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Multi-residue determination of micropollutants in *Phragmites australis* from constructed wetlands using microwave assisted extraction and ultra-high-performance liquid chromatography tandem mass spectrometry

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Bruce Petrie [a, b, 1], Benjamin D. Smith [a], Jane Youdan [c], Ruth Barden [c], Barbara Kasprzyk-Hordern [a, b, *]

[a] *Department of Chemistry, University of Bath, Bath, BA2 7AY, UK*
[b] *Water Innovation & Research Centre (WIRC), University of Bath, Bath, BA2 7AY, UK*
[c] *Wessex Water, Bath, BA2 7WW, UK*

HIGHLIGHTS

- First method developed for micropollutants in *Phragmites australis*.
- Multi-residue determination of 81 micropollutants.
- Method quantitation limits were <5 ng g^{-1} dry weight.
- 17 micropollutants (including metabolites) found up to 200 ng g^{-1}.

GRAPHICAL ABSTRACT

ABSTRACT

In constructed wetlands micropollutants can be removed from water by phytoremediation. However, micropollutant uptake and metabolism by plants here is poorly understood due to the lack of good analytical approaches. Reported herein is the first methodology developed and validated for the multi-residue determination of 81 micropollutants (pharmaceuticals, personal care products and illicit drugs) in the emergent macrophyte *Phragmites australis*. The method involved extraction by microwave accelerated extraction (MAE), clean-up using off-line solid phase extraction and analysis by ultra-high-performance liquid chromatography tandem mass spectrometry. Development of the MAE method found the influence of studied variables on micropollutant recovery to be: extraction temperature > sample mass > solvent composition. Validation of the developed extraction protocol revealed method recoveries were in the range 80–120% for the majority of micropollutants. Method quantitation limits (MQLs) were generally <5 ng g^{-1} dry weight demonstrating the sensitivity of the methodology. Application of the method to *P. australis* from a constructed wetland used to treat trickling filter effluent found 17 micropollutants above their MQL, up to concentrations of 200 ng g^{-1}. Other than uptake, the presence of several metabolites (carbamazepine 10,11 epoxide, desvenlafaxine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, *N*-desmethyltramadol and norketamine) indicated metabolism within the plant may also occur. This new analytical methodology will enable a process mass balance of the constructed wetland to be attained for the first time, and thus help understand the role of phytoremediation in micropollutant removal by such systems.

* Corresponding author. Department of Chemistry, University of Bath, Bath, BA2 7AY, UK.
  E-mail address: b.kasprzyk-hordern@bath.ac.uk (B. Kasprzyk-Hordern).
[1] Current address: School of Pharmacy and Life Sciences, Robert Gordon University, Aberdeen AB10 7JG, UK.

1. Introduction

Organic micropollutants such as pharmaceuticals, personal care products and illicit drugs are ubiquitous in surface waters at the low ng L^{-1} to μg L^{-1} concentration range [1,2]. This is of concern as they can exert adverse toxicological responses to aquatic biota [3]. The presence of these micropollutants in the aquatic environment is mainly attributed to their incomplete removal during conventional wastewater treatment [2]. Currently, low-cost tertiary treatment options such as constructed wetlands are being considered for effluent polishing. Their primary use is to enhance the removal of macropollutants such as suspended solids, organic matter and nutrients. However, fortuitous removal of micropollutants such as pharmaceuticals is also observed [4].

Constructed wetlands tend to be surface flow (water flows over a benthic substrate) or sub-surface flow (water flows through a porous medium such as gravel) [4]. These systems utilize macrophytes (such as *Phragmites australis* − the common reed) which are thought to contribute to the removal of micropollutants during treatment [5]. Several studies have investigated the removal of micropollutants by constructed wetlands [6−10]. This has involved comparing micropollutant concentrations or loads in receiving water and treated water. On the other hand, laboratories studies have been conducted using macrophytes and a synthetic water medium containing micropollutants of interest [11−13]. Such studies tend to rely on measuring the reduction in micropollutant concentration in the water medium to quantify uptake. However, little analysis has been conducted directly on plants grown in constructed wetlands treating real wastewater. This is due to the lack of good analytical approaches for the determination of micropollutants in plant matter found in constructed wetlands. To date, there are no fully validated methods reported in the literature specifically developed for the determination of micropollutants in plants found in constructed wetlands.

Numerous methods have previously been developed and validated for the determination of micropollutants in other plant types such as vegetables and cereals [14−18]. In such matrices, method quantitation limits (MQLs) range from <1 to ~1000 ng g^{-1}. Some studies have applied these methods to determine a limited number of micropollutants in plants from constructed wetlands. Zarate et al. [19] investigated the personal care products triclosan, methyltriclosan and triclocarban in three constructed wetland macrophytes: *Typha latifolia*, *Pontederia cordata* and *Sagittaria graminea*. Both triclosan and triclocarban were ubiquitous in the studied plant tissues at mean concentrations up to ~50 ng g^{-1} [19]. Hijosa-Valsero et al. [20] investigated several pharmaceuticals and personal care products (naproxen, ibuprofen, ketoprofen, salicylic acid, caffeine, diclofenac, carbamazepine, galaxolide, methyldihydrojasmonate and tonalide) in the roots of *P. australis* with concentrations up to 2500 ng g^{-1} found. The most commonly applied extraction methods for organic micropollutants from solid environmental matrices are ultra-sonic extraction (USE), microwave accelerated extraction (MAE) and pressurised liquid extraction (PLE) [20−23]. Dorival-Garcia et al. [21] found little difference in extraction efficiency of quinolone antibiotics from sewage sludge using USE, MAE and PLE. However, both MAE and PLE enable greater automation compared to USE [21]. Furthermore, MAE can provide further time savings as several samples can be extracted simultaneously.

Due to the lack of methods reported in the literature for the determination of micropollutants in plants grown in constructed wetlands, the aim of this study was to develop a robust and sensitive method for the extraction of a high number of micropollutants in *P. australis*. This is the first time a method has been developed and validated specifically for the determination of micropollutants in this matrix. A total of 81 micropollutants were investigated to encompass a range of physico-chemical and biological properties (Table S1). This was achieved by developing a new MAE method, and utilising off-line solid phase extraction (SPE) and ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

2. Materials and methods

2.1. Materials

A total of 81 micropollutants representing a varied range of physico-chemical and biological properties were investigated during the study (Table S1). The labelled internal standards acetaminophen-D4, ibuprofen-D3, bisphenol A-D16, carbamazepine-13C6, ketoprofen-D3, naproxen-D3, propranolol-D7 and atenolol-D5 were purchased from Sigma-Aldrich (Gillingham, UK). Bezafibrate-D6 was obtained from QMX laboratories (Thaxted, UK). Methylparaben-13C, amphetamine-D5, methamphetamine-D5, 3,4-methylenedioxy-methamphetamine-D5 (MDMA-D5), 3,4-methylenedioxy-amphetamine-D5 (MDA-D5), heroin-D9, codeine-D6, ketamine-D4, cocaine-D3, benzoylecgonine-D8, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine-D3 (EDDP-D3), morphine-D3, cotinine-D3, cocaethylene-D8, temazepam-D5, 1S,2R-(+) ephedrine-D3, mephedrone-D3, methadone-D9, norketamine-D4, estrone (2,4,16,16-D4), estradiol (2,4,16,16-D4) and quetiapine-D8 hemifumarate were purchased from LGC standards (Middlesex, UK). Citalopram-D6, metoprolol-D7, and mirtazapine-D3 were obtained from TRC (Toronto, Canada). All standards were purchased as 0.1 or 1.0 mg mL^{-1} solutions or in powder form. Chemicals in powder form were prepared at a concentration of 1 mg mL^{-1} in the recommended solvent and stored at −20 °C. Stock solutions of antibiotics were prepared monthly [23].

Methanol (MeOH) and toluene was HPLC grade and purchased from Sigma-Aldrich. Water (H$_2$O) was of 18.2 MΩ quality (Elga, Marlow, UK). All glassware was deactivated using 5% dimethylchlorosilane (DMDCS) in toluene (Sigma−Aldrich) to mitigate the loss of basic chemicals onto -OH sites present on glass surfaces. This consisted of rinsing once with DMDCS, twice with toluene and three times with MeOH. Ammonium acetate (NH$_4$OAc), ammonium fluoride (NH$_4$F) and acetic acid (1.0 M, CH$_3$COOH) used for the preparation of mobile phases were purchased from Sigma-Aldrich. Oasis HLB (60 mg, 3 mL) SPE cartridges were purchased from Waters (Manchester, UK).

2.2. Constructed wetlands

P. australis were collected from a constructed wetland in the South-West of the UK. The wastewater treatment plant itself consists of primary sedimentation, secondary treatment by trickling filters and sedimentation followed by constructed wetlands as a final polishing step. The site serves a population equivalent of 12,500. The constructed wetlands consisted of six different beds, all being non-aerated horizontal sub-surface flow with differing substrates planted with *P. australis*. The depth of substrate was approximately 1 m in each bed resulting in a hydraulic retention time of approximately 14 h under dry weather conditions. *P. australis* were collected on four consecutive days near both the inlet and outlet of the bed containing gravel as the substrate. This bed was considered well-established as *P. australis* had been growing for one year and were generally 1.5−2.0 m in height. *P. australis* were transported to the laboratory on ice, and roots washed with tap water to remove excess sediment. These were then frozen immediately at −20 °C prior to further processing. It is assumed that freezing reeds upon collection inhibits the degradation of target micropollutants until analysis.

2.3. Microwave assisted extraction

Once frozen, individual plants were freeze dried (ScanVac, CoolSafe freeze dryer, Lynge, Denmark). These were then homogenized using a mechanical blender (Kenwood, Havant, UK). Representative 0.5 g samples were spiked with 50 ng of all internal standards (in 50 μL MeOH) and left for a minimum of 2 h. Extraction was performed using 25 mL of 25:75 MeOH:H$_2$O at a temperature of 50 °C using a 800 W MARS 6 microwave (CEM, UK). Samples were heated to 50 °C over 10 min and maintained at this temperature for 30 min. Once cooled samples were filtered through glass fibre membranes (0.7 μm) and diluted with H$_2$O to achieve a final MeOH concentration of <5%, ready for SPE.

During the development process, extraction temperatures of 30, 50, 70, 90, 110 and 130 °C were tested. Extraction solvent compositions of 25:75, 50:50 and 75:25 MeOH:H$_2$O and sample masses of 0.5, 1.0 and 2.0 g were also investigated. When trialling different extraction solvent compositions, all samples were diluted to 500 mL using H$_2$O to avoid varying amounts of MeOH in the extract having any influence on SPE recovery. To validate the method and determine recoveries, samples were spiked at concentration levels of 25, 50 and 125 ng g^{-1}. All analysis was performed in triplicate.

2.4. Solid phase extraction

An existing SPE protocol was applied as it was known to be successful for the simultaneous extraction of all micropollutants investigated here [23]. Oasis HLB cartridges were conditioned using 2 mL MeOH followed by 2 mL H$_2$O at a flow rate of 1 mL min^{-1}. Samples were then loaded at a constant flow rate of 5 mL min^{-1} and dried under vacuum. Analytes were then eluted using 4 mL MeOH at a flow rate of 1 mL min^{-1}. These extracts were dried under nitrogen at 40 °C using a TurboVap evaporator (Caliper, UK, <5 psi).

Dried extracts were reconstituted in 500 μL 80:20H$_2$O:MeOH, filtered through pre-LC-MS 0.2 μm PTFE filters (Whatman, Puradisc) and transferred to polypropylene vials (Waters, Manchester, UK) ready for UHPLC-MS/MS analysis.

2.5. Ultra-high-performance liquid chromatography tandem mass spectrometry

Two chromatography methods were used to ensure maximum sensitivity was achieved for the varied range of micropollutants studied [23]. Acidic micropollutants were separated using a gradient of 1 mM NH$_4$F in 80:20H$_2$O:MeOH (mobile phase A) and 1 mM NH$_4$F in 5:95H$_2$O:MeOH (mobile phase B). Initial conditions of 100% A were maintained for 0.5 min before reducing to 40% over 2 min and further reduced to 0% over 5.5 min. This was maintained for 6 min before returning to starting conditions which were maintained for 8.4 min to re-equilibrate the column.

For basic micropollutants, separation was achieved using 5 mM NH$_4$OAc and 3 mM CH$_3$COOH in 80:20H$_2$O:MeOH (mobile phase A) and MeOH (mobile phase B). Starting conditions of 100% A were reduced to 10% over 20 min and maintained for a further 6 min. Following this, mobile phase conditions were returned to starting conditions and held for 7.5 min for re-equilibration. Both methods utilised a reversed-phase BEH C18 column (150 × 1.0 mm, 1.7 μm particle size) (Waters, Manchester, UK) fitted with a 0.2 μm, 2.1 mm in-line prefilter to remove particulates using a Waters Acquity UPLC system (Waters, Manchester, UK). The column was maintained at 40 °C and the mobile phase flow rate was 0.04 mL min^{-1}. The injection volume for both methods was 15 μL.

The UPLC system was coupled to a Xevo TQD Triple Quadrupole Mass Spectrometer (Waters, Manchester, UK), equipped with an electrospray ionisation (ESI) source. Acidic micropollutants were analysed in negative ionisation mode (ESI -) with a capillary voltage

Sample preparation
- *Plants frozen at -20 °C*
- *Frozen plants freeze dried and homogenized*
- *0.5 g samples spiked with 50 ng internal standards*
- *Left for 2 h*

Extraction: MAE
- *Extraction at 50 °C using 25 mL 75:25 H$_2$O:MeOH for 30 min*
- *Extracts filtered (0.7 μm) and diluted to 5 % MeOH using H$_2$O*

Clean-up: Oasis HLB SPE
- *Cartridges conditioned 2 mL MeOH then 2 mL H$_2$O*
- *Samples loaded at 5 mL min^{-1} and dried*
- *Elution using 4 mL MeOH*
- *Extracts dried and reconstituted in 80:20 H$_2$O:MeOH*

Analysis of acidic analytes: UHPLC-MS/MS
- *Reverse phase C$_{18}$ column (150 x 1.0 mm; particle size 1.7 μm)*
- *Gradient using 1 mM NH$_4$F in 80:20 H$_2$O:MeOH (A) and 1 mM NH$_4$F in 95:5 MeOH:H$_2$O (B)*
- *100 % A (0.5 min) → 40 % (2 min) → 0 % (5.5 min) → 0 % (6 min) → 100 % (0.1 min) → 100 % (8.4 min)*
- *Column temperature 25 °C*
- *Column starting pressure 8,500 psi*
- *Sample manager 4 °C*
- *Injection volume 15 μL*
- *Analysis in ESI – mode*
- *Total run time 22.5 min*

Analysis of basic analytes: UHPLC-MS/MS
- *Reverse phase C$_{18}$ column (150 x 1.0 mm; particle size 1.7 μm)*
- *Gradient using 5 mM NH$_4$OAc and 5 mM CH$_3$COOH in H$_2$O (A) and MeOH (B)*
- *100 % A → 10 % (20 min) → 10 % (6 min) → 100 % (0.5 min) → 100 % (7.5 min)*
- *Column temperature 25 °C*
- *Column starting pressure 8,000 psi*
- *Sample manager 4 °C*
- *Injection volume 15 μL*
- *Analysis in ESI + mode*
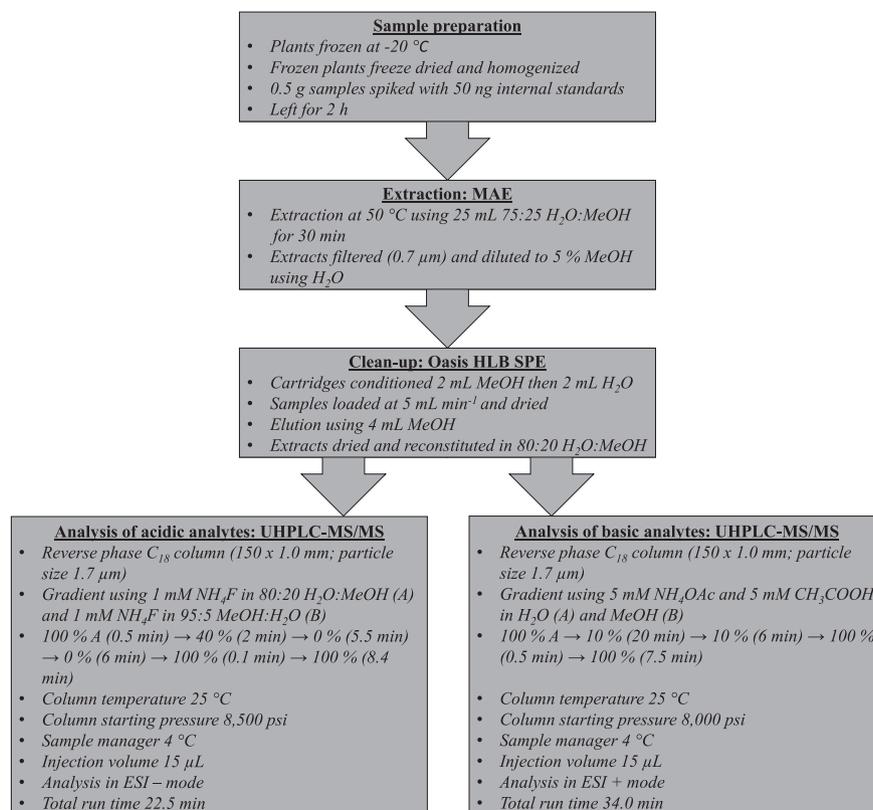- *Total run time 34.0 min*

Fig. 1. Overview of developed analytical protocol used to determine target micropollutants in *P. australis*.

of 3.20 kV. Basic micropollutants were analysed in positive ion-isation mode (ESI +) with a capillary voltage of 3.00 kV. The opti-mised MS/MS transitions can be found in Table S2. In both methods the source temperature was 150 °C whilst the desolvation tem-perature was 400 °C. A cone gas flow of 100 L h^{-1} and a desolvation gas flow of 550 L h^{-1} was used. Finally, the nebulising and des-olvation gases were nitrogen, and the collision gas was argon. An overview of the entire analytical protocol is detailed in Fig. 1.

3. Results and discussion

3.1. Ultra-high-performance liquid chromatography tandem mass spectrometry

The UHPLC-MS/MS method applied in this study was previously developed and optimised for the studied micropollutants as described in Petrie at al [23]. Briefly, instrument detection limits (IDLs) ranged from 0.01 to 1.16 ng mL^{-1} and instrument quantita-tion limits (IQLs) from 0.04 to 5.79 ng mL^{-1} (Table 1). A 17 level calibration curve was prepared with the majority of

micropollutants exhibiting linearity between the IQL and 500 ng mL^{-1} with r^2 of $\geq$0.997 (Table 1). Most micropollutants demonstrated intra- and inter-day precision of <10% (Table S3). Accuracy generally ranged from 90 to 110% both within the same day and between different days (Table S4). For further discussion on the instrument validation please refer to Petrie et al. [23]. To ensure quality of data, standard tolerances of ion ratio and chromato-graphic retention time were employed throughout the study [24].

3.2. Microwave assisted extraction method development

From previous experience and a review of the literature [21–23], three extraction parameters were considered to have the greatest impact on analyte recovery using MAE: (i) extraction temperature, (ii) extraction solvent composition, and (iii) sample mass. Therefore these were the focus of investigation during the development process.

Previously developed MAE methods for the extraction of micropollutants from solid environmental matrices (wastewater sludge, sediments and soils) have used extraction temperatures

Table 1
Instrument detail of studied micropollutants including instrument detection and quantitation limits (n = 81, ordered by micropollutant class).

Micropollutant class	Micropollutant	Rt (min)	ESI mode	Corresponding internal standard[b]	Linearity		IDL$_{S/N}$ (ng mL^{-1})	IQL$_{S/N}$ (ng mL^{-1})
					Range (ng mL^{-1})	r^2		
UV filters	Benzophenone-1	9.6	−	Bisphenol A-D16	0.06–500	0.995	0.01	0.06
	Benzophenone-2	7.9	−	Bisphenol A-D16	0.05–500	0.998	0.01	0.05
	Benzophenone-3	21.2	+	Methadone-D9	0.05–400	0.990	0.01	0.05
	Benzophenone-4	6.9	−	Methylparaben-13C	1.01–500	0.993	0.31	1.01
Parabens	Methylparaben	7.5	−	Methylparaben-13C	0.06–500	1.000	0.01	0.06
	Ethylparaben	8.3	−	Naproxen-D3	0.11–500	0.997	0.03	0.11
	Propylparaben	9.2	−	Naproxen-D3	0.12–400	0.998	0.04	0.12
	Butylparaben	10.1	−	Naproxen-D3	0.06–500	0.999	0.01	0.06
Plasticizer	Bisphenol-A	9.0	−	Bisphenol A-D16	0.10–500	0.998	0.03	0.10
Steroid estrogens	E1	9.8	−	E1-D4	0.49–500	0.999	0.10	0.49
	E2	9.7	−	E2-D4	0.47–500	0.999	0.09	0.47
	EE2	9.8	−	E2-D4	0.48–500	0.995	0.10	0.48
Antibacterials/antibiotics	Sulfasalazine	7.1	−	Naproxen-D3	0.90–500	0.996	0.27	0.90
	Clarithromycin	18.9	+	Methadone-D9	0.06–500	1.000	0.01	0.06
	Azithromycin	14.0	+	EDDP-D3	0.11–500	0.999	0.03	0.11
	Trimethoprim	8.4	+	Methamphetamine-D5	0.10–500	0.999	0.03	0.10
	Sulfamethoxazole	9.6	+	Benzoylecgonine-D8	0.10–500	0.999	0.03	0.10
Hypertension	Valsartan	7.6	−	Naproxen-D3	1.12–500	0.993	0.34	1.12
	Irbesartan	8.6	−	Bisphenol A-D16	0.50–500	0.998	0.10	0.50
NSAIDs	Ketoprofen	7.7	−	Ketoprofen-D3	0.54–500	0.998	0.11	0.54
	Ibuprofen	9.8	−	Ibuprofen-D3	0.05–500	0.999	0.01	0.05
	Naproxen	8.1	−	Naproxen-D3	0.49–500	0.998	0.10	0.49
	Diclofenac	9.0	−	Naproxen-D3	0.10–500	0.995	0.03	0.10
	Acetaminophen	5.1	+	Acetaminophen-D4	0.54–500	0.999	0.11	0.54
Lipid regulators	Bezafibrate	7.9	−	Bezafibrate-D6	0.10–500	1.000	0.03	0.10
	Atorvastatin	9.3	−	Naproxen-D3	0.05–500	0.990	0.01	0.05
Diabetes	Gliclazide	17.8	+	CBZ-13C6	0.05–500	0.998	0.01	0.05
Antihistamines	Fexofenadine	8.4	−	Bisphenol A-D16	0.09–500	0.998	0.03	0.09
	Cetirizine	18.7	+	Temazepam-D5	0.08–500	1.000	0.02	0.08
Cough suppressant	Pholcodine	3.7	+	Atenolol-D7	1.14–500	0.994	0.35	1.14
Beta-blocker	Atenolol	4.3	+	Atenolol-D7	0.10–500	1.000	0.03	0.10
	Metoprolol	11.2	+	Metoprolol-D7	0.05–500	0.999	0.01	0.05
	Propranolol	15.1	+	Propranolol-D7	0.09–500	0.999	0.03	0.09
H$_2$ receptor agonists	Ranitidine	4.6	+	Cotinine-D3	5.17–500	0.998	1.03	5.17
	Cimetidine	5.3	+	Codeine-D6	0.52–500	0.998	0.10	0.52
X-ray contrast media	Iopromide	4.9	+	Atenolol-D7	5.79–500	0.990	1.16	5.79
Drug precursor and metabolite	Ephedrine/pseudoephedrine	7.2	+	1S,2R-(+) Ephedrine-D3	0.10–500	1.000	0.03	0.10
	Norephedrine[a]	6.3	+	1S,2R-(+) Ephedrine-D3	0.50–500	0.999	0.01	0.50
Anti-cancer	Azathioprine	7.8	+	Cotinine-D3	0.10–500	0.998	0.03	0.10
	Methotrexate	7.9	+	Amphetamine-D5	0.92–500	0.996	0.28	0.92
	Ifosfamide	12.7	+	Metoprolol-D7	0.05–500	0.999	0.01	0.05
Anaesthetic and metabolite	Ketamine	10.6	+	Ketamine-D4	0.05–500	0.999	0.01	0.05
	Norketamine[a]	11.1	+	Norketamine-D4	0.10–500	0.999	0.03	0.10
Anti-depressants and metabolites	Venlafaxine	14.1	+	Metoprolol-D7	0.04–500	1.000	0.01	0.04
	Desmethylvenlafaxine[a]	10.8	+	Metoprolol-D7	0.10–500	0.998	0.03	0.10

Table 1 (*continued*)

Micropollutant class	Micropollutant	Rt (min)	ESI mode	Corresponding internal standard[b]	Linearity		IDL$_{S/N}$ (ng mL^{-1})	IQL$_{S/N}$ (ng mL^{-1})
					Range (ng mL^{-1})	r^2		
	Mirtazapine	13.5	+	Mirtazapine-D3	0.05–500	0.999	0.01	0.05
	Citalopram	15.1	+	Citalopram-D6	0.50–500	0.999	0.05	0.50
	Desmethylcitalopram[a]	15.2	+	Citalopram-D6	0.05–500	0.999	0.01	0.05
Anti-epileptic and metabolites	CBZ	16.2	+	CBZ-13C6	0.05–500	1.000	0.01	0.05
	CBZ 10,11-epoxide[a]	13.5	+	CBZ-13C6	0.10–500	0.998	0.03	0.10
	10,11-Dihydro-10-hydroxy CBZ[a]	13.5	+	CBZ-13C6	0.50–500	0.999	0.05	0.50
Human indicators	Nicotine	3.3	+	Codeine-D6	1.00–500	0.996	0.30	1.00
	Caffeine	8.3	+	Cotinine-D3	0.50–500	0.999	0.10	0.50
	Cotinine[a]	7.2	+	Cotinine-D3	0.05–500	0.999	0.01	0.05
Calcium channel blocker	Diltiazem	16.7	+	CBZ-13C6	0.10–500	0.999	0.01	0.10
Hypnotic	Temazepam	18.2	+	Temazepam-D5	0.05–500	0.999	0.01	0.05
Anti-psychotic	Quetiapine	18.2	+	Quetiapine-D8	0.05–500	0.999	0.01	0.05
Veterinary	Tylosin	17.6	+	Methadone-D9	0.56–500	0.997	0.11	0.56
Analgaesics and metabolites	Morphine	3.5	+	Morphine-D3	1.00–500	0.997	0.30	1.00
	Dihydromorphine[a]	3.3	+	Morphine-D3	0.05–500	0.998	0.01	0.05
	Normorphine[a]	3.4	+	Morphine-D3	1.00–500	0.997	0.30	1.00
	Methadone	17.6	+	Methadone-D9	0.05–400	0.998	0.01	0.05
	EDDP[a]	14.8	+	EDDP-D3	0.05–500	0.999	0.01	0.05
	Codeine	6.1	+	Codeine-D6	0.50–500	0.999	0.10	0.50
	Norcodeine[a]	6.5	+	Codeine-D6	1.00–500	0.999	0.30	1.00
	Dihydrocodeine[a]	5.5	+	Codeine-D6	0.10–500	0.999	0.03	0.10
	Tramadol	11.0	+	Metoprolol-D7	1.00–500	0.999	0.01	1.00
	N-desmethyltramadol[a]	11.9	+	Cocaine-D3	0.50–500	0.999	0.01	0.50
	O-desmethyltramadol[a]	8.3	+	MDA-D5	1.00–400	0.997	0.01	1.00
Stimulants and metabolites	Amphetamine	8.4	+	Amphetamine-D5	0.10–500	0.999	0.03	0.10
	Methamphetamine	8.5	+	Methamphetamine-D5	0.10–500	0.999	0.03	0.10
	MDMA	8.6	+	MDMA-D5	0.05–500	1.000	0.01	0.05
	MDA[a]	8.6	+	MDA-D5	0.10–500	0.999	0.03	0.10
	Cocaine	11.3	+	Cocaine-D3	0.05–500	1.000	0.01	0.05
	Benzoylecgonine[a]	9.7	+	Benzoylecgonine-D8	0.05–500	0.998	0.01	0.05
	AEME[a]	3.5	+	Acetaminophen-D4	0.50–500	0.997	0.10	0.50
	Cocaethylene[a]	12.9	+	Cocaethylene-D3	0.05–500	1.000	0.01	0.05
	Mephedrone	9.8	+	Mephedrone-D3	0.05–500	0.998	0.01	0.05
	MDPV	12.1	+	Cocaethylene-D3	0.05–500	0.999	0.01	0.05
Opioid and metabolite	Heroin	10.9	+	Heroin-D9	0.50–500	0.999	0.10	0.50
	6-acetylmorphine[a]	7.7	+	Cotinine-D3	0.10–500	0.998	0.03	0.10

Key: Rt, retention time; ESI, electro-spray ionisation; IDL, instrument detection limit; IQL, instrument quantitation limit; E1, estrone; E2, 17β-estradiol; EE2, 17α-ethiny-lestradiol; CBZ, carbamazepine; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MDMA, 3,4-methylenedioxy-methamphetamine; MDA, 3,4-methylenedioxy-amphetamine; AEME, anhydroecgonine methylester; MDPV, methylenedioxypyrovalerone.

[a] Metabolite.

[b] Micropollutants without their own labelled internal standard are considered semi-quantitative [24].

ranging from 87 to 150 °C, depending on the matrix and solvent used for extraction [21,25]. In our study, extraction temperatures of 30, 50, 70, 90, 110 and 130 °C were investigated. An extraction of temperature of 50 °C was found to give the highest recovery for the

majority of micropollutants (52 of 81) (Fig. 2). This temperature is lower than previously developed MAE methods for other environmental matrices (sludge, sediments and soils) [21–23,25,26]. Poorer recovery at ≥70 °C was attributed to increased matrix
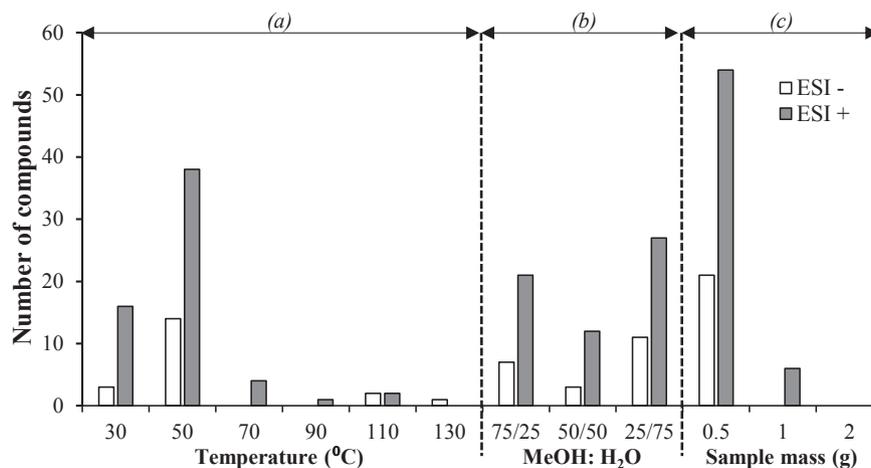

Fig. 2. Number of compounds exhibiting highest recovery at varying: temperature (a), extraction solvent composition (b) and sample mass (c) in ESI − (n = 20) and ESI + (n = 61) ionisation modes.
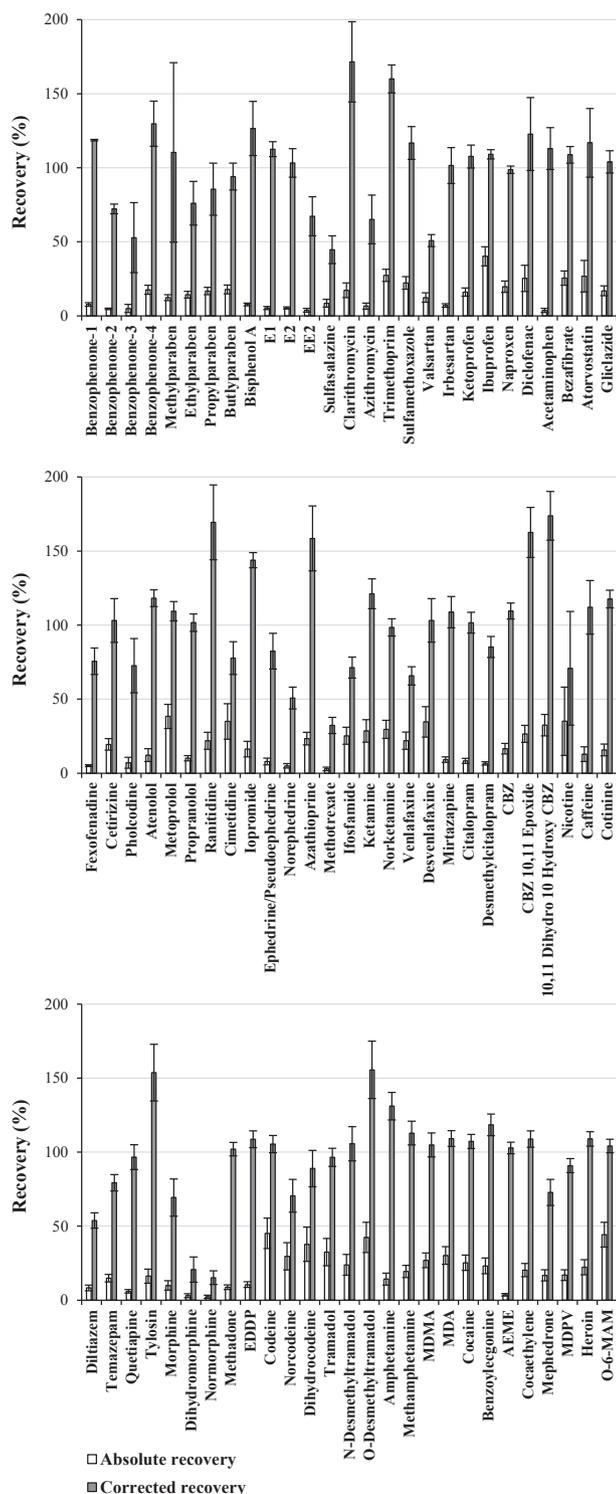
Fig. 4. Matrix suppression of studied micropollutants in *P. australis* extracts (ordered by micropollutant class − see Table 1). Key: E1, estrone; E2, 17β-estradiol; EE2, 17α-ethinylestradiol; CBZ, carbamazepine; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MDMA, 3,4-methylenedioxy-methamphetamine; MDA, 3,4-methylenedioxy-amphetamine; AEME, anhydroecgonine methylester; MDPV, methylenedioxypyrovalerone.
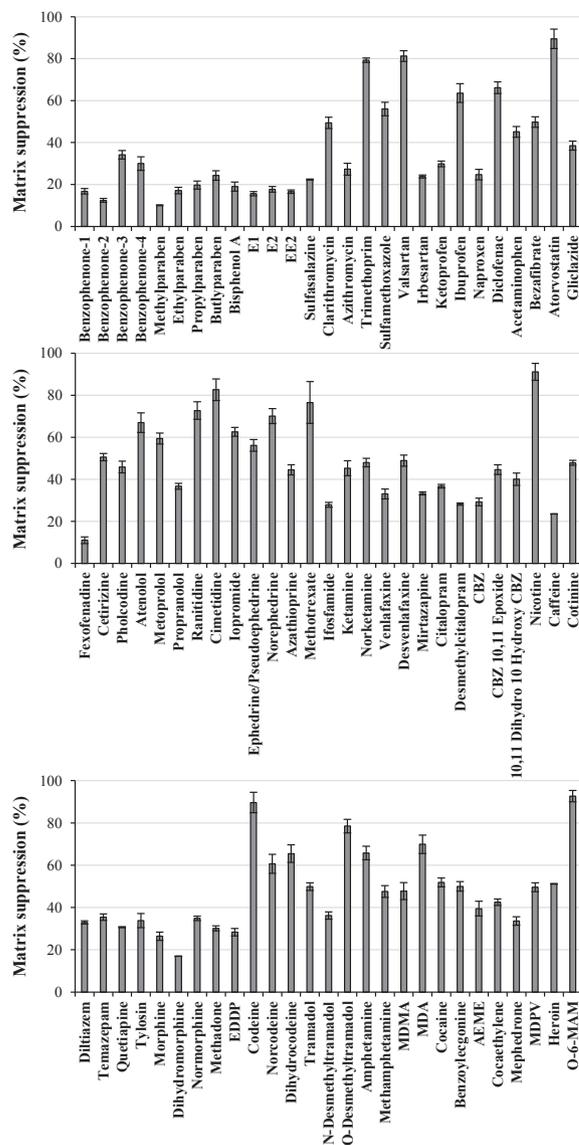
Fig. 3. Average absolute recoveries (not accounted for using internal standards) and corrected recoveries (accounted for internal standard losses e.g., method accuracy) of studied micropollutants from *P. australis* using optimised microwave assisted extraction method at three spike levels − 25, 50 and 125 ng g^{-1} (ordered by micropollutant class − see Table 1). Key: E1, estrone; E2, 17β-estradiol; EE2, 17α-ethinylestradiol; CBZ, carbamazepine; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MDMA, 3,4-methylenedioxy-methamphetamine; MDA, 3,4-methylenedioxy-amphetamine; AEME, anhydroecgonine methylester; MDPV, methylenedioxypyrovalerone.

suppression of analyte signal strength (over lower extraction recovery) caused by increasingly heterogeneous extracts as temperature was increased. This could be overcome by using more selective SPE sorbents. However, this would require more than one SPE protocol to encompass the range of micropollutants studied here. Micropollutants which are not thermally stable may also degrade at higher extraction temperatures [16]. Nevertheless, simultaneous extraction and satisfactory recovery of all micropollutants was achieved using the non-selective HLB sorbent at an extraction temperature of 50 °C.

In contrast to temperature, the solvent compositions tested for extraction had less impact to micropollutant recovery (Fig. 2). A solvent mixture containing MeOH:H$_2$O (and in some cases buffers/salts added) is a popular choice for extraction [21−23], and is easily made compatible with the SPE method applied. Other solvents such as hexane can be used, but it is considered to enhance the extraction of hydrophobic constituents which can increase matrix

Table 2
Method detection and quantitation limits of studied micropollutants in *P. australis* (n = 81, ordered by micropollutant class).

Micropollutant class	Micropollutant	MDL (ng g^{-1} dry weight)	MQL (ng g^{-1} dry weight)
UV filters	Benzophenone-1	0.13	0.78
	Benzophenone-2	0.21	1.06
	Benzophenone-3	0.20	1.01
	Benzophenone-4	1.76	5.74
Parabens	Methylparaben	0.08	0.49
	Ethylparaben	0.21	0.77
	Propylparaben	0.24	0.72
	Butylparaben	0.06	0.34
Plasticizer	Bisphenol-A	0.39	1.30
Steroid estrogens	E1	1.89	9.27
	E2	1.69	8.85
	EE2	2.68	12.88
Antibacterials/ antibiotics	Sulfasalazine	3.17	10.57
	Clarithromycin	0.06	0.35
	Azithromycin	0.45	1.67
	Trimethoprim	0.11	0.37
	Sulfamethoxazole	0.14	0.45
Hypertension	Valsartan	2.76	9.08
	Irbesartan	1.43	7.15
NSAIDs	Ketoprofen	0.69	3.37
	Ibuprofen	0.02	0.12
	Naproxen	0.51	2.49
	Diclofenac	0.12	0.40
	Acetaminophen	3.01	14.79
Lipid regulators	Bezafibrate	0.12	0.39
	Atorvastatin	0.04	0.19
Diabetes	Gliclazide	0.06	0.30
Antihistamines	Fexofenadine	0.58	1.75
	Cetirizine	0.10	0.41
Cough suppressant	Pholcodine	4.94	16.08
Beta-blocker	Atenolol	0.25	0.82
	Metoprolol	0.03	0.13
	Propranolol	0.30	0.89
H$_2$ receptor agonists	Ranitidine	4.71	23.64
	Cimetidine	0.29	1.49
X-ray contrast media	Iopromide	7.14	35.62
Drug precursor and metabolite	Ephedrine/ pseudoephedrine	0.38	1.27
	Norephedrine[a]	0.20	10.10
Anti-cancer	Azathioprine	0.13	0.43
	Methotrexate	9.84	32.33
	Ifosfamide	0.04	0.20
Anaesthetic and metabolite	Ketamine	0.04	0.18
	Norketamine[a]	0.10	0.34
Anti-depressants and metabolites	Venlafaxine	0.05	0.18
	Desmethylvenlafaxine[a]	0.09	0.29
	Mirtazapine	0.11	0.55
	Citalopram	0.60	6.02
	Desmethylcitalopram[a]	0.15	0.76
Anti-epileptic and metabolites	CBZ	0.06	0.30
	CBZ 10,11-epoxide[a]	0.11	0.38
	10,11-Dihydro-10-hydroxy CBZ[a]	0.15	1.54
Human indicators	Nicotine	0.86	2.85
	Caffeine	0.78	3.88
	Cotinine[a]	0.06	0.32
Calcium channel blocker	Diltiazem	0.12	1.21
Hypnotic	Temazepam	0.07	0.34
Anti-psychotic	Quetiapine	0.17	0.83
Veterinary	Tylosin	0.68	3.44
Analgaesics and metabolites	Morphine	3.02	10.06
	Dihydromorphine[a]	0.33	1.65
	Normorphine[a]	12.71	42.38
	Methadone	0.11	0.57
	EDDP[a]	0.10	0.48
	Codeine	0.22	1.11
	Norcodeine[a]	1.01	3.37
	Dihydrocodeine[a]	0.08	0.26
	Tramadol	0.03	3.07
	N-desmethyltramadol[a]	0.04	2.09

Table 2 (*continued*)

Micropollutant class	Micropollutant	MDL (ng g^{-1} dry weight)	MQL (ng g^{-1} dry weight)
Stimulants and metabolites	O-desmethyltramadol[a]	0.02	2.36
	Amphetamine	0.21	0.70
	Methamphetamine	0.15	0.51
	MDMA	0.04	0.19
	MDA[a]	0.10	0.33
	Cocaine	0.04	0.20
	Benzoylecgonine[a]	0.04	0.22
	AEME[a]	2.72	13.61
	Cocaethylene[a]	0.05	0.25
	Mephedrone	0.06	0.30
	MDPV	0.06	0.30
Opioid and metabolite	Heroin	0.45	2.25
	6-acetylmorphine[a]	0.07	0.23

Key: MDL, method detection limit; MQL, method quantitation limit; E1, estrone; E2, 17β-estradiol; EE2, 17α-ethinylestradiol; CBZ, carbamazepine; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MDMA, 3,4-methylenedioxy-methamphetamine; MDA, 3,4-methylenedioxy-amphetamine; AEME, anhydroecgonine methylester; MDPV, methylenedioxypyrovalerone.

[a] Metabolite.

suppression considerably [18]. The MeOH:H$_2$O ratios trialled were 25:75, 50:50 and 75:25. Using 25 mL of 25:75 MeOH:H$_2$O gave the highest recovery for the highest number of compounds (Fig. 2). Although the gains were marginal (few percent), it was also advantageous to use 25:75 MeOH: H$_2$O for sample processing as a lower volume of H$_2$O was required for dilution prior to SPE. This kept the analysis time required for SPE to a minimum.

When developing an extraction method for environmental matrices, it is essential to determine the greatest sample volume or mass which yields maximum recovery. This ensures maximum sensitivity of the method can be achieved. Of the three masses tested (0.5, 1.0 and 2.0 g), a sample mass of 0.5 g gave the highest recovery. This is similar to previously reported MAE methods developed for environmental matrices (Fig. 2) [21,23,26]. Poorer recovery was observed at sample masses of 1.0 and 2.0 g which is likely to be caused by increased matrix suppression and/or saturation of the HLB sorbent due to higher quantities of co-extractives. It can be expected that some improvement in recovery will be achieved at lower sample masses. However, 0.5 g ensured adequate sensitivity (with the recovery achieved) for the low ng g^{-1} concentrations of micropollutants anticipated to be found in *P. australis* here. Throughout the development process, no difference in trend was observed between micropollutants determined in ESI - or ESI + modes for the extraction variables studied (Fig. 2).

3.3. Method validation

To validate the method, micropollutant recovery was determined by spiking samples in triplicate at 25, 50 and 125 ng g^{-1}. Absolute recoveries (i.e., not accounting for the internal standard response) of the 81 studied micropollutants ranged from 2.4% to 45.1% (Fig. 3). Selection of internal standards for micropollutants which did not have their own deuterated or C13 analogues available was based on structural similarity and chromatographic retention time to best account for matrix suppression. Corrected recovery/method accuracy (i.e., analyte losses accounted for using internal standards) ranged from 15% for normorphine to 174% for 10,11-dihydro-10-hydroxycarbamazepine, with the majority of compounds exhibited corrected recoveries in the range 80−120% (Fig. 3, Table S5). This range of recoveries is typical for multi-residue methods investigating a high number of micropollutants (>50) in environmental matrices [23,27,28]. The overall precision of the

method was generally within ±20% (Fig. 3, Table S6).

Matrix suppression (%) of analyte signal strength was quantified by comparing extracted samples spiked post SPE and non-spiked samples with the standard solution used for spiking:

$$MS = \frac{PA\ spiked\ ex - PA\ ex}{PA\ std} x\ 100 \tag{1}$$

Where *PA spiked ex* is the peak area of the spiked extract post SPE, *PA ex* is the peak area of the extract (not spiked) and *PA std* is the peak area of the standard solution used for spiking. Therefore suppression of 0% shows no matrix suppression and complete suppression would be 100%. All compounds were subject to suppression which ranged from 10% to 93% (Fig. 4, Table S7). In general, lower signal suppression was observed for micropollutants determined in ESI - mode. These levels of signal suppression are typical of previously reported multi-residue methods investigating micropollutants in other solid environmental matrices [22,23,29]. Again, this was a result of extracting environmental matrices using the non-selective HLB sorbent. Nevertheless it was essential for the simultaneous extraction of the broad range of micropollutants studied. This also demonstrates that the use of labelled internal standards to correct for matrix suppression as well as analyte losses

during sample preparation was essential.

To determine the method's sensitivity, method detection limits (MDLs, ng g^{-1}) and MQLs (ng g^{-1}) were calculated according to [22]:

$$MDL = \frac{S\ x\ IDL\ x\ 100}{Abs\ Rec\ x\ CF} \tag{2}$$

$$MQL = \frac{S\ x\ IQL\ x\ 100}{Abs\ Rec\ x\ CF} \tag{3}$$

S is the volume of solvent used for extraction (mL) divided by the mass of sample (g). *IDL* and *IQL* are the instrument detection and quantitation limits, respectively (ng mL^{-1}). *Abs Rec* is the absolute recovery of the method (%) and *CF* is the concentration factor.

MDLs were <1 ng g^{-1} for 65 of the 81 micropollutants studied, demonstrating the sensitivity of the method developed (Table 2). The corresponding MQLs ranged from 0.2 to 42.4 ng g^{-1} with the majority of compounds being <5 ng g^{-1}. These MQLs are similar to other MAE methods used to extract organic micropollutants from digested sludge [22,23,26,30] and sediments [25,26]. From the very limited data available on the level of micropollutants in macrophytes used to treat municipal wastewater [19,20], the reported
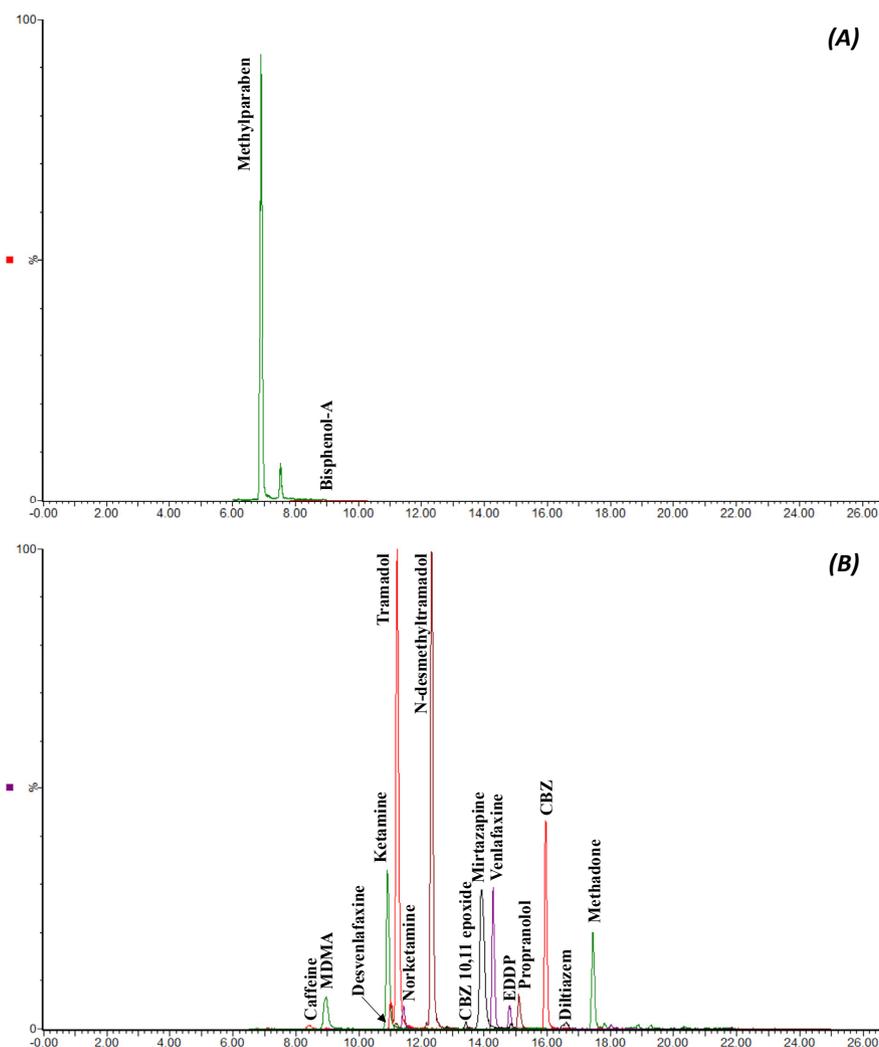

Fig. 5. UHPLC-MS/MS chromatograms of micropollutants found in *P. australis* (sample reed 1: outlet) in ESI - (A), and ESI + (B) ionisation modes. To see chromatograms of all micropollutants in spiked *P. australis* see Fig. S1. Key: CBZ, carbamazepine; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MDMA, 3,4-methylenedioxymethamphetamine.

MQLs are adequately sensitive for their determination at concentrations expected to be found in the field.

3.4. Method application

The developed method was applied to determine the concentration of micropollutants in *P. australis* from a constructed wetland treating secondary municipal (trickling filter) effluent in the UK. A total of eight plants were collected over four consecutive days. Of the 81 micropollutants investigated, 17 were found above their respective MQL at least once (Fig. 5). These compounds were methylparaben, bisphenol-A, propranolol, ketamine, venlafaxine, mirtazapine, carbamazepine, diltiazem, caffeine, methadone, tramadol and MDMA, as well as the metabolites norketamine,

desvenlafaxine, carbamazepine 10,11 epoxide, EDDP and *N*-desmethyltramadol. Such micropollutants are commonly found in trickling filter effluents in the UK [2].

Mean concentrations (n = 8 plants) of the 17 detected micropollutants ranged from <5 ng g^{-1} for desvenlafaxine, diltiazem, and EDDP to 197 ± 84 ng g^{-1} for methylparaben (Fig. 6). There was no apparent difference in micropollutant concentration from *P. australis* collected close to the inlet and outlet of the constructed wetland. There is a paucity of information on the concentration of micropollutants found in emergent macrophytes such as *P. australis* used to treated municipal wastewater. Hijosa-Valsero et al. [20] measured the concentration of the pharmaceuticals ibuprofen, naproxen and salicylic acid in the roots of *P. australis*. Concentrations in roots ranged from 100 to 2500 ng g^{-1} fresh weight. The
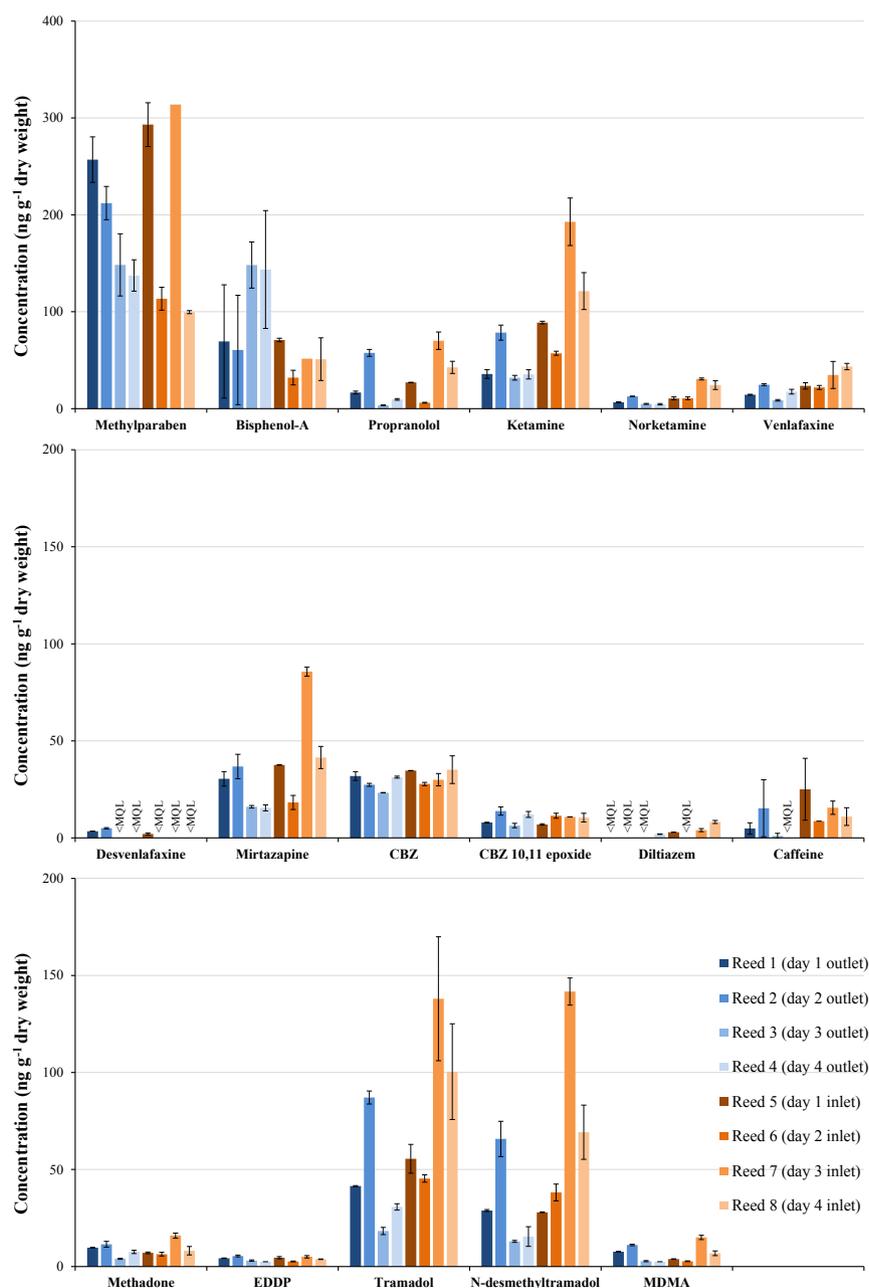

Fig. 6. Concentration of micropollutants found in *P. australis* collected from constructed wetland treating secondary (trickling filter) effluent (Table S8). Key: MQL, method quantitation limit; CBZ, carbamazepine; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MDMA, 3,4-methylenedioxy-methamphetamine.

comparatively higher concentrations in their study is attributed to the likelihood that these compounds were present in the receiving wastewater for treatment at greater concentrations. The receiving wastewater was settled sewage (post primary treatment) whereas our study treated secondary effluent (post trickling filter treatment). However, within plant variations in concentration may also contribute to this difference as in our study the whole plant was investigated (roots, stem and leaves). Future application of the method will address this issue by investigating micropollutant concentrations throughout the various sections of the plant. Furthermore, several of the detected micropollutants are chiral in nature. It is postulated that enantiomers of the same chiral compounds will be taken up, transported and metabolised within *P. australis* at different rates. The pesticide isofenphos-methyl has been found to be metabolised stereoselectively in the vegetable pak choi [31].

Interestingly, several of the micropollutants found in *P. australis* such as carbamazepine and ketamine are poorly removed by conventional secondary wastewater treatment processes such as trickling filters and activated sludge [2]. Their presence in *P. australis* indicates uptake is a viable removal pathway for these normally recalcitrant compounds. Furthermore, the occurrence of their metabolites carbamazepine 10,11 epoxide and norketamine suggests that metabolism of the parent micropollutant may also occur within the plant. The determination of metabolites has been previously used to suggest that metabolism of carbamazepine within plants such as vegetables [32,33] and other macrophytes (Cattail) [34] takes place. Depending on their rate of uptake and metabolism by *P. australis*, constructed wetlands could be successful for the removal of these normally recalcitrant micropollutants from wastewater. The focus of future work will be to establish a complete process mass balance of the constructed wetland for all micropollutants. This will enable the performance of the constructed wetland for micropollutant removal to be assessed whilst determining the dominant mechanisms of removal for each individual compound.

4. Conclusion

A new multi-residue method was developed and validated for the determination of 81 organic micropollutants including metabolites in the emergent macrophyte *P. australis*. The use of MAE, off-line SPE and UHPLC-MS/MS achieved MQLs <5 ng g^{-1} for the majority of studied compounds. Application of the method found 17 micropollutants at concentrations up to 200 ng g^{-1} in *P. australis* collected from constructed wetlands treating municipal wastewater. Other than uptake, the determination of several metabolites indicates plant metabolism may occur. This method will be used to further investigate the role of phytoremediation in the removal of micropollutants by constructed wetlands.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2016.12.042.

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