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Quantification of phenolic metabolites of environmental chemicals in human urine using gas chromatography–tandem mass spectrometry and isotope dilution quantification

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Abstract

We have developed a method to measure 12 urinary phenolic metabolites of pesticides or related chemicals. The target chemicals for our method are 2-isopropoxyphenol; 2,4-dichlorophenol; 2,5-dichlorophenol; carbofuranphenol; 2,4,5-trichlorophenol; 2,4,6-trichlorophenol; 3,5,6-trichloro-2-pyridinol; *para*-nitrophenol, *ortho*-phenylphenol, pentachlorophenol, 1-naphthol and 2-naphthol. The sample preparation involves enzyme hydrolysis, isolation of the target chemicals using solid phase extraction cartridges, a phase-transfer catalyzed derivatization, cleanup using sorbent-immobilized liquid/liquid extraction cartridges, and concentration of the sample. Derivatized samples are analyzed by capillary gas chromatography–tandem mass spectroscopy using isotope dilution calibration for quantification. The limits of detection are in the mid ng/L range and the average coefficient of variation was below 15% for most of the analytes. Using our method, we measured concentrations of the target chemicals in urine samples from the general population.
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Keywords: Phenolic metabolites; Human urine; GC–MS/MS

1. Introduction

Numerous analytical methodologies for biological monitoring of urinary phenolic chemicals in occupationally exposed populations have been published [1–5]. The limits of detection (LODs) of these methods range from 1 μg/L to 60 μg/L. However, biomonitoring of the general population typically requires LODs of 1 μg/L or less. In previous work, we reported the simultaneous measurement of 12 urinary phenols with average LODs about 1 μg/L in a 10-mL urine sample [6,7]. These methods have essentially eight major steps for sample preparation: (1) the addition of internal standards; (2) the enzyme hydrolysis of urine; (3) liquid–liquid extraction; (4) back extraction into a basic solution; (5) the formation of chloropropyl derivatives through the use of a phase-transfer catalysis reaction; (6) liquid–liquid extraction of the reaction mixture; (7) silica column cleanup; and (8) sample concentration. We analyzed concentrated derivatized samples by using gas chromatography coupled with tandem mass spectroscopy (GC–MS/MS). Although these methods are highly selective and relatively sensitive, they are still labor-intensive and require a great deal of time. Also, the properties of the method, such as sensitivity, precision, and extraction recoveries, needed further improvement.

We significantly refined the existing methods to produce a more streamlined method that was less labor-intensive and had a higher throughput, better precision and extraction recoveries, and lower LODs. The analytes that were measured using our method are listed in Table 1. These analytes

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are potential metabolites of 30 environmental chemicals including several contemporary pesticides. Our refined method involves (1) the addition of internal standards; (2) the enzyme hydrolysis of urine; (3) extraction of the metabolites by solid phase extraction (SPE) using OASIS® or STRATA® cartridges; (4) back extraction into a basic solution; (5) the formation of chloropropyl derivatives through the use of a phase-transfer catalysis reaction; (6) cleanup of the derivatized sample mixture using sorbent-immobilized liquid/liquid extraction cartridge ChemElut®; and (7) concentration of the sample. Concentrated derivatized samples are analyzed by GC–MS/MS. In addition to improvements in recovery, precision, and sensitivity, a reduced volume of urine is used, and the total analytical time (including sample preparation and analysis) is dramatically reduced.

2. Experimental

2.1. Materials

All solvents used were analytical grade with purity greater than 98%. Ethyl ether, butyl chloride (BuCl), methanol (MeOH), hexane, acetonitrile and toluene were purchased from Tedia Company Inc. (Fairfield, Ohio). Acetic acid, sulfuric acid, sodium acetate, sodium hydroxide, sodium sulfate, tetrabutylammonium hydrogen sulfate ($TBAHSO_4$), and hydrochloric acid (HCl) were obtained from J.T. Baker Co. (Phillipsburg, NJ). 1-Chloro-3-iodopropane and β-glucuronidase from *Helix pomatia* (G 0751, EC 3.2.1.31, type H-1) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo.). OASIS® HLB and Strata® X solid phase extraction cartridges were obtained from Waters Corporation, Milford, MA and Phenomenex, Torrance, CA, respectively. ChemElut® sorbent-immobilized liquid/liquid extraction cartridges were purchased from Sample Varian Preparation Products, Walnut Creek, CA.

The following native standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO): 2-isopropoxyphenol (IPP; 97%), 2,5-dichlorophenol (25DCP; 98+%), 2,4-dichlorophenol (24DCP; 99%), 2,4,5-trichlorophenol (245TCP; 99+%), 4-nitrophenol (PNP; 99+%), *o*-phenyl phenol (OPP; 99+%), and pentachlorophenol (PCP; 98%). 2,4,6-Trichlorophenol (246TCP; 98%) was obtained from Eastman Kodak Co. (Rochester, NY). 3,5,6-Trichloropyridinol (TCPY; 99%) was purchased from Dow Chemical Co. (Midland, MI). 1-Naphthol (1N; 99%) and 2-naphthol (2N; 99%) were obtained from Janssen Chimica (Geel, Belgium). Carbofuranphenol (CFP; 99.5%) was purchased from Chem Service (West Chester, PA). All labeled standards were custom synthesized by Cambridge Isotope Laboratories (Andover, MA), except for the $^{13}C_6$ 1-naphthol, which was synthesized in house at the Centers for Disease Control and Prevention (CDC). All labeled standards had chemical and isotopic purities of 99+%.

Gases used by the instrumentation had a minimum purity of 99.999% and were purchased from Holox (Atlanta, GA).

2.2. Reagent preparation

All reagents were prepared according to Hill et al. [7] using bioanalytical grade I water. An acetate buffer solution was prepared by combining 3.4 g sodium acetate, 1.1 mL acetic acid, and 700 mL of bioanalytical water. The buffer–enzyme solution was prepared by dissolving 0.358 g β-glucuronidase (338,000 units/g) in 100 mL 0.1 M acetate buffer (pH 5).

2.3. Standard preparation

2.3.1. Isotopically labeled internal standard

An isotopically labeled internal standard (ISTD) solution was prepared by weighing approximately 0.5 mg of each of the nine isotopically labeled analytes (Table 1) into a 2.5-mL volumetric flask and dissolving with acetonitrile to yield a 200-ng/μL solution. The individual stock solutions were stored at −20 °C until used. A multiple analyte ISTD solution was prepared by adding 250 μL of each of nine internal standard stock solutions into a 50 mL volumetric flask and diluting the solution with acetonitrile to make a final concentration of 1 ng/μL. This solution was used as an ISTD spiked in all unknown samples, quality control (QC) materials, and

Table 1
Analytes measured, their parent pesticides, and their corresponding labeled analogues

Analyte	Analyte status	Parent pesticide name	Internal standard
2-Isopropoxyphenol (2IPP)	Metabolite	Propoxur	$^{13}C_6$-ring 25DCP
2,5-Dichlorophenol (25DCP)	Metabolite	1,4-Dichlorobenzene	$^{13}C_6$-ring 25DCP
2,4-Dichlorophenol (24DCP)	Metabolite	1,3-Dichlorobenzene, nitrofen, dichlofenthion	$^{13}C_6$-ring 24DCP
Carbofuranphenol (CFP) (2,3-dihydro-2,2-dimethy-7-hydroxybenzofuran)	Metabolite	Carbofuran, benfuracarb, carbosulfan	$^{13}C_6$-ring 246TCP
2,4,6-Trichlorophenol (246TCP)	Metabolite	Chlorinated benzenes, chlorinated phenols	$^{13}C_6$-ring 246TCP
3,5,6-Trichloro-2-pyridinol (TCPY)	Metabolite	Chlorpyrifos, chlorpyrifos methyl	$^{13}C_5$, ^{15}N-ring TCPY
p-Nitrophenol (PNP)	Metabolite	Parathion, methyl parathion, EPN, other chemicals	$^{13}C_6$-ring PNP
2,4,5-Trichlorophenol (245TCP)	Metabolite	Chlorinated benzenes, chlorinated phenols	$^{13}C_6$-ring 245TCP
1-Naphthol (1N)	Metabolite	Napthalene, carbaryl	$^{13}C_6$-ring 1N
2-Naphthol (2N)	Metabolite	Napthalene	$^{13}C_6$-ring 1N
2-phenylphenol (OPP)	Parent pesticide	2-Phenylphenol	$^{13}C_6$-ring OPP
Pentachlorophenol (PCP)	Parent pesticide	Pentachlorophenol	$^{13}C_6$-ring PCP

calibration standards. For the three target analytes without analogous labeled internal standards (2IPP, CFP, and 2N), the labeled compounds for 25DCP and 246TCP were used as ISTDs.

2.3.2. Native standards and calibration plots

A native standard stock solution was prepared by weighing approximately 5 mg of the native standard into a 25-mL volumetric flask and dissolving with acetonitrile to yield a 200 ng/μL solution. Standard mixture solutions of the 12 target analytes were prepared by spiking 0.62, 1.25, 2.5, 5.0, 12.5, 25, 50, 125, and 250 μL aliquots of each individual stock standard into 10 mL volumetric flask and dissolving with acetonitrile. The individual stock solutions and the standard mixture solutions were stored at −20 °C until used. Calibration standards were prepared daily by spiking 2 mL of "blank" urine with 40 μL of the appropriate concentration of standard mixture solutions prepared in acetonitrile. The calibration standards were prepared according to the sample preparation procedure described below.

2.3.3. Quality control materials

Urine was collected from multiple (>30) donors, combined, diluted with water (1:1, v/v) to reduce endogenous levels of the analytes of interest, and mixed overnight at 20 °C. After pressure filtering with a 0.2-μm filter capsule, the urine was divided into three pools. The first pool (QC low pool) was spiked with the native standard stock solution to yield an approximate concentration of 10 μg/L for all of the metabolites. The second pool (QC high pool) was spiked with the native standard stock solution to yield an approximate concentration of 20 μg/L for all of the metabolites. The third pool was not spiked. After being screened for possible endogenous analytes, it was used as matrix material for calibration standards and blanks. All QC pools were characterized to determine the mean and 99th and 95th control limits by a consecutive analysis of at least 20 samples from each QC pool. After establishing the control limits of the pools, both QC high and low samples contained within each analytical run were evaluated for validity using the Westgard multirules [8] shown below:

(1) If both QC results were within the 95% confidence limits, then the run was accepted as valid.
(2) If one of two QC results was outside the 95% confidence limits, then the following rules were evaluated. It the QC failed one of these additional rules, the run was considered invalid and the entire analysis was repeated.

i. 1_{3s}: Either QC is outside of a 99% confidence limit.
ii. 2_{2s}: Both QCs are outside of 95% confidence limits on the same side of the mean.
iii. R_{4s} sequential: Both QCs are outside of 95% confidence limits on opposite sides of the mean.
iv. 10_x sequential: The previous nine QC results (for the previous nine runs) were on the same side of the mean.

The mean values and limits of each QC pool remained constant throughout each study; however, they were reestablished after each study to ensure the most accurate limits were used.

2.4. Sample preparation

All urine, reagents, and calibration standards were brought to room temperature. A 2 mL aliquot of the appropriate sample was pipetted into a 15 mL tube with screw cap and spiked with 50 μL of the combined internal standard spiking solution, using an automatic Gilson 215 liquid handler (Gilson, Middleton, WI), to give an approximate 12.5 μg/L concentration of the internal standard in the urine. To hydrolyze possible glucuronide or sulfate-conjugated metabolites, β-glucoronidase in 0.1 M acetate buffer (pH 4.5) was used. Samples were incubated for 17 h at 37 °C. The urine hydrolysates were extracted using SPE cartridges. First, samples were acidified with 250 μL of 2 M H_2SO_4 and mixed. Generally, 3 cm^3 Oasis® cartridges were used for SPE and were preconditioned with 1 mL of a 20% ethyl ether/BuCL solution followed by 1 mL MeOH, and 1 mL of 0.05N HCl solution. Samples were applied to the cartridges and the cartridges were washed with a 5% MeOH solution. Samples were eluted with 4 mL ethyl ether: BuCl (1:4) into conical centrifuge tubes. NaOH (1 mL, 3N) was added to each extract and vortex was mixed to extract the analytes from the organic phase back into the aqueous phase. The organic layer was discarded and the aqueous layer was transferred into a round bottom 15 mL centrifuge tube. The chloropropyl ether derivatives of the target analytes were made by adding 0.5 mL 0.4 M $TBAHSO_4$ and 0.5 mL 1-chloro-3-iodopropane:BuCl (1:5) and incubating in a 60 °C drybath for 1 h. The target analyte derivatives were separated from the reaction mixtures using 3 cm^3 ChemElut® sorbent-immobilized liquid/liquid extraction cartridges. The reaction mixtures were applied to the cartridges and allowed to sit for about 5 min. The analytes were eluted from the cartridges with 8 mL (2 × 4 mL) of hexane and collected in a conical 15 mL centrifuge tube. The samples were evaporated to dryness using a Turbovap LV Evaporator (Zymark, Hopkinton, MA) at 30 °C and 10 psi of nitrogen for approximately 30 min. Samples were reconstituted with 75 μL of toluene and transferred to autosampler vials, capped, and stored at −20 °C until analyzed.

2.5. Instrumental analysis

2.5.1. GC conditions

Samples (1 μL) were injected into the gas chromatograph (TraceGC, ThermoQuest, San Jose, CA) by splitless injection using an autosampler (CTC A200s, Carrboro, NC) with an injection purge delay of 60 s. The GC was coupled to a triple quadrupole mass spectrometer (FinniganTSQ-7000, ThermoFinnigan, San Jose, CA). A 30-m J&W (Folsom, CA) DB-5MS ([5% phenyl]-methyl polysiloxane, 0.25 μm

film thickness, 0.25 mm i.d.) capillary column was used for separation of the chloropropyl ethers of the target analytes. A guard column (deactivated fused silica column, Restek, Bellefonto, PA) was used to help extend the useful life span of the analytical column. The temperature of the injector was 250 °C and transfer line was 260 °C. The column temperature was initially 80 °C for 2 min and was then heated linearly using two ranges: to 160 °C at 10 °C/min and then to 260 °C at 4 °C/min. The final temperature of 260 °C was held for 2 min.

2.5.2. Mass spectrometric conditions

The chloropropyl ethers of the target analytes were analyzed using multiple reaction monitoring (MRM) except for pentachlorophenol. All of the precursor ions were the pseudomolecular ($[M+H]^+$) ions produced by chemical ionization in the positive ion mode Methane was used as a reagent gas with a pressure of 1500 mT and argon as a collision induced dissociation gas with a pressure of 2 mT. Pentachlorophenol was determined by using negative chemical ionization in selected ion mode (SIM). A full auto-tune of the mass spectrometer was performed before analysis of every set of samples. MS conditions were as follows: source temperature was 150 °C, electron energy was 200 eV, and the potential for the continuous dynode electron multiplier varied depending upon multiplier lifetime. Table 2 summarizes the characteristic precursor/product ion combinations and collision offsets used in measuring each analyte and ISTD with a width mass window of 0.4 amu and a scan rate of 0.03 s^{-1}. The product ions for ^{35}Cl precursor ions were selected to maximize specificity, sensitivity, and linear dynamic range. The product ions for ^{37}Cl precursor ions were used only for confirmation purposes and added to the selectivity of the analysis.

Peaks were automatically integrated using the Xcalibur® software (version 1.3) (ThermoFinnigan, San Jose, CA). The background signal was subtracted, and all data were smoothed (3-point smooth). The analyst checked and corrected any discrepancies in peak selection, yielding an accurate integration. Peak areas and other pertinent data were exported into a Microsoft Excel® file and loaded into a Microsoft Access® database for permanent storage. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC).

2.5.3. Quantification

Calibration plots were constructed for each analytical run with seven analyte concentrations, ranging between 0.20 ng/mL and 125 ng/mL, which were plotted against the area of the native analyte ion divided by the area of the labeled analyte ion. Calibration standard concentrations encompassed the entire linear range of the analysis. The lowest standard concentrations were at or below the LODs to ensure linearity and accuracy at the low concentration end. A linear regression analysis of the calibration plot provided a slope and intercept from which unknown sample concentrations could be determined. All contributions from the labeled ion to the native ion channels and vice versa were accounted for in the final calculations.

2.6. Method validation

2.6.1. Daily operating protocol

A typical sample batch included 1 blank urine sample, 36 unknown samples, 1 low QC, 1 high QC, and 7 standards. Before daily instrumental analysis, a known standard was analyzed to confirm acceptable chromatographic resolution and

Table 2
Multiple reaction monitoring analysis of phenolic metabolites

Analyte	Retention time window (min)	Precursor ion mass (Q)	Product ion mass (Q)	Precursor ion mass (C)	Product ion mass (C)	CO (−eV)
2IPP	10.0–11.5	229.2	187.1	231.2	189.1	10.0
25DCP	11.5–13.0	239.2	163.1	241.2	163.1	10.5
24DCP	11.5–13.0	239.2	163.1	241.2	163.0	10.5
$24DCP_L$	11.5–13.0	245.2	169.1	247.2	169.1	10.5
CFP	11.5–13.0	241.2	199.1	243.2	201.1	11.0
246TCP	13.0–15.0	273.5	198.0	275.5	200.0	11.5
$246TCP_L$	13.0–15.0	279.0	203.1	281.0	205.0	11.5
TCPY	13.0–15.0	274.1	198.0	276.0	200.0	13.0
$TCPY_L$	13.0–15.0	280.0	204.0	282.0	206.0	13.0
PNP	15.0–16.0	216.1	140.1	218.1	140.1	18.0
PNP_L	15.0–16.0	222.2	146.1	224.2	146.1	13.0
245TCP	15.0–16.0	273.2	196.9	275.2	198.9	11.5
$245TCP_L$	15.0–16.0	279.0	203.1	281.0	205.0	11.5
1N	16.0–18.0	221.2	145.1	223.0	145.0	12.5
2N	16.0–18.0	221.2	145.1	223.0	145.0	12.5
$1N_L$	16.0–18.0	227.10	151.2	229.0	151.0	12.5
OPP	16.0–18.0	247.1	171.2	249.1	171.2	13.0
OPP_L	16.0–18.0	253.2	177.2	255.2	177.2	13.0
PCP	18.0–22.0	228.9	NA	230.8	NA	NA
PCP_L	18.0–22.0	234.9	NA	236.9	NA	NA

CO, collision offset; Q, ion used for quantification; C, ion used for confirmation; L, labeled compounds; NA, not applicable.

mass spectral sensitivity. At the end of the run, we required that the data of blank and QC samples met clear specifications before we considered an unknown batch of sample data valid.

2.6.2. Limits of detection

The LODs for each analyte were calculated as $3s_0$, where s_0 is the standard deviation at zero concentration. s_0 was estimated as the *y*-intercept of a linear regression analysis of a plot of the standard deviation (in units of concentration) versus the concentrations of the four lowest standards. The calculated LOD was verified as a reasonable estimate by injecting concentrations of the analytes at the LOD.

2.6.3. Extraction recoveries

The extraction recoveries of the method were determined at three concentrations (6 μg/L, 25 μg/L and 100 μg/L) that spanned the calibration range and where at least one sample was near the expected range of unknown samples. The recoveries were measured by spiking six "blank" urine samples (2 mL) with the appropriate native standard spiking solution and preparing the samples according to the method. Six additional "blank" urine samples (unspiked) were prepared concurrently. After the SPE step, all extracts were spiked with 50 μL ISTD to correct for instrument variation, which resulted in a more accurate recovery calculation. The samples that were not spiked before preparation were then spiked with the appropriate native standard spiking solution to serve as control samples representative of 100% recovery. The sample preparation after the extraction step was completed according to the method and the samples were analyzed. The recovery was calculated by a comparison of the ratio of the native standard and ISTD areas in the recovery samples to those in the control samples.

2.6.4. Relative recovery

We define the relative recovery as the ability of the method to quantify the spiked value, regardless of analyte losses through the sample preparation procedure. The relative recovery of the method was evaluated by spiking "blank" urine samples at different concentrations spanning the range of expected uknown sample concentrations, processing through the method, and calculating the resulting concentration as if the sample had an unknown concentration. A linear regression analysis was performed on a plot of the measured concentration versus the expected concentration. The slope of the resulting line was evaluated. A slope of 1 would indicate 100% relative recovery.

2.6.5. Precision

The method precision was determined by calculating the coefficient of variation (CV) of repeat measurements of the QC materials at two concentrations (about 10 μg/L and 20 μg/L). At least 42 repeat measurements over a 2-month period were used in the calculations. These measurements were made in consecutive runs representing data from a single human study described below.

2.6.6. Human studies

Urine samples were collected as part of the National Health and Nutrition Examination Survey conducted by the National Center for Health Statistics at CDC [9]. Upon collection, samples were frozen within 4 h and were stored at −20 °C until analysis. All protocols were reviewed and approved by a human subjects review committee and complied with all institutional guidelines for the protection of human subjects. Approximately 2000 urine samples from adults and children were analyzed using this method to validate the speed and ruggedness of the analysis.

3. Results and discussion

One of the main goals of our laboratory is to develop analytical techniques for the biological monitoring of exposure to pesticides in general population samples. The selection of the analytes for this study was based on several factors previously discussed by Hill et al. [7]. In general, these phenolic compounds are potential metabolic products of a variety of environmental chemicals including several contemporary pesticides used in the United States. We previously developed a method for the measurement of most of the target chemicals discussed in this paper [6,7]. However, because we have a continuing need to reduce the sample volume used for analysis and increase the analytical throughput of our laboratory, we sought to modify the method to improve its performance characteristics. The previous method required 10 mL of urine for analysis, which seems a reasonable amount until samples from infants and small children are considered. Our aim was to dramatically reduce the volume for analysis, thus accommodating children studies, improve both the magnitude and consistency of extraction recoveries, decrease the analyst time involved with the sample preparation, and if possible, improve other performance characteristics such as LODs and precision.

To improve the extraction recoveries and reduce the labor-intensiveness of the method, we explored alternative extraction techniques. We evaluated two different polymeric cartridges (Oasis® HLB and Strata® X). Both cartridges are macro-porous copolymers made from a balanced ratio of two monomers, the lipophilic divinylbenzene and the hydrophilic *N*-vinylpyrrolidone. Several organic solvents of variable polarity were tested as eluents for the extraction. Both cartridges produced similar results and ethyl ether/BuCl (1:4) was the best elution solvent.

The extraction recoveries of target metabolites are shown in Table 3. The recoveries for the metabolites were generally greater than 75% with a standard deviation lower than 10% for most of the metabolites, except PCP which had consistently lower recoveries.

Our method uses phase-transfer catalysis for the derivatization of the metabolites with 1-chloro-3-iodopropane to yield the chloropropyl of the target analytes. The quaternary salt, $TBAHSO_4$, was used as the catalyst in a basic media

Table 3
Recovery of metabolites in 2 mL of urine at different concentrations

Analyte	Recovery (%)					
	6 ppb		20 ppb		100 ppb	
	OASIS®	STRATA®	OASIS®	STRATA®	OASIS®	STRATA®
2-Isopropyphenol (2IPP)	95 ± 12	90 ± 11	84 ± 9	88 ± 9	89 ± 9	97 ± 11
2,5-Dichlorophenol (25DCP)	82 ± 13	101 ± 8	93 ± 3	93 ± 4	94 ± 1	99 ± 2
2,4-Dichlorophenol (24DCP)	77 ± 14	88 ± 9	94 ± 3	88 ± 4	92 ± 2	83 ± 4
Carbofuranphenol (CFP)	99 ± 8	96 ± 5	92 ± 7	91 ± 6	95 ± 6	99 ± 5
2,4,6-Trichlorophenol (246TCP)	89 ± 9	99 ± 10	91 ± 6	95 ± 4	95 ± 3	89 ± 3
3,5,6-Trichloro-2-pyridinol (TCPY)	89 ± 7	93 ± 5	95 ± 3	91 ± 3	94 ± 2	90 ± 2
p-Nitrophenol (PNP)	81 ± 4	98 ± 6	80 ± 2	73 ± 7	84 ± 2	73 ± 2
2,4,5-Trichlorophenol (245TCP)	68 ± 7	81 ± 17	87 ± 3	81 ± 3	94 ± 3	64 ± 8
1-Naphthol (1N)	71 ± 5	83 ± 7	88 ± 2	84 ± 1	93 ± 4	81 ± 6
2-Naphthol (2N)	82 ± 8	95 ± 5	97 ± 3	88 ± 2	99 ± 4	81 ± 5
2-Phenylphenol (2PP)	84 ± 8	98 ± 9	94 ± 5	89 ± 4	93 ± 2	89 ± 4
Pentachlorophenol (PCP)	54 ± 14	67 ± 8	64 ± 3	65 ± 2	66 ± 1	67 ± 2

(3N NaOH), which increases the rate of the phase transfer as previously reported by Rabinovitz et al. [10] and fully observed in our results [6,7]. Also, we evaluated the efficiencies of the derivatization reactions at two temperatures (60 °C and 70 °C) and different reaction times from 30 min to 4 h. The optimal derivatization condition for most of the analytes was incubation for 1-h reaction at 60 °C. After derivatization, the reaction mixtures were cleaned using sorbent-immobilized liquid extraction instead of using formal liquid–liquid extraction coupled with silica gel cleanup, which enabled us to significantly reduce the amount of time required for the analysis. Hexane was the best elution solvent tested for this final step.

Filtered ion chromatograms of a blank sample, low level calibration sample, and an unknown urine sample are shown in Fig. 1. The clarity of this unknown chromatogram is typical of an unknown sample, although most samples do not have detectable levels of all of the target analytes. All of the analytes were resolved by time or mass between 9 min and 21 min.

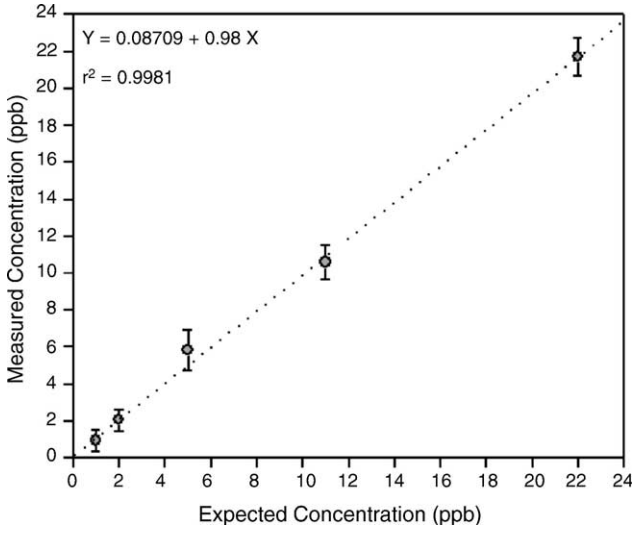

Fig. 2. A plot of expected concentration versus the measured concentration of 3,5,6-trichloro-2-pyridinol (TCPY) in urine. The circles represent the data points and the dot line represents a linear regression analysis of the data. The average relative recovery is 98.0%.

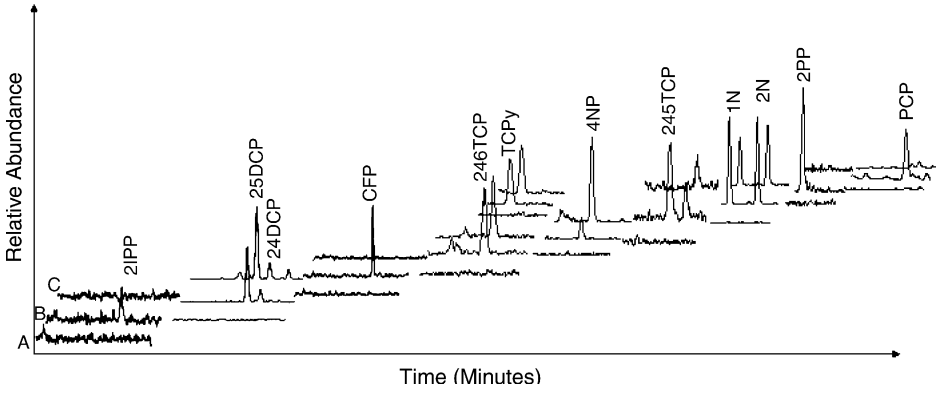

Fig. 1. Typical filtered ion chromatogram of the 12 phenolic chloropropyl esters in a (A) blank sample, (B) low level (1 ng/mL) calibration standard, and an (C) unknown sample. Each chromatogram is offset by a few seconds to show the peaks more clearly. The concentrations of the urinary phenolic metabolites in the unknown sample range are as follows: 2IPP (<LOD); 25DCP (2.5 ng/mL); 24DCP (2.5 ng/mL); CFP (<LOD); 246TCP (2.1 ng/mL); TCPY (3.1 ng/mL); PNP (6.0 ng/mL); 1N (2.3 ng/mL); 2N (1.5 ng/mL); OPP (<LOD); and PCP (<LOD).

Table 4
Summary of the method specifications

Analyte	LOD (μg/L)	%Error of slope	Relative recovery (%)	R.S.D. (%)	
				Low pool	High pool
2-Isopropyphenol (2IPP)	0.4	1.9	99.8	17.3	18.4
2,5-Dichlorophenol (25DCP)	0.1	3.0	100.0	10.9	14.8
2,4-Dichlorophenol (24DCP)	0.3	1.9	100.0	10.8	17.8
Carbofuranphenol (CFP)	0.4	2.6	99.9	13.1	15.4
2,4,6-Trichlorophenol (246TCP)	1.3	0.3	100.6	11.2	17.2
3,5,6-Trichloro-2-pyridinol (TCPY)	0.4	2.2	98.0	8.1	8.7
4-Nitrophenol (PNP)	0.4	2.2	99.9	14.2	15.7
2,4,5-Trichlorophenol (245TCP)	0.9	5.3	103.7	23.0	23.0
1-Naphthol (1N)	0.3	1.3	99.9	10.4	10.5
2-Naphthol (2N)	0.2	2.3	100.1	10.0	10.7
2-Phenylphenol (2PP)	0.3	1.8	100.0	10.0	9.4
Pentachlorophenol (PCP)	0.5	3.6	99.5	9.8	10.7

LOD, limit of detection calculated as $3s_0$; R.S.D., relative standard deviation.

Fig. 2 shows the spiked analyte concentrations plotted against the measured analyte concentrations (μg/L) in the fortified samples inserted among study samples. The circles represent the data points, and the dot line represents a linear regression of the data. The correlation coefficient (r^2) and the slope of the liner regression line were 0.9981 and 0.98, respectively, indicating excellent agreement between the spiked and the measured values of 3,5,6-trichloro-2-pyridinol (TCPY). The r^2 and slopes of similar plots for the others metabolites are reported in Table 4.

Fig. 3 is a plot of the quality control samples for 3,5,6-trichloro-2-pyridinol (TCPY) spiked into urine. The mean values established in multiple runs were 24.0 1 μg/L and 11.1 μg/L for low QCH and high QCL, respectively. Similar QC plots were generated for all analytes and were used to determine the validity of each analytical run and to guarantee appropriate analytical precision. Table 4 shows the total CVs for each analyte generated over a 2-month period for a single human study. The LODs of the method ranged from 0.1 μg/L to 1.3 μg/L. Our LODs were equal or lower than those reported in the literature for most methods, which typically range from about 1 μg/L to 20 μg/L.

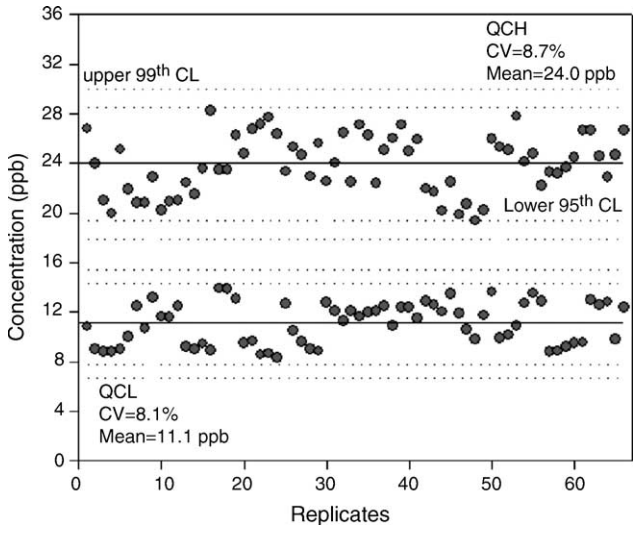

Fig. 3. A plot of the 3,5,6-trichloro-2-pyridinol (TCPY) quality control material replicates from a single study. The solid line represents the mean. The dashed lines represent the upper and lower 95th and 99th control limits.

Our modifications dramatically increased the analytical throughput of our method. By almost halving the GC–MS/MS analysis time, totally eliminating the need for a time-consuming traditional liquid–liquid extraction, and by cutting the derivatization time by about 80%, we increased our throughput three-fold. Previously, only 24 samples (including unknown, QC materials, a blank, and two standards used in a continuing calibration curve) could be prepared and analyzed in about 50 h, which averages more than 2 h per sample (2.5 h/unknown sample). Using the refined method, approximately 40 h are required to prepare and analyze 48 samples, which averages about 50 min per sample, representing a time savings of about 65%. This time savings allows us to process approximately 150 samples per week as compared to only 70 samples per week previously, effectively doubling our overall productivity.

This method was used to measure the target analytes in about 2000 samples collected from the general U.S. population [9,11]. All target analytes were detected in some percentage of the population verifying that our method is suitable for general population studies (Table 5). TCPY was detected the most frequently, in more than 91% of the samples tested. 24DCP, 25DCP, and 246TCP were also detected in more than 84% of the samples tested. 2IPP was detected the most infrequently in only 3% of the samples.

We had several limitations to our current analytical approach for measuring environmental chemicals. Ideally, we would have isotopically labeled internal standards for all analytes included in the method; however we were missing such standards for three analytes, which introduced some imprecision in their analysis. These three analytes were quantified using the labeled isotope standards of the nearest eluting analyte (Table 1). Also, because the LODs of the analytes are in the sub ng/mL range, and many of the chemicals measured in humans are at or near the LOD, we should have an indicator of quality control at the low end of our method. Because we were unaware that the human levels would be

Table 5
Distribution percentiles of selected target analytes in the U.S. population

Analyte	Age group (years)	*N*	Geometric mean	Median	95th percentile	Frequency of detection
25DCP	All	1989	6.01 (5.38)	6.50 (5.60)	440 (299)	85
	6–11	480	7.57 (8.17)	9.0 (11.3)	630 (516)	85
	12–19	680	5.85 (3.95)	4.8 (4.11)	382 (233)	86
	20–50	829	5.82 (5.36)	6.6 (5.60)	420 (280)	85
24DCP	All	1990	1.11 (0.994)	0.75 (0.794)	22.0 (13.9)	84
	6–11	481	1.27 (1.37)	0.82 (0.966)	29.0 (25.3)	81
	12–19	679	1.30 (0.877)	0.95 (0.645)	21.6 (10.3)	88
	20–50	830	1.05 (0.967)	0.70 (0.795)	21.0 (11.6)	83
OPP	All	1991	0.494 (0.441)	0.490 (0.413)	2.00 (2.93)	70
	6–11	480	0.506 (0.547)	0.490 (0.504)	2.20 (2.61)	70
	12–19	681	0.506 (0.342)	0.490 (0.319	2.00 (1.96)	68
	20–50	830	0.489 (0.450)	0.490 (0.420)	1.90 (3.28)	70
TCPY	All	1994	1.77 (1.58)	1.70 (1.47)	9.90 (8.42)	91
	6–11	481	2.88 (3.11)	2.70 (3.20)	16.0 (14.0)	97
	12–19	681	2.37 (1.60)	2.10 (1.45)	12.5 (6.16)	97
	20–50	832	1.53 (1.41)	1.50 (1.33)	8.60 (6.42)	89
PNP	All	1989	[a]	<LOD	5.0 (4.2)	22
	6–11	479	[a]	<LOD	4.2 (4.2)	26
	12–19	680	[a]	<LOD	5.7 (4.0)	25
	20–50	830	[a]	<LOD	4.5 (4.3)	21

Concentrations are expressed as ng/mL with creatinine-adjusted concentrations (μg/g creatinine) in parentheses. <LOD, less than limit of detection.
[a] Cannot be reliably calculated because frequency of detection <60%.

so low, in many instances, we did not include such a pool. Thus, we have developed another quality control pool at the low end range which will be used in subsequent studies; we are currently characterizing its mean and standard deviation. Though we have been successful at reducing the labor-intensiveness of the method, a great deal of analyst time is still required. We have successfully automated the SPE component using a Gilson 215 SPE unit (Gilson, Middleton, WI); however, the human data reported here were not generated using this automated component. The analyst time may be further reduced in the future by eliminating the need for a chemical derivatization step by transferring the analysis to a high performance liquid chromatography–MS/MS.

4. Conclusions

We report a method for quantifying 12 phenolic metabolites of environmental chemicals in urine by using SPE and chemical derivatization with analysis using isotope-dilution GC–MS/MS. Our method is characterized by its sensitivity, selectivity, and precision. The low analytical LODs of this method allow the determination of internal doses resulting from incidental or background exposures. The stability and precision of the measurement system over several months has demonstrated the robustness of the method.

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References

[1] M.T. Shafik, H.C. Sullivan, H.F. Enos, Int. J. Environ. Anal. Chem. 1 (1971) 23.
[2] T.M. Shafik, H.C. Sullivan, H.R. Enos, J. Agric. Food Chem. 21 (1973) 295.
[3] D. Knopp, S. Glass, Int. Arch. Occup. Environ. Health 63 (1991) 329.
[4] R. Grover, C.A. Franklin, N.I. Muir, A.J. Cessna, D. Riedel, Toxicol. Lett. 33 (1986) 73.
[5] R. Grover, A.J. Cessna, N.I. Muir, D. Riedel, C.A. Franklin, K. Yoshida, Arch. Environ. Contam. Toxicol. 15 (1986) 677.
[6] D.B. Shealy, M.A. Bonin, J.V. Wooten, D.L. Ashley, L.L. Needham, Environ. Int. 22 (1996) 661.
[7] R.H. Hill Jr., D.B. Shealy, S.L. Head, C.C. Williams, S.L. Bailey, M. Gregg, S.E. Baker, L.L. Needham, J. Anal. Toxicol. 19 (1995) 323.
[8] J.O. Westgard, Basic QC Practices: Training in Statistical Quality Control for Health Care Laboratories, Westgard QC Inc., Madison, WI, 2002.
[9] CDC National Health and Nutrition Examination Survey, Available online: http://www.cdc.gov/nchs/nhanes.htm [accessed 6-5-2003].
[10] M. Rabinovitz, Y. Cohen, M. Halpern, Angew. Chem., Ed. Engl. 25 (1986) 960.
[11] CDC Second National Report on Human Exposure to Environmental Chemicals, Available online: http://www.cdc.gov/exposurereport [accessed 6-5-2003].