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Multiclass analysis of emerging organic contaminants in tropical marine biota using improved QuEChERS extraction followed by LC MS/MS

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ABSTRACT

Marine biota is one of the important components of an ecosystem, and it has been widely used as a pollution indicator for emerging organic contaminants (EOCs) in the coastal zone. Previous studies divulged the concentration of EOCs in various types of biota matrices at trace level detection. EOCs have been associated with several adverse human health effects. Bioaccumulation of EOCs in the food web has been the main concern, and thus a sensitive and selective method for trace quantification of EOCs in biota matrices need to be developed and optimized. Considering various matrix interferences for EOC analysis in marine biota, a combination technique of QuEChERS and solid phase extraction cleanup with detection based on liquid chromatography with tandem mass spectrometry (LC MS/MS) is presented in this study. A method for 16 compounds grouped into four different classes, namely, pharmaceutically active chemicals, phenolic endocrine disrupter compounds, estrogenic hormones, and pesticides, was developed and validated for biota extraction. Satisfactory extraction was obtained for the optimized method with percentage of recovery from 64% to 114% and excellent sensitivity with detection limit in the range of 0.02–3.50 ng/g. Linearity of the standards (in the solvent) in the LC MS-MS analysis ranged from 0.991 to 0.999. The relative standard deviation for intra-day and inter-day repeatability was less than 20%, indicating good-precision analysis. Assessment on the matrix effects showed ionization suppression for all the developed compounds. The developed method was verified by analyzing biota matrices collected from the Klang River estuary. Trace concentrations of EOCs, ranging from 0.05 to 10.76 ng/g, were found in those matrices. Of the 16 targeted compounds, 10 were detected, namely, diclofenac, bisphenol A, sulfamethoxazole, amoxicillin, E2, E1, progesterone, testosterone, primidone, and 4-octylphenol. The other compounds were below the method detection limit.

1. Introduction

Emerging organic contaminants (EOCs) have been attracting scientific as well as public attention in the recent decade because of their adverse effect to humans and ecosystems. Research and monitoring of EOCs in various components of ecosystems such as in the surface water, sediment, and biota have been extensively carried out by a number of researchers for the past few years. Different classes of EOCs such as estrogenic hormones, pharmaceutically active chemicals, and endocrine disrupter compounds (EDCs), have been detected in those matrices, prompting for an extensive research on their toxicological effect and fate in the environment. Trace concentrations of EOCs found in those matrices have been linked to the possible uptake by humans that may lead to various health consequences such as cancer, reduced fertility/semen quality, disruption in reproductive and development function, and colorectal and bladder tumors [1]. In addition, biomagnification and bioaccumulation of EOCs were also observed in aquatic biota such as fish, mollusk, and bivalves, suggesting that trophic magnification may take place along the food web, which eventually reaches humans, who are higher-level consumers [2–4].

One of the important components of an ecosystem is biota matrices, which include numerous living organisms in either the marine or freshwater habitat. Biota has been used as a potential biological indicator for emerging organic pollution in marine and coastal ecosystems.

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The application of biota as a pollution indicator have been described by numerous researchers, with several biota species have been used for monitoring the presence of various EOCs in the marine and coastal zone [5–8]. Sun et al. [8] evaluated the status of emerging halogenated organic contaminations in the Pearl River estuary by using fish and invertebrates, whereas Cunha et al. [5] reported the potential use of mussel as a bioindicator for pharmaceutical pollution in the Portuguese coastline. Bayen et al. [6] used bivalves to monitor the presence of pharmaceutically active chemicals such as caffeine, carbamezapine, and sulfamethoxazole in the Singaporean mangrove ecosystem, whereas Moreno-González et al. [9] suggested the golden gray mullet fish as a potential indicator for pharmaceutical contamination in Mar Menor Lagoon, Spain. The latest study was reported by Ismail et al. [10], when a different type of fish species was used as an indicator for alkylphenol pollution in the mariculture area around Pulau Kukup, Johor, Malaysia.

Several types of analytical procedures have been developed and applied for determination of EOCs in biota samples, ranging from the conventional soxhlet extraction technique to automated and miniaturized techniques such as pressurized liquid extraction as well as the easy and quick extraction technique (QuEChERS). The main challenge for the determination of EOCs in biota is the complexity of these matrices, which may have high organic/lipid content as well as be rich in undesirable components. This challenge has also been highlighted by Huerta et al. [11] in a comprehensive review on the development of analytical methodologies for determination of pharmaceutical compounds in biota. To overcome this drawback, a combination of extraction and cleanup techniques was suggested by Omar et al. [12], in order to provide cleaner extract for trace level analyte detection. Several studies on the application of two or more combination techniques to extract trace organic EOCs from various types of biota matrices have also been reported [13–16], and this extraction method was proven efficient for complex biota samples.

The QuEChERS technique offers several advantages over the conventional extraction method such as its simplicity, cost effectiveness, and less solvent usage. Owing to its growing popularity, an increasing trend in publication on applying QuEChERS for trace determination of EOCs in biota samples has been noted for the past few years. The primary secondary amine (PSA) is one of the main components in the QuEChERS methodology, and it is mainly used for removing polar matrices such as organic acids, sugar, and pigments. The other sorbent used is silica-based C18, which has excellent capacity for lipid removal. However, the application of silica-based C18 has its own limitation, as it is not chemically stable at alkaline pH value, and PSA is not suitable for acidic analyte [17]. Therefore, in order to obtain efficient extraction, which can accommodate for both acidic and basic compounds, a number of researchers have made some improvements to the existing QuEChERS method by incorporating several processes such as liquid–liquid extraction (LLE) [5], cleanup sorbents [18], silica gel cleanup [19], polystyrene–divinylbenzene (PS-DVB) sorbent [17], and enhanced matrix removal (EMR) [20]. These additional cleanup processes have helped in providing better analyte purification as well as extraction efficiency for trace quantification of EOCs.

In this work, QuEChERS in combination with offline solid phase extraction (SPE) employing a polymeric sorbent material was developed for the extraction of multiclass EOCs from biota species collected from field monitoring work. The polymeric SPE is an excellent choice as a cleanup sorbent as it can cover a wider range of pH and polarity. In this study, the targeted compounds consist of 16 multiclass EOCs categorized into four groups: pharmaceutical compounds (diclofenac, primidone, progesterone, sulfamethoxazole, amoxicillin, testosterone, and dexamethasone), phenolic EDCs (bisphenol A, 4-octylphenol, and 4-nonylphenol), estrogenic hormones (estrone, 17β-estradiol, and 17α-ethynylestradiol), and pesticides (quinalphos, diazinon, and chlorpyrifos). To the best of the authors' knowledge, the present work is the first to provide an improved QuEChERS method for multiclass analysis of EOCs in tropical marine biota matrices. The optimized extraction and cleanup protocol were evaluated using validation parameters such as linearity, extraction efficiency (% recovery), precision, method detection limit, and matrix effects. The optimized protocol was then verified by determining the concentration of multiclass EOCs in tropical marine biota species comprising of fish and mollusk collected from the Klang River estuary, Malaysia.

2. Materials and methods

2.1. Standards, chemicals, and materials

Analytical standards of high purity for all targeted compounds were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Deuterated labeled compounds, used as surrogates and internal standards such as 17β-estradiol, 17α-ethynylestradiol, progesterone, primidone, and testosterone, were acquired from Cambridge Isotope Laboratories, MA, USA. Polymeric reversed-phase C18 SPE cartridges (Strata-X, 200 mg, 6 mL) were from Phenomenex (CA, USA), whereas the QuEChERs extraction kit was from Agilent Technologies (CA, USA). Hydromatrix, a high-purity inert diatomaceous earth sorbent was procured from Agilent Technologies (CA, USA), whereas aluminum oxide 90 active neutral was obtained from Merck (Darmstadt, Germany). High-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC MS) grade methanol (MeOH), acetonitrile (ACN), and acetone were purchased from Fisher Scientific (NJ, USA), whereas ammonium hydroxide (NH_4OH, 25% ammonia solution) and formic acid (CH_2O_2, HPLC-grade), used as chemical additives for mobile phases, were purchased from Fisher Scientific (Loughborough, UK). Ultrapure water (UPW) with water sensitivity > 18.2 MΩ·cm at 25 °C was produced from a Milli-Q water purification system (Millipore, MA, USA).

2.2. Sample collection

Wild fish samples were caught using a fishing net at several sampling points along the Klang River estuary. The sea catfish *Arius thalassinus* and croaker *Pennahia anea*, two different fish species that were abundantly present in this estuary, were collected. The collected fish samples were dissected using a stainless-steel dissection apparatus (previously soaked and cleaned with methanol) before being homogenized in a grinder. As for mollusk samples, the mangrove snail *Nerita lineata* samples were handpicked at several locations along the estuary. For each sampling location, 30–40 individual mollusk samples were collected, with their flesh carefully taken out, homogenized, and pooled as one sample. All samples were wrapped in aluminum foil and stored at −20 °C.

2.3. Extraction and cleanup

The freeze-dried tissue muscle of the marine biota specimen was weighed precisely to be about 0.500 ± 0.001 g and mixed with 1.00 ± 0.001 g Al_2O_3 and Hydromatrix, before being grounded into finely divided particles. Samples were transferred into a 50 mL polypropylene tube and spiked with deuterated labeled surrogate standards. UPW (10 mL) was poured into the samples, followed by the addition of acetonitrile (10 mL). The mixture was hand shaken for 15 s and vigorously shaken using a vortex mixer for another 15 s, before it was allowed to stand until two layers of solution was formed. The mixture was added with acetate buffer (QuEChERS Extract Pouches, AOAC Method, Agilent Technologies, USA), vortex shaken for 30 s, and centrifuged at 4000 rpm for 5 min. The aliquot of the organic phase was then mixed with dispersive SPE (Agilent Technologies, USA), hand shaken, and vortexed for 30 s before it was centrifuged again at 4000 rpm for 5 min. The aliquot was transferred into a glass tube for SPE cleanup, which was carried out on the basis of the SPE protocol reported by Omar et al. [21]. The extracts from SPE cleanup were reduced to approximately 1–2 mL using a rotary evaporator and further concentrated to near dryness

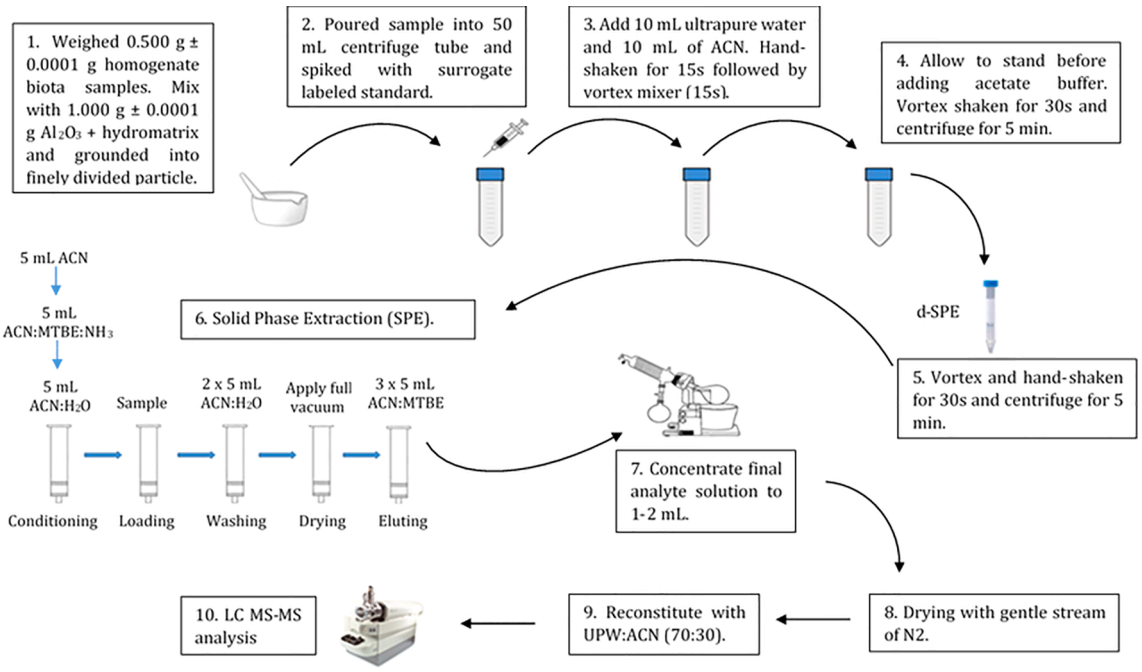

Fig. 1. Analytical procedure for analysis of multiclass EOC in biota sample.

Table 1
Optimization of source dependent parameters for the developed compounds.

Compounds	Ionisationmode	PrecursorIon	ProductIon	DP,v	EP, v	CE, v	CXP, v
Amoxicillin	Positive	366.0	349.0	86.35	7.13	25.10	8.02
			113.9	81.96	7.05	23.16	4.82
Primidone	Positive	219.0	162.1	50.0	6.00	19.80	3.70
			91.2	50.0	6.00	19.80	3.70
Primidone_D5	Positive	224.0	167.10	42.96	11.32	17.08	3.02
			124.10	42.03	11.27	24.88	2.43
Sulfamethoxazole	Positive	254.0	108.2	60.82	9.46	36.71	2.98
			156.0	59.74	9.21	21.87	5.08
Dexamethasone	Positive	393.0	147.0	55.57	4.29	9.45	2.33
			236.9	71.85	4.05	39.05	4.92
Testosterone	Positive	289.0	109.0	72.00	11.00	37.40	4.00
			271.0	72.00	11.00	37.40	4.00
Testosterone_D5	Positive	294.0	100.0	76.00	9.00	29.00	4.00
			113.0	76.00	9.00	37.00	4.00
Progesterone	Positive	315.0	109.0	75.00	9.00	40.80	4.00
			297.0	75.00	9.00	40.80	4.00
Progesterone_D9	Positive	324.5	109.0	75.00	9.00	40.80	4.00
			297.0	75.00	9.00	40.80	4.00
Quinalphos	Positive	299.2	242.8	49.88	7.04	20.97	4.16
			146.9	72.27	11.05	25.92	4.94
Diazinon	Positive	305.2	169.0	62.55	7.17	28.56	1.14
			153.10	52.12	10.39	26.12	3.87
Chlorpyrifos	Positive	351.6	199.9	51.86	11.31	26.66	6.64
			310.8	29.35	3.05	11.74	4.21
Estrone	Negative	269.0	144.9	102.00	6.00	49.90	13.60
			143.0	109.00	7.00	88.00	14.40
Diclofenac	Negative	294.0	249.9	13.00	2.00	17.60	24.90
			214.1	43.00	5.00	28.20	23.30
Bisphenol A	Negative	227.1	211.0	71.00	6.40	29.00	28.00
			133.0	66.00	6.40	34.00	19.30
4-nonylphenol	Negative	219.1	133.0	64.00	6.00	39.80	13.40
			146.7	74.00	6.00	30.10	19.70
4-octylphenol	Negative	205.1	132.9	62.90	8.00	43.00	47.10
			117.0	90.00	10.90	73.00	75.00
17β-estradiol	Negative	271.0	143.0	124.00	8.90	76.90	12.10
			145.0	129.90	7.10	49.80	16.00
17β-estradiol_D4	Negative	275.2	145.0	124.00	8.90	76.80	16.00
			147.0	129.00	7.10	49.80	12.05
17α-ethynyl estradiol	Negative	295.0	145.0	108.80	9.60	58.20	10.20
			143.0	105.00	8.10	85.50	20.60
17α-ethynyl estradiol_D4	Negative	299.0	147.0	74.28	8.00	52.00	151.0
			145.0	72.00	8.00	71.50	69.0

under a gentle stream of nitrogen blow. The concentrated extracts were then reconstituted with UPW:ACN (70:30) to 1 mL, followed by filtration using a 0.20 μm PTFE membrane filter (Agilent Technologies, USA), before being introduced for LC MS-MS analysis. Fig. 1 shows the flow process for the analysis of multiclass EOCs in biota matrices optimized in this study.

2.4. HPLC MS-MS analysis

Analytical determination was accomplished using a Spark Holland HPLC apparatus attached to an AB Sciex 3200 Q-trap triple quadrupole system as an MS analyzer (AB Sciex, MA, USA). Reversed-phase C18 analytical columns were used as stationary phases for analyte separation (Gemini NX, 50 mm × 2.0 mm, 3 μm, Phenomenex, CA, USA), whereas mobile phases were composed of UPW (aqueous) and a mixture of ACN/MeOH (60:40) (organic). Both positive and negative ionization modes were applied for analyte determination as shown in Table 1. The ionization modes were improved by adding chemical additives, ammonium hydroxide (NH_4OH, 0.2%, v/v) and formic acid (CH_2O_2, 0.2%, v/v), in mobile phases for negative and positive ionization modes, respectively. The HPLC elution program was gradient for both ionization modes with mobile phase B (organic phase) concentration starting from 5% to 95% in 4 min and being held constant for 2 min. Upon reaching the initial concentration, the system was left to equilibrate for 2 min before proceeding to the next run. The LC parameters were set as follows: flow rate for the mobile phase was 0.30 mL/min; oven column temperature was fixed at 40 °C; and injection volume was set at 20.0 μL. Meanwhile, the optimum operating condition for the MS analyzer were as follows: curtain gas, 20 psi; CAD gas 1, 40 psi; CAD gas 2, 40 psi; temperature, 600 °C (positive) and 550 °C (negative); and ion spray voltage, 4500 V (positive) and 5500 V (negative).

2.5. Quality assurance and quality control

Quality assurance and quality control (QC) steps were employed to ensure the reliability of results obtained. Cross-contamination from the laboratory apparatus and glassware was avoided by soaking them using laboratory detergent (Decon 90), washing them with deionized water, and rinsing them with an organic solvent. The apparatus and glassware were then dried in the oven at a temperature of 90 °C to remove any traces of water and the solvent. HPLC-grade chemical and solvents were used for sample preparation, whereas high-purity (>95%) native and labeled standards were used for standard preparation. For every batch of analysis, a procedural blank was analyzed to monitor any possible cross-contamination from samples, whereas a QC spike was carried out to check the efficiency of sample extraction and cleanup. Analyses were carried out in duplicate, and the mean concentration was calculated as a final analytical result.

3. Results and discussion

3.1. Optimization of analytical methods for biota samples

Optimization of analytical methods for determination of multiclass EOCs in biota samples covered two important aspects: optimization of LC MS-MS parameters and sample extraction and cleanup. Optimization of targeted compounds in LC MS-MS was carried out by introducing the individual standard into the MS analyzer using a direct infusion technique. Each of the targeted compounds was checked for ionization in the MS system; then, the fragmentation pattern was determined using the product ion scan mode. Table 1 shows the product ion for each of the targeted compounds developed in this study. After that, a multiple reaction monitoring (MRM) method was developed by optimizing compound-dependent parameters such as declustering potential, entrance potential, cell exit potential (CXP), and collision energy (Table 1). The MRM method was used for quantitation of targeted

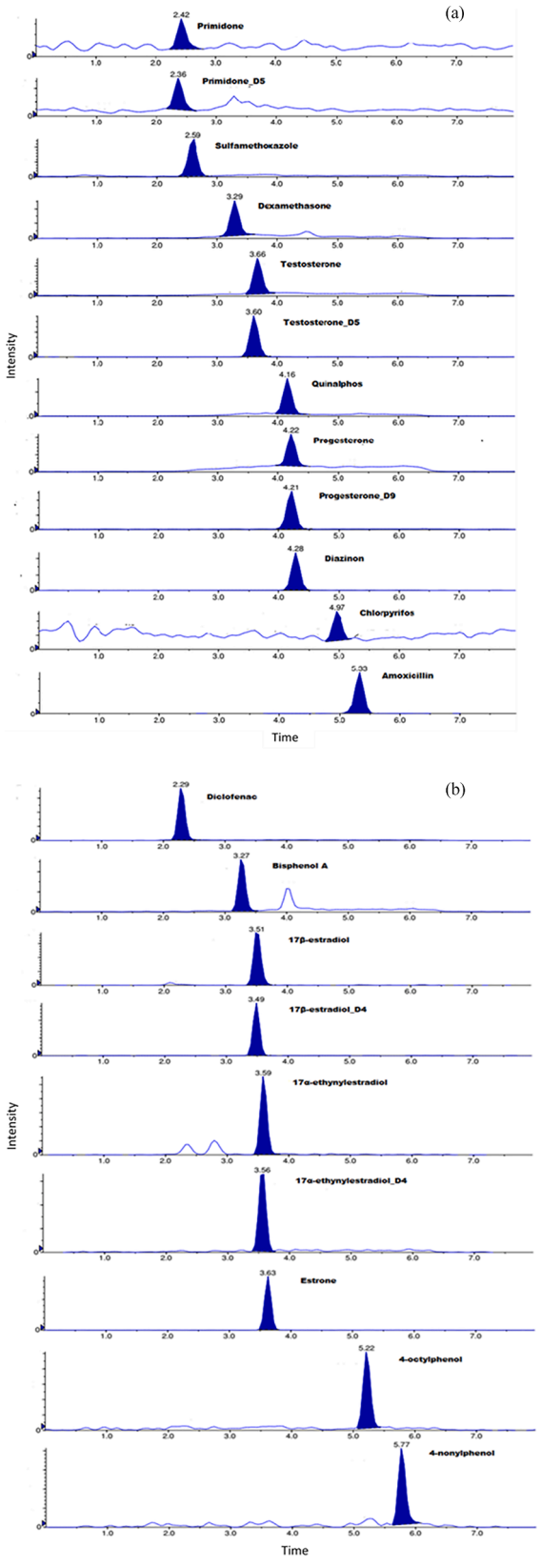

Fig. 2. Chromatographic separations for (a) positive and (b) negative ionization mode compounds spiked at 50 ng/g.

Table 2
Extraction recoveries, retention time, method detection limit and linearity of the targeted compounds optimized for biota sample.

Compounds	Retention time, min	% Recovery(%RSD, n = 3)50 ng/gQuEChERS		% Recovery(%RSD, n = 3)50 ng/gQuEChERS + SPE clean up*		% Recovery (%RSD, n = 3)100 ng/gQuEChERS		% Recovery (%RSD, n = 3)100 ng/gQuEChERS + SPE clean up*		MDL, ng/g	Linearity, *r*
Diclofenac	2.29	62	(8.94)	83	(3.03)	61	(15.70)	96	(4.17)	0.35	0.999
Bisphenol A	3.27	84	(4.19)	90	(6.15)	90	(4.44)	96	(6.25)	0.29	0.998
17β-estradiol	3.51	85	(5.88)	110	(5.94)	88	(2.86)	103	(7.32)	0.18	0.993
EE2	3.59	91	(4.97)	98	(8.16)	96	(5.21)	90	(11.11)	0.12	0.999
Estrone	3.63	95	(10.05)	87	(15.61)	103	(3.88)	93	(8.11)	0.02	0.994
4-octylphenol	5.22	51	(8.91)	61	(6.56)	58	(13.04)	69	(15.33)	0.15	0.997
4-nonylphenol	5.77	48	(4.17)	65	(8.53)	52	(7.69)	64	(14.06)	0.45	0.997
Primidone	2.42	103	(2.44)	92	(3.83)	108	(2.33)	114	(3.08)	0.06	0.996
Sulfamethoxazole	2.59	66	(6.06)	73	(7.59)	69	(5.11)	82	(3.66)	0.05	0.995
Dexamethasone	3.29	68	(3.70)	79	(4.46)	65	(7.69)	88	(8.57)	1.50	0.998
Testosterone	3.66	112	(6.25)	108	(1.85)	109	(3.23)	111	(4.98)	0.06	0.999
Quinalphos	4.16	42	(3.61)	72	(4.17)	54	(4.67)	76	(3.31)	0.14	0.994
Progesterone	4.22	92	(13.04)	110	(3.20)	98	(7.69)	108	(2.33)	0.07	0.998
Diazinon	4.28	52	(3.85)	67	(11.94)	61	(4.92)	81	(7.41)	0.08	0.997
Chlorpyrifos	4.97	55	(3.64)	78	(7.69)	57	(8.77)	77	(4.58)	3.50	0.999
Amoxicillin	5.33	79	(2.53)	93	(4.30)	86	(2.92)	91	(6.59)	0.23	0.995

*SPE clean up based on Omar et al. [21]

compounds analyzed in the LC MS-MS system. Peak separation was also optimized by evaluating the mobile phase composition used for compound elution in the LC system. Basically, the mobile phase composition for gradient elution used in this study followed the method described by Omar et al. [21]. However, some modifications were made by adding chemical additive, formic acid (CH_2O_2, 0.2%, v/v) into the mobile phase composition to improve the ionization of compounds as well as for better peak separation. Besides the mobile phase composition, the final reconstitution solvent was also determined to find the best peak separation. Four reconstitution solvents were assessed: (1) 100% ACN, (2) 100% MeOH, (3) a mixture of UPW:MeOH (90:10), and (4) a mixture of UPW:ACN (70:30). It was observed that the best peak separation was achieved using the mixture of UPW:ACN (70:30), which was used as the final reconstitution solvent for samples as well as for the preparation of a standard solution before being injected into the LC MS-MS system. Fig. 2 shows the separation of targeted compounds in both positive and negative ionization modes.

Th extraction recovery for this method was evaluated using the fish tissue muscle that was previously analyzed, and no targeted compounds were present in the specimen. Sample extraction and cleanup were optimized on the basis of the QuEChERS technique. This technique, which was originally developed for determination of pesticide residue in food samples [22], has been gaining popularity for analysis of multiclass EOC in biological matrices [19,23,24]. Sample extraction and cleanup were evaluated by spiking two levels of the mixed EOC solution, at 50 and 100 ng/g; two extraction cleanup protocols were used. The first extraction protocol assessed was QuEChERS extraction without additional SPE cleanup, whereas the second protocol involved QuEChERS with additional offline SPE cleanup. The extraction and cleanup of biota matrices were a challenging part for method development because of the nature of these matrices; that is, they may have high organic carbon and lipid content as well as very low analyte concentration, usually present at trace levels. Therefore, Omar et al. [12], in a review of analytical strategies for determination of EDCs in environmental and biota matrices, suggested a combination of a few extraction and cleanup techniques for purification and analyte enrichment, particularly for biological samples. In this assessment, the QuEChERS technique in combination with offline SPE cleanup was evaluated in order to provide better extraction recoveries for biota matrices in this study. Poor extraction recoveries were observed for some of the developed compounds using QuEChERS without additional SPE cleanup as shown in Table 2. However, after combination with offline SPE cleanup, an improvement in extraction recoveries was noted for some of the developed compounds, particularly for diclofenac, 4-octylphenol, 4-nonylphenol, sulfamethoxazole, dexamethasone, diazinon, chlorpyrifos, and quinalphos. Therefore, the QuEChERS in combination with offline SPE was chosen as the extraction and cleanup protocol for both fish and mollusks samples in this study. Satisfactory extractions were achieved for all targeted compounds spiked at both spiking levels. Extraction recoveries for developed compounds spiked at 100 ng/g range from 64%

Table 3
The application of QuEChERS as an extraction technique for emerging organic contaminants in various types of biota matrices.

Sample matrix/weight	Extraction and clean-up protocol	Compounds	% Recovery	Detection limit, ng/g	Detection	References
Mussel/2.0 g	QuEChERS + clean up sorbent	UV filter, UV stabilizer and musk (9 compounds)	50–126	0.50–50.00	GC MS-MS	Groz et al. [18]
Fish, mussel, seaweed/2.0 g	QuEChERS + LLE	Phenolic EDCs (2 compounds)	67–107	0.02–0.80	LC MS-MS	Cunha et al. [5]
Fish/10.0 g	QuEChERS	Pesticides (52 compounds)	71–120	0.01–50.00	LC MS-MS	Barbeiri et al. [31]
Mollusk/0.01 g	QuEChERS	Pharmaceuticals (2 compounds)	85–100	18.00–128.00	LC MS-MS	Berlioz-Barbier et al. [23]
Bivalves/1.0 g	QuEChERS + silica gel clean up	Pharmaceuticals (7 compounds)	35–77	5.00–100.00	LC MS-MS	Núñez et al. [19]
Fish, mussels/2.0 g	QuEChERS + PS DVB sorbent	Perfluorinated compounds (13 compounds)	71–116	0.001–0.070	LC MS-MS	Yin et al. [17]
Fish/1.0 g	QuEChERS + EMR lipid removal	Pharmaceuticals (21 compounds)	25–86	0.25–25.43	LC QTOF-MS	Peña-Herrera et al. [20]
Fish/3.0 g	Modified QuEChERS	Polychlorinated biphenyls (19 compounds)	73–96	less than 1.00	GC MS-MS	Chamkasem et al. [32]
Fish and mollusk/0.5 g	QuEChERS + SPE	Pharmaceuticals, estrogenic hormones, pesticidesand phenolic EDCs (16 compounds)	64–114	0.02–3.50	LC MS-MS	This study

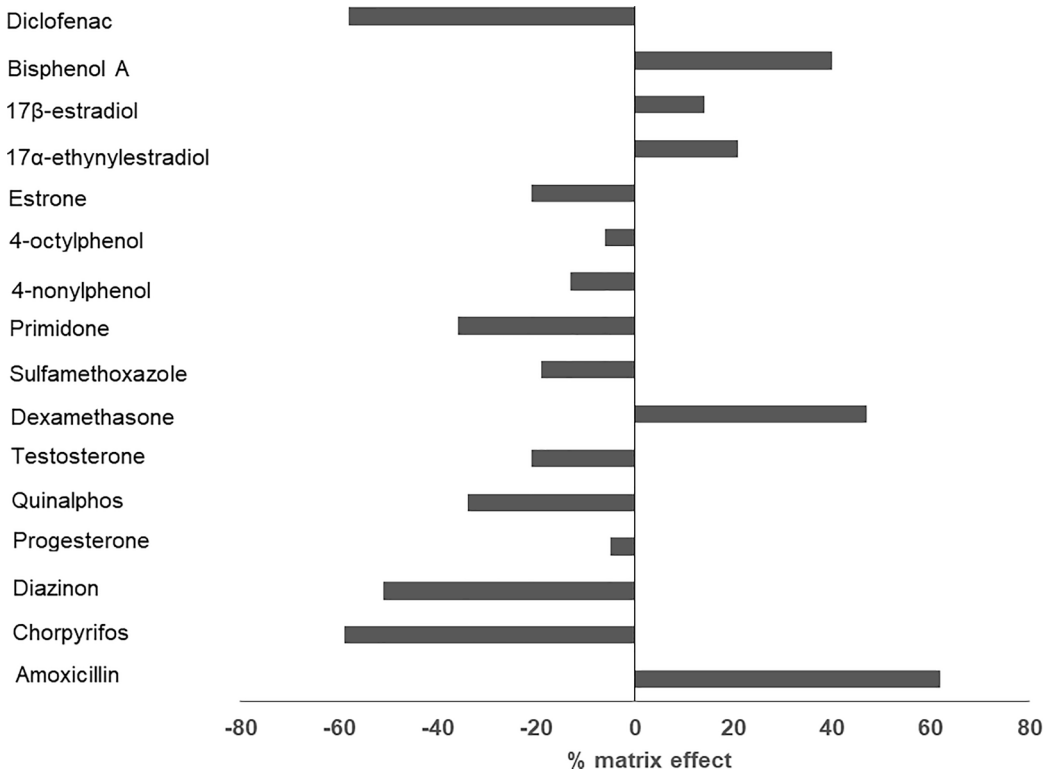

Fig. 3. Matrix effects for the developed compounds.

to 114%, whereas for those at the spiking level of 50 ng/g, the recoveries were in the range of 61% to 110%.

On the basis of previously reported studies, researchers opted to combine QuEChERS with several other cleanup alternatives such as LLE, silica gel cleanup, cleanup sorbents, PS-DVB sorbents, and EMR-lipid to remove lipid and undesirable components from complex biota matrices. A comparison of the application of QuEChERS in combination with other cleanup techniques for biota extraction is shown in Table 3. Satisfactory extraction efficiency, as well as low detection limit, was achieved for the developed method, particularly for the PS-DVB sorbent, when Yin et al. [17] reported the recoveries were in the range of 71–116% with a detection limit from 0.0001 to 0.0070 ng/g for extraction of 13 perfluorinated compounds in fish and mussel samples. However, the recovery was slightly lower for QuEChERS in combination with EMR-lipid and silica gel for extraction of pharmaceuticals compounds in fish and bivalves as reported by Peña-Herrera et al. [20] and Núñez et al. [19], respectively. It was also noted that most of the researchers developed the extraction method for a single class of compounds, and thus, this present work provides an improved QuEChERS technique for determination of multiclass EOCs in biota matrices with better extraction efficiency and a low method detection limit.

3.2. Method validation

Validation is an important part of method development as it is an indicator for reliability of the method to be used for real sample analysis. Linearity, extraction recovery, precision, method detection limit, and matrix effects are the most common parameters that should be included when method validation is concerned. This validation exercise is in line with the Eurachem guideline [25], which states that the developed analytical technique including instrumentation should be tested and verified to ensure that it is fit for purpose. In addition, method validation exercise is necessary to conform to the European Commission [26] regulation, which requires that a newly optimized method be checked in terms of analytical testing performance before it can be used for routine analysis.

Validation of this method was carried out by checking the linearity of compounds in LC MS-MS as well as determining the detection limit for the developed compounds. Linearity of compounds, evaluated from a series of standard solution (0.1 to 200.0 ng/mL), ranges from 0.990 to 0.999, whereas the method detection limit, calculated on the basis of a 3:1 signal-to-noise ratio, ranges from 0.02 to 3.50 ng/g. Method precision was determined through intra-day and inter-day repeatability. Intra-day repeatability, evaluated from the triplicate experiment of spiked biota extraction in a single day, was less than 15%, which is suggested as the acceptable precision for field sample analysis. Meanwhile, inter-day repeatability was also assessed by analyzing the spiked biota sample for three consecutive days, and the relative standard deviation (RSD) for all of the targeted compounds was less than 18%. The required RSD value for method precision should be at or less than 20%, as stipulated in the European Commission [26] regulation.

The influence of a biota matrix on the efficiency of electrospray ionization in the MS system was also evaluated by determining the matrix effect for each compound. The matrix effect was evaluated by spiking the fish tissue muscle with the mixed EOC standard solution after extraction, and the signals (A_{biota}) were compared with the signals (A_{solvent}) of the standard in the solvent at 50 ng/mL. Equation (1) was used to calculate the percentage of matrix effects:

$$\%\ matrix\ effect\ (ME) = \left(\frac{A_{biota}}{A_{solvent}} - 1\right) \times 100 \quad (1)$$

Fig. 3 shows the percentages of matrix effects for the developed compounds. The matrix effects for all the developed compounds were in the range of −59% to 47%, indicating the ionization suppression occurred for all of the compounds in the MS analyzer. A matrix effect of less than 100% is called ionization suppression, whereas a matrix effect that is >100% is known as signal enhancement [27,28]. Therefore, isotope labeled surrogate standards were used in this study to compensate for this ionization suppression as well as for recovery correction and analyte quantification as described by Wu et al. [28] and

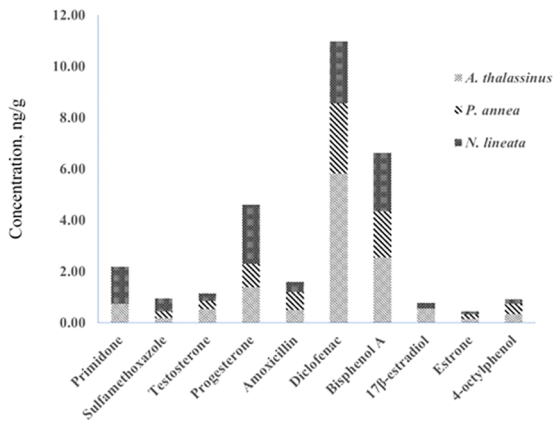

Fig. 4. Concentration of multiclass EOCs in biota matrices analysed using the optimized method.

van de Steene and Lambert [29].

3.3. Application to real samples

As part of the validation and verification exercise, the optimized method was then applied to determine the concentration of biota collected from the field sampling. The concentration of EOCs in biota samples analyzed from the Klang River estuary is depicted in Fig. 4. The trace level of EOCs was detected in the three biota samples analyzed, with *A. thalassinus* having the highest concentration of diclofenac, at 10.76 ng/g, and *P. annea* having the lowest concentration of E1, at 0.05 ng/g. The detailed results for the analyzed biota samples from the Klang River estuary were reported in a previous study by Omar et al. [30]. Of the 16 targeted compounds developed in this study, 10 were detected in those matrices, namely, diclofenac, BPA, E1, E2, amoxicillin, progesterone, testosterone, sulfamethoxazole, primidone, and 4-octylphenol. Meanwhile, the other six compounds were below the method detection limit for all of the biota samples analyzed. The distribution pattern of EOCs showed that diclofenac is dominantly present in all of the biota samples, followed by BPA and progesterone. This suggests that the biota matrices analyzed from this study can be used as potential biological indicators for organic pollution assessment in the tropical coastal and marine ecosystems.

4. Conclusion

An improved analytical methodology based on the QuEChERS technique was successfully optimized for simultaneous determination of 16 multiclass EOCs in tropical marine biota matrices. Analytical optimization covered two important aspects: optimization of LC MS-MS parameters and sample extraction and cleanup. Peak separation was optimized by evaluating the mobile phase composition used for compound elution in the LC system, as well as the final reconstitution solvent for a better peak shape. The QuEChERS technique in combination with SPE employing a polymeric material as a cleanup sorbent was found to provide improvement on the overall extraction recoveries of the targeted compounds. The optimized method showed satisfactory accuracy and sensitivity for all the targeted compounds. Verification of the newly optimized method was carried out by analyzing biota matrices collected from field sampling, and this method was successfully applied for determining multiclass EOCs at trace concentration. Therefore, this study illustrated that the optimized method could be used for simultaneous analysis of trace levels of multiclass EOCs in marine biota species with high accuracy and precision.

CRediT authorship contribution statement

Omar, T.F.T.: Conceptualization; Formal analysis; Validation; Writing - original draft. **Aris A.Z.:** Funding acquisition; project administration; Supervision; Writing - review and editing. **Yusoff, F.M.:** Supervision; Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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