500-μL eject into the needle seat" commands. Tube connections inside the autosampler were modified in-house to connect the needle seat directly to the SPE column. In this way, the execution of the "eject into the needle seat" command resulted in loading of the sample directly onto the SPE column by the autosampler syringe (Figure 1B). A needle rinse, performed by lowering the needle into a vial containing MeOH, was included before the second ejection. After the sample loading was complete, the SPE column was washed for 3.3 min while unbound urine components were carried to waste by a flow (1 mL/min) of 20% MeOH/80% water (Figure 1C, Table 1). The collection of the HPLC−MS/MS data lasted 16.6 min, after which the HPLC pump was equilibrated for 2.2 min for the next elution cycle while the flow through the SPE column was brought to a complete stop (Table 1).

Mass Spectrometry. The API 4000 mass spectrometer was used in negative ion APCI mode with the following settings: curtain gas (N_2) 20 psi, heated ion source gas 50 psi, heated gas temperature 500 °C, nebulizer current −3 μA. Declustering potential, focusing potential, and collision energy were optimized for each analyte. All channels were monitored with a 50-ms dwell time adding up to 1.65-s cycle time. The negative fragment ions used for quantitation and the retention time for the analytes are listed in Table 2.

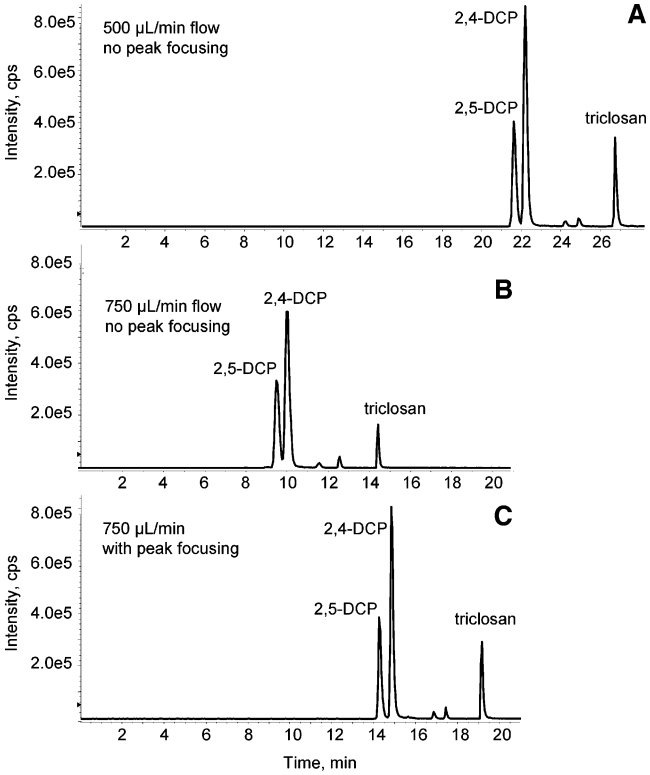

Figure 2. Separation of the 2,4- and 2,5-dichlorophenol isomers: (A) 0.5 mL/min without peak focusing, (B) 0.75 mL/min without peak focusing, and (C) 0.75 mL/min with peak focusing (dilution of 0.5 mL/min SPE eluate with 0.25 mL/min 20% MeOH during the first 2 min).

RESULTS AND DISCUSSION

One of the most common limitations of on-line SPE is compromised HPLC resolution.[39] Diluting the sample with water before the HPLC separation was suggested for the analysis of chlorophenols in wastewater.[31] The usual six-port valve setup of on-line SPE was used with a modified binary pump. One line of the binary pump (mobile phase B) was used to elute the analytes from the SPE column with a 100% organic eluent the other line of

(38) Gustafsson, J. E.; Uzqueda, H. R. *Clin. Chim. Acta* **1978**, *90*, 249−57.
(39) Wissiack, R.; Rosenberg, E.; Grasserbauer, M. *J. Chromatogr., A* **2000**, *896*, 159−70.

Table 3. Solid-Phase Extraction Recoveries, Spiked Standard Concentration Recoveries, and Limits of Detection with the On-line SPE Method

analyte	SPE rec	(std concn) (ng/mL) spiked rec (%)				LOD (ng/mL)
Bisphenol A	100	(1) 113	(10) 102	(50) 98	(100) 100	0.4
4-*tert*-octylphenol	86	(1) 125	(10) 104	(50) 102	(100) 99	0.2
benzophenone-3	98	(10) 101	(50) 105	(100) 97	(200) 97	0.3
o-phenylphenol	72	(1) 113	(5) 108	(10) 112	(50) 102	0.1
triclosan	65	(10) 92	(50) 121	(100) 114	(500) 102	2
2,4-dichlorophenol	85	(1) 91	(5) 98	(10) 99	(50) 98	0.2
2,5-dichlorophenol	90	(1) 116	(10) 125	(100) 119	(500) 98	0.1
2,4,5-trichlorophenol	85	(1) 103	(5) 104	(10) 98	(50) 99	0.1
2.4,6-trichlorophenol	70	(1) 98	(5) 104	(10) 92	(50) 102	0.3

the binary pump (mobile phase A) was used for the dilution through a mixing Tee.[31] This setup would not be adequate for the extraction of the phenols from urine because 100% MeOH would elute both the analytes of interest and the unwanted urine components that could interfere with the HPLC−MS/MS analysis. However, by replacing the 6-port with a 10-port switching valve, the elution of the analytes and dilution of the eluate can be easily performed. With a system including a 10-port switching valve, the HPLC pump (pump 2) can be used to run a gradient through the SPE column while a solvent with low organic content from the other pump (pump 1) could be used for dilution. Because the lines used during the sample transfer and dilution are also flushed together with the SPE column between sample injections (Figure 1), cross contamination from the mixing Tee is eliminated. The elution of the nonpolar phenols from the C18 SPE column required 0.5 mL/min 50% MeOH/50% water for 2 min. Therefore, the HPLC gradient elution from pump 2 had to start with at least 50% MeOH content. Without dilution, baseline separation of the 2,4- and 2,5-dichlorophenol chromatographic signals (Figure 2A) was possible only with a slow (0.5 mL/min) gradient, from 50% MeOH to 90% MeOH, extending each SPE-HPLC run to 28 min. Increasing the flow of the HPLC gradient to 0.75 mL/min reduced the SPE-HPLC time to 21 min, but the separation of the 2,4- and 2,5-DCP signals was compromised (Figure 2B). Peak focusing resulting from the dilution of the SPE eluate improved peak width and resolution of the isomer signals and allowed baseline separation in a 21-min run (Figure 2C).

The MS instrument throughput was increased by having concurrent SPE separation and HPLC−MS/MS acquisition. While one sample was injected and loaded onto the SPE column, the HPLC−MS/MS data from the previous sample was being collected. The analysis time per sample was limited only by the length of the HPLC run, which had to be at least 21 min for the elution of all analytes and separation of the dichlorophenol isomers. For each sample, at the end of the SPE step, the flow from pump 1 going on the SPE column was brought to a complete stop to ensure that the analytes remained on the column. The flow to the SPE column was restarted by pump 2 at the beginning of the next sample run to transfer the analytes into the HPLC column.

Table 4. Precision of Concentration Measurements in Spiked Quality Control (QC) Samples

	QC low		QC high	
analyte	mean	CV (%)	mean	CV (%)
Bisphenol A	2.9	17	18.7	8
4-*tert*-octylphenol	5.9	24	21.5	13
benzophenone-3	17.0	20	41.3	15
o-phenylphenol	1.6	21	7.1	8
triclosan	47.8	20	259	14
2,4-dichlorophenol	2.1	14	23.5	7
2,5-dichlorophenol	34.1	10	265	7
2,4,5-trichlorophenol	2.2	6	16.2	6
2.4,6-trichlorophenol	6.4	16	29.8	11

Diluting the urine aliquots 10 times and acidifying after incubation were necessary to minimize matrix effects and maximize recovery. Acidic pH was especially important for the chlorophenols. Furthermore, the optimal performance of the method was obtained with nonbuffered aqueous HPLC eluents. Ammonium acetate, even at concentrations as low as 0.5 mM in the HPLC eluent, suppressed the ionization of all phenols by 60−70%.

One other limitation of conventional on-line SPE is that particles from the matrix may accumulate on the front loading end of the SPE column.[36] During the back-flush elution of the analytes from the SPE column, these particles may transfer to the front end of the HPLC column and may result in increased back pressure and poor analytical chromatographic performance, especially when a large number of samples are to be analyzed. To prevent this from occurring, we used conical bottom autosampler vials, centrifuged all urine samples, and put a 0.2-μm prefilter between the needle seat and the SPE column (Figure 1). Replacing the prefilter after ~100 urine injections was a sufficient preventive measure.

We tested the ionization of the analytes with both APCI and turbo ion spray (TIS), a variant of electrospray ionization. 2,4-DCP, 2,5-DCP, and 2,4,6-TCP ionized 10, 100, and 4 times better with APCI than with TIS, respectively, while the other analytes ionized between 2 and 4 times better with TIS than with APCI.

Table 5. Urinary Phenol Levels (Number of Samples with Detectable Levels, Mean, and Selected Percentiles) in 30 Anonymous Adult Volunteers with No Known Occupational Exposure to Phenols[a]

	detectable	mean	fifth	10th	50th	90th	95th
Bisphenol A	26	3.5	<LOD	<LOD	2.4	10	11.5
4-*tert*-octylphenol	25	0.6	<LOD	<LOD	0.5	0.9	1.5
benzophenone-3	30	437	6.8	7.6	82.3	1425	2120
o-phenylphenol	30	0.5	<LOD	0.1	0.4	1.0	1.3
triclosan	24	127	<LOD	<LOD	12.5	567	702
2,4-dichlorophenol	23	1.9	<LOD	<LOD	0.6	3.3	4.9
2,5-dichlorophenol	30	44.6	0.4	1.1	3.1	77.7	78.9
2,4,5-trichlorophenol	8	0.1	<LOD	<LOD	0.1	0.3	0.4
2,4,6-trichlorophenol	5	0.5	<LOD	<LOD	0.3	1.3	1.6

[a] Concentrations are given in ng/mL. Samples with concentrations <LOD were given a value equal to LOD/$2^{0.5}$ for the statistical calculations.

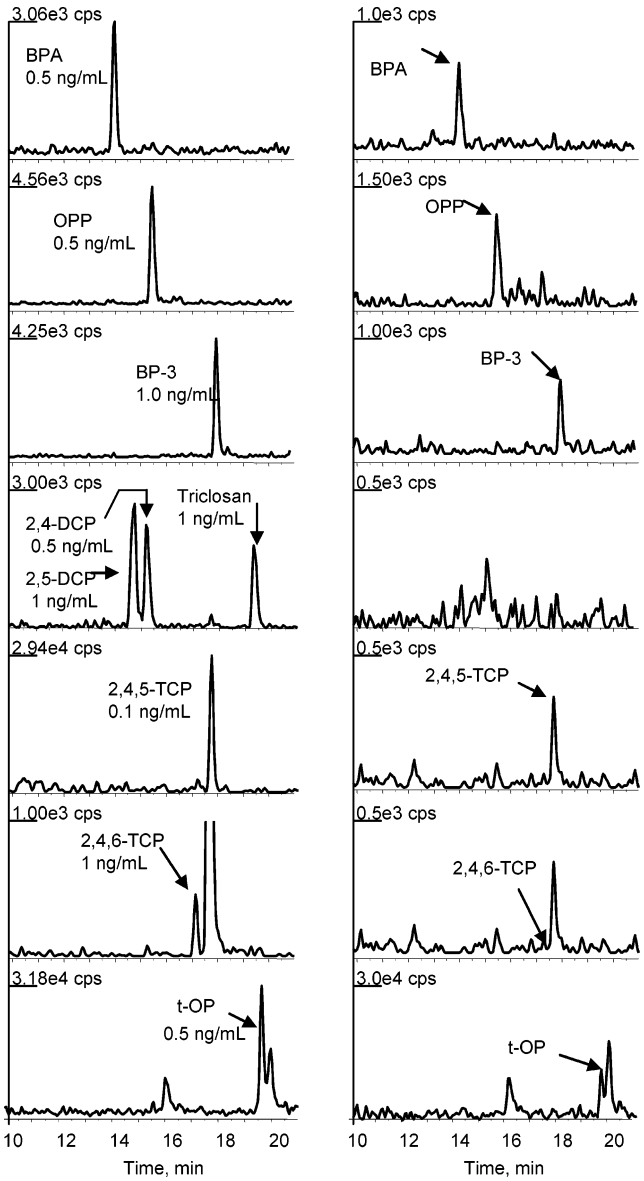

Figure 3. Typical HPLC−MS/MS extracted ion chromatograms for a low concentration (ng/mL in urine) calibration standard (left) and a reagent blank (right).

As a compromise, we chose APCI since it provided the most efficient ionization for most analytes. In the harsher APCI mode, the m/z 161 → 125 and 163 → 125 dichlorophenol transitions were also observed at the retention time of triclosan (Figure 2), suggesting in-source fragmentation of triclosan into dichlorophenol. The m/z 161 → 125 transition was 20 times more intense than the m/z 287 → 142 (triclosan molecular ion → product ion) transition observed with TIS, thus resulting in a lower limit of detection (LOD). Therefore, for triclosan, we monitored the m/z 161 → 125 transition for quantitation and the m/z 287 → 142 transition for confirmation.

Spiked urine was analyzed repeatedly to determine the 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.[40] S_0 was determined from five repeated measurements of the low-level standards prepared with synthetic urine.[38] Except for triclosan (2 ng/mL), the LODs were 0.1−0.4 ng/mL in urine (Table 3). These values reflect the good sensitivity of the method, which is comparable to more sensitive, but less selective GC/MS methods.[12] Typical chromatograms for a reagent blank and a low concentration standard are shown in Figure 3. The interday variation of the calibration curve slopes, measured as the coefficient of variation (CV), was less than 10%. The method accuracy was assessed by five replicate analyses of urine spiked at four different concentrations and expressed as a percentage of the expected value (Table 3). The intraday variability, reflected in the method accuracy, was very good (92−125%) for all analytes at all spike levels (Table 3). We determined the method precision by calculating the CVs of 60 repeated measurements of the QCL and QCH materials over a period of 4 weeks (Table 4). These CVs, which reflect the intraday and interday variability of the method, show good precision (6 and 25%) even for BP-3 for which a labeled internal standard was not available. SPE recoveries were calculated from the area count ratios obtained after on-line SPE injection of spiked urine and after direct injection of an aqueous standard using the same HPLC method.

We tested the usefulness of our method by analyzing 30 urine samples collected from anonymous adult volunteers with no known occupational exposure to phenols (Table 5). We frequently detected BPA (in 87% of the investigated samples), 2,5-DCP (100%), BP-3 (100% of the samples), and triclosan (80% of the samples) at a wide range of concentrations, thus confirming the sensitivity of our analytical method and its value for future studies.

(40) Taylor, J. K. *Quality Assurance of Chemical Measurements*; Lewis Publishers: Chelsea, MI, 1987.

In summary, using a unique column switching setup, we developed an automated on-line SPE-HPLC-MS/MS method for the simultaneous measurement of nine environmental phenols in human urine. We achieved sensitivity, selectivity, and precision comparable to previous off-line SPE GC/MS methods.[12,24,41] The on-line SPE method is rugged, labor and cost-effective, and allows for the analysis of large number of samples for epidemiologic studies to assess the prevalence of human exposure to environmental phenols.

(41) Matsumoto, A.; Kunugita, N.; Kitagawa, K.; Isse, T.; Oyama, T.; Foureman, G. L.; Morita, M.; Kawamoto, T. *Environ. Health Perspect.* **2003**, *111*, 101−4.

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