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NOTE

Improvement of bisphenol A quantitation from urine by LCMS

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Abstract Bisphenol A (BPA) is a synthetic chemical extensively used in many consumer products. It mimics estrogen activities and is related to developmental disorders and metabolic diseases. The current challenge of BPA detection is their low circulating levels at 0.1~10 ng/mL which is close to the detection limit of most of current analytical methods. In this report, we developed a simple, sensitive, and accurate liquid chromatography mass spectrometry (LCMS) method after 1-methylimidazole-2-sulfonyl chloride derivatization. The method significantly improves sensitivity 5~9-fold over dansyl derivatization and approximately 100-fold without derivatization.

Keywords Bisphenol A (BPA) · Urine · ESI-LCMS · Orbitrap MS · 1-Methylimidazole-2-sulfonyl derivatization · Dansyl derivatization

Introduction

Bisphenol A (BPA) is an industrial chemical present in many synthetic plastic materials, particular polycarbonates. It is the main intermediate in the synthesis of polycarbonate polymers and epoxy resins extensively used in many consumer products such as baby bottles, lining of food and beverage containers, and dental fillings. Human exposure to BPA is prevalent, as it migrates from plastic products into food or water during the process of heating, allowing for the direct exposure to humans [1, 2]. Exposure through medical supplies is also possible since BPA can leach into liquids from polycarbonate or PVC-based medical containers or other devices. BPA was also shown to be absorbed by the skin, leading to significant exposure via ink from cashier receipts. Once absorbed, BPA is glucuronidated and/or sulfated followed by elimination in urine and feces [3, 4].

The biological activities of BPA have been extensively studied. It was reported that BPA exhibited endocrine-disrupting properties and can cause reproductive and developmental defects in animals [5]. BPA is also associated with many medical disorders in humans such as cardiovascular diseases, diabetes, cancer, and liver enzyme abnormalities [6]. Better knowledge about its adverse health effects and recommended exposure thresholds was hindered by the lack of sufficient epidemiologic data. Consequently, monitoring human exposure to this prevalent environmental hazardous material gained increasing interests in recent years. Urine is the preferred matrix to measure environmental exposures since it integrates over long periods of exposure times and is noninvasive; however, it contains BPA levels in the sub-nanogram per milliliter range only which is difficult to measure.

The preferred analytical technique for BPA measurements in biological fluids is online solid phase extraction (SPE) for the isolation and enrichment followed by liquid chromatography mass spectrometry (LCMS) in negative mode to monitor the negatively charged analytes [7]. The drawback of these methods is the low sensitivity with levels in healthy populations being measured near the limit of detection. Recently, BPA derivatization with dansyl chloride or ethyl chloroformate was reported to improve sensitivity during LCMS [8] or gas chromatography mass spectrometry (GCMS) analysis [9]. An electrochemical bisphenol A sensor

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based on N-doped graphene sheets was proposed for BPA analysis with a detection limit of 5.0×10^{-9} mol/L [10], but whether this method works for urine is not known.

In this report, we intended to apply a new derivatization method to improve LCMS-based sensitivity and overall analytical efficiency.

Experimental

Chemicals and reagents

BPA and isotope-labeled BPA-$^{13}C_{12}$ used as an internal standard were purchased from Cambridge Isotope Laboratories (Andover, MA) as 100 μg/mL in acetonitrile solutions. Dansyl chloride (DSCl), 1-methylimidazole-2-sulfonyl chloride (ISCl), and sulfatase powder (from *Helix pomatia*, type H-1, >10,000 units/g) were purchased from Sigma-Aldrich (St. Louis, MO). β-Glucuronidase (*Escherichia coli* K12, solution in 50 % glycerol, >140 U/mL) was obtained from Roche Applied Science (Indianapolis, IN). A standard reference material for BPA in urine (SRM 3673 "organic contaminants in nonsmokers' urine") was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). LCMS-grade water and formic acid were purchased from Sigma-Aldrich, and LCMS-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ).

Calibration standards

Calibration standards were prepared from the stock solution by serial dilution in acetonitrile. An eight-point calibration curve was prepared covering a range from 0.1 to 200 ng/mL. The working internal standard was prepared at 1 μg/mL in MeOH. One hundred microliters of calibration standard and 10 μL of internal standard (BPA-$^{13}C_{12}$, 1 μg/mL) were mixed, dried under nitrogen, and subjected to derivatization.

Sample preparation

One hundred microliters of the human urine was mixed with 50 μL of an enzyme mixture (containing β-glucuronidase 2 % *v*/*v* and sulfatase 2 mg/mL in 1 M ammonium acetate, pH 6.5) and 10 μL of the internal standard (BPA-$^{13}C_{12}$, 1 μg/mL) and was incubated at 37 °C for 90 min with horizontal rotation at 100 rpm. After the addition of 5 μL of glacial acetic acid for pH adjustment and extraction with 1 mL of methyl *t*-butyl ether (MtBE) by vortexing at 2000 rpm for 30 s, the upper organic layer was transferred to an HPLC vial and dried under a nitrogen flow.

Derivatization of bisphenol A with DSCl

Dried urine extracts or dried standards were mixed with 75 μL of dansyl chloride (2 mg/mL in acetone) and 75 μL of sodium bicarbonate (100 mM, pH 9) in glass vials, then tightly capped, and heated at 65 °C for 15 min. The resulting mixture was transferred to HPLC inserts, and 10 μL was injected for LCMS analysis.

Derivatization of BPA with ISCl

Dry urine extracts or standards were treated as above except for replacing dansyl chloride with 75 μL ISCl (2 mg/mL in acetone).

LCMS analysis

The analysis was carried out on a model Accela ultra-HPLC system (Thermo Electron, Waltham, MA) including a model HTC Pal autosampler (Leap Technologies, Carrboro, NC) and a Q Exactive Orbitrap mass spectrometer (Thermo Electron). Ten microliters of the reaction mixture was injected onto a Hypersil Gold C18 column (50×2.1 mm, 1.9 μm; Thermo, Waltham, MA) with a mobile phase consisting 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) at a flow rate of 800 μL/min. For the analysis of BPA-1-methyl imidazolesulfonyl (IS) derivatives, the following gradient was used: A/B (*v*/*v*) 70/30 to 35/65 from 0 to 5 min, then back to initial condition 70/30, and equilibrate for 3 min. The total run time was 8 min. The analysis of BPA-dansyl (DS) derivatives was carried out on an Agilent SB-C18 column (50×3.0 mm, 1.8 μm; Agilent, Santa Clara, CA) using the same mobile phase at 800 μL/min flow rate with the following gradient: A/B (*v*/*v*) 60/40 to 10/90 from 0 to 10 min, kept at the same ratio for 1 min then back to initial condition, and equilibrate for 5 min. The total run time was 16 min. Mass detection was carried out under positive electrospray ionization (ESI) mode with spray voltage of 4.5 kV, capillary temperature of 350 °C, S-lens RF value of 50, heater temperature of 300 °C, and in-source CID of 5 eV. Nitrogen was used as sheath gas (pressure 45 units) and auxiliary gas (pressure 10 units). Two MS methods were applied: full scan was performed in a scan range of 150 to 1000 *m*/*z*, maximum injection time 100 ms, microscan 1, resolution 35,000, and AGI target 1e6 and targeted SIM (tSIM) scans the exact masses of BPA and BPA-$^{13}C_{12}$ (see Table 1 for details), isolation width 1.5 mass units, maximum injection time 100 ms, microscan 1, resolution 35,000, and AGI target 1e6.

Method validation

Linearity was measured using an eight-point calibration curve by plotting the ratio (peak area of the analytes/peak area of the

Table 1 Parameters of BPA-1-methylimidazole-2-sulfonyl (IS) and dansyl (DS) adducts using Orbitrap mass spectrometry in full scan (FS) and targeted SIM (tSIM) mode

	IS derivatization				DS derivatization			
	$[M+H]^+$	t_R (min)	LOD (pg/mL)		$[M+H]^+$	t_R (min)	LOD (pg/mL)	
			tSIM	FS			tSIM	FS
BPA	517.12101	3.8	0.12	1.9	695.22441	11.6	1.1	10
BPA-$^{13}C_{12}$	529.16126	3.8			707.26466	11.6		

LOD was defined as the concentration yielding a signal-to-noise ratio of 3 in standards

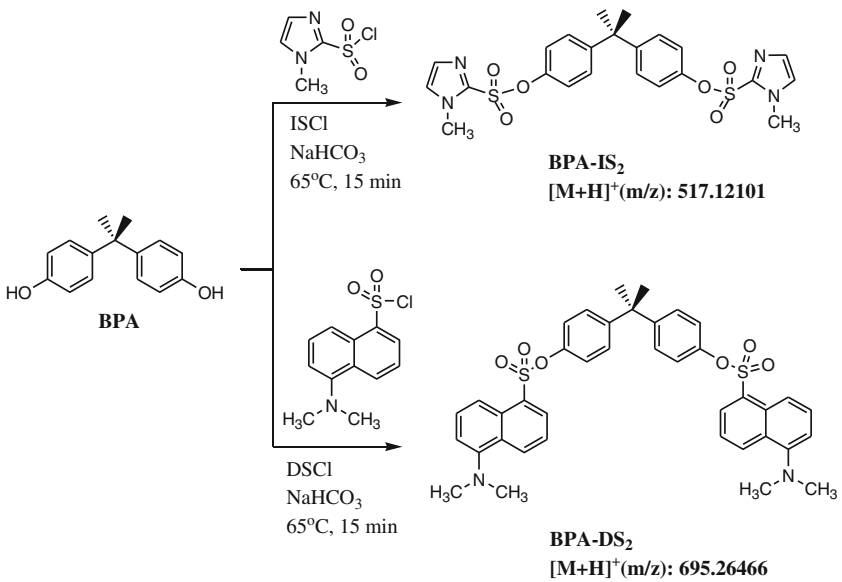
internal standard) versus analyte concentration. Precision was evaluated by repeated analysis of quality control (QC) urine samples. Two sets of internal urine pools, one high and the other low in BPA, were analyzed every day for 4 days, and the intra- and inter-day coefficient of variation (CV) was computed. Accuracy was evaluated by comparisons of our measured values versus defined concentrations of the standard reference material obtained from NIST (SRM 3673, urine).

Results and discussion

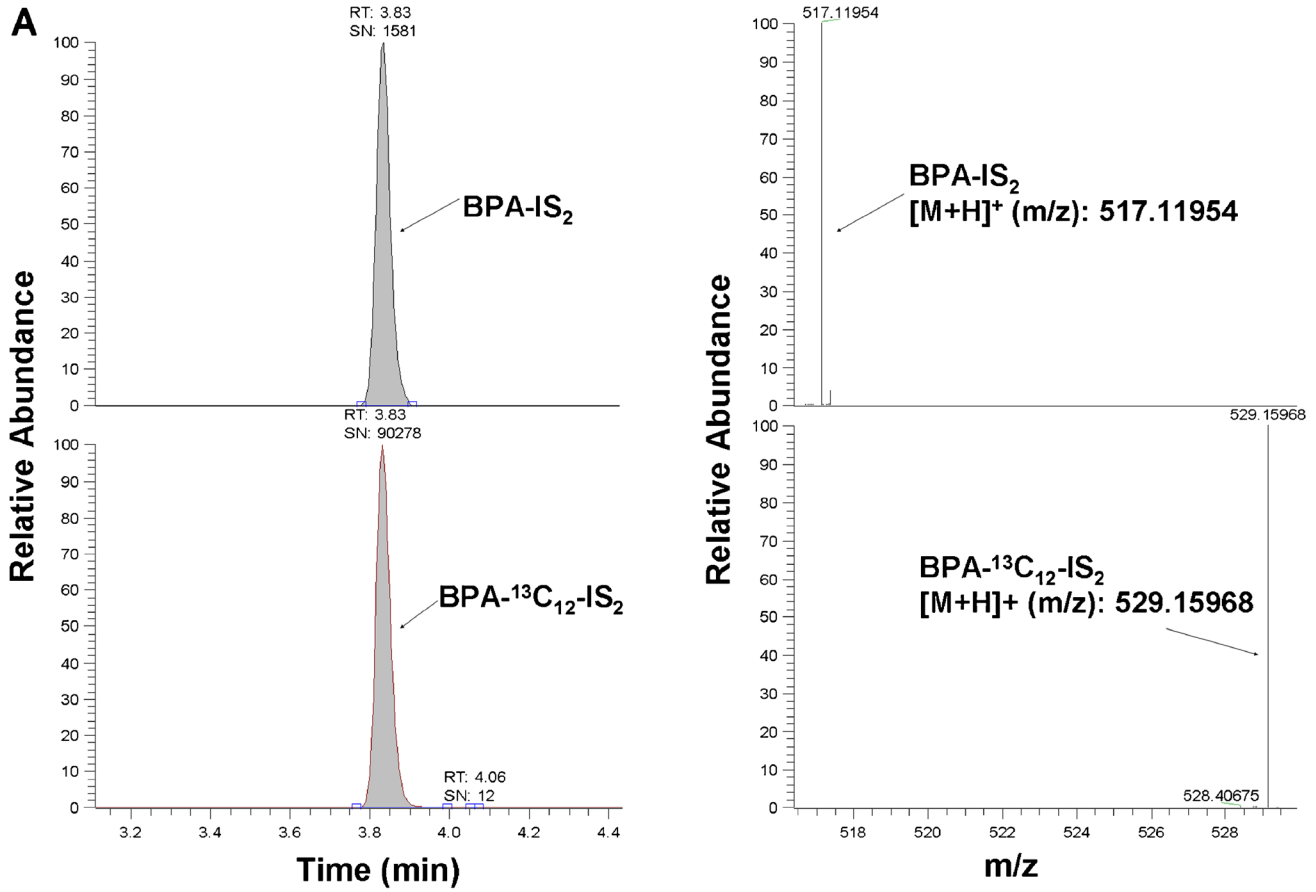
BPA is present in urine at very low levels, often below 1 ng/mL, in healthy populations and therefore poses great challenges for the accurate quantitation. Traditionally, BPA analysis required extensive sample preparation procedures to enrich BPA in order to be accurately detected. GCMS was previously reported for the analysis of BPA, but this requires a large amount of starting material, extensive cleanup procedures, and a derivatization step to yield a volatile product that can be ionized. In contrast, LCMS is more advantageous because the sample preparation steps are much less complicated and turnaround is much faster.

On-line SPE coupled to HPLC followed by (−)APCI MS was reported for the analysis of urinary BPA with a limit of detection of 0.1~0.4 ng/mL [7]. However, this method requires the injection of a large amount of urine samples, thus leading to frequent change of online SPE cartridges. Moreover, repeated injection of a large amount of urine will decrease MS sensitivity and cause high CVs. Therefore, this method is less favorable for epidemiology studies that require thousands of samples to be analyzed with short turnaround times. Herein, our goal is to develop a sensitive, fast, and efficient method to detect BPA with a small volume of biological samples.

Dansylation has been widely used as an effective derivatizing reagent for modifying phenolic compounds because the sulfonyl chloride moiety readily and quantitative reacts with phenols [11] and the resulting dansyl (DS) adduct exhibits enhanced sensitivity for fluorescence analysis [12] and also for (+)ESI-LCMS detection. The latter reaches limits of detection of 5 pg/mL due to the high proton-affinity of the dansyl tag [8]. During our most recent studies to improve the



Scheme 1 Derivatization reactions of bisphenol A (BPA) with 1-methylimidazole-2-sulfonyl chloride (ISCl) and dansyl chloride (DSCl)


A
RT: 3.83
SN: 1581
BPA-IS2
RT: 3.83
SN: 90278
BPA-13C12-IS2
RT: 4.06
SN: 12
Relative Abundance
Time (min)
517.11954
BPA-IS2
[M+H]+ (m/z): 517.11954
529.15968
BPA-13C12-IS2
[M+H]+ (m/z): 529.15968
528.40675
m/z

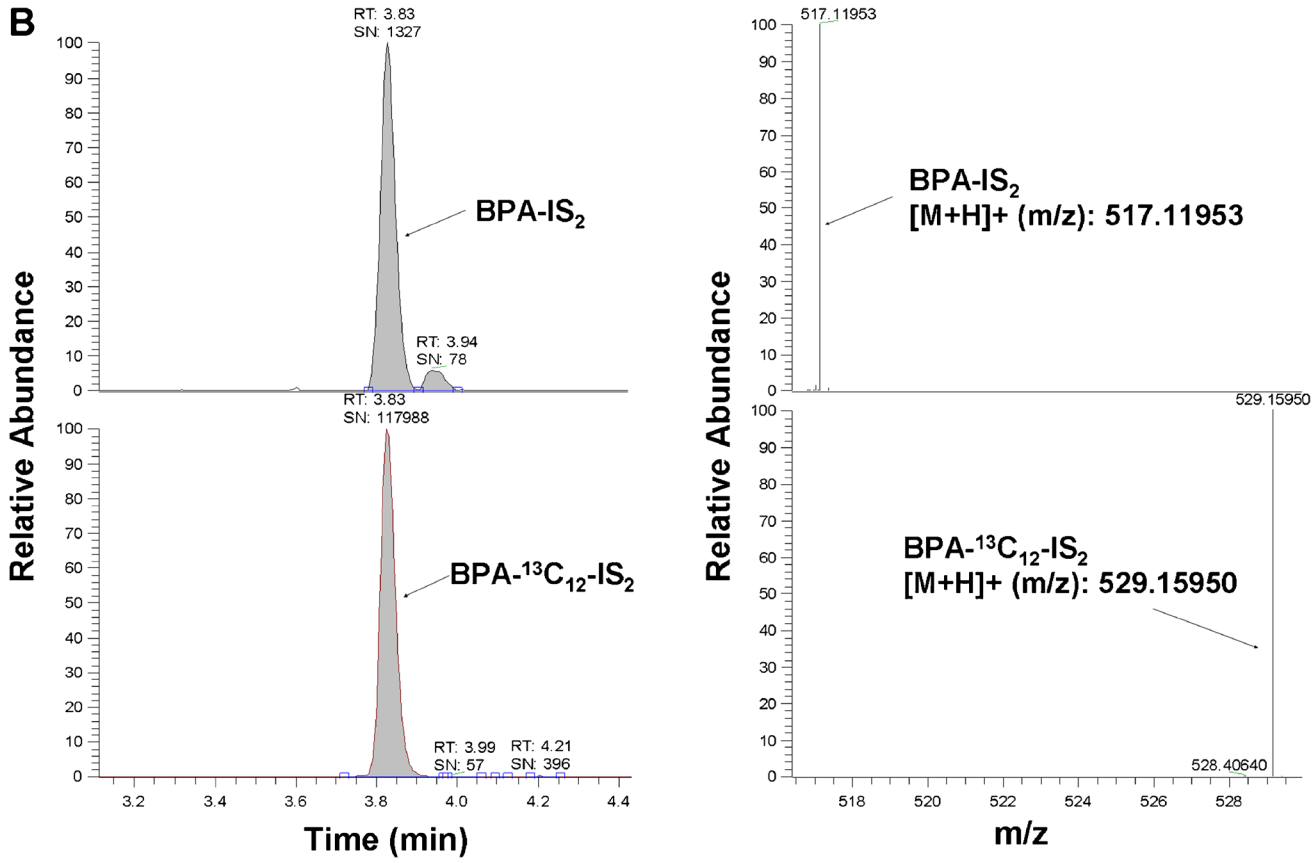

B
RT: 3.83
SN: 1327
BPA-IS2
RT: 3.94
SN: 78
RT: 3.83
SN: 117988
BPA-13C12-IS2
RT: 3.99
SN: 57
RT: 4.21
SN: 396
Relative Abundance
Time (min)
517.11953
BPA-IS2
[M+H]+ (m/z): 517.11953
529.15950
BPA-13C12-IS2
[M+H]+ (m/z): 529.15950
528.40640
m/z

Table 2 Validation of the IS method with the urine samples by tSIM MS

	Concentration found (ng/mL)	Precision (CV) (%)		Accuracy (%)
		Intra-day (n=3)	Inter-day (n=4)	
QC1[a] (low)	2.00±0.22	7~13	11	–
QC2[b] (high)	9.34±0.45	2~7	4	–
SRM 3673[c]	2.00±0.10	1~4	5	104

[a] *QC1* low-level internal urine pool

[b] *QC2* high-level internal urine pool

[c] Standard reference urine purchased from NIST certified for 1.92±0.11 ng/mL BPA

LCMS sensitivity of phenolic steroids, we found a structurally similar sulfonyl compound that exhibits better sensitivity over DS [13]. In the current study, we translated this experience to BPA analyses by tagging the new 1-methylimidazolyl-sulfonyl moiety to BPA and compare that product to the DS derivative.

Similar to dansyl chloride, 1-methylimidazole-2-sulfonyl chloride (ISCl) contains a sulfonyl chloride functional group that underwent rapid and quantitative reaction with phenolic alcohols. The derivatization reaction of extracted urine with ISCl was carried out in a similar fashion as DSCl in the presence of aqueous sodium bicarbonate to afford quantitatively a double-tagged BPA product (Scheme 1). We optimized the reaction by variations of bases, solvents, reaction times, and temperature ranges. An important factor that we found to affect derivatization is the quality of the sulfonyl chloride reagent. By keeping dansyl chloride and 1-methylimidazole sulfonyl chloride in desiccators, we could maintain their reactivity. The obtained product was directly subjected to HPLC analysis using a 50-mm Hypersil Gold C18 column. We found that this column is more selective and faster than an Agilent SB C18 column. BPA-IS and interferences carrying the same mass were well separated, and BPA-IS was eluted in less than 4 min with a total run time of 8 min (Fig. 1). On the other hand, BPA-DS was best chromatographed on an Agilent SB C18 column and eluted at around 11 min with a total run time of 16 min. Since the IS tag is smaller than the DS tag (ca. 60 % by MW) and its product is less lipophilic, its elution is faster, thereby requiring less solvent.

MS analysis was carried out with Orbitrap (+)ESI MS (model Q Exactive) because we found this instrument in most of our applications to be more sensitive compared to tandem MS [11]. The limit of detection (LOD) was determined for full scan (FS) and targeted SIM (tSIM) using a signal-to-noise ratio of 3. Table 1 shows the specific ion masses targeted in tSIM mode and the parameters used for quantitation of BPA, BPA-$^{13}C_{12}$-IS, and BPA-DS. Our results indicate that IS products can be measured more sensitively than DS products: in FS MS and tSIM mode, the LOD of BPA-IS was 1.9 and 0.12 pg/mL, which is five times and nine times lower than BPA-DS, respectively. The BPA-IS calibration curve is highly linear (R^2>0.999) in the experimental concentration range of 0.1~200 ng/mL. Moreover, we found that in the lower calibration range of 0.1~10 ng/mL, the IS curve (R^2>0.99) showed a better linearity than the DS curve (R^2<0.98). Figure 1 shows typical chromatograms and mass spectra of the BPA-IS derivatives.

The IS method was validated for precision and accuracy using internal urine pools and a NIST urinary standard reference material (SRM 3673) under tSIM MS conditions (Table 2). Precision was evaluated using two sets of internal urine pools: one quality control (QC) at low (QC1) and one at high (QC2) BPA level, all analyzed in triplicates for four consecutive days. The low-level QC1 (mean level=2.00 ng/mL) showed an intra-day CV of 7~13 % (mean 11 %) and an inter-day CV of 11 %; the high-level QC2 (mean level=9.34 ng/mL) showed better CVs, namely 2~7 % (intra-day) and 4 % (inter-day). SRM 3673 showed even better precision (1–4 %). The accuracy of our IS method was evaluated using SRM 3673 which was found to have a BPA level of 2.00± 0.10 ng/mL. Therefore, high accuracy of our proposed method was proven with a deviation of only 4 % from the certified concentration (1.92±0.11 ng/mL).

◀ **Fig. 1** LC/MS chromatogram and spectrum of BPA-IS derivatives by (+ ESI) tSIM from standards (**A**, 0.1 ng/ml) and a urine pool (**B**)

Conclusions

In this report, we developed a sensitive and accurate LCMS method for the determination of BPA in urine after derivatization with 1-methylimidazole-2-sulfonyl (IS) chloride in a simple and quantitative reaction. The resulting BPA-IS adduct showed a LOD of 0.12 pg/mL by tSIM and was nine times more sensitive than the BPA-dansyl derivative. The precision and accuracy of the method was validated using internal quality control and SRM urine samples from NIST. The IS method requires much less turnaround time and may also be useful for improving MS sensitivity of other phenol-containing compounds.

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