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Solid phase analytical derivatization of anthropogenic and natural phenolic estrogen mimics with pentafluoropyridine for gas chromatography–mass spectrometry

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ABSTRACT

A simple, low cost, fast and sensitive method is reported for the determination of the four endocrine disrupting chemicals (EDCs) 4-*tert*-butylphenol, 4-*tert*-octylphenol, bisphenol A and 17β-estradiol using pentafluoropyridine as the derivatizing reagent. These EDCs were determined by simultaneous extraction and derivatization in a solid phase analytical derivatization (SPAD) technique without the aid of any phase transfer catalyst (PTC) or an ion-pair mechanism. Recoveries of analytes as their tetrafluoropyridyl derivatives from water ranged from 71% for 4-*tert*-butylphenol to 106% for 17β-estradiol; from urine they ranged from 61% for 17β-estradiol to 91% for 4-*tert*-octylphenol; and from humic acids solution the ranged from 59% for 17β-estradiol to 104% for 4-*tert*-octylphenol in humic acid solutions. Calibration curves were constructed from a matrix of human male urine in the range 1–40 ng/mL and had coefficients of correlation greater than 0.99. For 4-*tert*-butylphenol, bisphenol A and 17β-estradiol the limits of quantitation were 5 ng/mL and for 4-*tert*-octylphenol it was 1 ng/mL. This method was applied to determine EDCs and detected 4-*tert*-octylphenol, bisphenol A and 17β-estradiol in concentrations comparable to those found in the literature. The method offers advantages in speed of analysis, reduced reagent and specificity of derivatization.

1. Introduction

Compounds that possess endocrine activity have been and remain – a concern in environmental toxicology. It was recognized as the cause of reproductive failure in North American birds [1]. The basis of these ideas was the electron capture detector (ECD) [2] which, when coupled to the gas chromatograph (GC) first met the sensitivity and selectivity necessary to identify that DDT was a persistent contaminant responsible for decimating this class of animals. Other compounds originating from industrial activity are now known to have estrogenic activity (xenoestrogens) capable of disrupting endocrine function in wild life and humans [3–5]. Notably EDCs in the environment are a potential risk for breast cancer in men [6]. The alkylphenols are one such class of compounds. In addition natural estrogens, such as estradiol, are also a concern if present at higher concentrations in the environment [7,8]. As in the case with DDT, detection of xenoestrogens and natural estrogens in the environment and in humans puts a premium on sensitivity and specificity of analysis.

Along with detection by mass spectrometry (MS), sample preparation is an important factor in providing sensitivity necessary for the study of endocrine disrupting compounds (EDC). In this context, analytical derivatizations (AD) can increase sensitivity by up to three orders of magnitude even with the high technology capabilities of the MS [9].

The most common methods for detecting EDCs are based on GC–MS and [10–12] using wall-coated open-tubular (WCOT) columns which combine high sensitivity of detection and high separation power. Gas chromatography of the EDCs requires silylation, to addresses the issues interaction with free silanol groups and volatility [13–15].

Other reagents, however, produce derivatives that also enhance volatility but can increase detection sensitivity by several orders of magnitude even for mass spectrometric detection [16–20]. The additional steps required, long reaction times, residual excess reagent and general extra sample handling impede their widespread use.

One method to overcome some of these impediments is to combine AD with extraction. This is exemplified by extractive alkylation (EA) which classically involves use of counter-ions to transfer ionized species into a water immiscible organic solvent (typically dichloromethane) containing the reagent [21–24]. While effective, it is a time consuming technique, difficult to automate

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and is not environmentally friendly. An alternative is a combination of solid phase extraction (SPE) and AD, termed solid phase analytical derivatization (SPAD) [25–29]. As an SPE technique, it is fast, consumes relatively small amounts of solvent and is easy to automate. SPAD has been used to detect fatty acids, phenols [29–33], amines [34,35] and carbonyls [27,36,37] from biological tissue homogenate, plasma and urine.

For detection of EDCs, Kuklenyik et al. [38] as well as Kojima et al. [24,39,40] developed a variant of SPAD by adding quaternary ammonium counterions to the reaction mixture either as soluble phase transfer catalysts or tethered to the solid phase. The rationale was that this class of analyte was more hydrophilic than those previously studied [30,32,41,42]. Consequently counter-ions would be required to transfer the more hydrophilic ionized alkylphenolates onto the surface of the solid phase which contained the reagent.

While there are a large variety of reagents, derivatization of organic acids with pentafluorobenzylbromide (PFBBr) to form pentafluorobenzyl (PFB) ethers or esters provides the greatest increase in detection sensitivity in GC–MS with negative ion chemical ionization (NICI) [38,43–46]. Although it is a very useful reagent, its high boiling point (174–175 °C) makes it difficult to remove from the final isolate and its numerous reaction side products sometimes require pre-purification of the isolate by HPLC [47] before analysis.

Perfluorinated aromatic compounds are an alternative to PFBBr. These compounds are effective electrophiles for ionized hydroxyls and for amines [48–50]. Kojima's group tested a pool of potential fluorinated aromatics and reported that pentafluoropyridine (PFPy) had a reactivity profile, volatility, and produced derivatives that were well suited to analysis [24]. The boiling point of PFPy is 84 °C and is more easily removed from the final isolate than PFBBr. Furthermore tetrafluoropyridyl (TFP) derivatives have shorter retention times than the PFB ethers/esters and this is a desirable trait. Finally, although the fluorines at the 2 and 3 positions are reactive, mild conditions produce regioselective reaction only at the 4 position.

The present work further investigated the SPAD reaction of PFPy as a reagent for detection of EDCs using XAD-4 as a solid phase. This material is a polystyrenedivinylbeznene cross linked macroreticular resin that is stable to alkali, base and elevated temperature. In addition, of all the XAD resins it has the highest specific surface area. These characteristics improve recovery and precision [51]. We determined 4-*tert*-butylphenol, 4-*tert*-octylphenol, bisphenol A and 17β-estradiol from humic acid solutions and urine. Unlike earlier work, however, SPAD using XAD-4 does not require a phase transfer catalyst or any other ion-pairing mechanism. An additional and important characteristic is that under SPAD conditions PFPy is specific in its reaction towards phenols, particularly alkylphenols, in the presence of carboxylic acids. Such specificity can be effective at reducing interferences from matrices such as plasma, urine or environmental samples which are rich in carboxylic acids [32,42].

2. Experimental

2.1. Materials

4-*tert*-Butylphenol, 4-*tert*-octylphenol, bisphenol A, 17β-estradiol, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), pentafluoropyridine, humic acid and Amberlite XAD-4 were all purchased from Sigma–Aldrich (Oakville, ON, Canada). Doubly distilled water and all solvents used were from Caledon (Georgetown, ON, Canada). The 30 m × 0.25 mm I.D. (film thickness 0.25 μm) DB-5ms ultra inert wall-coated open-tubular column for gas chromatography was obtained from J &W Scientific (Folsom, CA, USA).

2.2. Instrumentation

Gas chromatographic analysis were performed using an Agilent Technologies HP-6890 gas chromatograph (Palo Alto, CA, USA) fitted with a split/splitless injector and a single quadrupole mass selective detector (HP 5973). Helium was used as carrier gas at a constant linear velocity of 40 cm/s. Injections of 1 μL were introduced into a splitless injector (purge time of 2 min and a purge flow of 50 mL/min), inlet temperature 280 °C and temperature gradient of 80 °C for 1 min, then ramped at 15 °C/min to 260 °C followed by another ramp at 20 °C/min to 290 °C and held for 6 min. The GC–MS transfer line was maintained at 280 °C, and the MS quadrupole and source heaters were maintained at 150 °C and 230 °C, respectively. The HP 5973 mass selective detector (MSD) was equipped with electron impact ionization at which was set at 70 eV. For quantitation the MSD operated in the selective ion monitoring (SIM) mode. For structure identification the injection aliquot contained 1 μg of TFP derivative and the MSD scanned the range from $m/z = 50$ to $m/z = 550$.

2.3. Preparation of TFP derivatives for use as standards

The ethers of EDCs were synthesized by the standard procedure outlined below [52]. Twenty five mg of the parent compound of the analyte was dissolved in 2 mL of acetone. Equi-molar amount of PFPy and 50 mg of powdered potassium carbonate were added to this solution and the reaction mixture was shaken for 2 h at ambient temperature. At this point GC–FID analysis of the reaction mixture showed only one peak which was not starting material. Using 5 mL of pentane and 5 mL of water, the ether derivatives were extracted into pentane. The pentane phase was washed three times with 5 mL of doubly distilled water to remove any acetone or potassium carbonate which could be carried over (either in solution or as particles) it was then dried with sodium sulfate and transferred to a weighed vial and evaporated to dryness in a stream of nitrogen till constant weight was achieved. The residue was dissolved in a volume of heptane sufficient to produce a concentration of 1 mg/mL in this solvent. GC–FID analysis of the heptane solutions also showed a single peak for each derivative. This solution served as a standard for determination the mass spectrum and of recovery.

2.4. Preparation of working solutions

2.4.1. Preparation of analyte stock and spiking solutions

Stock solutions of analytes were prepared by dissolving measured amounts of the analytes of in acetone at a concentration of 1 mg/mL. These were kept tightly sealed at 4 °C and warmed to room temperature until use. A 20 mg/L humic acid stock solution was prepared in doubly distilled water. Spiking solutions were prepared by diluting the analyte stock solutions by 100 fold to provide a concentration of 100 ng/mL.

2.4.2. Collection of urine samples

For studies on recovery and preparation of calibration curve, urine was collected from a healthy drug free male volunteer. Urine for determination of EDC in humans was obtained from a female was a house painter [38] and had been occupationally exposed to paints for 13 years and was stored at 4 °C for 24 h before use.

2.4.3. Preparation of spiked solutions

Three aqueous matrices were studied: doubly distilled water; urine from a male donor who was not occupationally exposed to EDCs; urine from an occupationally exposed human female donor in her mid thirties; humic acid solution containing 20 mg/L of humic acid. Fifty microliters of spiking solution were pipetted into 90 mL of aqueous matrix and 1.0 M NaOH was added till 100 mL mark is

Table 1
m/*z* of ions used to identify analyte derivatives.

Analyte	*m*/*z*
4-*tert*-Butylphenol	284,299
4-*tert*-Octylphenol	284,355
Bisphenol A	511,526
17β-Estradiol	493,403,362,129

reached in a volumetric flask. The flasks were thoroughly mixed and the solutions were used immediately. Calibration curves were prepared spiking analytes into urine of the male donor in the range of concentrations from 1 to 40 ng/mL.

2.5. Preparing the resin for derivatization by SPAD

In studies with 1 mL of sample 24 mg of XAD-4 phase was measured in a siliconized glass tube. The resin was sequentially washed with 100 μL of acetonitrile, 1.0 M HCl and followed by two times of 200 μL doubly distilled water. The resin was impregnated with 4 μL of PFPy in 56 μL of acetonitrile and shaken for 2 min to distribute the reagent solution throughout the pores and interstitial spaces. In studies with 4 mL of sample, 48 mg of XAD-4 phase was measured in a siliconized glass tube. The resin was sequentially washed with 200 μL of acetonitrile, 1.0 M HCl and followed by two times of 200 μL doubly distilled water. The resin was impregnated with 8 μL of PFPy in 112 μL of acetonitrile and shaken for 2 min to distribute the reagent solution throughout the pores and interstitial spaces.

2.6. Recoveries, accuracy and precision

Calibration curves were constructed to cover the concentration range from 1 ng/mL to 40 ng/mL [38,53]. These were used to determine the concentrations. Accuracy was determined as the percentage between experimentally determined concentrations and the prepared concentrations. Recoveries for derivatization of 4-*tert*-butylphenol, 4-*tert*-octylphenol, bisphenol A and 17β-estradiol were calculated by comparison of peaks areas obtained from injection of 1 μL containing 10 ng of synthetic TFP derivative determined by GC–MS with SIM monitoring.

2.7. Derivatization from aqueous matrix by SPAD

Four milliliters of alkalinized matrix containing 20 ng (5 ng/mL) of each analyte was added to the tube containing the prepared XAD-4 resin as described in Section 2.4. This deposited the reagent on the surface and initiated the reaction. Reaction conditions comprised shaking the tube in a horizontal position, at 150 cycles/min for 20 min. The supernatant was then aspirated and the XAD-4 resin was dried using a stream of nitrogen. Eight sequential washes with 100 μL of pentane eluted the derivatized analytes. The pentane extract was evaporated to dryness using a stream of nitrogen. The derivatives were taken up in 25 μL of BSTFA and 1 μL was injected into the GC–MS with the mass spectrometer operating in the SIM mode. The ions monitored for quantitation appear in Table 1. The response for each TFP derivative was measured as the sum of the peak areas for its ions determined in SIM.

2.8. Test of reactions of PFPy with carboxylic acids and chlorophenols

Straight chain carboxylic acids were subjected the same conditions that were used for derivatization of phenols. On the synthetic scale, the reactions were those described in Section 2.3. On the analytical scale the reaction conditions were those described in Section 2.7. In a third experiment the conditions in Section 2.7 were modified by the addition tetrabutylammonium hydroxide at a concentration of 0.1 Molar.

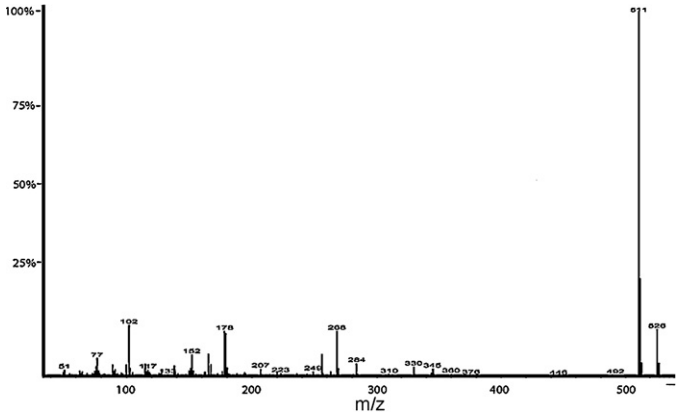

Fig. 1. Mass spectrum of bisphenol A derivative.

3. Results and discussion

3.1. Mass spectrometric identification of derivatives and determination of response

The TFP ethers 4-*tert*-butylphenol and 4-*tert*-octylphenol fragmented via the benzyl cleavage [24] as evidenced by the ion at *m*/*z* = 284. In both instances the molecular ion was also present. For quantitative analysis response for 4-*tert*-butylphenol–TFP ether were measured by the sum of the areas of the base peak *m*/*z* 284 and the molecular ion *m*/*z* 299 while for 4-*tert*-octylphenol-TFP ether it was determined using the sum of the areas of the base peak *m*/*z* 284 and the molecular ion *m*/*z* 355. In the present work, the greater volatility of the derivatives facilitated the study of bisphenol A and 17β-estradiol since the TFP derivatives eluted at lower temperatures and shorter retention times than the corresponding pentafluorobenzyl derivatives. In this study the mass spectrum of bisphenol A-TFP ether had two major ions (Fig. 1). The ion at *m*/*z* 526 corresponded of the bis-TFP derivative of the parent compound and a base peak *m*/*z* 511 formed by the loss of one methyl group ($[M–CH_3]^+$). Both these ions were summed and used for bisphenol A determination. The mass spectrum of the 17β-estradiol-TFP-TMS mixed ethers showed four mass fragments *m*/*z* 493, 403, 362 and 129 (Fig. 2). The molecular ion was observed at *m*/*z* 493. The base peak *m*/*z* 403 was formed by the 1,4-elimination of

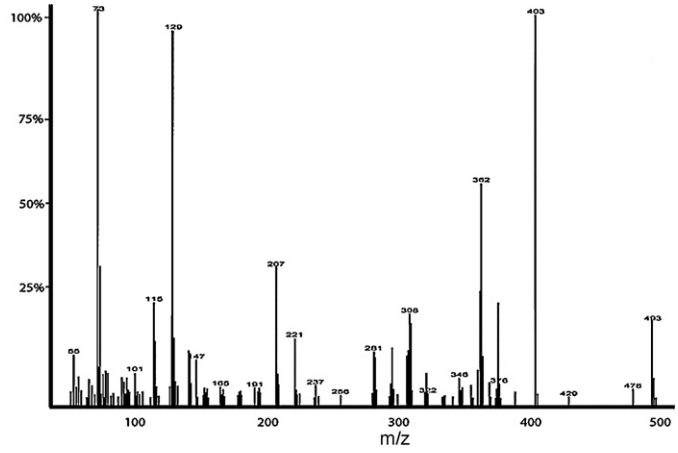

Fig. 2. Mass spectrum of 17β-estradiol derivative.

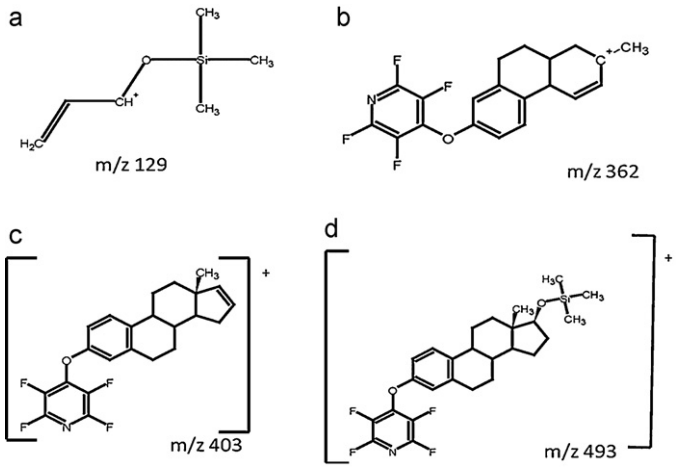

Fig. 3. Mass fragments and molecular ions of 17β-estradiol. (a) *m/z* 129 (b) *m/z* 362 (c). *m/z* 403 and (d) *m/z* 493.

trimethylsilanol, $HOSi(CH_3)_3$. The possible structures of these are shown in Fig. 3. 17β-estradiol was determined by summing all four ions.

3.2. Method development

3.2.1. Effect of elution solvent on recovery

Known amounts of the TFP ethers were coated on 48 mg of XAD-4 resin by adding them in acetone solvent which upon evaporation deposited these compounds on the surface. Pentane, acetone and ethyl acetate were tested for elution efficiency. Four 100 μL washes with acetone followed by another four 100 μL washes with diethyl ether produced recoveries of greater than 90% with RSD lower than 10%. However for samples containing humic acid this procedure gave low recoveries less than 50% and RSD greater than 20%. Eight 100 μL washes with pentane also showed quantitative extraction of derivatives even in the presence of humic acid. When the elution procedure had less than eight washes of 100 μL of pentane there was high variance in recoveries therefore eight 100 μL washes of pentane became the standard procedure.

3.2.2. Derivatization conditions

Derivatization occurred under mild reaction conditions with the temperature at 30 °C and from aqueous medium. Derivatization with electrophiles including PFPy requires ionization of the phenolic EDCs. The pK_a values for these EDCs are around 10 and thus require a pH of at least 12 for complete ionization. NaOH was added to a final concentration of 0.10 M and was used for all studies in this work [24].

3.2.3. Effect of aqueous phase volume, weight XAD-4 and volume pentafluoropyridine on recovery

The initial reaction conditions used 24 mg of XAD-4, PFPy 4 μL and 1.0 mL of sample volume. In order to increase sensitivity the sample volume was increased to 4.0 mL, and the amounts of XAD-4 and PFPy were only doubled. Despite the disproportionate increases in sample size relative to reagent and solid phase, the recoveries were the same in both instances.

3.2.4. Optimization of time

Reaction times 5, 10 and 20 min were compared in 4 mL of alkalinized aqueous phase. Reaction times of less than 20 min produced lower recoveries and high variance. For all experiments the reaction time was 20 min. With a reaction time of 20 min and at concentration of 5 ng/mL in doubly distilled water the method produced high and precise recoveries (Table 2).

3.3. Method Characterization

Calibration curves covered the range of expected concentrations [38,53] and were prepared by spiking analytes into urine from the male donor. Aliquots were analyzed according to the sample preparation procedure in Section 2.7. Responses were determined from peak areas because the markedly different structures and fragmentation patterns rendered structural analogues ineffective as internal standards. For instance use of 4-*tert*-butylphenol an internal standard for 4-*tert*-octylphenol, bisphenol A and 17β-estradiol did not improve precision over the use of peak areas as the response. The problem is further exacerbated by the complexity of the matrix when studying humans. Kukelenyik et al. noted [38] that the correct blank for human urine is obtained by combining samples from "multiple anonymous donors". Compounds present in such a

Table 2
Recovery, accuracy, precision.

Analyte	Low	Intermediate	High
4-*tert*-Butylphenol			
Spiked	5 ng/mL	20 ng/mL	30 ng/mL
Found	5.15 ng/mL	20.48 ng/mL	28.98 ng/mL
Accuracy	1.03	1.02	0.97
Recovery	0.70	0.705	0.747
RSD (N = 4)	3.22	10.03	3.22
4-*tert*-Octylphenol			
Spiked	1 ng/mL	20 ng/mL	30 ng/mL
Found	0.90 ng/mL	18.95 ng/mL	31.80 ng/mL
Accuracy	0.90	0.95	1.06
Recovery	0.91	0.86	0.77
RSD (N = 4)	6.80	17.68	2.94
Bisphenol A			
Spiked	5	20	30
Found	5.20 ng/mL	18.67 ng/mL	33.14 ng/mL
Accuracy	1.04	0.93	1.10
Recovery	0.7	0.78	0.66
RSD (N = 4)	4.85	10.62	0.62
17β-Estradiol			
Spiked	5 ng/mL	20 ng/mL	30 ng/mL
Found	4.46 ng/mL	19.51 ng/mL	38.02 ng/mL
Accuracy	0.89	0.98	0.95
Recovery	0.61	0.56	0.43
RSD (N = 4)	5.60	16.99	9.08

Table 3
Recovery from 4 mL alkalinized matrix spiked at a concentration of 5 ng/mL.

Analyte	Matrix					
	Water		Urine		Humic acid	
	Recovery	% RSD ($N = 5$)	Recovery	% RSD ($N = 4$)	Recovery	% RSD ($N = 4$)
4-*tert*-Butylphenol	71	4	70	11	75	6
4-*tert*-Octylphenol	83	4	91	14	104	3
Bisphenol A	72	13	70	11	66	5
17β-Estradiol	106	10	61	13	59	8

sample are representative of those found in the local populations and these will affect selection of an internal standard. The resources for obtaining the local population blank were not available. Despite these limitations, the precision of the method using absolute calibration, remained acceptable (Tables 2–4). It is clear, however, that deuterated analogues of the analytes would be preferred for determination of EDC.

The urine from the male donor showed no background concentrations of 4-*tert*-butylphenol, 4-*tert*-octylpheno and bisphenol A. Estradiol was detected at a concentration of 1.6 ng/mL. The area corresponding to this value was subtracted from those in the calibration curve. The curves were linear over the concentration range studied and the coefficients of correlation were based on absolute response measured as the area of the chromatographic peak and exceeded 0.99 for all three compounds studied. The intercepts for 4-*tert*-butylphenol, 4-*tert*-octylphenol and bisphenol A were between 15 and 22% of the limit of quantitation. For estradiol the intercept was 32% of the detection limit.

Accuracy varied from a low of 0.89% for 5 ng/mL for 17β-estradiol to a high of 1.10% indicating that the absolute calibration technique – although not ideal – provided a reliable measure of the concentration. Limit of quantitation was 5 ng/mL for 4-*tert*-butylphenol, bisphenol A and 17β-estradiol but because of a higher response factor, limit of quantitation (LOQ) for 4-*tert*-octylphenol was 1 ng/mL. Precision for all four analytes was within FDA guidelines (Table 3) at – and above – the LOQ.

A typical chromatogram from urine spiked to a concentration of 5 ng of each analyte in 1 mL is shown in Fig. 4. The urine was from a male donor who had no known exposure to alkylphenols or bisphenol A and would be expected to have very low concentrations of 17β-estradiol.

Finally, the effect of matrix on recovery was determined using doubly distilled water, urine and humic acid. Urine is a readily available biofluid and is frequently used in clinical analysis. Its collection is not invasive. Humic acid is a macromolecule rich in phenol, carboxylic acid and sugar residues. It is the degradation product of plant material and is a contaminant of natural water.

There was marginal effect on recovery of 4-*tert*-butylphenol, 4-*tert*-octylphenol and bisphenol A when the matrix changed from doubly distilled water, to urine or humic acid. 4-(*tert*-octyl) phenol showed a slightly higher recovery compared to doubly distilled water. Liu et al. [54] in their work reported higher recoveries for solid phase extraction of 4-*tert*-octylphenol from humic acid solution compared to that from water. These results may reflect the presence of this compound in the humic material or be the result of interferences from this complex matrix. Estradiol appears to be more susceptible to matrix effects. Since this is the most lipophilic analyte the reduction in recoveries may be due to the increased ionic strength of urine or humic acid solutions which would reduce the solubility of the steroid in an aqueous solution. In the latter case there may also be binding to the lipophilic regions of the macromolecule.

Table 4
Detection in urine from a human female occupationally exposed to EDCs.

Analyte	Urinary concentrations ng/mL	% RSD ($N = 4$)
4-*tert*-Butylphenol	ND	–
4-*tert*-Octylphenol	5.6	7
Bisphenol A	15.4	14
17β-Estradiol	13.7	10

3.4. Selectivity of pentafluoropyridine in SPAD

Pentafluoropyridine is well known to under-go nucleophilic substitution reactions with electron rich nucleophiles in a stepwise manner first replacing the 4-fluorine atom then 2-fluorine atom and finally at 3-fluorine atom [48]. Amines [48] and ionized alcohols [47] react with this reagent although the latter does so slowly. We investigated the reaction of PFPy with alkylphenols, chlorophenols and fatty acids in both SPAD and standard conditions outlined in Section 2.3. The reagent reacted with alkylphenols to produce tetra fluoroypyridyl (TFP) derivatives in greater than 70% recovery (Table 2). The recoveries for reaction with mono and dichlorophenol were considerably lower and variable. There was no reaction with higher chlorinated phenols and carboxylic acids. This allows derivatization of phenols at alkaline pH in the presence of carboxylic acids which simplifies chromatograms from biological materials [31].

3.5. Application of method: determination of EDC resulting from occupational exposure

The developed method was applied to the analysis urine samples from a healthy, human female donor. As a professional house painter this donor was exposed to paints for over 13 years. The detected 4-*tert*-octylphenol and bisphenol A levels are within the range for an individual exposed to paint [38] and the 17β-estradiol

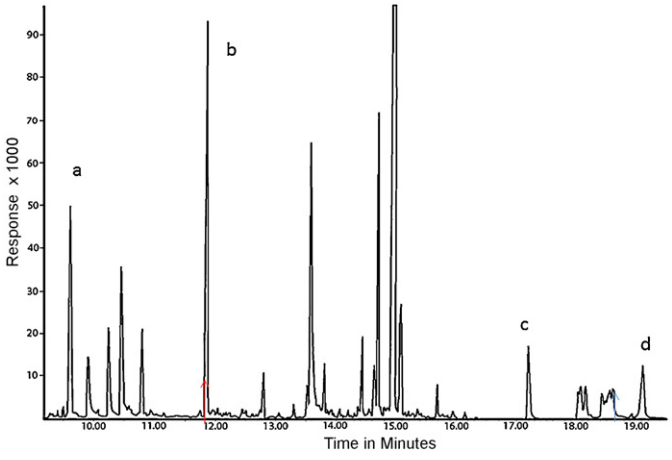

Fig. 4. Sample of urine from human male donor spiked with each analyte at 5 ng/mL and isolated as the TFP derivatives. In any given run, the ions for each TFP ether were monitored by SIM: (a) 4-*tert*-butylphenol-TFP ether (b) 4-*tert*-octylphenol-TFP ether (c) bisphenol A-TFP ether (d) 17β-estradiol-TFP ether.

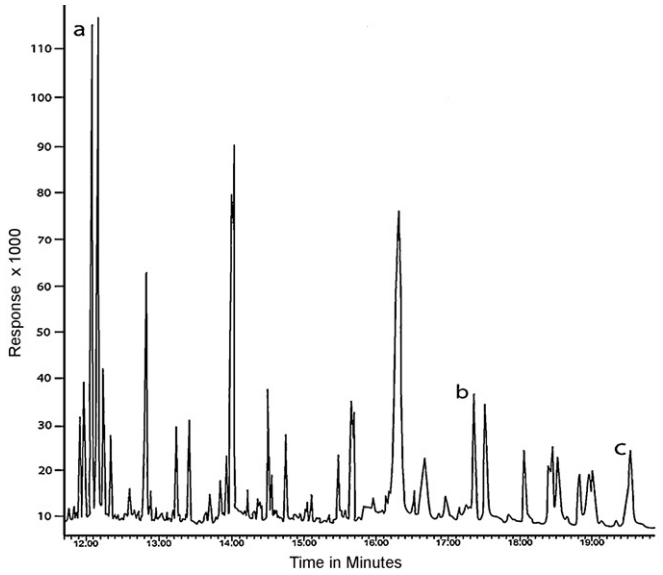

Fig. 5. Sample of urine from human female donor occupationally exposed to paint fumes and analytes isolated as the TFP derivatives. In any given run, the ions for each derivative were monitored by SIM: (A) 4-*tert*-octylphenol-TFP ether (B) bisphenol A-TFP ether (C) 17β-estradiol-TFP ether.

detected are acceptable for a female in her mid thirties [55]. The matrix was considerably richer (Fig. 5). While this is to be expected from the occupational exposure the additional compounds could also come from the environment [56] or diet [57]. Since electron impact was the ionization technique some of the peaks may not be the TFP derivatives of phenols. While column efficiency remained the same, we note a small consistent increase in retention times for all peaks (Fig. 5) compared to the chromatogram in Fig. 4 which is attributed to a flow controller problem.

3.6. Discussion of the proposed method and comparison with published procedures

Kojima et al. [24,39,40] previously reported three methods for the determination of alkylphenols using pentafluoropyridine as an analytical reagent to derivatize specifically aryl hydroxyl groups. In their first method liquid–liquid (L/L) extractive derivatization [24] using tetrabutylammonium cation as the phase transfer catalyst and CH_2Cl_2 as the extraction solvent. Liquid/liquid extraction methods are time consuming processes particularly when they require larger volumes of sample and organic solvent.

To circumvent the issues with L/L extractive derivatizations, Kojima's group investigated the option of SPAD using quaternary ammonium cations as the phase transfer catalyst [24,39,40]. Coating a C_{18} SPE cartridge with tetra-n-hexylammonium bromide provided an ion-exchange surface which sorbed the phenolates from alkalinized aqueous solution [39]. After drying the cartridge the analytes on the surface were contacted with 2 mL of a 0.1% solution of PFPy in acetone which also served as an elution solvent. Following elution from the column and concentration of the eluate, normal phase chromatography further purified the sample. The volume of the final injection solution was 0.5 mL. In another variant the solid phase was a strong anion-exchange resin [40] in which sorbed the phenolates. After drying the solid phase, the sorbed analytes were contacted with pentafluoropyridine solution (200 μL in 2 mL hexane) for 10 min to allow for derivatization. Following elution and concentration the volume of the volume of the final injection solution was again 0.5 mL.

The present method does not require the use of a tethered or soluble phase transfer catalyst and is simple to use. Soluble phase transfer catalysts can co-elute with derivatized analyte and appear in the final isolate [58–60]. It is sometimes necessary to remove this material by SPE [58]. Eliminating the use of counterions removes interferences and extra steps. Although use of ion-exchange phase MAX™ achieves the same end of eliminating counterion in the final isolate, XAD-4 is less expensive and can be used under a wide range of conditions [27,36,61]. It is also stable to alkali and eliminates the concern the instability of silica based to base [39]. In addition, the volumes of sample involved are smaller than those used by Kojima's group. The high concentration factor reduces the volume of the final isolate by 20 (from 500 μL to 25 μL) compared to that of previous work thus minimizing the losses in detection limits from using only 4 mL rather than 20–100 mL of sample [24,39,40]. Finally, since the mass of solid phase is approximately one tenth of that used in previous investigations, the elution volume is less than half of that reported in published work [39,40].

As in previous studies reported by Kojima et al. [39], the present work found that PFPy reacts specifically with phenolates in the presence of carboxylate. In our hands, carboxylic acids did not react with PFPy whether under SPAD conditions from any matrix or under conditions used to prepare the TFP derivatives of the phenols (Section 2.3). Nor was there any reaction in SPAD when tetrabutylammonium cation was added to the reaction mixture.

This is an interesting and useful finding whose mechanism is unknown. It is worth considering the potential mechanism(s) as the specificity allows a significant clean-up of the isolate [39]. In previously reported work, extraction/derivatization of phenols with PFPy occurred in the presence of tetrahexylammonium cation. Under these conditions, carboxylates may sorb, but not react with PFPy at room temperature and in the short reaction times used for derivatization of phenols [39]. There are several lines of evidence suggesting this mechanism. The sorption of the carboxylates is demonstrated by the fact that following derivatization of the phenols with PFPy, the sorbed carboxylates were derivatized according to the method by Field and Monahan [62] using methyl iodide at 80 °C for one hr and recovered as the methyl esters [23,39]. The need for these elevated temperatures, however, could arise from the lower reactivity of methyl iodide rather than other components of the system (analyte, tetrahexylammonium cation and solid phase). But under liquid/liquid phase transfer catalysis, methyl iodide rapidly reacts with carboxylates at room temperature [58,63] and so the reactivity of methyl iodide itself is not a factor in slowing reaction rates. This demonstrates that interactions between analyte, reagent, quaternary amine and solid phase can slow the reaction rate of methyl iodide with carboxylic acids. Similar mechanisms may slow reaction of carboxylates with PFPy.

The present work adds further information regarding the possible mechanism. The reaction specificity occurs both in SPAD and in synthetic reactions for preparation of the derivatives. In SPAD on XAD-4 there is no quaternary amine thus removing the phase transfer as a factor inhibiting reaction between the carboxylate ion and PFPy [39]. In the synthetic reactions, there XAD-4 is eliminated. It appears that the specificity of reaction of PFPy towards phenols may be due to physicochemical characteristics of the reagent and carboxylate, possibly the relative hardness/softness of the electrophile and nucleophile. Regardless of the mechanism it is a useful characteristic of PFPy for determination of phenols in complex biological or environmental matrices [32,39,42].

The use of both PFPy and BSTFA in this method extends the range of analytes detected and we report the first analysis of bisphenol A and 17β-estradiol by derivatization with PFPy and found both these analytes it in the urine of a human female exposed to paint fumes [38].

4. Conclusions

The SPAD method presented here used PFPy to determine four important EDCs 4-*tert*-butylphenol, 4-*tert*-octylphenol, bisphenol A and 17β-estradiol. Recoveries of the TFP derivatives from humic acid and urine matrices were high and reproducible. The method does not require an ion-pairing as phase transfer catalysts. This result simplifies the procedure and provides new information on the mechanism for the specificity of PFPy towards phenols. Although already sufficiently sensitive to determine alkylphenols from humans exposed to these compounds, there is further room for improvement in this GC–MS method. Use of a large volume injector to inject a higher percentage of the final isolate would increase sensitivity. Although there is little information on the negative ion chemical ionization spectra of the tetrahydorpyridyl derivatives it is likely that this ionization technique would even further lower detection limit.

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