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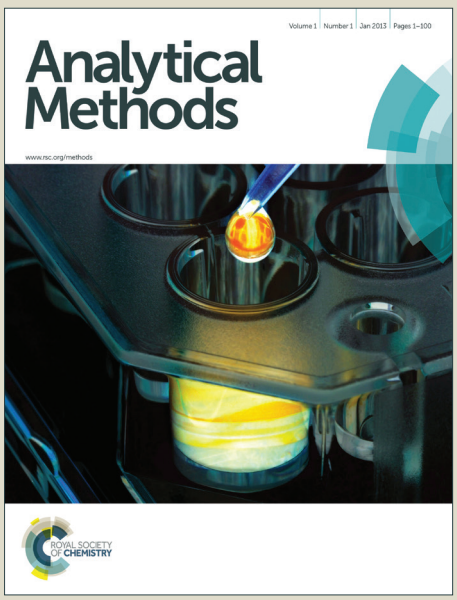

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Determination of five estrogens in wastewater using a comprehensive two-dimensional gas chromatograph

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Abstract

Comprehensive two dimensional gas chromatography (GCxGC) hyphenated with rapid quadrupole mass spectrometry was successfully used to develop a novel method for the determination of trace level estrogens in influent and effluent wastewater. Five estrogens used for the study were 17β-estradiol (βE2), 17α-estradiol (αE2), estrone (E1), 17α-ethynylestradiol (EE2) and estriol (E3). Two orthogonal columns and thermal modulation result in enhanced separation, while the rapid scanning quadrupole mass spectrometer gives high resolution peaks. Samples were extracted with Hydrophilic-Lipophilic Balance (HLB) cartridges and derivatized with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) prior to analysis. The method uses a single extraction step and ng/L method detection limits were achieved using a relatively low sample volume of 500 mL. Elimination of additional cleanup steps make the method time effective. Furthermore, the method has less initial cost as the instrument is far less expensive than a tandem mass spectrometer. A parallel conventional gas chromatographic-mass spectrometric (GCMS) study was carried out to compare the results. Detection limits were 2 to 4 times improved with the GCxGC over the GCMS.

Keywords: Comprehensive two-dimensional gas chromatography, Estrogens, Environmental trace analysis.

1. Introduction

Comprehensive two-dimensional gas chromatography (GCxGC) was first introduced by Zaiyou Liu and John Phillips in 1991.[1] In this technique, two different open tubular GC columns are serially connected with a modulator, an interface between the two columns. Each solute band spreads as it travels through the capillary column. The bands are then refocused by the thermal modulator that alternates between cooling and rapid heating. Thus the thermal modulation accumulates each eluting chemical and then releases a series of concentrated, narrow pulses into the second column.[2-4] Typical modulation time intervals are in the range of 2-6 seconds. Modulated fractions are quickly separated on the second column and directed towards the detector. First dimension columns in GCxGC are usually longer than second dimension columns, whereas shorter second dimension columns commonly have reduced inner diameters to enhance separation efficiency.[3, 4]

In principle, analyte retention times on the second column are required to be less than or equal to the modulation period in order to prevent overlapping peaks in subsequent fractions of modulation.[3, 5] GCxGC instruments are usually equipped with detectors having rapid acquisition rates in order to be compatible with the fast second dimension separations and narrow elution peak widths. Similar to conventional gas chromatography, mass spectrometric (MS) detectors are widely used in GCxGC due to their ability to provide structural information. Among MS detectors, rapid scanning quadrupole mass spectrometer (qMS) detectors have become the most popular detectors because of their user friendliness and affordability.[6, 7]

When compared to conventional one-dimensional gas chromatography, GCxGC produces improved separations in terms of peak capacities, dynamic ranges, sensitivities and specificities. The overall peak capacities in GCxGC are the product of peak the capacities of both columns.[8] Therefore, peak capacities are higher because any unresolved eluents from the first column have an opportunity to be refocused and separated on the second column with a different stationary phase.[9] Sensitivities and dynamic ranges are increased as a result of analyte refocusing.[9, 10] Moreover, when compared to heart-cut multidimensional gas chromatography where only a fraction of insufficiently separated components from the first column are introduced to the second column, GCxGC provides time effective, enhanced resolutions for all sample components.[5, 11] Therefore, with the increasing need for the separation and analysis of complex samples, GCxGC has rapidly gained attention in recent years.

Estrogen hormones excreted to the environment by humans and wildlife are capable of deleterious impacts on aquatic organisms even at extremely low concentrations.[12] They are known to be the most potent endocrine disruptors (EDs) in the environment due to their high affinity to estrogen receptors.[13-16] For example, vitellogenin production in male rainbow trout by 17β-estradiol (βE2) has been reported at ppt level environmental concentrations.[13, 17] Vitellogenin is an egg yolk precursor protein expressed in the female fish but, in the presence of estrogenic endocrine disruptive chemicals, male fish can express the vitellogenin gene in a dose dependent manner. In general, humans use significant amounts of estrogens as medicine.[18, 19] Humans and livestock both secrete significant quantities of natural hormones. Excreted in urine and feces, these hormones are eventually discharged into municipal wastewaters or into the environment.[20-22] Therefore, hazardous amounts of estrogens can be accumulated in

wastewaters in urban areas with high population densities and in agricultural regions. Wastewater influents undergo a treatment process at a wastewater treatment plant (WWTP). Any estrogens not removed during treatment are released to the environment, making WWTPs potential pathways of environmental EDs.

The most frequently employed methods for determining estrogens in environmental aqueous matrices are chromatography combined with MS, such as GC/MS, LC/MS, GC /MS/MS and LC/MS/MS. Among these techniques, chromatography coupled with tandem mass spectrometry (MS/MS) has been used most extensively during the last few decades despite the high cost of instrumentation. Selectivity of the analysis is increased using MS/MS because both the specific mass of the precursor ion and product ion can be used for the quantification. This produces improved results compared to single MS detectors by reducing matrix interferences in a complicated sample mixture. None of these instruments however can reliably quantify trace concentrations of estrogens in a complex matrix without pretreatment. Thus, preconcentration of a larger sample volume into a smaller volume is often required. During the preconcentration process however, a considerable amount of matrix is also concentrated into this small volume. Hence, multiple clean-up techniques are typically necessary to eliminate matrix interferences as much as possible before injecting in to the instrument. Additional clean-up steps lead to cleaner chromatograms, but they are generally time-consuming and often result in loss of analyte recovery.

We have found that the two dimensional separation associated with GCxGC reduces matrix interferences enough to eliminate the need of additional cleanup procedures. We also have determined that the sample concentration associated with GCxGC and the enhanced performance of the rapid scan quadrupole mass spectrometer (QP 2010 ultra) result in improvements in signal to noise ratios and thus reduced limits of detection. Even though tandem mass spectrometry possesses better selectivity, a

GCxGC-qMS method is far less expensive, yet maintains the sensitivity to detect ng/L levels of estrogens. Moreover, a method without additional cleanup steps will obviously be faster, use fewer resources and be less costly than most existing methods. Therefore the objective for this study was to obtain acceptable detection limits, using the GCxGC-qMS, with a single extraction step from a relatively small initial sample volume.

The most prevalent sample preconcentration method for estrogenic compounds is solid phase extraction (SPE). Reversed Phase SPE cartridges, such as C18, and polymeric SPE cartridges, such as hydrophilic lipophilic balanced (HLB) are commonly used due to the non-polar nature of analytes. We had previously developed a method for estrogen analysis of wastewater using LC/MS/MS detection and HLB SPE cartridges were successfully employed for the preconcentration of the samples.[23] The same extraction method was used in the presented method. Advantages of HLB extractions over other SPE techniques include the ability to retain both hydrophilic and hydrophobic compounds with high capacity through van der Waals and H donor-acceptor interactions and the ability to remove moisture after sample loading through the action of a dry air stream.

In order to obtain good GC detection, intermolecular hydrogen bonds of the analytes were reduced through analyte derivatization and hence the volatility is increased by silylating the active hydrogen atoms (Figure 1). Derivatization also increases the thermal stability of the estrogens.[24, 25] Silylation was performed by reacting estrogens with BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) + 1% TMCS (trimethylchlorosilane) according to procedures described in Yi-qi *et al.* (2007)[26] and Shareef *et al.* (2006)[25] with slight modifications. Experimental details are described in the experimental section. Derivatized samples were used for the GCxGC-qMS analyses. The flow diagram of the method is shown in Figure 1. A one dimensional GC analysis was also carried out with identical samples parallel to the GCxGC experiment in order to compare results.

2. Materials and methods

2.1 Reagents, solutions, apparatus and equipment

The internal standard anthracene and the required analytical standards of βE2, αE2, E3, E1, and EE2 were obtained as powders from Sigma- Aldrich (St. Louis, MO). A 250 mg/mL solution of each standard was prepared by dissolving accurately weighed powders in a corresponding amount of methanol (MeOH) to obtain the required concentrations. Calibration solutions of the required concentrations were subsequently prepared by diluting standard solutions with MeOH. HPLC grade MeOH and water, anhydrous DMF, and BSTFA + 1% TMCS were also purchased from Sigma-Aldrich. HLB SPE cartridges (500 mg) were purchased from Waters Corp. (Milford, MA). Glass fiber filter papers were purchased from EMD-Millipore (Billerica, MA). A Visiprep vacuum manifold was obtained from Supelco (Bellefonte, PA) and a Vortex mixer was purchased from Fisher Scientific (Pittsburgh, PA). An N-EVAP 111 Nitrogen evaporator was obtained from Organomation Associates Inc. (Berlin, MA). GC Image software by Zoex Corporation (Houston, TX) was used to generate GCxGC images.

2.2 Sample pretreatment

Sample collection

Influent and effluent waste water samples were collected from a wastewater treatment plant situated in northern Mississippi. Target hormones were not detected in any of the limited samples collected and analyzed during method development (Supporting information). Samples were collected in amber brown glass bottles, and 1 mL of formic acid was added on-site to prevent microbial degradation. Collected samples were filtered using glass fiber filter papers soon after transportation to the laboratory and were kept under refrigeration (~4.4 ^{0}C) until analysis.

Solid phase extraction

Solid phase extraction was carried out similarly to our previous study.[23] Briefly, samples were removed from refrigeration and allowed to sit at room temperature for one hour. 500 mL portions of water samples were measured and placed in glass bottles and the pH was adjusted to between 3-4 using 1 M HCl. According to the manufacturer's instructions, Oasis HLB cartridges (500 mg) were preconditioned using 5 mL MeOH and 5 mL water. Samples were loaded and eluted with a flow rate of 1-2 mL/min. To ensure the extraction of the whole sample, sample bottles were subsequently washed with 10 mL distilled water and eluted with the same flow rate. For the removal of salts and proteins and to prevent the wells from clogging, cartridges were rinsed with 3 mL 5% MeOH in water[27] and then dried under high vacuum for 30 min. We have optimized the volume of the eluent in our previous study and it showed that 10 mL of MeOH with a flow rate of 1 mL/min gives complete extraction. Therefore, trapped hormones were eluted with the same solvent and conditions.

Derivatization with BSTFA

MeOH eluent was evaporated to ~1 mL using a gentle nitrogen stream and transferred to a 3 mL amber brown vial. Subsequently, the test tube used to collect eluent was washed with 1 mL of MeOH and added to the same vial. Transferred MeOH eluent and washings were then evaporated to dryness using a gentle nitrogen stream. 100 µL of DMF and 100 µL of BSTFA + 1% TMCS were added and heated in a water bath to 65 ^{0}C for one hour. The derivatized sample was then used for GCxGC-qMS analysis.

2.3 Instrumental analysis

Chromatography

GCxGC analyses were performed on a Shimadzu 'GCxGC-MS-QP 2010 ultra' comprehensive two dimensional gas chromatograph coupled to high scan speed quadrupole mass spectrometer. Shimadzu GCMS solution software was used to control the instrument and process data while Zoex GC Image R2.4 software was used to generate 2D and 3D GC images. A 30 m X 0.25 mm X 0.50 µm, 50% Phenyl

Polysilphenylene siloxane (BPX 50) column was used as the first-dimension column and a 2 m X 0.1 mm X 0.1 µm, 100% Polydimethylsiloxane (BPX 1) column was used as the second-dimension column. The two serially connected columns were housed in the same oven. The oven was programmed to heat as follows; initially at 80 ^{0}C for 1 min, from 80 to 200 ^{0}C at 20 ^{0}C/min, from 200 to 280 ^{0}C at 8 ^{0}C/min and hold at 280 ^{0}C for 3 min. Total program time was 20 min. Using an auto sampler, 1 µL injections were done in splitless (1 min) mode at 280 ^{0}C. The carrier gas was He at a pressure of 253.2 kPa. The thermal modulation interval was 3 sec with a hot jet temperature of 330 ^{0}C. Cooling was caused through the Joule-Thompson effect of expanding liquid nitrogen.

For the 1D-GC analyses, an Agilent 7890A GC / 5975C MS system was used. A BPX 50 column, identical to the first dimension column employed in the GCxGC experiments, was employed for 1D-GCMS analysis. The temperature program was also identical to that in the GCxGC experiment.

Mass spectrometry

In the GCxGC experiments, an electron impact ion source was operated at 70 eV. A quadrupole mass analyzer was operated in SIM mode at an event time of 0.03 seconds (Scan rate of 33.3 Hz). For high SNR, the analysis was done in three groups; (1) 6 – 10 min for m/z 178, (2) 10.01 -14 min for m/z 416 and (3) 14.01-16.0 min for m/z 342, 440, and 504. The ion source temperature was 200 ^{0}C and the interface temperature was 275 ^{0}C.

In 1D-GC experiments, the scan rate of the MS was kept at 8.33 Hz. Analysis was done in two groups, (1) 6 – 9 min for m/z 178, (2) 9.01 -20 min for m/z 416, 342, 440, and 504. Other parameters were identical to those used in the GCxGC experiments.

3. Results and Discussion

3.1 Recovery

Recovery tests were performed with the purpose of evaluating the extraction efficiency of the method. Our previous study gave excellent percent recoveries for similar wastewater samples at 20 and 200 ng/L spike levels.[23] We performed a recovery test at 100 ng/L level to confirm. Five hundred milliliter portions of estrogen-spiked and unspiked wastewater samples were extracted, derivatized and quantified. Recoveries were calculated using the following equation.

$$R\% = (S - U)/T \times 100$$

Where, R% = percent recovery, S = observed amount of the spiked sample, U = observed amount of the unspiked sample, and T = amount spiked. Recovery tests were carried out for influent, effluent and pure water. Recovery values for influent, effluent and pure waters were 87-94%, 88-96% and 90-98% respectively. Moreover, standard deviations (SD) of three replicate analyses were less than 10%. Recovery values and their SDs for all five hormones are listed in Table 1. Acceptable recovery values and low standard deviations are indications of the accuracy and precision of the method, thus, reliable quantification is expected. Anthracene was used as the internal standard to correct for injection volume variations and matrix matched calibration curves were generated to confirm a linear response in a typical sample matrix, and to ascertain that the linear dynamic ranges are satisfactory. Linear regressions for all generated calibration curves (LOQ - 1000 ng/L) ranged from 0.993 to 0.999. Therefore, the internal standard method can be successfully used in the application of the method for quantification of estrogens in real samples. Retention times for the most intense peaks of βE2, αE2, E1, EE2 and E3 were 12.93, 13.26, 14.45, 14.34 and 14.48 minutes respectively.

3.2 Detection limits

The method has a concentration factor of 2500x when the entire sample preparation scheme is considered. Method detection limits (MDL) were calculated by correcting the instrument detection limits (IDL) by the concentration factor of the method. In the presented method, MDL of E1, αE2 and

βE2 ranged from 0.1 – 0.3 ng/L for pure water and from 1.4 – 2.9 ng/L for influent and effluent waters. For EE2 and E3 in pure water, MDLs were 0.4 and 2.4 ng/L respectively. For influent and effluent waters the MDL ranged from 3.6 - 8.6 ng/L. Compared to 1D-GC, observed detection limits for GCxGC experiments were approximately reduced by half (improved) for pure water and reduced by a factor of 2 to 4 for waste water (Table 2). Chromatographic peaks normally show Gaussian shapes as a narrow chromatographic band is broadened during its movement through the column. In gas chromatographic separations with open tubular columns, the longitudinal diffusion and the resistance to mass transfer are the main reasons for the band broadening.[28] In GCxGC, the broadened band at the end of the longer first column is periodically trapped and released by the thermal modulator. Therefore, the sample is introduced to the shorter second column as very narrow pulses.[1, 7, 29] Band widths of the focused bands are decreased by a factor of 10–50 (Table 3) and hence the signal intensity increases considerably.[10] This result in higher signal to noise ratios, thus improved (lower) detection limits. Therefore GCxGC is expected to give better detection limits than 1D-GC.[10, 30] Mostafa *et al.* (2013)[30] reported that for hydrocarbons, with FID and TOF-MS, GCxGC gives enhanced detection limits compared to 1D-GC. In contrast, Engel *et al*. (2013)[31] reported in a study that utilized many different detectors, that for some organochlorine or organophosphorus pesticides, in some circumstances, 1D-GC gives better detection limits than GCxGC. However in our work which used a rapid quadrupole mass spectrometer, for all five estrogens, we observed improved detection limits in GCxGC experiments. Figure 2 shows the chromatogram of βE2 and αE2 separation (m/z = 416) in GCxGC-qMS and GC/MS experiments.

Previously reported methods often require large initial volumes of sample and multiple cleanup steps to achieve ng/L detection limits.[32-34] However in the presented method, acceptable detection limits were obtained using a relatively small initial sample volume and without any additional cleanup steps. The baseline of each chromatogram had a sinusoidal waveform with a time interval equal to the instruments thermal modulation. Analysis to analysis variations in magnitude of this waveform were

observed when monitoring different selected ions and were more pronounced whenever matrix chemicals were present. These variations did not significantly change estrogen peak intensities or calculated limits of detection. This results in high standard deviation of the baseline, which causes lower SNRs of the desired peaks. Figure 3 shows the difference of baselines in chromatograms of EE2 in pure and influent water matrix.

3.3 GCxGC images

GCxGC images are graphical representations of the two dimensional separations achieved by comprehensive two dimensional gas chromatography. In these images, retention times for chemicals to move across the first column are displayed on X-axis and the retention times for chemicals to move across the second column are displayed on Y-axis. GCxGC images allow visualization of two dimensional separations of complex mixtures. This can aid in the elucidation of the composition of unknown complex mixtures.[35] In the presented work, GC Image software was used to generate two dimensional images. The use of GC images in this project did not provide a significant advantage as the method was carried out to identify five selected pre-targeted hormones in SIM mode, yet the technique can be used to determine co-eluting matrix interferences and help visualize the two dimensional separation of the five derivatized estrogens. Figure 4 shows the GCxGC image and the chromatogram of separation of βE2 and αE2 in an influent matrix (MS group 2, m/Z 416) which shows no co-eluting spots in the same retention time. However in the image of the group 3 MS separation, three masses were included to the same image as the software did not allow generating separate images of each ion. Therefore the image did not provide a clear idea about the purity of the peaks, yet showed minimum matrix interferences (Supporting information).

4. Conclusion

A method for the trace-level analyses of five estrogens in influent and effluent wastewater were successfully developed using a GCxGC-qMS. The detection limits of E1, αE2 and βE2 ranged from 1.4-2.9 ng/L for influent and effluent waters. For EE2 and E3 in influent and effluent waters, MDL ranged from 3.6-8.6 ng/L. GCxGC produced 2-3 times better detection limits than a conventional GC/MS. Additional cleanup steps and a larger initial sample volume would be needed in order to carry out the same analysis using a conventional GC/MS. The initial sample volume used was 500 mL, which is relatively small compared to most of the methods reported in literature. We previously reported a LC/MS/MS method which used a 200 mL sample volume and produced a detection limit below 1 ng/L. The currently presented method, however, is more affordable. A GCxGC-qMS is less expensive than a tandem mass spectrometer and provides ng/L detection limits. Moreover, the presented method requires no additional cleanup steps after the HLB extraction, making the analysis faster.

5. Acknowledgements

Authors would like to acknowledge Shimadzu Corporation for providing the comprehensive two-dimensional gas chromatograph and Zoex Corporation for providing the modulator and the software. Next, we would like to thank the staff members of the wastewater treatment plants for their support. Furthermore, we would like to express our appreciation the Mississippi State Chemical Laboratory for use of instrumentation and the Mississippi State University Chemistry Department for financial support of this project. A special thank goes to Dr. Debra Mlsna for proof reading the manuscript and Mr. Joey Raines for his help on the instrument use. We would also like to thank undergraduate research students David Fulton and Andrew Hanna for help in the lab and with editing the manuscript.

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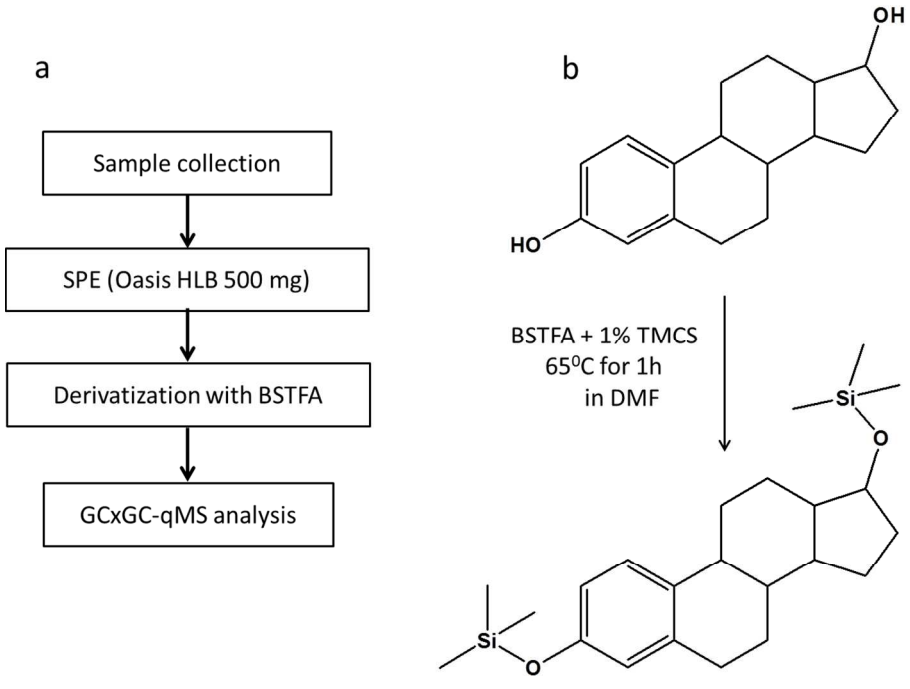

Figure 1: (a) Flow diagram summarizing the method for estrogen quantification. (b) Silylation of active hydrogen atoms in E2 with BSTFA.

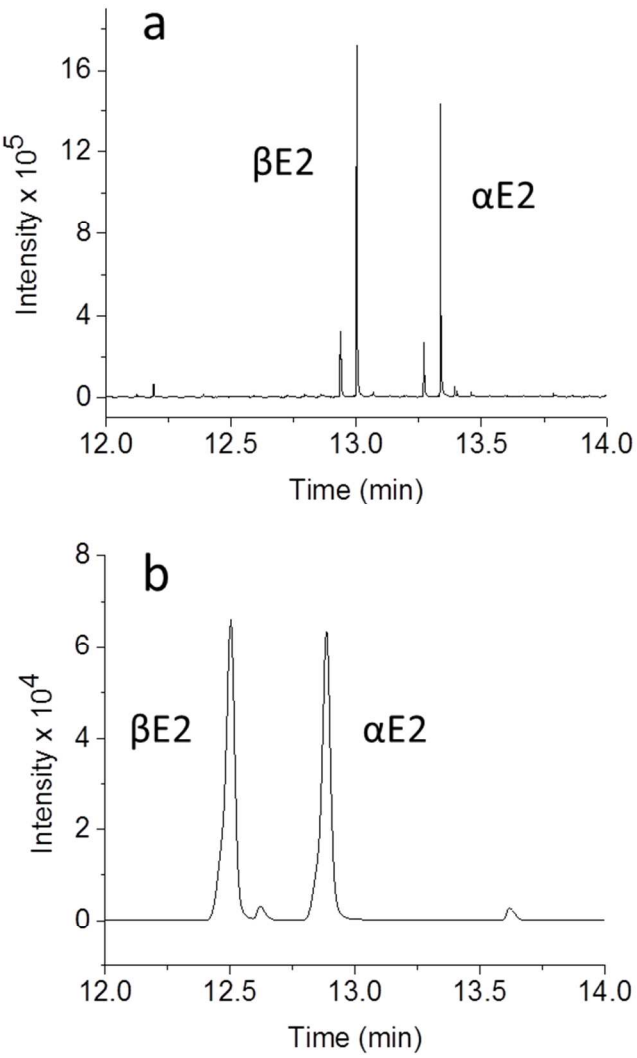

Figure 2: Chromatogram of βE2 and αE2 separation (m/z = 416) in (a) GCxGC-qMS and (b) GC/MS experiments.

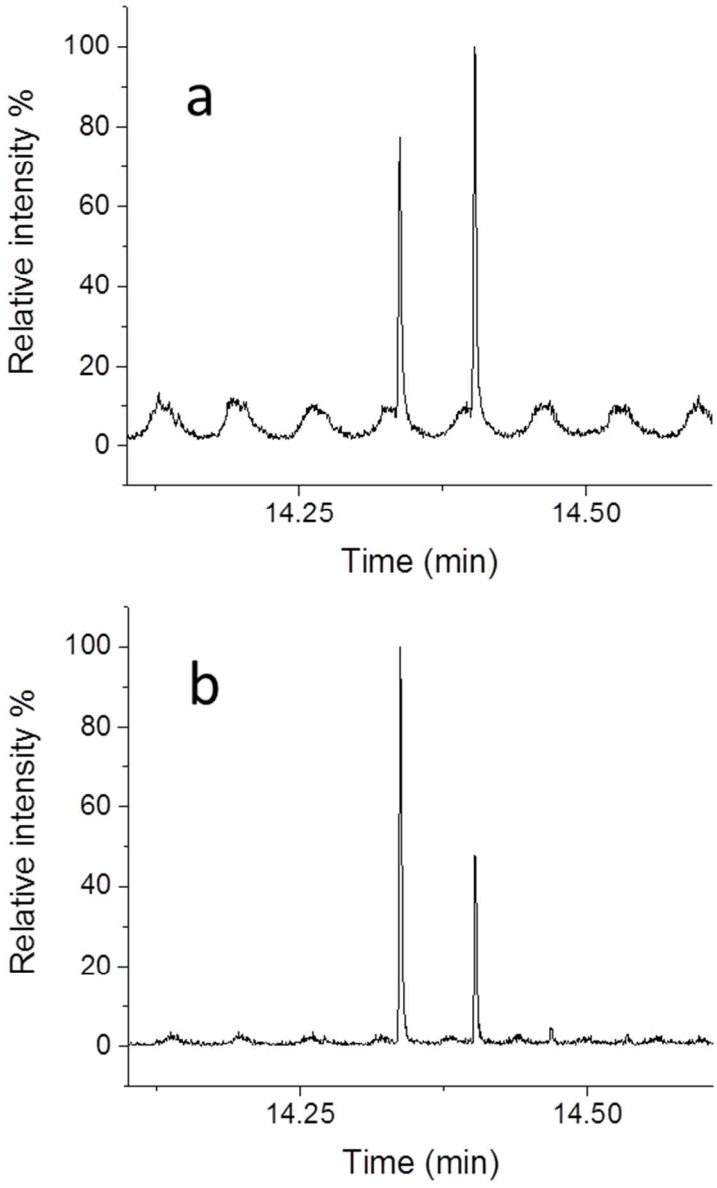

Figure 3. SIM chromatograms when 50 ng of EE2 spiked to 500 mL of (a) influent and (b) pure water extract.

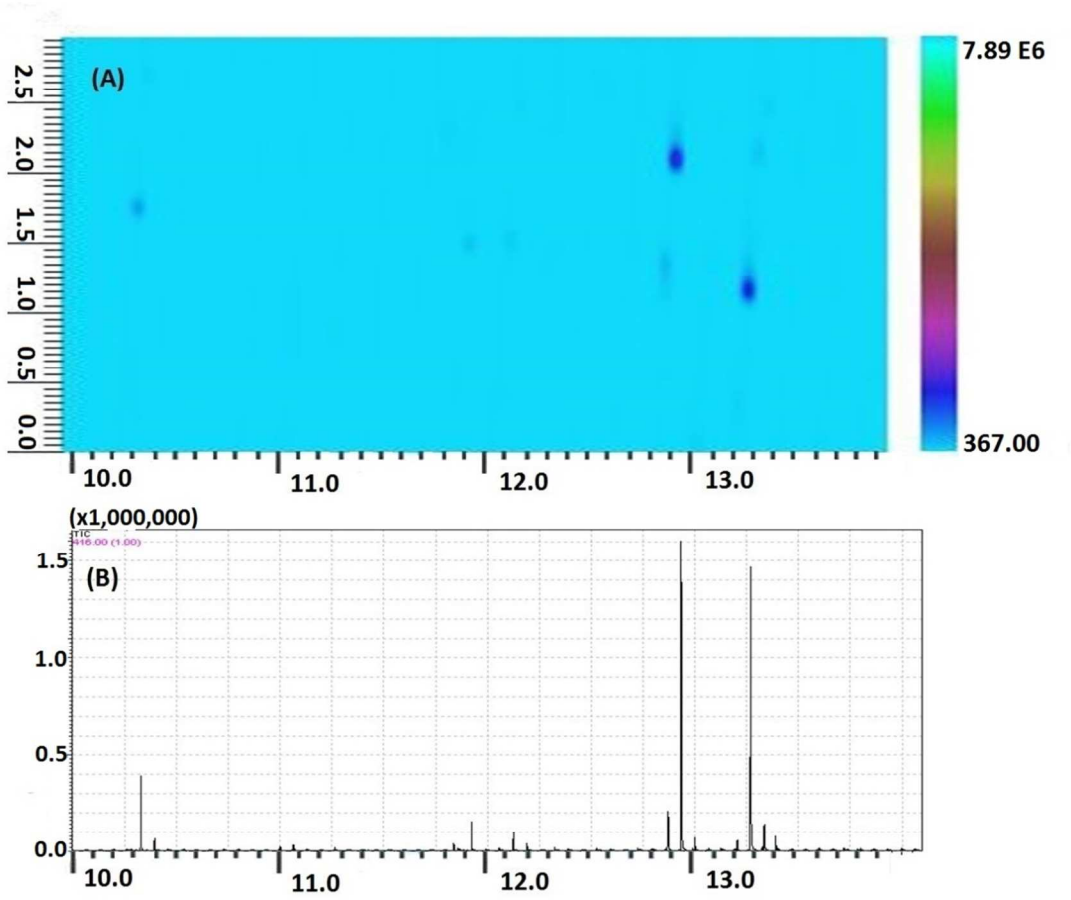

Figure 4: GCxGC image and the chromatogram of separation of βE2 and αE2 spiked 50 ng to 500 mL of influent extract.

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Table 1: Compiled recovery values.

	100 ng/L spiked (n=3)		
	R% (SD)		
	Influent	Effluent	Pure
E1	89 (3)	93 (5)	98 (3)
βE2	90 (5)	94 (4)	90 (2)
αE2	90 (3)	92 (5)	94 (5)
EE2	88 (4)	94 (6)	90 (1)
E3	96 (4)	87 (7)	97 (2)

Table 2: Method detection limits in GCxGC-qMS and GC-MS methods.

		GCxGC-qMS		GC-MS	
Matrix	Estrogen	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)
Pure	E1	**0.1**	0.3	**0.2**	0.6
	βE2	**0.1**	0.3	**0.2**	0.6
	αE2	**0.3**	1.1	**0.6**	2.1
	EE2	**2.4**	8.0	**5.4**	18.1
	E3	**0.4**	1.4	**0.8**	2.7
Effluent	E1	**1.4**	4.8	**5.7**	19.0
	βE2	**1.7**	5.6	**6.0**	20.2
	αE2	**1.9**	6.5	**8.0**	26.6
	EE2	**6.7**	22.2	**11.3**	37.5
	E3	**3.6**	12.1	**6.8**	22.5
Influent	E1	**1.9**	6.3	**6.4**	21.4
	βE2	**2.0**	6.8	**6.1**	20.2
	αE2	**2.9**	9.5	**9.4**	31.4
	EE2	**8.6**	28.6	**12.6**	41.9
	E3	**4.6**	15.4	**7.3**	24.3

Table 3: Calculated peak widths in GCxGC-qMS and GC-MS experiments.

Estrogen	Peak widths (W) / min		$W_{GC/MS}$ /$W_{GCxGC-qMS}$
	GC/MS	GCxGC-qMS	
βE2	0.116	0.006	19
αE2	0.177	0.006	29
E1	0.119	0.004	30
EE2	0.226	0.006	38
E3	0.131	0.006	22