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Simultaneous determination of UV-filters and estrogens in aquatic invertebrates by modified quick, easy, cheap, effective, rugged, and safe extraction and liquid chromatography tandem mass spectrometry

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ABSTRACT

Ultraviolet-filters (UV-filters) and estrogens have attracted increased attention as contaminants of emerging concern (CECs) due to their widespread occurrence in the environment. Most of these CECs are hydrophobic and have the potential to accumulate in aquatic organisms. To date, co-analysis of UV-filters and estrogens has not been reported due, in part, to the complex environmental matrices. Here, a multi-residue method has been developed for simultaneous determination of five UV-filters and three estrogens in tissue from aquatic and marine organisms. The procedure involved a modified Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction with a novel reverse-solid-phase extraction (reverse-SPE) cleanup in place of dispersive-SPE, followed by liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis. The tissue mass, acetonitrile content, and salt conditions for QuEChERS extraction, along with the reverse-SPE cartridge material and elution conditions, were thoroughly investigated and optimized. Five UV-filters (*i.e.*, 3-(4-methylbenzylidene) camphor, benzophenone-3, ethylhexyl-methoxycinnamate, homosalate, and octocrylene) and three estrogens (*i.e.*, estrone, 17β-estradiol, and 17α-ethinylestradiol) were simultaneously analyzed by taking advantage of wrong-way-round ionization in LC–MS/MS. The optimized analytical protocol exhibited good recoveries (>80%) for target compounds and enabled their detection at concentrations as low as 0.2 ng/g in 50 mg tissue samples. The method was applied to determine concentrations of target analytes in four invertebrates (*i.e.*, *Orconectes virilis*, *Procambarus clarkii*, *Crassostrea virginica*, and *Ischadium recurvum*). All eight target analytes were detected at least once in the tissue samples, with the highest concentration being 399 ng/g of homosalate in *O. virilis*. These results highlight the ubiquitous bioaccumulation of CECs in aquatic and marine invertebrates.

1. Introduction

In recent years, organic ultraviolet-filters (UV-filters) and estrogenic hormones have attracted increased attention as contaminants of emerging concern (CECs) due to their widespread occurrence in the environment and potentially adverse effects on aquatic ecosystems and human health [1–5]. UV-filters are widely used in personal care products to protect skin from harmful effects of solar irradiation [6] and in food packaging, pharmaceuticals, and textiles to prevent photodegradation of polymers and pigments [7]. After use, these chemicals reach the environment through recreational activity or wastewater effluent. Most organic UV-filters are hydrophobic (*e.g.*, log K_{ow} > 3, see Table S.1 in the Supporting Information) and poorly degraded during wastewater treatment [8,9]. The presence of these compounds in the environment has resulted in concerns about their potential effects on aquatic organisms [3,4,10]. Five UV-filters (*i.e.*, 3-(4-methylbenzylidene) camphor (4-MBC), benzophenone-3 (BP-3), ethylhexylmethoxycinnamate (EHMC), homosalate (HMS), and octocrylene (OC)) were selected for this study due to their toxicity [10–12], consumption [13], and previous detection in aquatic organisms [4,14,15].

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Estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2) are considered priority chemicals since large amounts of these potent [3,5] estrogens are excreted by both humans and animals [16]. E2 is the main female sex hormone synthesized by vertebrates, while E1 is its primary degradation product [17]. The synthetic estrogen EE2 has been widely used in oral contraceptives and estrogen replacement therapy [18]. Moreover, these estrogens are hydrophobic (Table S.1 in the Supporting Information) and bioaccumulate in the food web [19–21]. For these reasons, E1, E2, and EE2 were also added to the European Union Commission watchlist [10,22] mentioned above. The US Environmental Protection Agency has included E1 and E2 on the Drinking Water Contaminant Candidate List [23].

Simultaneous determination of UV-filters and estrogens will provide a better understanding of the impacts of these chemicals on aquatic organisms without the need for undue analytical workloads. However, no currently reported analytical methods simultaneously detect multiple UV-filters and estrogens in biological tissues. Moreover, previous reports on the occurrence of CECs in aquatic biota have mainly focused on vertebrates [14,24–32], with few studies on accumulation of UV-filters and estrogens in invertebrates [21,28,33]. In this work, an effective analytical method was developed for simultaneous determination of three estrogens (*i.e.*, E1, E2, and EE2) and five UV-filters (*i.e.*, 4-MBC,BP-3, EHMC, HMS, and OC) in biological tissues from aquatic and marine invertebrates.

Available gas chromatography (GC) and liquid chromatography (LC) coupled to mass (or tandem mass) spectrometry (MS or MS/MS) methods for determination of UV-filters in aquatic biota were reviewed in 2012 [34] and 2015 [35]. GC–MS was less sensitive to matrix effects during ionization; however, GC–MS based methods only apply to volatile and highly hydrophobic (or derivatized) UV-filters, and the derivatization process can affect precision and accuracy [36]. In contrast, LC-based methods allow analysis of multiple analytes with a wide range of physicochemical properties. Only four LC–MS or LC–MS/MS methods have been developed for determination of multiple UV-filters in biota [14,24–26]. In general, these protocols involved solvent-based extraction followed by solid-phase extraction (SPE) cleanup.

Barreiros et al. [37] reviewed analytical techniques for E2 and EE2 and emphasized MS/MS as the most attractive detection method due to high specificity, minimal cross reactivity, and the potential for simultaneous determination of multiple analytes. Due to their low molecular weight and volatility, estrogens often require derivatization to improve sensitivity and selectivity during GC–MS/MS analysis. This process is time-consuming and may result in analyte loss [36,38]. For these reasons, LC–MS/MS is increasingly becoming the preferred technique for measurement of estrogens [37]. Matrix effects (MEs) are critical during LC–MS/MS analysis, and these effects represent a challenge for determination of estrogens in tissue samples. Only four LC–MS/MS protocols [21,27,28,39] have been applied to biological tissue. Similar to UV-filters, these methods have employed solvent extraction, followed by SPE cleanup.

Although UV-filters and estrogens have been successfully recovered from tissue samples by solvent extraction (*e.g.*, Soxhlet extraction, accelerated solvent extraction (ASE)) and cleanup (*e.g.*, SPE, gel permeation chromatography (GPC)), these procedures are tedious, time-consuming, and require large solvent volumes. To simplify extraction, the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) concept was introduced by Anastassiades et al. [40]. This protocol has been successfully applied to determine a variety of CECs (*e.g.*, E1, EHMC, and OC) in benthic invertebrates (*e.g.*, *Potamopyrgus antipodarum*, *Gammarus fossarum*, and *Chironomus riparius*) [33], marine mussels (*Mytilus galloprovincialis*) [41], and fish (*Acipenser transmontanus*) [42].

Following pretreatment, LC–MS/MS exhibits the greatest potential for simultaneous determination of multiple UV-filters and estrogens in biological tissue. Most UV-filters have been analyzed in positive mode [14,24–26], while estrogens are preferentially analyzed in negative mode [21,27,28,33,39]. Ionization efficiency is dependent on mode and mobile phase composition. For that reason, different elution methods have been used for measurement of UV-filters and estrogens [21,26–28,33]. This limitation increases the required sample volume and doubles the analytical workload of the instrument.

The objectives of this study were as follows: (1) optimize a QuEChERS-based protocol for simultaneous extraction of multiple UV-filters and estrogens in tissue from aquatic organisms; (2) improve the LC–MS/MS analytical workload through combination of positive and negative ionization modes without sacrificing sensitivity; and, (3) employ the developed methods to determine the concentration of UV-filters and estrogens in representative macroinvertebrate organisms (*i.e.*, crayfish, oysters, and mussels). Overall, this work addresses the challenges of effective co-extraction of chemically-diverse estrogens and UV-filters from complex tissue matrices and their associated compatibility issues during LC–MS/MS analysis.

2. Materials and methods

2.1. Standard chemicals and reagents

E2, EE2, BP-3, EHMC, HMS, and OC were purchased from Sigma-Aldrich (St. Louis, MO). E1 was acquired from Acros Organics (Morris Plains, NJ), and 4-MBC was obtained from Alfa Aesar (Stoughton, MA). Seven deuterated compounds were used as surrogates or internal standards. Specifically, EE2-d_4 (for EE2) and 4-MBC-d_4 (for 4-MBC) were acquired from CDN Isotopes (Pointe-Claire, Canada). E2-d_3 (for E1 and E2), BP-3-d_5 (for BP-3), EHMC-d_{15} (for EHMC), HMS-d_4 (for HMS), and OC-d_{15} (for OC) were purchased from Sigma-Aldrich. Physicochemical properties are summarized for the target analytes and deuterated compounds in Tables S.1 and S.2 (see Supporting Information), respectively.

Stock solutions of the eight analytes and seven deuterated surrogates were individually prepared at 400 mg/L in methanol (MeOH). Two working solutions (*i.e.*, one for the eight analytes and one for the seven deuterated compounds) were prepared weekly at 20 mg/L in MeOH. The final solutions were prepared in 100% MeOH for spiking (*i.e.*, 1 mg/L) purposes, and in 50% MeOH for standard calibration (*e.g.*, 0.5–25 μg/L) analysis. All solutions were stored in amber glass containers at −20 °C.

Inorganic chemicals, including anhydrous magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), ammonium hydroxide (NH_4OH), and ammonium formate (NH_4COOH), and LC–MS grade water, MeOH, and acetonitrile (ACN) were obtained from Fisher Scientific. Formic acid (HCOOH, 99%) was purchased from Sigma-Aldrich. Hydrophilic-lipophilic balanced (HLB, as 60 mg, 3 cm^3; 150 mg, 6 cm^3; and, 500 mg, 6 cm^3) and Sep-Pak Vac C18 (as 200 mg, 3 cm^3) cartridges were acquired from Waters (Milford, MA). Isolute C18 cartridges (as 100 mg, 3 cm^3 and 500 mg, 3 cm^3) were purchased from Biotage (Uppsala, Sweden).

2.2. Preparation of tissue samples from aquatic organisms

Virile crayfish (*Orconectes virilis*) were collected from seven different sites in urban streams (Baltimore, MD) using a backpack electrofisher. Red swamp crayfish (*Procambarus clarkii*) and eastern oysters (*Crassostrea virginica*) were purchased from Aquatic Research Organisms (ARO; Hampton, NH). Hooked mussels (*Ischadium recurvum*) and eastern oysters were collected using a stainless

steel dredge from two sites in the Chesapeake Bay at the mouth of the Chester River (CBCR). Crayfish were rinsed with DI water several times to remove sediment and other residue on the outer surface, and then whole crayfish were transferred into amber-glass containers and sacrificed at −20 °C. The frozen crayfish were thawed slowly at 4 °C and dissected to collect the tail tissue. Eastern oyster and hooked mussel samples were rinsed with DI water and then dissected to collect the tissue. The water and lipid contents of the tissues were determined by the gravimetric method and a modified Folch extraction protocol [43], respectively. Detailed information is provided in Texts S.1 and S.2, and the results are summarized in Table S.3 (see Supporting Information). Tissue samples were lyophilized, then homogenized by mortar and pestle. The final freeze-dried tissues were stored in sealed containers at −20 °C.

2.3. Modified QuEChERS extraction with reverse-SPE cleanup

The tissue mass, ACN content, salt conditions, and cleanup strategy were thoroughly investigated; the complete extraction protocol for UV-filters and estrogens is summarized in the Supporting Information (Fig. S.1). Briefly, aliquots (50 mg) of the lyophilized tissue samples were placed in 15 mL centrifuge tubes. Before extraction, 10 ng (10 μg/L × 1 mL in MeOH) of the deuterated compounds (or analytes for standard addition analysis) were spiked into the tube and allowed to equilibrate overnight, during which time the residual MeOH evaporated. The centrifuge tube was then filled with 5 mL DI water, vortexed for 30 s, filled with 5 mL ACN, and vortexed for 30 s. Then, 2.5 g $MgSO_4$ and 1.0 g NaCl were placed in the tube. This mixture was vortexed for 30 s and then centrifuged at 6000*g* for 10 min. The extract was cleaned by a reverse-SPE process (described below in Section 3.2.2), by processing 2.5 mL of the upper ACN layer through HLB cartridges (60 mg, 3 cm^3). An additional 2 mL of ACN was added to elute any remaining target compounds. The final eluent (~4.5 mL) was evaporated to dryness under nitrogen in the dark to minimize photodegradation of analytes and surrogates. To reconstitute the analytes for LC–MS/MS analysis, 0.5 mL MeOH (containing internal standards for standard addition analysis) was mixed with 0.5 mL 0.1% NH_4OH.

2.4. LC–MS/MS analysis

All analytes were measured using an UltiMate 3000 LC coupled to a Thermo TSQ Quantum Access Max triple quadrupole tandem mass spectrometer (Thermo; Waltham, MA). The LC–MS/MS operating conditions were systematically studied to achieve low limits of detection. LC separation of analytes was achieved with a Waters XBridge C18 column (2.1 × 150 mm, 2.5 μm) with a guard column (2.1 × 10 mm, 3.0 μm) containing the same material. The mobile phase flow rate was set to 0.2 mL/min and the column compartment was maintained at 40 °C. The mobile phase was comprised of (A) LC–MS grade water with 0.1% NH_4OH (pH 10.5) and (B) MeOH with 0.1% NH_4OH. Fresh mobile phase was prepared before every analysis. The mobile phase flow gradient was as follows: 70% B for 2.5 min, linear increase to 100% B within 1 min, constant for 5 min, linear decrease to 70% B within 1 min, and constant for 5.5 min to re-equilibrate the column chemistry. The overall method run time was 15 min, and 100 μL of sample was injected.

Both positive and negative electrospray ionization (ESI) modes were employed. The spray voltage was 3000 V, capillary temperature was 350 °C, vaporizer temperature was 300 °C, sheath gas pressure was 35 (arbitrary units, nitrogen), auxiliary gas pressure was 10 (arbitrary units, nitrogen), and collision-induced dissociation pressure was 1.5 mTorr (argon). The collision energy and tube lens offsets were optimized in selected reaction monitoring (SRM) mode through auto-loop injection. The precursor ion and two characteristic product ions were identified for each analyte. The most abundant product ion was used for quantitation, while the second most abundant was used for confirmation. The optimized detector parameters and ion transition information are summarized in Table 1 .

Table 1
ESI–MS/MS operational parameters and instrumental/method performance metrics.

Analyte	Ion transition[a]	CE[b] (V)	TLO	Linear range (μg/L)	R^2	IDL (pg)	IQL (pg)	Reproducibility[c] (% RSD; $n = 6$)		MDL[d] (ng/g)	MQL[d] (ng/g)	IS
								Intra-day	Inter-day			
EE2	**295.1 → 145.1** *295.1 → 159.1*	−41 −37	−105	0.5–25	0.9986	7.6	25	3.4	7.6	1.0	3.3	EE2-d_4
E2	**271.1 → 183.0** *271.1 → 145.1*	−44 −42	−104	0.25–25	0.9984	3.8	13	3.0	3.8	0.5	1.7	E2-d_3
E1	**269.1 → 145.0** *269.1 → 143.0*	−40 −58	−106	0.1–25	0.9974	1.5	5	2.9	1.5	0.2	0.7	E2-d_3
BP-3	**229.1 → 151.1** *229.1 → 105.1*	19 19	73	0.1–25	0.9993	1.5	5	2.1	1.5	0.6	2.0	B*P*-3-d_5
4-MBC	**255.1 → 105.2** *255.1 → 212.1*	31 17	87	0.1–25	0.9995	1.5	5	2.6	1.5	0.6	2.0	4-MBC-d_4
OC	**362.2 → 232.0** *362.2 → 250.2*	19 7	90	0.1–25	0.9997	1.5	5	4.9	1.5	1.0	3.3	OC-d_{15}
EHMC	**291.2 → 161.1** *291.2 → 179.0*	18 5	68	0.1–25	0.9974	1.5	5	2.7	1.5	0.6	2.0	EHMC-d_{15}
HMS	**261.1 → 137.0** *261.1 → 93.1*	−20 −38	−78	0.75–25	0.9989	11	38	3.1	11	2.0	6.7	HMS-d_4

Acronyms: CE, Collision Energy; TLO, Tube Lens Offset; IDL, Instrumental Detection Limit; IQL, Instrumental Quantitation Limit; RSD, relative standard deviation; MDL, Method Detection Limit; MQL, Method Quantitation Limit; IS, Internal Standard.

[a] The first product ion (**bold**) was used for quantitation, and the second product ion (*italics*) was used for confirmation.

[b] Positive and negative values represent ESI mode.

[c] 500 pg injection.

[d] Based on 50 mg red swamp crayfish tissue samples.

2.5. Quality assurance and quality control

The method developed for concurrent analysis of UV-filters and estrogens was evaluated for linear range, sensitivity, selectivity, reproducibility, and matrix effects. A ten-point calibration curve, ranging from 0.1 to 25 μg/L, was established for each analyte. Blank injections, quality controls, and internal standards were added to each analytical batch to check for possible contamination, instrument performance, and internal quality control. Analyte recovery was thoroughly examined in the extraction and purification steps using standard additions. Specifically, analytes were spiked into (i) 50, 150, and 500 mg of lyophilized tissue to determine extraction efficiency and (ii) pure ACN and the extracted ACN layer (from tissue samples) to determine recovery during cleanup. MEs were examined by comparing the absolute response for spiked samples with those for blank samples (Eq. (1)).

$$ME = \frac{R_{SO} - R_O}{R_S} - 1 \quad (1)$$

In Eq. (1), R_{SO} is the response of the spiked analyte in the sample extract, R_O is the response of the unspiked sample extract, and R_S is the response of the spiked analyte in the mobile phase. An *ME* of 0 indicates no impact from the background matrix. If $ME > 0$, then the sample matrix enhanced the signal; however, if $ME < 0$, then the matrix suppressed the analyte signal.

Background contamination is a common issue for environmental analysis of UV-filters [14,36] due to their ubiquitous presence in personal care products. To minimize contamination, all glassware underwent rigorous washing protocols followed by baking (2 h) at 450 °C and rinsing with HPLC-grade MeOH. During experimentation and sample handling, all project personnel avoided using products containing the analytes of concern.

3. Results and discussion

3.1. Optimization of LC–MS/MS conditions

As summarized in previous analytical reviews for UV-filters [35] and estrogens [37], ACN and MeOH containing volatile additives have been commonly used for optimizing LC separation and improving ionization efficiency. Low concentrations of HCOOH (*i.e.*, 0.15–0.20%, *v/v*) improve the peak shape [14] and sensitivity of UV-filters [26]. NH_4OH has been frequently incorporated into the mobile phase for estrogen analysis to increase negative mode ESI efficiency [28,44]. For those reasons, the addition of HCOOH, NH_4COOH, and NH_4OH to ACN and MeOH was evaluated for simultaneous analysis of UV-filters and estrogens by LC–MS/MS. Specifically, HCOOH (0.1% *v/v*, pH ~2.7), NH_4COOH (10 mM, pH ~6.3), and NH_4OH (0.1% *v/v*, pH ~10.5) were added to both organic solvents and LC–MS grade water. For all combinations, the elution conditions described in Section 2.4 were employed. A mixture of the UV-filter and estrogen analytes was injected at 10 μg/L, and the corresponding responses are summarized in Fig. 1.

For the three estrogens and the HMS UV-filter, negative mode ionization efficiencies were significantly suppressed by HCOOH and NH_4COOH (Fig. 1a). Signals improved with NH_4OH in MeOH,

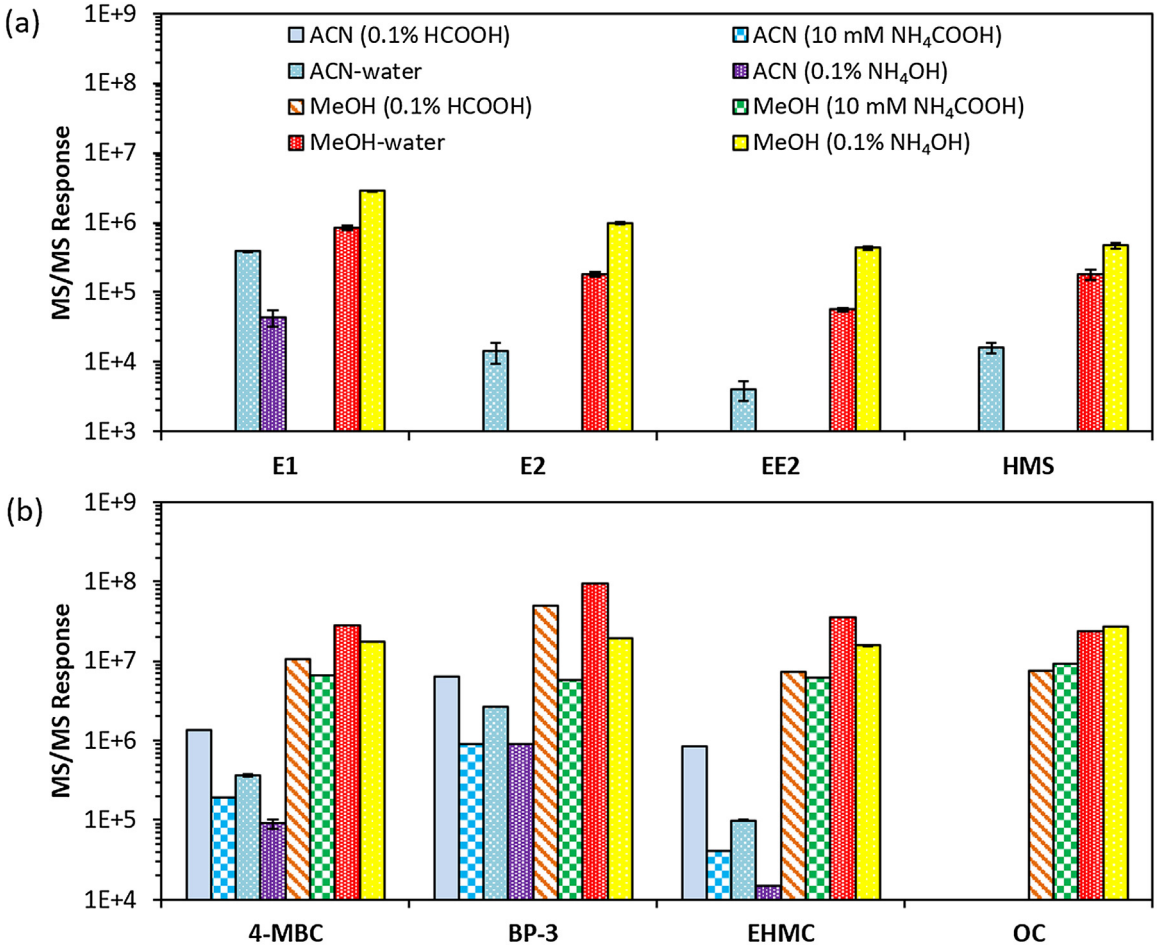

Fig. 1. MS/MS response for 10 μg/L of individual analytes in (a) negative and (b) positive ESI modes for different mobile phase compositions ($n = 4$; error bars are standard deviation).

but the response was minimal for the same conditions in ACN. These results were somewhat expected as incorporation of bases into the mobile phase facilitates deprotonation of analytes during negative mode ESI. This trend was also observed for the other UV-filters (*i.e.*, 4-MBC, BP-3, EHMC, and OC) in ACN for positive mode ESI (Fig. 1b). The highest ionization efficiency was achieved using the HCOOH additive in ACN, because the acidic mobile phase conditions promote protonation. Similar observations were noted in previous methods [14,26,45]. However, with the exception of BP-3, higher ionization efficiencies were obtained using NH_4OH in MeOH. As these conditions should inhibit the protonation of UV-filters, these findings were unexpected. This phenomenon, namely the abundant generation of $[M+H]^+$ ions during positive mode ESI in base-modified mobile phases, has been previously described as "wrong-way-round" ionization [46]. Fig. 1b is the first observation of wrong-way-round ionization for UV-filters. The wrong-way-round ESI behavior of UV-filters in MeOH is especially important to improve the compatibility of mobile phase selection and enable combined analysis of UV-filters and estrogens. Although the MeOH-water combination provided a slightly higher analyte response in positive ESI mode, method reproducibility may be problematic for the MeOH-water mobile phase due to the lower buffer intensity. For these reasons, MeOH was selected as the organic mobile phase and 0.1% NH_4OH was employed as an additive to buffer pH and improve ionization.

For each analyte, the collision energy and tube lens offset MS/MS parameters were optimized in the various mobile phase compositions. Standard solutions (1 mg/L) of individual analytes were injected by syringe, mixed with the mobile phase, and delivered to the ESI chamber. For this analysis, mobile phase composition was adjusted based on the retention time of the analyte and the mobile phase gradient (see Section 2.4). The optimized MS/MS parameters are summarized in Table 1; furthermore, analogous information is provided in Table S.4 (see Supporting Information) for the internal standards. The identified precursor and product ions are similar to previous reports for UV-filters [11,14,26] and estrogens [39,47], but the instrumental detection limits (IDLs) for UV-filters are at least 3–5 times lower than previously reported methods [11,14,26]. By taking advantage of the wrong-way-round ionization of UV-filters for combined analysis with estrogens, the analytical workload was increased and solvent consumption was decreased.

C18 columns have been widely employed for LC separation of both UV-filters [11,14,26] and estrogens [47,48]. The XBridge C18 column was used due to its wide pH operating range (*i.e.*, 1–12), high separation efficiency, and normal peak shapes. Regardless of the larger injection volume (*i.e.*, 100 μL *vs.* 5 μL [26]) and particle size (*i.e.*, 2.5 μm *vs.* 1.7 μm [26]), improved peak shapes were achieved for EHMC and OC, as shown in Fig. 2. Although LC separation is not critical for MS/MS detection, separation of the analytes prevents ion suppression and cross-interference [21]. Here,

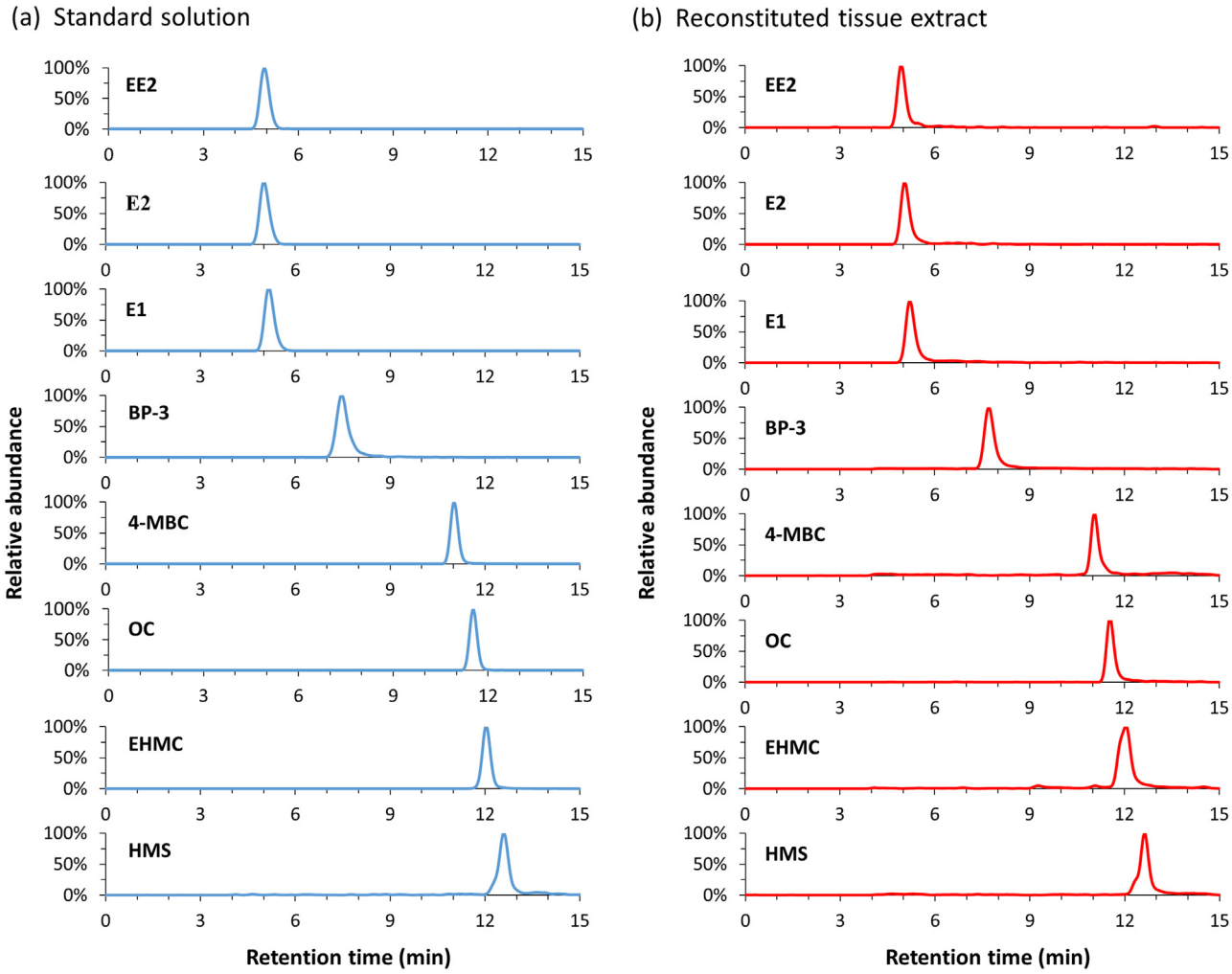

Fig. 2. Selected ion transition chromatograms for (a) standard solution containing 10 μg/L of estrogens and UV-filters and (b) reconstituted crayfish tissue extract spiked with 10 ng of analytes.

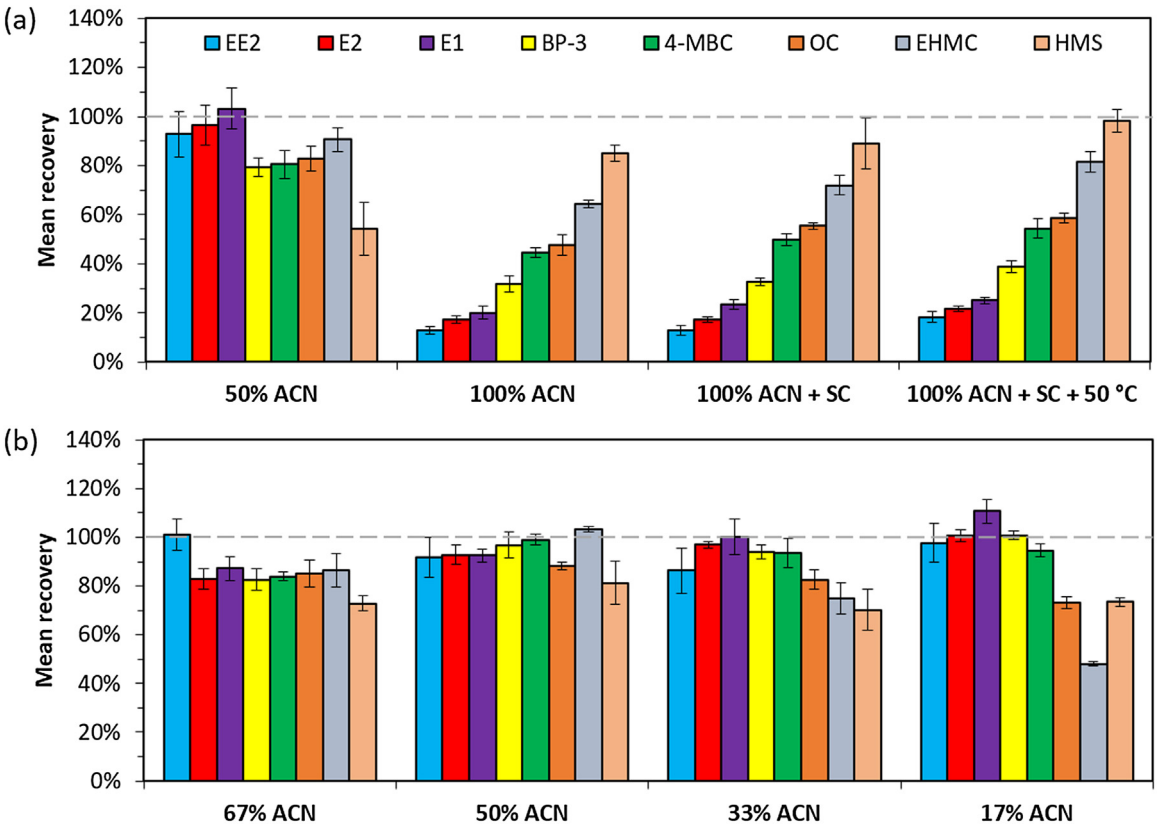

Fig. 3. Percent recovery ($n = 3$; error bars are standard deviation) for 500 ng of analytes spiked into 50 mg tissue (a) without salting out and (b) with salting out using 2.0 g $MgSO_4$ and 0.5 g NaCl per 5 mL DI. The SC label indicates sonication. Analytes are ordered from left-to-right according to their retention within the LC system.

the gradient elution conditions were optimized to balance analyte separation and method run time. As indicated in Fig. 2, the final gradient conditions provide sufficient resolution of individual UV-filters within 15 min. Complete separation of estrogens can be achieved by decreasing the initial composition of the mobile phase to 40% MeOH (not shown); however, this change increases method run time. As ESI interference in negative mode is much lower than in positive mode, co-elution of the estrogens does not cause significant cross-interference [48].

3.2. Optimization of the extraction and cleanup process

3.2.1. Modified QuEChERS extraction

UV-filters and estrogens have been successfully extracted from tissues separately. Meinerling and Daniels determined concentrations of four UV-filters in rainbow trout using a Soxhlet extraction followed by GPC and a Florisil treatment [24]. Zenker et al. [25] developed a simple solvent extraction with vigorous mixing followed by fraction collection for analysis of nine UV-filters in fish. Gago-Ferrero et al. [34] noted the high solvent consumption and time requirements of the above methods and built [14] an ASE protocol followed by SPE with C18 cartridges for determination of eight UV-filters in fish. Peng et al. [26] reported measurement of six UV-filters and seven benzotriazole UV-stabilizers using ultrasonic-assisted extraction with MeOH, purification using GPC coupled to silica gel column fractionation. For estrogens, Kwon et al. [27] extracted EE2 with a mixture of water and MeOH (1:4, *v/v*) from fish liver, with subsequent cleanup by C18 and HLB SPE cartridges in series. Kaklamanos et al. [39] established a protocol for determination of 20 anabolic steroids in bovine muscle using hydrolysis, extraction by methyl *tert*-butyl ether, hexane-based defatting, and cleanup by HLB and Amino SPE cartridges. The extraction and concentration protocols were simplified into one step by Chen et al. [28], who used matrix solid-phase dispersion with C8, followed by an alumina cartridge for cleanup.

Soxhlet, accelerated solvent, and ultrasound-, sonication-, and microwave-assisted extraction techniques all decrease solvent consumption and increase extraction efficiency by speeding up the time to equilibrium. All of these techniques involve one step, namely solid-liquid extraction with a specific solvent. As a result, optimization of the extraction efficiency is mainly a function of solvent composition and cycling [14,25–29,39]. The QuEChERS protocol [40] works differently. Briefly, pesticides were extracted from a 10 g fruit sample using 10 mL ACN. Then, 4 g of $MgSO_4$ and 1 g NaCl were added to induce phase separation. A 1 mL aliquot of the upper ACN layer was transferred for cleanup by dispersive SPE (dSPE). As such, the QuEChERS protocol includes two extraction steps: (1) extraction with 54–60% ACN and (2) salting out of analytes in 92% ACN [40]. Red swamp crayfish tissue was used for protocol development. The QuEChERS extraction performance on UV-filters and estrogens was first investigated by spiking 500 ng of each analyte into 50 mg of lyophilized tissue and extracting those analytes with different concentrations of ACN. The extract was diluted with mobile phase to minimize MEs, and results are summarized in Fig. 3.

As shown in Fig. 3a, higher extraction recoveries were achieved with 50% ACN compared to 100% ACN, with the exception of HMS. In 100% ACN, extraction increased from EE2 to HMS, and followed the same trend as LC retention time (see Fig. 2). Retention times in C18 reversed-phase chromatography align with hydrophobicity. As expected, sonication (20 min) and heating (50 °C) improved extraction efficiency in pure ACN; however, recoveries were still lower than the 50% ACN condition, suggesting that extract composition is more important. These results indicate that pure ACN is

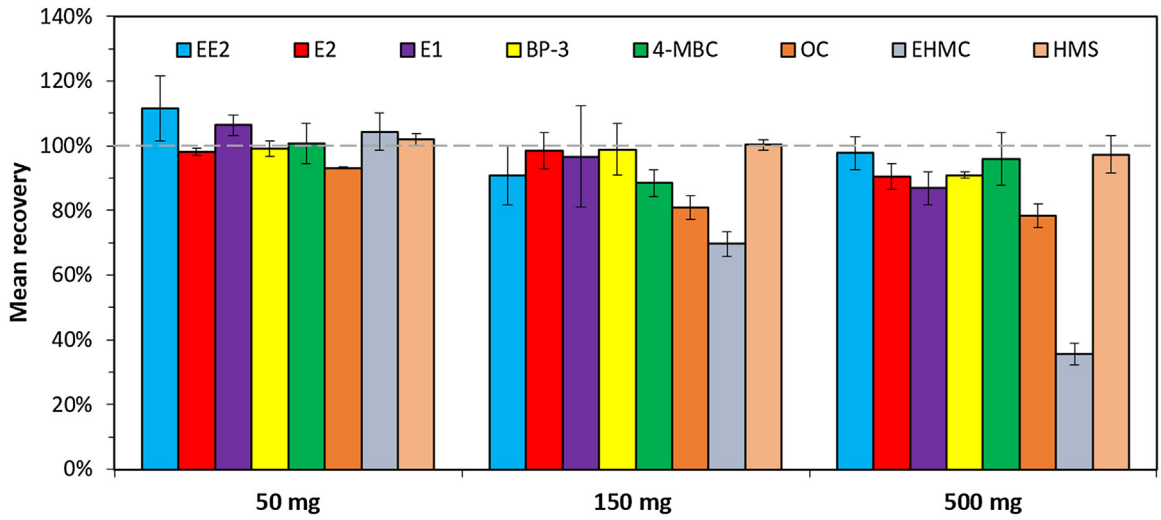

Fig. 4. Recovery of 500 ng of the UV-filter and estrogen analytes from 50, 150, and 500 mg of tissue for extraction with 5 mL DI and 5 mL ACN, containing 2.5 g $MgSO_4$ and 1.0 g NaCl.

too nonpolar to effectively extract the wide-range of UV-filters and estrogens, while 50% ACN is not suitable for HMS extraction.

The two extraction steps associated with QuEChERS protocols were hypothesized to provide improved recovery of a wide variety of estrogens and UV-filters. Indeed, Fig. 3b shows that greater than 80% recovery was attained for EE2, E2, E1, BP-3, and 4-MBC for all tested ACN concentrations, while the recoveries of OC, EHMC, and HMS decreased in 17–33% ACN. The relatively low recovery of the more hydrophobic compounds at the 17–33% ACN condition likely stems from the lower ACN content in the solvent layer formed during the salting out process. These trends should also apply to other hydrophilic and hydrophobic CECs. Specifically, a QuEChERS-based protocol can be effectively used to extract CECs that are less hydrophobic than 4-MBC through adjustment of the initial ACN content. If target CECs are more hydrophobic than HMS, direct solvent extraction is sufficient. This conclusion explains previous observations of low recovery efficiencies for polar pharmaceuticals in QuEChERS systems that use 67% ACN [49].

The type and concentration of salt used during QuEChERS is critical for the formation of the binary system and the purity of the ACN layer. As good recovery (>80%) was achieved with $MgSO_4$ and NaCl, this investigation focuses on optimization of their concentrations in the 50% ACN scenario. From Fig. S.2a (see Supporting Information), the mean recoveries of analytes were 93%, 101%, and 94% for 0.5, 1.0, and 1.5 g NaCl, respectively, with 2.0 g $MgSO_4$. As the NaCl concentration increased, better separation of the binary system was achieved, resulting in a purer ACN layer. The existence of this optimal condition agrees with previous observations [33,40]. This trend was also observed with 2.5 g $MgSO_4$, for which mean recoveries of 89%, 102%, and 95% were observed with 0.5, 1.0, and 1.5 g NaCl, respectively (Fig. S.2b, see Supporting Information). No statistical difference was noted in recovery efficiency for 1.0 g NaCl with 2.0 or 2.5 g $MgSO_4$; however, lower MEs were observed after salting out with 2.5 g $MgSO_4$, as shown in Fig. S.3 (see Supporting Information). Higher $MgSO_4$ concentrations were not considered due to salt precipitation. The optimized salting out conditions were 1.0 g NaCl and 2.5 g $MgSO_4$ per 5 mL DI, providing excellent recoveries (>93%) for all the analytes.

Because analyte extraction depends on solvent volume and tissue mass, partitioning experiments were conducted with different ratios of extract volume to sample mass. Specifically, 5 mL DI and 5 mL ACN were used to extract analytes spiked into 50, 150, and 500 mg tissue samples, corresponding to organic solvent-to-mass ratios of 100, 33, and 10 mL/g, respectively. The resultant extraction efficiencies are summarized in Fig. 4. With the exception of EHMC, greater than 70% recovery was obtained for all analytes in 500 mg tissue. The relatively low recovery of EHMC agrees with previous observations in fish liver [42] and reinforces the need for suitable internal standards for each analyte. Nevertheless, the observed extraction efficiency is comparable to ASE [14] and higher than vigorous mixing [25,27,39], Soxhlet [24], and ultrasonic-assisted [26] extraction protocols. As smaller sample sizes facilitate method transferability to other organisms, decrease solvent consumption, and lower the introduction of interfering substances to the LC–MS/MS system, 50 mg tissue samples were selected for use in this method.

3.2.2. *Extract cleanup with reverse-SPE*

As tissue extracts are complex matrices and LC–MS/MS techniques are sensitive to MEs, a cleanup step was added to remove interference. Although SPE and GPC have been successfully applied to reduce MEs for UV-filters [14,26] and estrogens [27,28,39], these techniques involve tedious operation and potential analyte loss during the loading and washing steps. The dSPE process, which is typically included in QuEChERS protocols, simplifies cleanup by absorbing interfering substances [40]. The main difference between SPE and dSPE is the targeted affinity of the sorbent. For SPE, the sorbent is designed to have a high affinity for the analytes, whereas dSPE sorbents preferentially sorb interfering substances. The principle of dSPE was adopted here. Specifically, the extract was directly added to an SPE cartridge to remove interference, filter insoluble compounds/salts, and prevent volume loss. This simple cleanup approach is termed reverse-SPE.

As nonpolar compounds like lipids are the main source of interference in tissue extracts [33,42], hydrophobic interaction-based SPE chemistries were tested for reverse-SPE application. The retention of UV-filters and estrogens was investigated by directly passing 5 mL ACN containing analytes through different SPE cartridges. ACN was collected from the bottom of the cartridge for analyte analysis, and the resulting recoveries are shown in Fig. 5. For 60 mg HLB cartridges, good recovery (>80%) was achieved for all analytes; however, the recovery of estrogens decreased significantly as the sorbent mass increased, in agreement with previous observations that HLB strongly retains estrogens [27,39]. Approximately 60–75% recovery was achieved for UV-filters with the 500 mg cartridges. The high recovery of UV-filters indicates that HLB provides

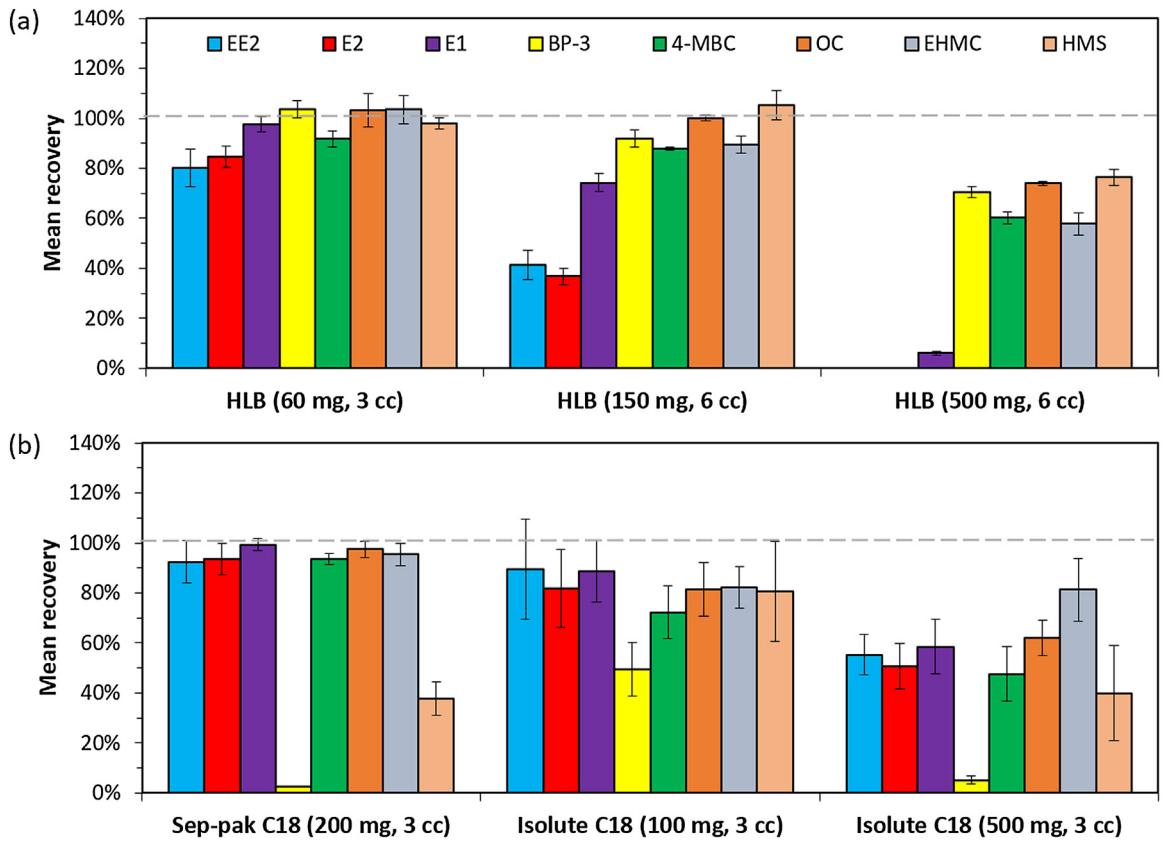

Fig. 5. UV-filter and estrogen recovery measured after passing 5 mL ACN with 2 μg/L of each analyte through (a) HLB and (b) C18 cartridges. Error bars are standard deviation ($n = 3$); the "cc" label is cm^3.

relatively weak retention of these chemicals in ACN, in agreement with previous observations [14]. The Sep-Pak and Isolute C18-based cartridges showed a strong retention of BP-3 and HMS over other analytes. In general, the retention of estrogens was similar to the 4-MBC, OC, and EHMC UV-filters. Further investigation focused on HLB (60 mg, 3 cm^3) and Isolute C18 (100 mg, 3 cm^3) because these two cartridges were the only ones that demonstrated acceptable recovery (>50%) for all analytes.

Application of the HLB (60 mg, 3 cm^3) and Isolute C18 (100 mg, 3 cm^3) cartridges was studied using tissue extracts spiked with analytes. In particular, 2.5 mL of tissue extract with analytes was passed through the cartridges, and then additional ACN was used to further elute the analytes. Fig. 6 presents the overall recovery of UV-filters and estrogens during reverse-SPE. Analyte recovery with Isolute C18 and HLB after the initial pass ranged from 49% for HMS to 90% for EHMC and 57% for E2 to 97% for OC, respectively. After an additional elution step with 2 mL of ACN, at least 80% recovery was achieved for all analytes with HLB cartridges, while the recovery of BP-3 and HMS was still below 70% for the Isolute C18 cartridges. Although recovery could be further improved with additional ACN, this step increases solvent consumption and could potentially introduce more interference from the tissue extract.

The removal of interference was investigated by quantifying MEs on internal standards reconstituted in tissue extract, as shown in Fig. 7. As indicated, both HLB and Isolute C18 cartridges reduce MEs after reverse-SPE, particularly for 4-MBC-d_4, OC-d_{15}, and EHMC-d_{15}. The reduced MEs obtained for internal standards agree with direct observation of the change in extract color following reverse-SPE operation (see Fig. S.4 in the Supporting Information). Signal suppression of internal standards in negative mode (*i.e.*, EE2-d_4, E2-d_3, and HMS-d_4) was lower than positive mode (*i.e.*, BP-3-d_5, 4-MBC-d_4, OC-d_{15}, and EHMC-d_{15}). HLB provided better recovery and similar reductions in MEs. Therefore, the optimized reverse-SPE procedure involved passing 2.5 mL tissue extract through the HLB (60 mg, 3 cm^3) cartridges. For UV-filters, the signal suppression ranged from 21% to 49%, comparable to previous reports that employed SPE [14,26].

3.3. Method validation

Analyte identification involved two carefully selected product ions. The relative responses of these ions were compared at a tolerance of 5% to confirm detection of the precursor ion (*i.e.*, the analyte). Seven surrogates/internal standards were used to compensate for instrumental variability and MEs. This report provides the first documented use of the internal standards, HMS-d_4, OC-d_{15}, and EHMC-d_{15}, for analytical purposes. As indicated in Figs. 7 and S.3 (see Supporting Information), the MEs on different compounds, especially UV-filters, are significantly different. Therefore, the use of deuterated internal standards for each individual UV-filter becomes critical to properly correct for MEs and provide reliable analyte quantitation.

Instrumental analytical performance metrics, including the coefficient of determination (R^2) for calibration curves, linear range, IDL, instrumental quantitation limit (IQL), and inter- and intra-day reproducibility, are summarized in Table 1. The calibration curves for all analytes were linear over a wide range of concentrations with $R^2 > 0.99$. Limits of detection and quantitation were defined as the analyte concentrations with signal-to-noise ratios of 3 and 10, respectively; these concentrations were multiplied by the injection volume to calculate the IDLs and IQLs. The relative standard deviation of the inter- and intra-day reproducibility tests was below 10%, indicating good method precision for all analytes. Overall method performance was further investigated through standard

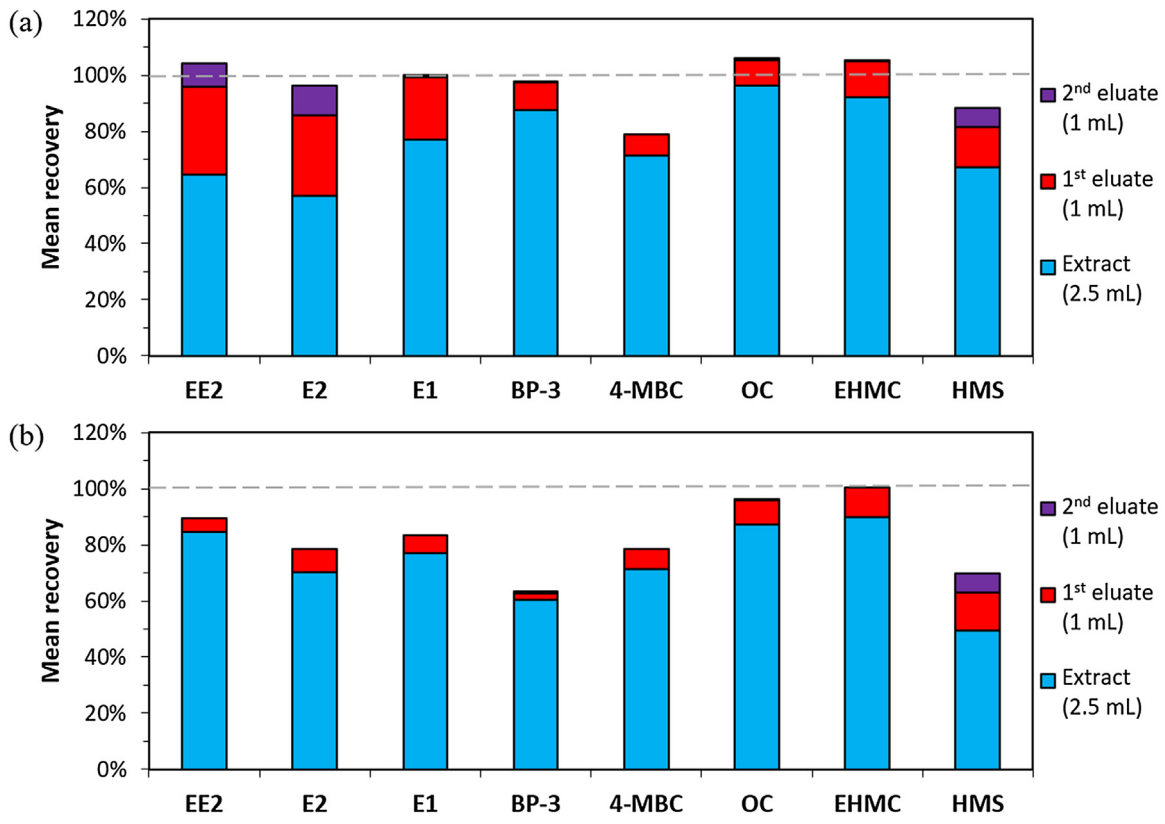

Fig. 6. Analyte recoveries from spiked tissue extracts (4 μg/L of each analyte) after reverse-SPE with (a) HLB (60 mg, 3 cm^3) and (b) Isolute C18 (100 mg, 3 cm^3) cartridges.

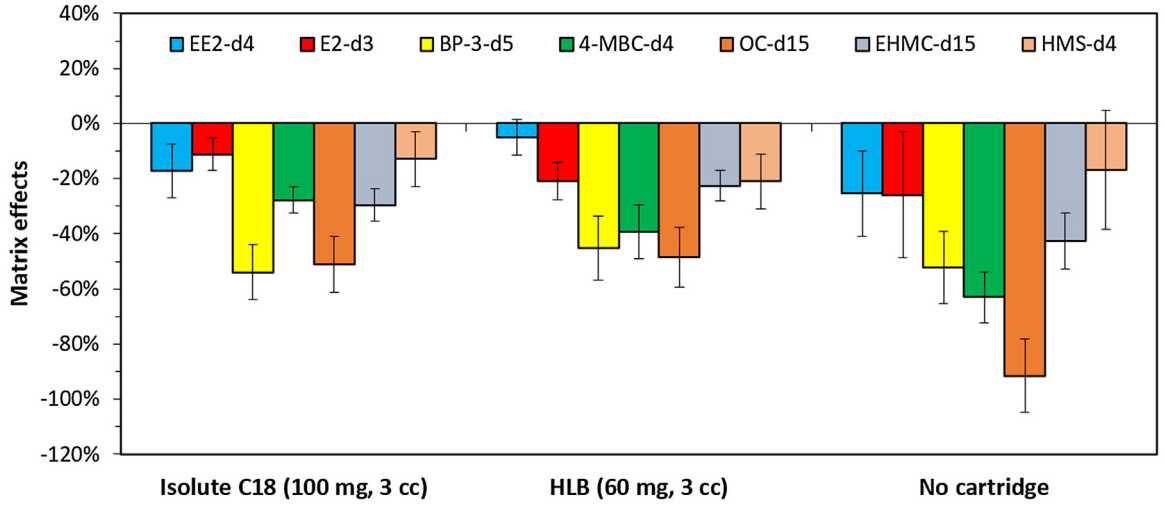

Fig. 7. Cleanup performance of reverse-SPE on tissue extracts containing 5 μg/L of each internal standard.

addition of analytes into tissue samples. Fig. S.5 in the Supporting Information shows that analyte recovery was high for tissue samples collected from eastern oysters, red swamp and virile crayfish, and hooked mussels, confirming the broad applicability of the optimized method for aquatic and marine organisms.

3.4. Application to environmental samples

The optimized sample extraction, cleanup, and analysis protocols were applied to determine UV-filter and estrogen concentrations in invertebrates collected from the Chesapeake Bay watershed and purchased from a scientific supplier. Tissue-phase concentrations are summarized in Table 2. All target analytes were detected at least once, and HMS was present in all samples. The 4-MBC (75–352 ng/g), OC (3–113 ng/g), and HMS (78–399 ng/g) UV-filters were present in all virile crayfish collected from an urban watershed (Baltimore, MD). In addition, BP-3 and EHMC were detected for select sites at concentrations up to 51 and 83 ng/g, respectively. For one site, 17 ng/g of the synthetic estrogen EE2 was present in crayfish tissue. This measured concentration was similar to previously reported values in fish [29,32], suggesting potential wastewater contamination. Many of these CECs were also measured in red swamp crayfish procured from ARO: 16 ng/g EE2; 43 ng/g BP-3; 3 ng/g OC; and, 174 ng/g HMS.

Table 2
Concentrations (ng/g lyophilized tissue) of analytes in the tissue of aquatic organisms. Error is standard deviation (n = 3).

Organism	Site[a]	EE2	E2	E1	BP-3	4-MBC	OC	EHMC	HMS
Eastern crayfish	BARN	n.d.[b]	n.d.	n.d.	n.d.	214 ± 23	60.6 ± 9.0	63.5 ± 7.2	399 ± 48
	DR1	n.d.	n.d.	n.d.	37.9 ± 4.4	352 ± 12	5.0 ± 0.1	n.d.	113 ± 7
	DR2	n.d.	n.d.	n.d.	n.d.	75.3 ± 11	37.1 ± 3.9	83.0 ± 5.1	263 ± 43
	DR3	n.d.	n.d.	n.d.	51.4 ± 2.2	97.8 ± 11	6.7 ± 0.3	n.d.	108 ± 3
	DR4	n.d.	n.d.	n.d.	n.d.	106 ± 17	113 ± 6	n.d.	260 ± 16
	DR5	17.1 ± 1.6	n.d.	n.d.	23.7 ± 0.3	112 ± 12	4.5 ± 0.4	n.d.	201 ± 20
	DRKR	n.d.	n.d.	n.d.	29.5 ± 0.3	190 ± 18	3.4 ± 0.2	n.d.	77.6 ± 7.5
Red swamp crayfish	ARO	15.5 ± 0.8	n.d.	n.d.	42.8 ± 5.1	n.d.	2.6 ± 0.3	n.d.	174 ± 7
Eastern oyster	ARO	n.d.	n.d.	n.d.	51.7 ± 2.5	n.d.	21.5 ± 3.8	n.d.	211 ± 21
	CBCR-2	n.d.	n.d.	n.d.	40.6 ± 7.5	n.d.	n.d.	241 ± 35	143 ± 40
	CBCR-3	19.1 ± 1.2	n.d.	n.d.	36.8 ± 2.5	n.d.	6.6 ± 0.7	155 ± 20	56.1 ± 5.6
Hooked mussel	CBCR-3	15.3 ± 0.7	15.5 ± 0.5	70.3 ± 3.2	35.4 ± 1.5	n.d.	14.4 ± 0.6	240 ± 13	107 ± 4

[a] BARN, Baisman Run; DR1-5, Dead Run Sites 1–5; DRKR, Dead Run at Franklintown; ARO, Aquatic Research Organisms; CBCR sites were located at the mouth of the Chester River, Chesapeake Bay.
[b] n.d. = not detected.

Eastern oysters and hooked mussels collected from the Chesapeake Bay demonstrated accumulation of estrogens and UV-filters. The higher CEC levels present in organisms from CBCR-3 may stem from land use impacts, since CBCR-2 was further downstream and, therefore, more dilute. The hooked mussel tissue collected from CBCR-3 contained all analytes except 4-MBC. Measured concentrations of EHMC were similar to a previously reported value for marine mussels [41]. Similar to the red swamp crayfish, BP-3, OC, and HMS were also detected in eastern oysters from ARO. The source of the UV-filters and synthetic estrogens in these organisms is not clear, but reinforces the fact that these CECs are ubiquitously present in the environment. Given the toxicity concerns associated with these two important contaminant classes, adoption of this optimized reverse-SPE-LC–MS/MS method to monitor estrogen and UV-filter levels in macroinvertebrates will provide important information to secure ecological health.

4. Conclusions

This study involved the development and validation of an LC–MS/MS method for simultaneous determination of multiple UV-filters and estrogens in aquatic invertebrates. Target compounds were extracted through a modified QuEChERS extraction, cleaned up with a novel reverse-SPE process, and simultaneously analyzed in one LC–MS/MS method that exploited wrong-way-round ionization and combined positive and negative modes. Satisfactory and reproducible recoveries were achieved for all analytes, and the IDLs were lower than previously reported methods, indicating improved method sensitivity. This novel method was successfully applied to measure UV-filters and estrogens in tissue from aquatic and marine organisms with a wide range of lipid content. The frequency of detection of UV-filters in collected biota demonstrated the ubiquitous presence and accumulation of these CECs in the environment. These findings highlight the need for further studies to concurrently investigate the accumulation, toxicity, and risk associated with CEC uptake in macroinvertebrates. The fast and effective analytical method reported here enables those future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.06.039.

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