UPLC-QTOF Mass Spectrometry Detection of Four Endocrine Disrupting Chemicals (Methyl Paraben, 2,4-Dichlorophenoxyacetic acid, Monobutyl Phthalate, and Bisphenol A) in Urine of Filipino Women

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ABSTRACT

Background and Objective. Endocrine Disrupting Chemicals (EDCs) are ubiquitously found as low-level contaminants and pose serious threat to women's health. EDCs may result in various reproductive disorders, fetal birth and developmental abnormalities, and endocrine and metabolic disorders. EDCs can be detected in body fluids of exposed individuals including blood and urine. This study aimed to detect four EDCs — Methyl Paraben (MP), 2,4-Dichlorophenoxyacetic acid (2,4-D), Monobutyl Phthalate (MBP), and Bisphenol A (BPA) in urine samples of women using Ultra-Performance Liquid Chromatography – Quadrupole Time-of-Flight (UPLC-QTOF) mass spectrometry.

Methods. Sequential steps of enzymatic deconjugation, liquid-liquid extraction, solid phase extraction, and liquid chromatography separation and mass spectrometry detection were optimized in urine samples. The method was used to analyze 70 urine samples from women of reproductive age.

Results. The sample preparation method showed a recovery ranging from 86.6% (MBP) to 100 % (2,4-D). The method demonstrated limits of quantitation ranging from 1.52 ng/m(MP) to 6.46 ng/mL(2,4D). Intra-day precisions expressed as relative standard deviation were all below 15% while accuracy was shown to range from 67.10% (2,4-D) to 102.39% (MBP). MP was detected in nine samples (12.86%) with a geometric mean value of 10.15 ng/ml (range: 3.62-52.39 ng/ml). MBP was detected in 68 samples (97.14%) with a geometric mean value of 97.62 ng/ml (range: 15.32-698.18 ng/ml). BPA was detected only once (9.58 ng/ml) while 2, 4-D was not detected in all samples.

Conclusion. A UPLC-QTOF mass spectrometry method to detect four EDCs at parts per billion level (ng/ml) was adapted and applied for analysis of urine samples. This method can find applicability in routine testing of clinical specimens as well as surveillance and other epidemiological studies.

Keywords: endocrine disruptors, Methyl Paraben, 2,4-Dichlorophenoxyacetic acid, Monobutyl Phthalate, Bisphenol A, UPLC-QTOF

eISSN 2094-9278 (Online)
Published: February 28, 2025
https://doi.org/10.47895/amp.vi0.9007

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INTRODUCTION

Endocrine Disrupting Chemicals (EDCs) are a group of compounds that are known to mimic, block, and/or interfere with the normal hormonal homeostasis which adversely affect, among others, the neurological, immunological, developmental, and reproductive aspect of the mammalian life.[1-3] Because of the ubiquitous nature of these compounds, being known to be present in a wide range of daily products—e.g., plastic bottles, metal food cans, detergents, flame retardants, food, toys, cosmetics, and pesticides—they pose a serious threat since even at low concentrations, a number of researches have shown its ability to reduce fertility and increase progression of some diseases, including obesity, diabetes, endometriosis, and some cancers.[3,4] In women, EDCs may result in reproductive disorders, fetal birth defects, fetal developmental abnormalities, endocrine and metabolic disorders, and even gynecological malignancies.[5]

Bisphenol A is one of the most commonly studied EDCs.[6] Its prevalence has been correlated to fertility.[7] Along with this, other Bisphenols, such as Bisphenol S and F, are also noted due to their similar uses, hence similar disposition once accumulated in the body.[8] They are primarily present in plastic food storage, canned goods, and thermal receipts.[9,10]

Pesticides also constitute a large family of EDCs which can be classified further as organochlorines, organophosphates, carbamates, triazines, and pyrethroids.[11] According to the Philippine Rice Research Institute, the ester form of 2,4-dichlorophenoxyacetic acid is one of the most used herbicides in 2017.[12] Undeniably, their use is highly important in order to increase food production and sustain the growing population; however, the hazard it poses to human health should be the primary concern.[13]

Another class of EDCs are parabens which are primarily present in personal care products and cosmetics.[14] As reported by previous work, parabens are more frequently detected in women than in men which can be attributed to their more frequent use of cosmetic products.[15] Reported effects of parabens include metabolic disorder and irregularity of sex hormones.[16] Methyl paraben, ethyl paraben, propylparaben, and butyl paraben are four of the most common parabens used in the said products.[17]

Phthalates is another class of EDCs present in a wide array of industrial and consumer products.[17,18] Exposure to phthalates may result in various reproductive disorders such as infertility, alteration of puberty, and cancer.[19] Phthalates have so many derivatives and one of the most commonly included types in phthalate studies is monobutyl phthalate.[20]

In the body, one of the routes of elimination of xenobiotics including EDCs is the urine.[21] These compounds are glucuronidated to help increase the polarity thereby allowing them to be excreted through the urine.[22] Hence, one good sampling source in the study of EDC levels among humans is by testing their urinary levels.

The variability of the classes of compounds that consist of the umbrella of EDCs in addition to their low levels in bodily tissues becomes one of the most challenging factors in large epidemiological studies. This can be addressed by the use of Liquid Chromatography coupled with Mass Spectrometry (LCMS)—an analytical tool highly regarded for its high resolution and sensitivity.[23]

In this paper, a UPLC-QTOF method for the simultaneous detection and quantitation of EDCs Bisphenol A (BPA), 2,4-Dichlorophenoxyacetic acid (2,4-D), Methyl Paraben (MP), and Monobutyl Phthalate (MBP) was investigated for potential utility of detection in women's urine samples.

MATERIALS AND METHODS

Reagents and Analytical Standards

For the enzymatic deconjugation, β-glucuronidase enzyme used was obtained from Megazyme while HEPES buffer was obtained from Sigma-Aldrich. Type I, from Milli-Q Plus ultrapure water system (Millipore,Milford, MA, USA), was used as distilled water. Analytical reagent (AR) grade ethyl acetate and acetic acid were obtained from RPI. SPE cartridge was procured from Waters. Reference standards of BPA (purity: 99%), MP (100%), and MBP (99%) were obtained from Sigma-Aldrich, while 2,4-D (95%) was from Titan Media. LCMS-grade methanol and acetonitrile were obtained from Duksan.

Sample Preparation

Urine sample (500 uL) was incubated with β-glucuronidase (1 uL; 250 kU/mL) in HEPES buffer (10 mM; pH 6.8) for 2.5 hours. Then, acetic acid (50 uL) was added and the mixture was vortexed. The sample was extracted with ethyl acetate twice (800 uL), where upper phases were removed and pooled, followed by solid-phase extraction using Oasis® HLB extraction cartridge assisted by a vacuum manifold. Sample was dried in SpeedVac for 4.5 hours and reconstituted with 120 uL of methanolic standard solution composed of 10 ng/ml MBP, 25 ng/ml MP, 50 ng/ml BPA, and 50 ng/ml 2,4-D which served as spike. Samples were centrifuged and supernatants were transferred to LCMS vials with insert.

Standard Preparation

Each standard was separately prepared by accurately weighing in 10-mL volumetric flasks. The standards were com-bined in a separate 10-mL volumetric flask. This pre-mixed stock solution was used in the preparation of a set of standards via serial dilution for the establishment of calibration curve—BPA and 2,4-D: 50, 100, 250, 500 and 1000 ng/mL; MP: 25, 50, 125, 250 and 500 ng/mL; and MBP: 10, 50, 100, 1000, and 5000 ng/mL—and quality control (QC) solutions (BPA and 2,4-D: 50 ng/mL; MP: 25 ng/mL, and MBP: 10 ng/mL)(see Appendix A).

LC-MS/MS Detection

Detection was carried out using Waters UPLC I-Class coupled with Xevo G2-XS Qtof mass spectrometer (Waters Corp., USA). The column used was a 2.1 × 100 mm x 1.8-Micron Acquity HSS T3 set at 40°C. The mobile phases consisted of 0.02% acetic acid in water (A) and 0.02% acetic acid in acetonitrile (B) with the gradient time program of 0 → 0.5 min 80% A, 0.5 → 4 min 60% A, 4 → 7 min 60% A, and 7 → 14 min 0% A. The flow rate was set at 0.3 mL/min with an injection volume of 5 µL. For the MS method, the following parameters were used: MS^E small molecules screening acquisition mode, Capillary voltage: 3 kV, Source temperature: 120°C, Desolvation temp: 550°C, Cone voltage: 30V, Cone gas flow: 50 L/hr, desolvation gas flow: 950 L/hr. The negative electrospray ionization (ESI) mode was used within the range of 50-1,200 m/z. Leucine enkephalin was used as a reference fluidics for mass correction.

Data Processing

Accurate mass screening was carried out using the UNIFI data analysis software. The precursor ion was set to be the deprotonated molecule whose criteria for identification include mass accuracy error of ≤10 mDa, retention time (RT) tolerance of ±0.03 min, and response of ≥500 counts. Precursor ions were subjected to library matching using the customized library based on the study of Gerona et al. and the fragmentation patterns were verified through their MS/MS fingerprint (see Appendix B).[24]

Sensitivity

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to the following formula:

$$LOD = 3.3 \times (SD/m)$$

$$LOQ = 10 \times (SD/m)$$

where SD is the standard deviation of the response and m is the slope of the linear calibration curve.

Precision and Accuracy

The QC solution was used to describe precision and accuracy. Intra-day precision was based on the relative standard deviation (RSD) of the 'detector counts' of the QC run every after eight samples on the same day. Inter-day precision was the RSD of the detector counts from three different days. Accuracy was based on the ratio of the calculated concentration using the calibration curve and the actual concentrations of the prepared QC solution.

Selectivity and Carry-over Effect

Blank (LCMS-grade Methanol) was analyzed to evaluate selectivity and the same blank was used to assess the carry-over effect. The carry-over effect was analyzed from the blank injected after the injection of the calibration standard's highest concentration. The type I water used was also subjected to the same sample preparation as mentioned above but without spiking.

Recovery

Three urine samples were used in the recovery test where two sets were prepared—one was spiked with the prepared mixture of the EDC standards before the sample preparation and another after the sample preparation. The liquid-liquid extraction was carried out two times. Recovery (%) was calculated using the responses as

$$\% \text{ Recovery} = [(\text{pre-spiking})/(\text{post-spiking})] \times 100$$

Study Subject

The method was applied in the detection of EDCs from 70 urine samples from anonymized pregnant and non-pregnant women participants aged 18-59 years old residing in Tondo, Metro Manila in the period January to December 2021 following a convenience series sampling. Samples were stored in urine cups at –20°C until analyzed.

RESULTS

The overlaid base-peak chromatograms of the standards with their respective RTs are shown in Figure 1. Among the four analytes, MP was eluted first at RT 3.80, followed by 2,4-D at RT 5.64, MBP at RT 6.44, and lastly BPA at RT 6.90. Using a C-18 column, with flow rate of 0.3 mL/min, and injection volume of 5uL, the analytes can be separated in 13 minutes.

Sensitivity

The LOD and LOQ are shown in Table 1. The LOD values ranged from 0.50 ng/ml (MP) to 2.13 ng/ml (2,4-D),

Table 1. Method Validation Parameters (i.e., RT, Sensitivity, Precision, Accuracy, and Recovery) for Simultaneous Detection of Four EDCs as Obtained in this Study

EDC Analyte	Retention time, min	LOD, ng/mL	LOQ, ng, mL	SD, ±ng,mL	Average R^2	Intra-Day Precision (Average % RSD; n=3)	Intra-Day Precision (%RSD)	Average Accuracy	%Recovery
MP	3.80	0.50	1.52	0.04	0.9963	8.29	25.72	86.28	87.24
2,4-D	5.64	2.13	6.46	1.04	0.9988	7.96	47.65	67.10	100.00
MBP	6.44	1.42	4.30	0.54	0.9999	12.32	42.52	102.39	86.59
BPA	6.87	1.10	3.34	0.04	0.9976	14.23	51.65	100.93	86.69

while the LOQ values ranged from 1.52 ng/ml (MP) to 6.46 ng/ml (2,4-D).

Precision and Accuracy

Table 1 also showed the results for precision and accuracy. The intra-day precision ranged from 8.29% (MP) to 14.23% (BPA) while the inter-day precision ranged from 25.72% (MP) to 51.65% (BPA). The average accuracy ranged from 67.10% for 2,4-D to 102.39% for MBP.

Selectivity and Carry-over Effect

Figure 2 showed the chromatograms of the blank and type I water. The analysis of the blank and the type I water according to the data processing method described above

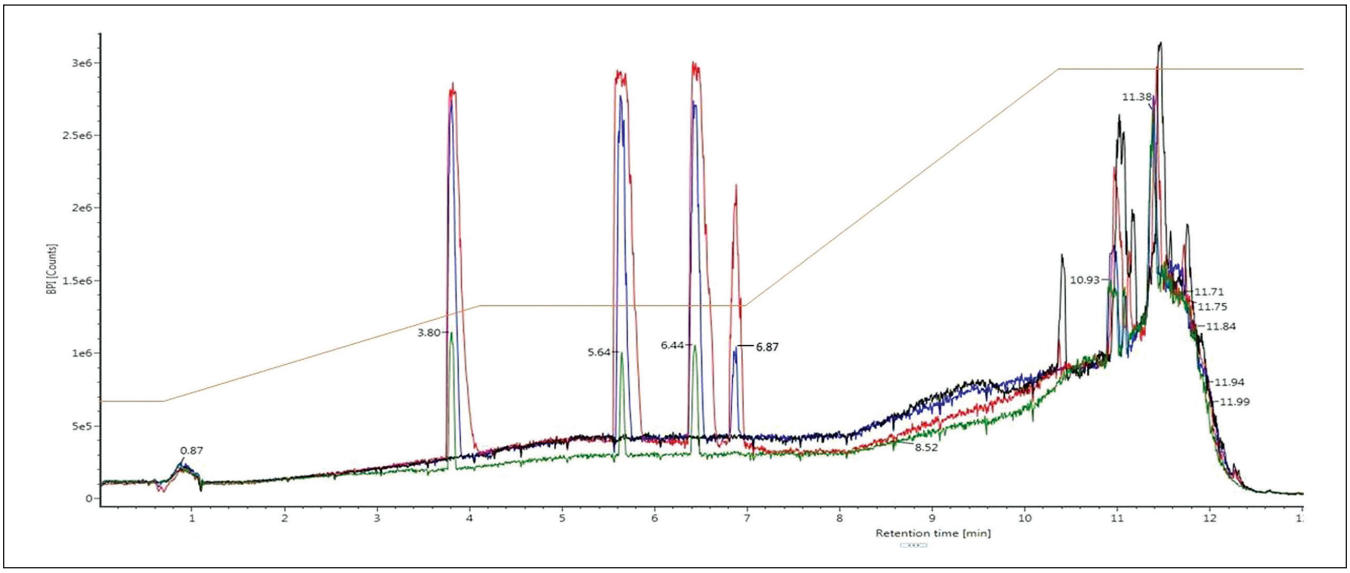

Figure 1. The overlaid base-peak ion (BPI) chromatograms of the EDC standards and their respective retention times; Methyl Paraben (3.80 min), 2,4-Dichlorophenoxyacetic acid (5.64 min), Monobutyl Phthalate (6.44 min), and Bisphenol A (6.87 min). Black: Blank, Green: 10^3 ng/ml, Blue: 10^4 ng/ml, Red: 10^5 ng/ml.

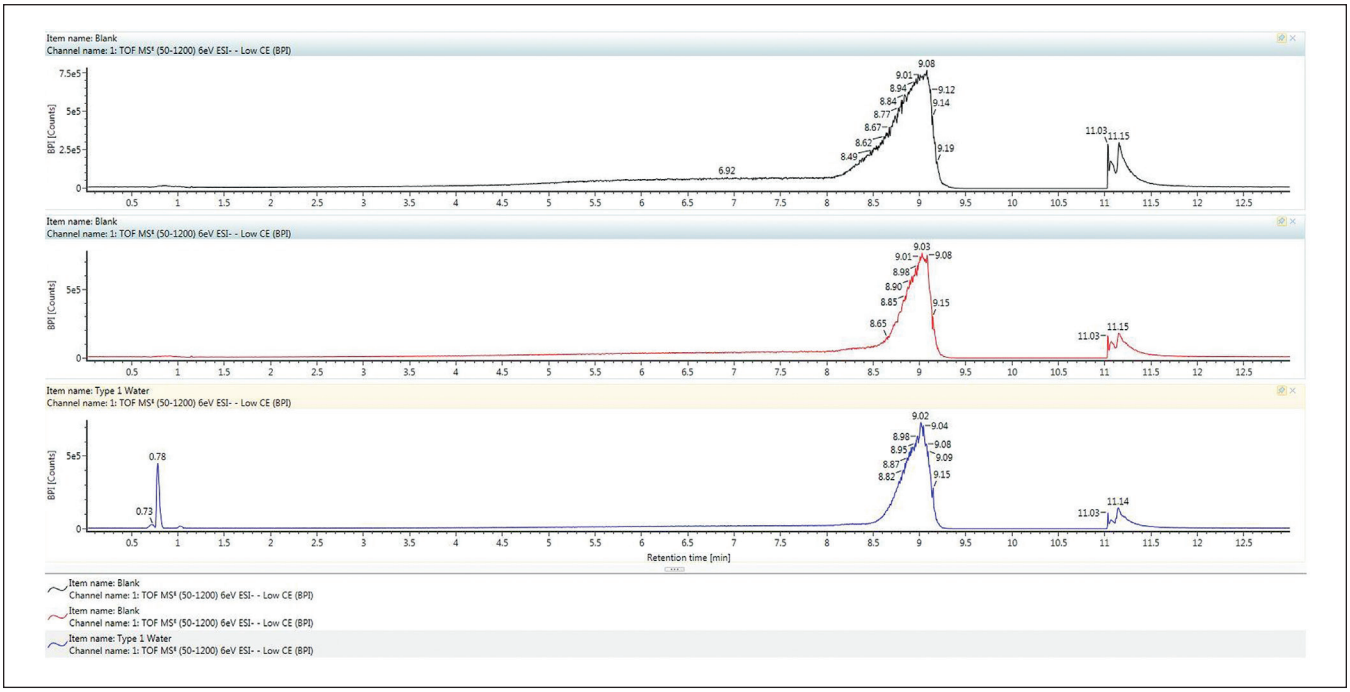

Figure 2. The BPI chromatograms of the blank (methanol) and Type I water injections for selectivity (black and blue, respectively) and carry-over assessments (red).

Table 2. Detection Frequency (DF), Mean Detection, and Standard Deviation (SD) Values of the Four EDCs in 70 Women Urine Samples

EDC Analyte	DF, %	Mean, ng/mL	SD, (±) ng/mL
MP	12.86	10.15	7.42
2,4-D	0.00	0.00	0.00
MBP	97.14	97.62	157.80
BPA	1.43	9.58	1.14

**Geometric mean*

showed no detectable levels of the analytes. Carry-over effect, which indicates the chromatographic system's clean-up and suitability status, was also not observed.

Recovery

The results of the recovery test as shown in Table 1 with extraction carried out two times were 87.2% (MP), 100% (2,4D), 86.6% (MBP), and 86.7% (BPA), respectively.

Detecting EDCs in Urine Samples

Seventy urine samples from women of reproductive age were tested for the four EDC compounds. The results are summarized in Table 2. The detection frequency (DF) was highest in MBP with 97.14%, i.e., 68/70 samples. The DF for MP was 12.86%, i.e., 9/70. BPA was detected in one sample while 2,4-D was undetected.

The geometric mean concentration was also highest in MBP at 97.62 ng/ml urine. Relative to this, MP and BPA showed lower mean levels at 10.15 and 9.58 ng/ml, respectively. Figure 3 shows the graphical representation of the EDC levels across the 70 participants.

DISCUSSION

The detection of EDCs from women urine samples has become a necessity as more EDC-associated disorders are emergent in the recent times. While conventional clinical chemistry allows single molecule identification, mass spectrometry-based method enables simultaneous detection of these EDC compounds. In this work, Methyl Paraben, 2,4-Dichlorophenoxyacetic acid, Monobutyl Phthalate, and Bisphenol A, each representing different families of EDCs, are analyzed using UPLC-QTOF.

Xenobiotics, including EDCs, exist in the human body in their glucuronidated form, thus, it would be necessary for them to be released in deconjugated form to allow downstream detection.[22] Here, samples were deconjugated with β-glucuronidase to liberate the EDCs into their free form and the EDC moiety be maximally recovered from the urine matrix.

The MBP data reported herein demonstrating the highest DF of 97.14%, mean concentration of 97.62 ng/ml, and a range of 0 to 698.18 ng/ml showed similar results with data obtained from Chinese population for the analysis of MBP, using Waters Quattro Micro LC–MS-MS system where the detection frequency was 99%, mean concentration was 96.76 ng/ml, with a range of 0 to 663.70 ng/ml.[25] The LOD and LOQ obtained in this study approximates the reported LOD and LOQ ranges of 0.85–5.33 ng/ml and 2.82–17.76 ng/ml, respectively. Also, the recovery rates were within the reported range of 81.84-125.32%. The intra-day precision expressed in %RSD obtained in this study were within the reported values of 1.74% to 14.24%. Meanwhile, only the inter-day precision for MBP for this method was within the reported range as values for the other three EDCs were either slightly higher or lower than the reported values. The consistently high R^2 values for each of the run with an average of 0.9999 indicated a good fit for the linear regression line calculated from the calibration curve.

For methyl paraben, though the DF is lower (12.86%) compared to the reported DF from a Brazilian study at 50%, the range (3.63 ng/ml to 52.39 ng/ml) agrees with their reported range of 0.82-33.16 ng/ml.[26] The geometric mean concentration is slightly higher at 10.15 ng/ml as compared to their mean at 8.57 ng/ml. The method validation parameters for intra-day precision approximates the reported range of 5.8-15% and the LOQ value was almost similar.

BPA was detected in only one out of 70 samples at a concentration of 9.58 ng/ml which falls within the previously reported value.[27]

2,4-D was not detected in the samples studied consistent with its low detection frequency reported in the literature. In a South African study with children urine samples, 2,4-D was detected only in 3 out of 20 samples with a maximum concentration of 0.20 ug/g creatinine.[28]

In a previous Philippine study in 2018 on the association of EDCs to breast cancer, MBP was detected in all samples for both control and case groups while BPA and MP were detected in 95.86 and 94.48%, respectively. The geometric mean for MBP, BPA, and MP among healthy participants were 153.2, 1.81, and 16.51 ng/ml, respectively.

The references cited in this study which are mass spectrometry-based, while not entirely similar to QTOF, but due to the limited data, are the closest comparison that can be made to benchmark the possible EDC values in Filipino biological sample.

With regard to the LOD and LOQ values of the different EDCs and their structural correlation, it is possible to presume that the higher the LOD and LOQ values, the more stable the precursor molecules and their fragments.[29] Among the EDCs analyzed, 2,4-D has the highest LOD while MP has the lowest value. Correspondingly, the possible structural stability correlation may not be reliable as LOD and LOQ are utilized mainly to measure instrument's sensitivity.[30]

Association for breast cancer was reported for elevated levels of perfluoroalkyl substances but not for MBP, BPA, or MP.[31] In this study, the MBP, BPA, and MP geometric means were 97.62, 9.58, and 10.15 ng/ml, respectively. Only

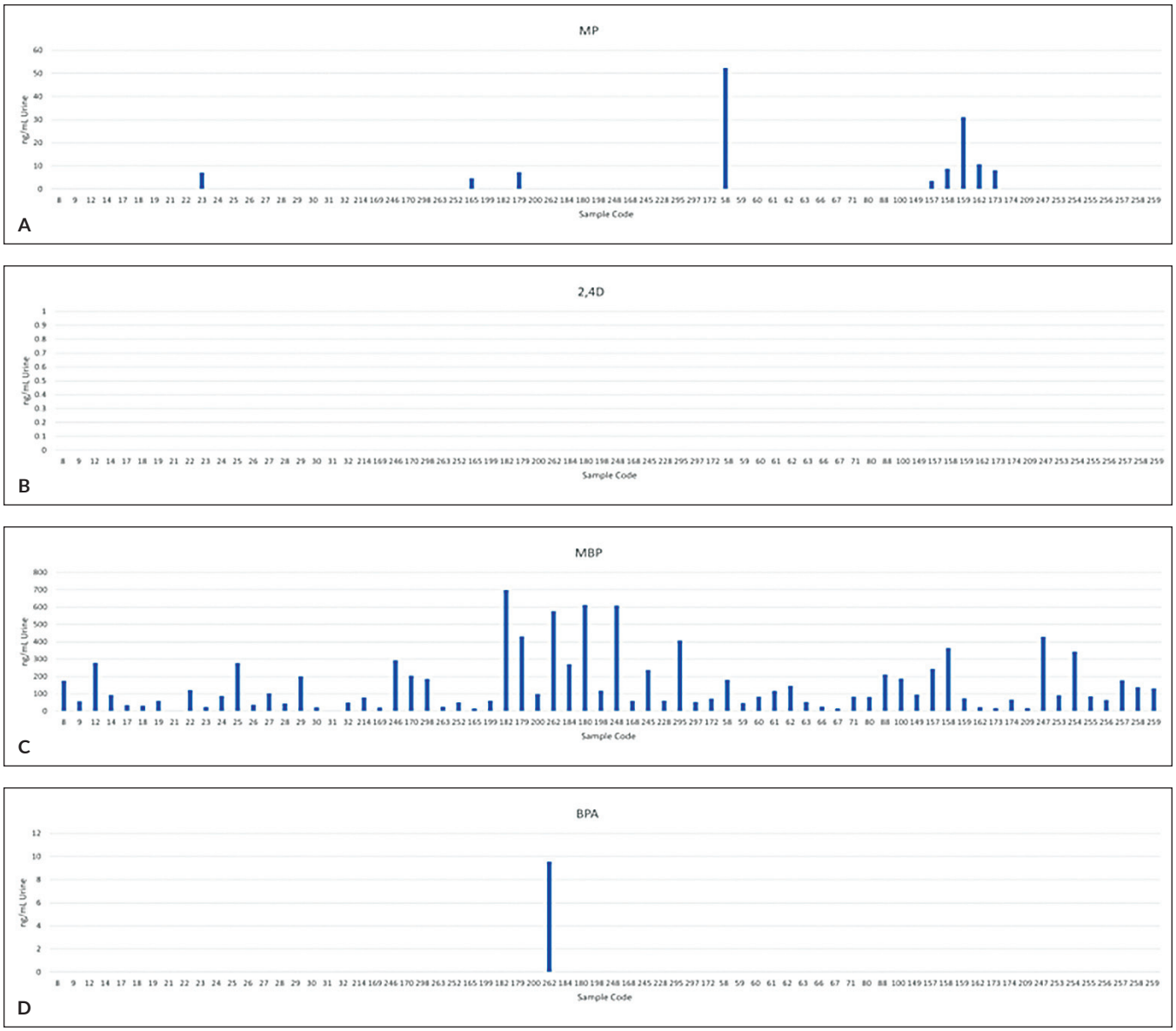

Figure 3. Comparative levels of endocrine disrupting compounds **(A)** Methyl Paraben, **(B)** 2,4- Dichlorophenoxyacetic acid, **(C)** Monobutyl Phthalate, **(D)** Bisphenol A detected through UPLC-QTOF in the urine samples of 70 Filipino women expressed in ng/mL.

BPA from a single participant was observed to have 5-folds higher value.

The DF for MBP obtained in this study is equally high at 97.14%. The geometric mean was 97.62 ng/ml although, no breast cancer association analysis was done.

Meanwhile for BPA, the DF reported in the previous study was 95.86% in normal control and breast cancer groups.[31] Most of the participants in their study belong to low-income families, with around 30% working in hospitals, 20% in households, and 10% in factories. The low detection frequency for BPA and MP in the present study could be due to a number of reasons. The participants in this study were pregnant and non-pregnant women mostly from Tondo, Manila area and were recruited and sampled at the time of the COVID-19 pandemic in January to December 2021. The frequent lockdowns and quarantines enforced during the period may have limited their exposure to occupational and environmental BPA, MP, and MBP.

This study was able to observe the same high detection frequency of MBP in women urine samples as previously reported. MBP is the first breakdown metabolite of dibutyl phthalate (DBP) which is mainly found in daily encountered products like plastics, varnishes, and cosmetic and personal care products.[32,33] The toxicity levels of MBP in endocrine and other systems are not yet fully established especially chronic exposure among pregnant women. Several endocrine studies

of MBP have shown to cause embryonic loss in rats, inhibit steroidogenesis in Leydig cells, and compromise human sperm function.[34-36] It has also been reported to possess a disruptive role in human pancreatic beta cells, and energy metabolism and antioxidant system in Zebra fish.[37-39]

CONCLUSION

A method for the simultaneous detection of four EDCs—MP, 2,4-D, MBP, and BPA—in women urine samples at parts per billion level (ng/ml) was adapted using UPLC-QTOF mass spectrometer. This method can find applicability in testing of clinical specimens as well as surveillance especially with the limited number of laboratories in the Philippines conducting this analysis. The high detection frequency of MBP may also add to the limited data of phthalates exposure in the country and may serve as a basis for deeper studies regarding this environmental contaminant and EDCs in general.

Acknowledgments

The authors would like to thank the Department of Biochemistry and Molecular Biology, UP Manila where the analyses were conducted, Ms. Janjhaylyn Hora and Ms. Jasmine Rule for helping in the preparation of urine samples, and Ms. Mara Joy Paulete, the Project Development Officer.

Informed Consent

Subjects had read, signed, and possessed a copy of their Informed Consent Form (ICF). The ICF indicates that research results can be used for publication, but all personal identification remains confidential.

Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

All authors declared no conflicts of interest.

Funding Source

The study was funded by the Department of Science and Technology - Philippine Council for Health Research and Development.

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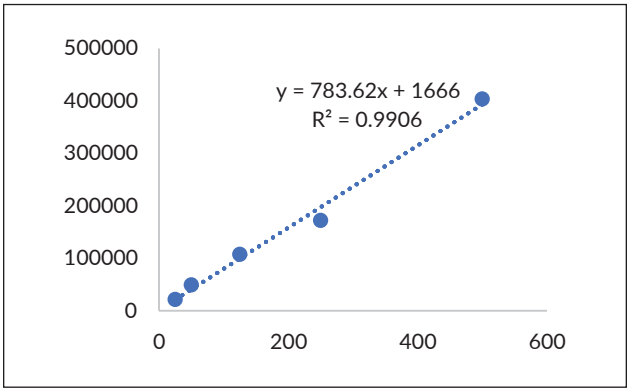
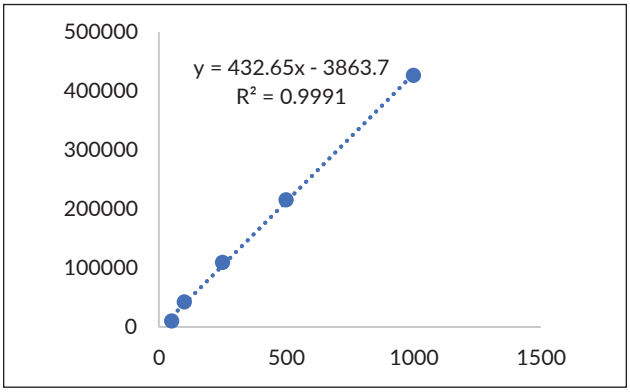
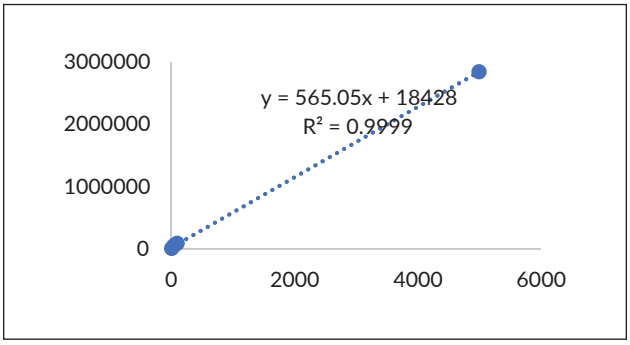
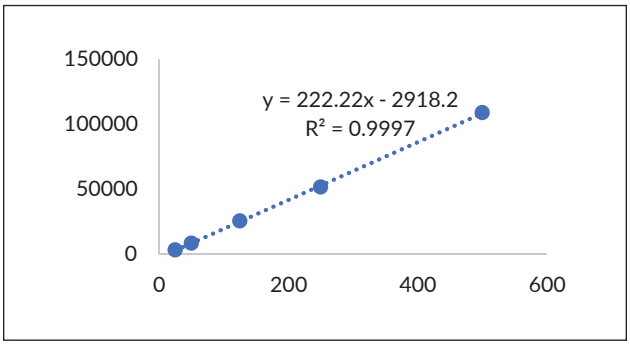
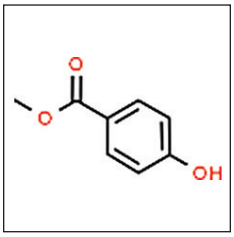
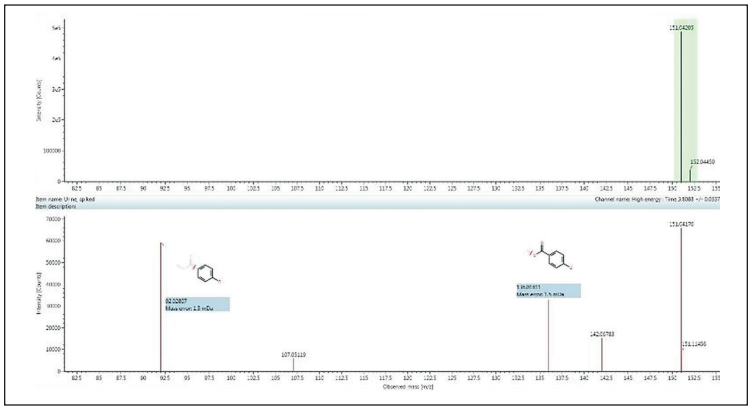
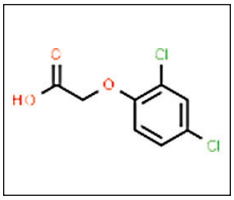
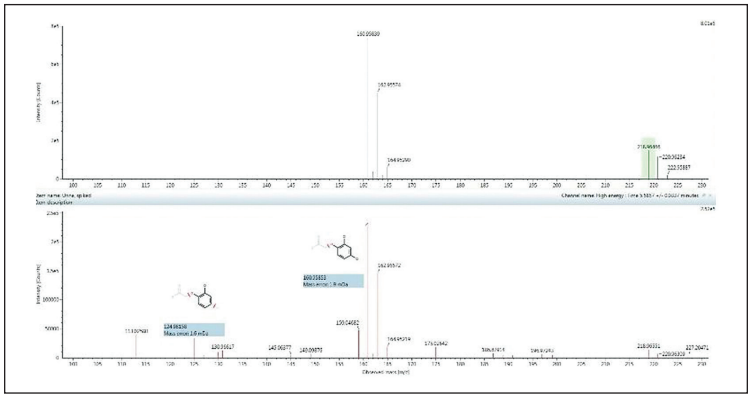
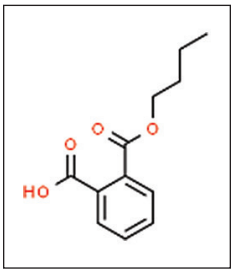
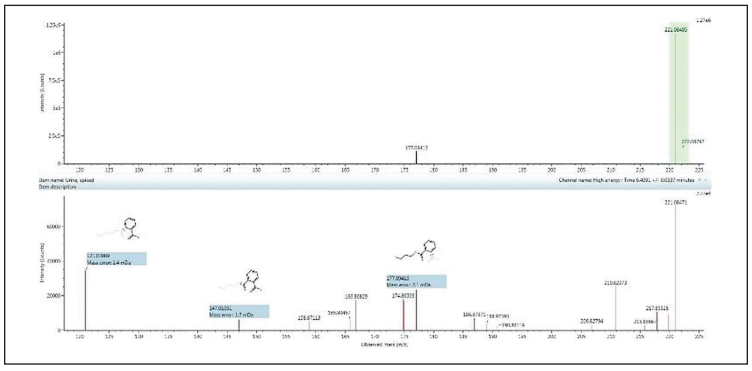
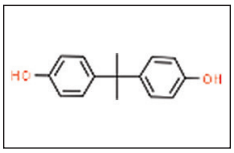
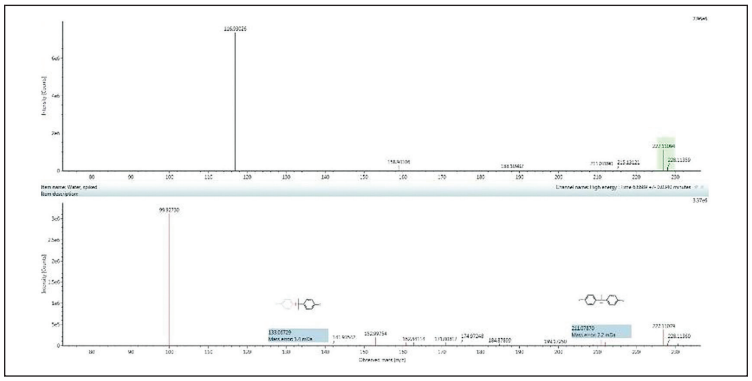
APPENDICES

Appendix A. Calibration plot, correlation coefficient, and linear regression of the calibrators of the four analytes

EDC Analyte	Correlation Coefficient	Linear Regression	Calibration Plot
MP	R = 0.9953	y = 783.62x + 1666	
2,4-D	R = 0.9995	y = 432.65x - 3863.7	
MBP	R = 0.9999	y = 565.05x + 18428	
BPA	R = 0.9998	y = 222.22x - 2918.2	

Appendix B. Chemical description, structure, and spectra of the four EDC analytes tested in this study

EDC Analyte	Monoisotopic Mass; Mass error	Structure	MS and MS/MS spectra
Methyl Paraben ***($C_8H_8O_3$)***	152.0473 Da (2.0 mDa; 13.1 ppm)		
2,4-Dichloro-phenoxyacetic acid ***($C_8H_6Cl_2$)***	219.9694 Da (2.5 mDa; 11.6 ppm)		
Monobutyl Phthalate ***($C_{12}H_{14}O_4$)***	222.0892 Da (3.0 mDa; 13.7ppm)		
Bisphenol A ***($C_{15}H_{16}O_2$)***	228.1150 Da (3.2mDa; 14.1 ppm)		

**The upper and lower spectra are the precursor and fragmentation spectra, respectively. The peaks highlighted with green are the precursor ions.*