Application of liquid chromatography-mass spectrometry methods to pharmaceuticals and personal care products in environmental samples

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Table of Contents

I. Abstract

This thesis focuses on developing different sample preparation techniques to extract pharmaceuticals and personal care products (PPCPs) in different matrices and optimising liquid chromatography-mass spectrometry (LC–MS) methods to identify and quantify PPCPs in environmental samples.

The first experimental chapter of this thesis highlights an application of ultra high pressure liquid chromatography hyphenated with a quadrupole Orbitrap mass spectrometer (UHPLC–Q-Orbitrap-MS) for analysis of PPCPs extracted from water samples. Several PPCPs were detected at trace levels indicating the sensitivity of the optimised method using UHPLC– Q-Orbitrap-MS system, which was also used in the MS/MS mode for confirmation purposes. The study also investigates different Orbitrap MS parameters such as AGC target and resolution which results in improving the mass accuracy and to obtain sufficient data points per peak to support identification and quantification of PPCPs.

A simple extraction method based on the quick, easy, cheap, effective, rugged and safe (QuEChERS) approach to extract PPCPs from freshwater invertebrates was developed. Different parameters of the QuEChERS extraction method were investigated such as use of hexane, acetonitrile and dilution of salt content which affects the recovery of the studied PPCPs. The extracts were then analysed by UHPLC–Q-Orbitrap-MS after optimising the MS method which includes using switching polarity mode. Some PPCPs were detected in the freshwater invertebrate samples which indicates the potential environmental impacts of these contaminants. The MS/MS procedure was further investigated by studying different collision energies and by consideration of structural feature of the molecules, it was possible to explain the fragmentation patterns for each PPCP.

PPCPs were also analysed using ultra high performance liquid chromatography triple quadrupole mass spectrometry (UHPLC–QqQMS) after optimising collision energies for different product ions of each PPCP. In this method, three product ions of each PPCP were used respectively for quantification and confirmation. The compounds were extracted from water samples using solid

phase extraction (SPE) as a reliable sample preparation technique and they were separated using a Cogent Diamond Hydride column under aqueous normal phase mode. A carryover problem was also investigated in this study, arising from an apparent instrumental problem that was not adequately preventing sample carryover, which was resolved by proposing that three blanks were needed between injections to reduce the carryover. Different washing solvents and percentage compositions were studied in order to choose the most effective way to reduce this problem.

In the last chapter, two advanced LC-MS systems, UHPLC–Q-Orbitrap MS and HPLC–QTOFMS, were compared in full scan mode. Both systems were used to analyse PPCPs in spider samples after extracting them using the QuEChERS approach. Although both instruments are high resolution mass spectrometers and can be used to detected contaminants at low levels, UHPLC–Q-Orbitrap MS shows higher sensitivity and lower detection limits for most of the studied PPCPs. In general, both systems proved to be sensitive and the mass accuracy was < 5 ppm for all compounds using both systems.

II. Publications during enrolment

Published works

2017:

Althakafy, J. T., Kulsing, C., Grace, M. R., Marriott, P. J., Liquid chromatography – quadrupole Orbitrap mass spectrometry method for selected pharmaceuticals in water samples. *J. Chromatogr. A* 2017, 1515, 164–171.

2018:

J.T. Althakafy, C. Kulsing, M.R. Grace, P.J. Marriott, Determination of selected emerging contaminants in freshwater invertebrates using a universal extraction technique and liquid chromatography accurate mass spectrometry, *J Sep Sci*, (2018) 1–10 https://doi.org/10.1002/jssc.201800507.

III. Selected conferences presentations

- Analysis of some PPCPS and selected illicit drugs in water by using UPLC–Orbitrap mass spectrometry. *23rd Annual RACI R&D Topics Analytical and Environmental Chemistry Conference 2015*, 6-9 December 2015, University of Melbourne, Melbourne – Oral presentation.

- Developing methods to analyse PPCPs in different matrices using UHPLC-Orbitrap mass spectrometry, *Symposium on Pharmaceuticals and Personal Care Products in Aquatic Ecosystems*, Water Studies Centre, Monash University, 11 May, 2016, Melbourne - Oral presentation.

- Determination of some emerging contaminants in freshwater invertebrates using a developed QUECHERS extraction technique and an advanced Orbitrap system. *40th International Symposium of Capillary Chromatography and 13th GC×GC Symposium*, 29 May-3 June 2016, Congress Centre, Riva del Garda, Italy – Poster presentation.

- Analysis of some pharmaceutical and personal care products in invertebrate samples using QUECHERS extraction technique and advanced LC/MS systems. *ASASS 2 - ACROSS International Symposium on Advances in Separation Science,* 30 November – 2 December 2016, Hobart Function and Conference Centre, Hobart - Oral presentation.

- Quantitative PPCP analysis in invertebrates using LC high-resolution mass spectrometry. *2017 RACI Centenary Congress,* 23-28 July 2017, Melbourne Convention and Exhibition Centre, Melbourne – Oral presentation.

IV. Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 2 original papers published in peer reviewed journals and 2 unpublished publications. The core theme of the thesis is to develop and apply sample preparation techniques and advanced liquid chromatography and mass spectrometric techniques for identification and quantification of pharmaceuticals and personal care products in different environmental matrices. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Chemistry, Faculty of Science under the supervision of Professor Philip J. Marriott (main supervisor), and Associate Professor Michael Grace (co-supervisor).

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 2 – 5, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status*	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Monash student Y/N*
2	*Liquid chromatography – quadrupole Orbitrap mass spectrometry method for selected pharmaceuticals in water samples*	*Published*	*80%. Developing methods and validation, preparing environmental samples, conducting the analytical instrumental study, collecting data and interpreting results, writing draft manuscript*	1) *Chadin Kulsing, helping interpreting results, editorial assistance. 5%* 2) *Michael R. Grace, supervision, editorial assistance. 5%* 3) *Philip J. Marriott, supervision, assisted interpretation results, editorial assistance. 10%*	1) *No* 2) *No* 3) *No*
3	*Determination of selected emerging contaminants in freshwater invertebrates using a universal extraction technique and liquid chromatography accurate mass spectrometry*	*Accepted; Manuscript Proofs submitted*	*80%. Developing methods and validation, preparing environmental samples, conducting the analytical instrumental study, collecting data and interpreting results, writing draft manuscript*	1) *Chadin Kulsing, helping interpreting results, editorial assistance. 5%* 2) *Michael R. Grace, supervision, editorial assistance. 5%* 3) *Philip J. Marriott, supervision, assisted interpretation results, editorial assistance. 10%*	1) *No* 2) *No* 3) *No*
4	*Investigation of carryover effects on quantification of PPCPs in environmental samples using silica hydride stationary phase and ultra high performance liquid chromatography hyphenated with triple quadrupole mass spectrometer*	*Not submitted*	*80%. Developing methods and validation, preparing environmental samples, conducting the analytical instrumental study, collecting data and interpreting results, writing draft manuscript*	1) *Chadin Kulsing, helping interpreting results, editorial assistance. 5%* 2) *Michael R. Grace, supervision, editorial assistance. 5%* 3) *Philip J. Marriott, supervision, assisted interpretation results, editorial assistance. 10%*	1) *No* 2) *No* 3) *No*

| 5 | *High resolution liquid chromatography mass spectrometry for PPCPs analysis in freshwater invertebrates: a comparison of quadrupole time of flight and quadrupole Orbitrap mass spectrometers* | *Not submitted* | *80%. Developing methods and validation, preparing environmental samples, conducting the analytical instrumental study, collecting data and interpreting results, writing draft manuscript* | *1) Chadin Kulsing, helping interpreting results, editorial assistance. 5%*
2) Michael R. Grace, supervision, editorial assistance. 5%
3) Philip J. Marriott supervision, assisted interpretation results, editorial assistance. 10 % | *1) No*

2) No

3) No |

* Status *(published, in press, accepted or returned for revision)*

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:

Date: 14/09/2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature:

Date: 14 Sept 2018

V. Acknowledgements

VI. List of Abbreviations

1D HPLC	One dimensional high performance liquid chromatography
1D LC	One dimensional liquid chromatography
ACN	Acetonitrile
AGC	Automatic gain control
ANP	Aqueous normal phase
APCI	Atmospheric pressure chemical ionisation
CET	Cetirizine
CID	Collision – induced dissociation
CIM	Cimetidine
DLs	Detection limits
dMRM	Dynamic multiple reaction monitoring
Dual AJS ESI	Dual jet stream electrospray ionisation source
EIC	Extracted ion chromatogram
ESI	Electrospray ionisation source
FLU	Fluoxetine
FWHM	Full width at half maximum
GC	Gas chromatography
GC×GC	Comprehensive 2D gas chromatography
GC×GC-TOFMS	Comprehensive 2D gas chromatography time-of-flight mass spectrometry
GC–ECD	Gas chromatography-electron capture detector
GC–MS	Gas chromatography-mass spectrometry
GC–MS/MS	Gas chromatography- tandem mass spectrometry
GC–TOFMS	Gas chromatography- time-of-flight mass spectrometry
HESI	Heated electrospray ionisation source
HILIC	Hydrophilic interaction liquid chromatography
HLB	Hydrophilic-lipophilic balance
HPLC–DAD	High performance liquid chromatography- diode array detection
HPLC–ESI-MS/MS	High pressure liquid chromatography- electrospray ionisation- tandem mass spectrometry
HPLC–MS/MS	High pressure liquid chromatography-tandem mass spectrometry
HRAM	High resolution accurate mass

HXN	Hexane
IT	Maximum injection time
LC	Liquid chromatography
LC×LC	Comprehensive two-dimensional liquid chromatography
LC–MS	Liquid chromatography-mass spectrometry
LC–MS/MS	Liquid chromatography- tandem mass spectrometry
LC–QQQMS	Liquid chromatography -triple quadrupole mass spectrometry
LC–QTOFMS	Liquid chromatography- quadrupole time-of-flight mass spectrometry
LLE	Liquid/liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MDLC	Multi-dimensional liquid chromatography
MeOH	Methanol
MQW	Milli-Q water
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NP	Normal phase chromatography
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
ppb	Parts per billion
PPCPs	Pharmaceuticals and personal care products
ppm	Parts per million
ppq	Part per quadrillions
ppt	Parts per trillion
PRM	Parallel reaction monitoring
PuLE	Pulverised liquid extraction
Q-GC–MS	Quadrupole gas chromatography-mass spectrometry
QMS	Quadrupole mass analyser
QQQMS	Triple quadrupole mass spectrometry
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe extraction method
RP	Reversed-phase chromatography
RP-HPLC	Reversed-phase-high performance liquid chromatography
SPE	Solid phase extraction

SRM	Selected reaction monitoring
TIC	Total ion chromatogram
tMRM	Triggered multiple reaction monitoring
TOFMS	Time-of-flight mass spectrometry
TQ-MS	Triple quadrupole-mass spectrometry
UHPLC	Ultra high performance liquid chromatography
UHPLC–QqQMS	Ultra high performance liquid chromatography- triple quadrupole mass spectrometry
USEPA	United States Environmental Protection Agency
WWTPs	Wastewater treatment plants

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Chapter 1

Introduction

1.1 Brief introduction

Pharmaceuticals and personal care products (PPCPs) are a group of chemicals which represent a wide range of chemicals that are used for different purposes such as prescription drugs, fragrances, sun screen products, veterinary drugs, fungicides, disinfectants and nutraceuticals [1]. The chemical nature of PPCPs – medicines, active ingredient compounds – means that their direct disposal to the environment presents a risk to biota in waterways. PPCPs belong to a group of chemicals called "emerging contaminants" which are chemical substances that have not been extensively investigated as contaminants and it is believed they can lead to environmental impacts and other health issues [2]. Although pharmaceuticals can be detected at trace levels, some previous studies mentioned that these contaminants lead to another risk which is the danger of continuous release to water ways [3]. There are numerous studies which have been done to investigate PPCPs in different matrices around the world. **Table 1** summarises analytical methods and sample preparation techniques used to investigate these contaminants in different environmental samples; these techniques are further discussed in Section 1.3. One good example to show the importance of this field of research is a reconnaissance which was done in the US between 1999 and 2000 about the concentrations of the organic wastewater contaminants, which includes pharmaceuticals and other contaminants, in water streams across the US [4]. This work has been cited more than 5100 citations (according to Scopus) which shows the growing concerns regarding these environmental contaminants that are continually released to the environment through different routes such as wastewater treatment plants (WWTPs).

Table 1 Analytical methods and chromatographic techniques used to analyse PPCPs in environmental matrices (selected common sample preparation and chromatographic techniques)

PPCPs subgroups	Sample	Methodology	Instrument	Reference
Antibiotics, analgesics, antiepileptic, blood lipid regulators drugs, and antimicrobial agents	Drinking water, groundwater, surface water, and wastewater	Solid Phase Extraction (SPE)	LC–QTOFMS	[5]
Fibrates, anti-inflammatory drugs and their metabolites	Rivers, ponds and tap water	SPE	GC–MS	[6]
Anti-inflammatory drugs, antimicrobial agents, fibrates and their metabolites, antidepressants and steroids	Surface water and river water samples	SPE	GC–MS	[7]
Anti-inflammatory drugs	Sewage water	SPE	GC–MS	[8]
Anti-inflammatory drugs, fibrates and antidepressants,	Influent and effluent samples (STP or Sewage Treatment Plants)	SPE	GC–MS	[9]
Anti-inflammatory drugs, fibrates, anxiety drugs and stimulants	Groundwaters	SPE	GC–MS	[10]
Analgesics, anti-inflammatory drugs, steroids, antibiotics and other pharmaceuticals such as drugs used for heart diseases and blood pressure	Groundwaters	SPE	HPLC–ESI-MS/MS	[10]
Anti- anxiety drugs	a comparison study using standard solutions + human whole blood	-	GC (GC–TOFMS, Q-GC–MS, GC–ECD) and LC (HPLC–DAD)	[11]
Pain killers, asthma drugs, antibiotics, stimulant drugs, veterinary drugs, neuropathic pain killers, histamine, steroids,	Drinking water & wastewater	SPE	LC–MS/MS	[12]

heart disease drugs, antihistamine drugs, antidepressants, fibrates, antibacterial agents, anticoagulant drugs, anti-inflammatory drugs, Anti-diabetic and antifungal drugs				
Plant hormones, pain killers, anti-inflammatory drugs, stimulants,	Wastewaters and surface waters	SPE	GC×GC–TOFMS	[13]
Anti-bacterial agent	Wastewaters and biosolids	SPE	GC–MS	[14]
Anti-diabetic drug	Dietary supplements and herbal medicines	SPE	LC–MS/MS	[15]
Anti-bacterial agents, antibiotics, heart diseases and blood pressure drugs, anticoagulant drugs, insomnia drugs, anti-inflammatory drugs, fibrates, muscular pain drugs and antidepressants.	Freshwater invertebrate, (Gammarus pulex) + surface waters	Pulverised liquid extraction (PuLE) + SPE	LC–MS/MS	[16]
Antidepressants, antihistamine, antihypertension, antiseizure, antimicrobial and fragrances	Fish tissue	Liquid/liquid exraction (LLE)	HPLC–MS/MS and GC–MS/MS	[17]
Antimicrobials, fungicides, analgesics, Stimulants and steroids	Feather meal and chicken tissue	LLE + SPE	LC–MS/MS	[18]
Analgesics/anti-inflammatory, lipid regulators, antibiotics, phsychiatrics-antiepileptics, psychomotor stimulants, Glucocorticoid steroids, disinfectants and hypolipidemic statins	Wastewater	SPE	LC–MS	[19]
Heart diseases and blood pressure drugs, histamine-2 blockers drugs, pain killers, stimulant, antibiotics, antidepressants, antihistamines, anti-inflammatory drugs, anti-fungal agents, anticoagulant,	Macroalgae, barnacle and fish	LLE + SPE	LC–MS/MS	[20]
Pain killers, antidepressant	Aquarium water	Syringe filter	LC–MS/MS	[21]
Antidepressants, stimulant, heart diseases and blood pressure drugs, antibiotics,	drinking-water sludge	QuEChERS	LC–MS/MS	[22]

fibrates, antidiabetic drug, antifungals, anti-inflammatory drugs, cosmetic and antimicrobials				
Antibiotics	Milk and honey samples	QuEChERS	LC–MS/MS	[23]
Anti-inflammatory drugs, fibrates, peeling agent	Invertebrates (bivalves)	QuEChERS	LC–MS/MS	[24]
Steroids, veterinary antibiotics, pain killers, antibiotics, anti-inflammatory drugs	Invertebrates (earthworms)	QuEChERS	LC–MS/MS	[25]
Anti-inflammatory drug and metabolites	Invertebrates (bivalve tissue)	QuEChERS	LC–MS/MS	[26]

This PhD project focuses on studying a group of different PPCPs, which are common drugs used in daily life, as potential contaminants in the environment. In addition, some of PPCPs in this thesis such as caffeine and fluoxetine have been investigated in several studies in terms of their toxicity, environmental effects and removal technologies; therefore, in this PhD project, these contaminants were investigated in different matrices using advanced analytical techniques. Different sample preparation techniques were also developed to extract these compounds from different samples. On the other hand, some of them such as amoxicillin and triclosan have not been extensively studied compared to other PPCPs in terms of detecting them in some environmental samples and developing LC– mass spectrometry (MS) method to analyse them in different matrices. Many investigations on these contaminants are always recommended which include but are not limited to optimisation in sample preparation techniques, separation using different approaches and employment of new advanced MS systems. This project mainly aims to develop simple sample preparation techniques, to employ advanced MS systems and to compare their efficiencies such as resolution and mass accuracy.

1.2 Environmental effects of PPCPs
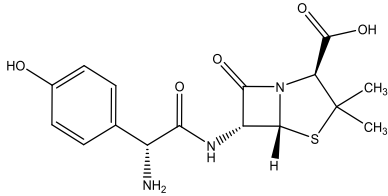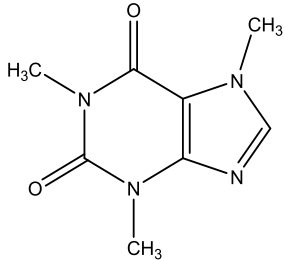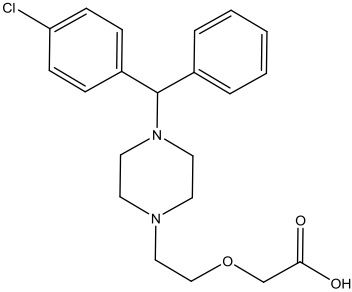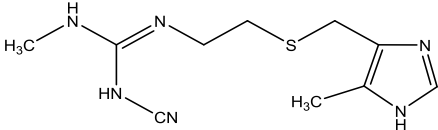
As chemicals, there is no doubt that PPCPs, which have different chemical and physical properties, can be expected to have some environmental impacts if they are released to the environment. This concern has recently started to appear [27, 28] around the world. Thus, there is still more research going on in this field to address the health effects of PPCPs in water which means that drinking water needs to be clean from these contaminants that can cause health problems [29]. There is a need to study PPCPs in detail in terms of their concentration levels or environmental impacts as these chemicals are continuously released to the environment, and in some cases in larger quantities compared to other contaminants [30]. One of the main sources of PPCPs in the environment is human excretion which includes some PPCPs with low percentage excretion of the original parent compound (such as aspirin, ibuprofen, and paracetamol) because they can be found in higher levels in water ways. This means that even low excretion percentages might show a resistant behaviour in the environment [31].
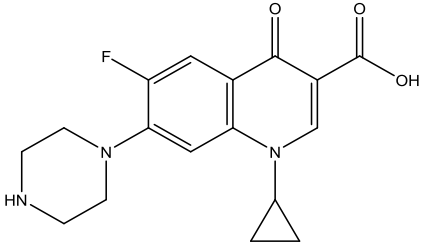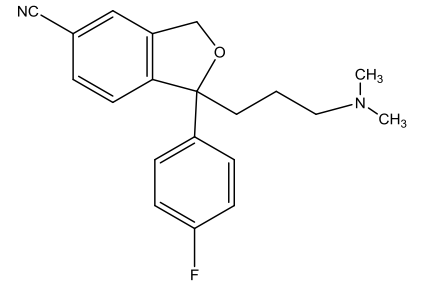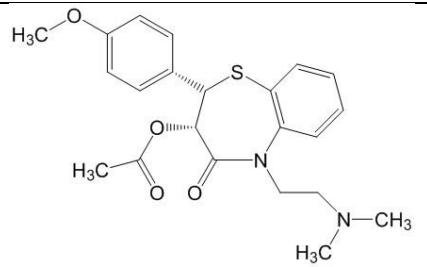
PPCPs might be frequently detected in different matrices due to their continuous discharge to the environment. For example, in some large populous countries such as China with their expected high use of drugs, PPCPs can be found in concentrations up to ppb levels in surface water and up to ppm levels in soil [32]. The main problem with pharmaceuticals for example is that they are chemicals designed to be active even at low doses and also the possibility that they can be effective for a long time, depending upon their metabolism in various matrices [33]. In addition, studies show that many PPCPs cannot be biodegraded [1]. For instance, diatrizoate and iopromide are considered resistant to biodegradation and they both have unidentified metabolites [1, 34] although biodegradation and bioconcentration processes need to be extensively studied.
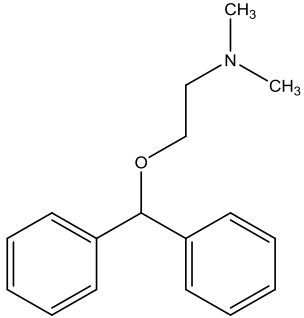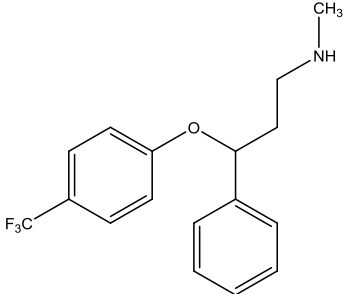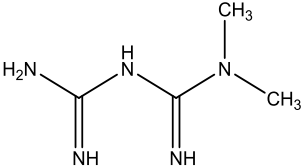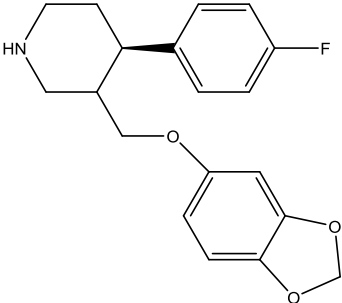
Lack of comprehensive research is one of the biggest problems to understand the behaviour of these contaminants. For instance, more studies need to be done in order to address
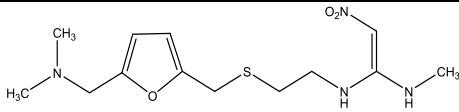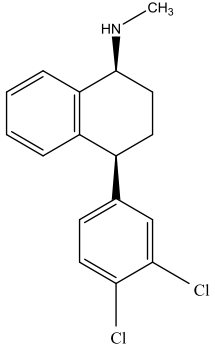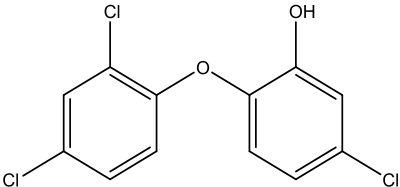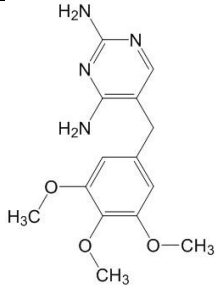
the seasonal behaviour of these contaminants in different environmental matrices [33]. Moreover, the polarity of most PPCPs, which may be in general higher than other contaminants, and the difficulty of detecting these pollutants are a big challenge in terms of applying both removal and analytical technologies [35]. As most PPCPs are polar (see structures in **Table 2**), the separation of these compounds needs to be appropriately chosen and optimised, for example, using reversed-phase chromatography, which is the most common in LC applications, with a non-polar stationary phase (C18 column for example) and polar mobile phase to separate PPCPs. In this case, less polar PPCPs will strongly retain, whereas more polar ones will elute earlier, and may interfere with the unretained peak, and cross-interfere due to matrix suppression. To generate a good separation and have interaction of all PPCPs with the C18 column, the mobile phase, which is generally classified as polar, should combine two or more solvents such as water and acetonitrile (ACN). The optimisation of separation can be done by developing a suitable gradient method for different analytes. Since PPCPs have different structures and polarities, this adds a challenge to find a proper LC method to elute these compounds in different matrices. **Table 2** shows information and structures of some PPCPs.

Table 2 Selected information and structures of some PPCPs

Compound	CAS no.	Accurate mass of [M+H$^+$]	Molecular formula	Compound (pKa // log Kow)*	BCF**	Structure
Amoxicillin	61336-70-7	366.1118	$C_{16}H_{19}N_3O_5S$	9.6 // 0.9	3	
Caffeine	58-08-2	195.0877	$C_8H_{10}N_4O_2$	-0.92 // -0.1	3	
Cetirizine	83881-52-1	389.1627	$C_{21}H_{25}ClN_2O_3$	7.6 // 2.8	3	
Cimetidine	51481-61-9	253.1230	$C_{10}H_{16}N_6S$	6.9 // 0.4	1.2	

Ciprofloxacin	85721-33-1	332.1405	$C_{17}H_{18}FN_3O_3$	5.8 and 8.7 // 0.3	3	
Citalopram	59729-32-7	325.1711	$C_{20}H_{21}FN_2O$	9.8 // 3.5	NA	
Diltiazem	33286-22-5	415.1686	$C_{22}H_{26}N_2O_4S$	12.9 and 8.2 // 2.7	NA	

Diphenhydramine	147-24-0	256.1696	$C_{17}H_{21}NO$	9.9 // 5.1	NA	
Fluoxetine	56296-78-7	310.1413	$C_{17}H_{18}F_3NO$	9.8 // 4.1	NA	
Metformin	1115-70-4	130.1087	$C_4H_{11}N_5$	12.3 // -0.5	3	
Paroxetine	78246-49-8	330.1500	$C_{19}H_{21}ClFNO_3$	9.8 // 3.6	3	

Ranitidine	66357-59-3	315.1485	$C_{13}H_{22}N_4O_3S$	8.1 // 0.3	NA	
Sertraline	79559-97-0	306.0811	$C_{17}H_{17}NCl_2$	9.9 // 5.1	NA	
Triclosan	3380-34-5	286.9439	$C_{12}H_7Cl_3O_2$	7.9 // 5.0	2.7-90	
Trimethoprim	738-70-5	291.1452	$C_{14}H_{18}N_4O_3$	17.3 and 7.2 // 0.9	3	

* All information obtained from https://www.drugbank.ca/drugs

** Bioconcentration factor (BCF) based on bioconcentration in the aquatic environment (mostly fish samples). The information obtained from

https://pubchem.ncbi.nlm.nih.gov

1.2.1 Measures to stop PPCPs and other contaminants from reaching the environment?

Most WWTPs are not specifically designed to remove PPCPs from water although PPCPs will most likely be diluted when they reach water ways after WWTP processing. As detailed below, several studies showed that some removal techniques can be employed inside WWTPs in order to reduce the PPCP pollutant content in water. A study by Yang et al. [36] found that some PPCPs such as caffeine and ibuprofen can be successfully reduced by 90% or more in water if a removal processes such as membrane filtration and activated sludge treatment were applied. For example, the average concentrations of caffeine and ibuprofen in the primary effluent were 80000 and 11000 ng L^{-1} respectively. Using membrane filtration, the average concentrations were reduced to 65 and 64 ng L^{-1} respectively. On the other hand, other PPCPs such as erythromycin and carbamazepine were decreased by 74 and 88% respectively using a granular activated carbon adsorption process. Other removal processes which include biodegradation, oxidation and bioaugmentation can also be employed to help reduce PPCPs in the effluent [28]. In addition to WWTPs, there are other facilities that can be employed to reduce or remove PPCPs from wastewater, such as constructed wetlands which are preferable in terms of low cost and maintenance [37]. Another study by Boyd et al. [38] mentioned that some treatment processes for rivers water such as ozonation, dual filtration and chlorination decreased some PPCPs concentrations such as naproxen and clofibric acid. On the other hand, the removal efficiency will be improved by more than 80% if a combination of removal technologies such as conventional activated sludge, sand filtration and ozonation is used [39].

Although these removal technologies illustrated some success to eliminate these contaminants, the variety of PPCPs and their numbers can be a big challenge against applying these technologies for all PPCPs. For instance, a tertiary treatment process in a WWTP, which includes using chlorine to destroy microorganisms, can lead to changing some PPCP chemical

structures which can subsequently be discharged through treated water to the environment [40]. Moreover, the activated sludge process, which is widely used in many WWTPs, are not expected to remove PPCPs from wastewater; therefore, it is crucial to enhance this process or use additional treatment in order to overcome this problem [28].

1.3 Analytical techniques used to analyse PPCPs

Most of the PPCPs which are detected in environmental samples are expected to be found in trace levels. Thus, it is crucial to use cutting edge analytical instrumental techniques that have the ability to detect these chemicals at very low concentrations. Of the several analytical techniques which can be used to identify and quantify these contaminants, the most appropriate are gas chromatography (GC) and liquid chromatography (LC), hyphenated with detection provided by mass spectrometry (MS). Using a combination of these technologies is a common approach in order to have a very clear picture about the sample mixture and to quantify PPCPs in different environmental matrices. This combination can be gas chromatography hyphenated with mass spectrometry (GC–MS) or liquid chromatography hyphenated with mass spectrometry (LC–MS). Both innovative separations and mass spectrometry techniques may also be applied to this problem. There are several advanced systems based on GC–MS and LC–MS which are commercially available and designed for many applications. Other advanced systems have recently been developed based on multi-dimensional GC, LC or MS, which may be defined as, for instance, GC^1MS^1, meaning separation based on one-dimensional GC and one-dimensional MS detection, and in the same manner GC^2MS^2 or GC^nMS^n. This approach has been used to analyse a variety of compounds such as pesticides [41].

1.3.1 Liquid chromatography (LC)

Ultra-high performance liquid chromatography (UHPLC) was introduced to the market in 2004. This LC system reduces the volume of mobile phase needed for separation without compromising the sensitivity of the chromatographic method [42]. UHPLC also has many advantages compared to HPLC which includes increased sensitivity and higher resolution with fast separation resulting in short analysis time [42, 43]. In this type of LC systems, short columns with smaller particle size can be effectively used. This might cause higher back pressure which can be overcome in elevated pressure capabilities of UHPLC systems.

Normal phase (NP) mode uses a polar stationary phase and nonpolar mobile phase which are reversed chromatographic conditions to those in reversed-phase (RP)-HPLC. In NP mode, the stationary phase can be silica gel, aluminium oxide, or polar polymer materials whereas the mobile phase can be nonpolar organic solvents such as hexane, dichloromethane, acetone or isopropanol. The stationary phase in NP is rather sensitive to water, which can come from the sample, and its association with the column stationary phase can lead to reduced efficiency, or variation in retention times. Moreover, pH of the mobile phase needs to be carefully adjusted because the charge state of the compound is important for its retention [44].

In reversed-phase (RP) mode, a nonpolar stationary phase and polar mobile phase are employed. The popular C18 stationary phase comprises octadecyl silica groups bound to hydroxyl groups. Mobile phase polarity in RP mode comprise water commonly modified with methanol (MeOH) or acetonitrile (ACN). A common LC gradient in RP separation mode is a mixture of ACN and water, with the amount of ACN increased over the gradient time, thus eluting polar analytes first, and progressively eluting more hydrophobic compounds later. In this case, since many of the target analytes exhibit different relative hydrophobic properties, therefore, they are suited to gradient analysis in RP mode. [44]. Although there are some limitations of RP-HPLC, such as being poorly suited to analysing highly polar analytes and

ensuring their adequate retention, and ensuring acceptable peak shapes, RP is still widely used in LC compared to other separation modes such as NP. This is due to the flexibility, relative simplicity and the multipurpose nature of this mode, in addition to the continual development of new instruments, stationary phases and mobile phase additives or adjustment [45].

One of the separation methods that has increased in popularity in the recent years and can be used to analyse a wide range of polar analytes is hydrophilic interaction liquid chromatography (HILIC). This separation method was introduced in 1990 and it is unusual since unlike the above phase combinations, it combines a NP stationary phase and RP mobile phase. In this case, the stationary phase can be hydrophobic such as polar polymers, and the mobile phase can be ACN or MeOH with 30% water. This can achieve an excellent separation of polar and ionisable analytes compared to RP mode [45]. In addition, using HILIC can address the issues related to retention and peak asymmetry of polar compounds which is common in NP separation mode [45, 46]. The mechanism of this mode is based on partition, although the initial suggested mechanism was similar to NP, which is adsorption, but the latter has limitations to compounds that have hydroxyl groups. In general, HILIC separation mode has proven that analytes can have different mechanisms of retention with stationary phases that depend on the chromatographic conditions. Understanding the mechanism and using it to inform method optimisation can be a challenge when this mode is used for separation, as opposed to RP or NP. Moreover, this separation mode is not recommended to be used with hydrophobic analytes as they do not allow enough stationary phase interaction [45].

1.3.2 Liquid chromatography–mass spectrometry (LC–MS)

LC–MS has proved to be a flexible analytical tool that can be used either for research or routine analysis. It is widely used to analyse different types of chemical substances such as those in metabolomics [47], lipidomics [48], flavonoids [49], plant hormones [50], pesticides

and veterinary drugs [51] and other synthetic compounds. LC, which is a preferable chromatographic technology to separate different types of polar and non-polar compounds in different matrices, has been considered even more successful when it is hyphenated to MS. One of the most important components in LC–MS interfaces is the ionisation interface which is realised by using several ionisation techniques. Most advanced LC–MS systems can be equipped with one or dual ionisation interfaces such as atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI). Today, ESI is more popular in LC–MS systems. It is a soft ionisation process that can produce charged ions from a droplet 'spray' which can be maintained in the interface without fragmentation and then be injected to the mass analyser [52]. This ionisation process can also be employed in both positive and negative modes.

After dissolving the sample in mobile phase, charged aerosol droplets, which can be generated by applying high voltage to a capillary flow outlet, are transmitted through reduced pressures by a heated gas which results in formation of ions in the gas phase – often called the 'single ion in droplet' mechanism. In this case, ions can have multiple charges generally governed by the size of the compound [53]. These ions can then be directed to the mass analyser. The ionisation process can sometimes generate additional ions such as $[M+Na]^+$ and $[M+K]^+$ instead of simply the protonated molecular ion $[M+H]^+$ because of the noncovalent forces that can be generated between the ions in the gas phase and solvent molecules. This leads to confounding of the pseudo-molecular ion identification, and might lead to insufficient ions in the form of $[M+H]^+$ – which often is normally preferred – for the analyte. Different conditions may be needed in this case, in order to enhance the $[M+H]^+$ ion, for instance by adding acid to the eluent. In addition, it is important to pay attention to the mobile phase solvents because the ESI process can be sensitive to solvent composition and as the gradient method changes, it could affect the ionisation process [52].

LC–MS has been tremendously developed in recent years. For instance, the resolution has been improved for different mass analysers which can provide more sensitivity and higher mass accuracy. The continuous development of LC platforms such as HPLC and UHPLC, which provides faster and better separation for many compounds, is also considered an important step to deal with different mobile phases and flow rates. Two-dimensional LC is also becoming popular especially for separating complex mixtures. It can also be hyphenated with MS to provide more data about complex samples. The use of MS/MS mode for fragmentation of the precursor ion to generate different products ions has also becoming more advanced and sophisticated by using different MS/MS modes from different mass analysers.

1.3.3 MS/MS

The MS/MS technique was proposed around 1980, with the triple quadrupole as the first instrument to implement this type of technology. In general, the MS/MS process starts through selection of the precursor ion and then fragmenting it by collision–induced dissociation (CID). The next stage is to scan product ions that are produced from the precursor ion. For successful fragmentation of the precursor ion, the precursor ion needs to gain additional energy through an ionisation step. This energy needs to overcome the reaction activation barrier in order to complete the fragmentation process. The use of CID can be obtained by collision with a neutral gas such as helium, which results in converting translational energy into internal energy, thence fragmentation. In general, all product ions obtained from the ion source during the ionisation process are available for MS/MS analysis. However, some information about the precursor ion can only be obtained in some applications without performing MS/MS fragmentation, by using soft ionisation technologies [54]. MS/MS is not limited to the triple quadrupole because it can be used with other mass analysers such as quadrupole-Orbitrap and quadrupole-time-of-flight MS.

Using MS/MS, fragmentation is a key in order to confirm the identity of the compound, which normally considers the uniqueness of a precursor / product ion mass-based relationship, which may be also at a specific retention time in the HPLC analysis. This can be done by optimising the collision energies of each compound which ideally leads to increase the intensity of the overall signal and hence, the compound can be both confirmed and also better quantified. Although full scan mode with accurate mass can be useful for the identification of the compound, it is still not enough as some analytes might share the same mass which can lead to misleading results. Using MS/MS can overcome this problem by using two or three product ions of the same precursor ion for both quantification and confirmation purposes. Several MS/MS modes can be used in different MS analysers. Some of these MS analysers are explained below with some details.

1.3.4 Orbitrap mass analyser

The Orbitrap mass spectrometer was invented by Makarov in 2000 [55]. **Figure 2** shows an example of an Orbitrap mass analyser system. The quadrupole filter in this case transfers the ions to the Orbitrap mass analyser which can measure the accurate mass of these ions [56]. This concept was developed based on the Kingdon device in 1923 which traps ions in an orbital motion using an electrostatic field [57]. An example of the Orbitrap mass analyser system is the Q-Exactive Plus which is a high resolution MS which offers resolving power up to 280,000 full width at half maximum (FWHM) in addition to being a very sensitive method to perform MS/MS analysis [56]. The resolution can be defined as the value $m/\Delta m$ for two peaks separated at 10 percent valley. For instance, if two peaks, which have the same height, can be separated by Δm and the valley between the two peaks is 10% of the peak height, the resolution is equal to $m/\Delta m$ where m is the mass for one of the two peaks [58]. The mass accuracy can be defined as

$$10^6 \times [(\text{exact mass} - \text{accurate mass})/\text{exact mass}]$$

and it can be expressed in parts per million (ppm) [56]. In general, the mass accuracy can be reported using 4 or 5 decimal places but the most common practice (usually new mass spectrometers) use 4 decimal places or 7 significant figures especially for masses between 100 and 900 Da [59]. The system provides accurate mass measurement which can be used to detect analytes at trace levels using either full scan or MS/MS modes. These types of mass analysers can be effectively used to tackle complex matrices and to provide accurate measurements for different class of compounds, due to their mass-discrimination possibilities. There is a strong relationship between mass accuracy and resolving power (which is the ability to separate the two adjacent peaks) because mass accuracy will be better with higher resolving power or resolution. In this case, it is expected to have a good mass accuracy especially for small molecules by using Orbitrap technology. However, resolving power is normally decreased with an increase of mass of the molecule. This is due to collision with gas molecules in the background which will be increased in this case, leading to fragmentation of the ions and then eliminating them from the trap, or from changes in the kinetic energy of the ions [57]. The Orbitrap mass analyser has proven to be an excellent tool that can be used for either screening (using mass accuracy within a 5 ppm window, database comparison and MS/MS fragmentation patterns) [60] or quantification [56] which can provide confidence in analysis whether it is employed for research or routine analysis. All instruments based on Orbitrap technology have been tested and used to perform different types of analysis for drugs, proteomics and food samples. The Orbitrap analyser has been employed for multi-dimensional LC (MDLC) which means a wide range of applications can be involved to use this type of design. For instance, analysing peptide mixtures is generally rather complicated, arising from the many peptides produced which overlap on the column in one dimensional (1D) HPLC. In this case, MDLC with usually two separation columns operated in series with a valve sampling operation can be

used to separate the complex mixture of peptides - 1D LC cannot minimise the co-elution problem [61]. For PPCPs analysis, MDLC can also be useful to separate a wide range of coextracted polar and nonpolar compounds by employing two types of columns, such as HILIC and C18. It is important to ensure that resolution is maximised when MDLC or comprehensive two-dimensional LC (LC×LC) are used, by use of suitable interface methods. For example, resolution in LC×LC can be calculated using the following formula:

$$Rs2D = \sqrt{R^2s\ ^1D + R^2s\ ^2D}$$

Where *Rs2D* is the total resolution of the LC×LC system, Rs^1D is the resolution in the first dimension between two peaks and Rs^2D is the resolution in the second dimension. The resolution can be firstly monitored in the first dimension to ensure it is not lost in the second one [62].

Parallel reaction monitoring (PRM) mode can be used to perform MS/MS analysis using the Orbitrap system. Precursor ions of PPCPs are fragmented in this mode resulting in different product ions for each precursor ion. The MS parameters in PRM mode are different compared to full scan mode which include resolution, automatic gain control (AGC) target and maximum injection time (IT). For example, the resolution is usually reduced to 17,500 FWHM instead of 70,000 FWHM in full scan mode which allows faster data collection in this mode in addition to obtaining sufficient data points per peak. Collision energies are also optimised in this mode which improves intensities for each analyte resulting in selecting the collision energy which provides the highest intensity of product ion.
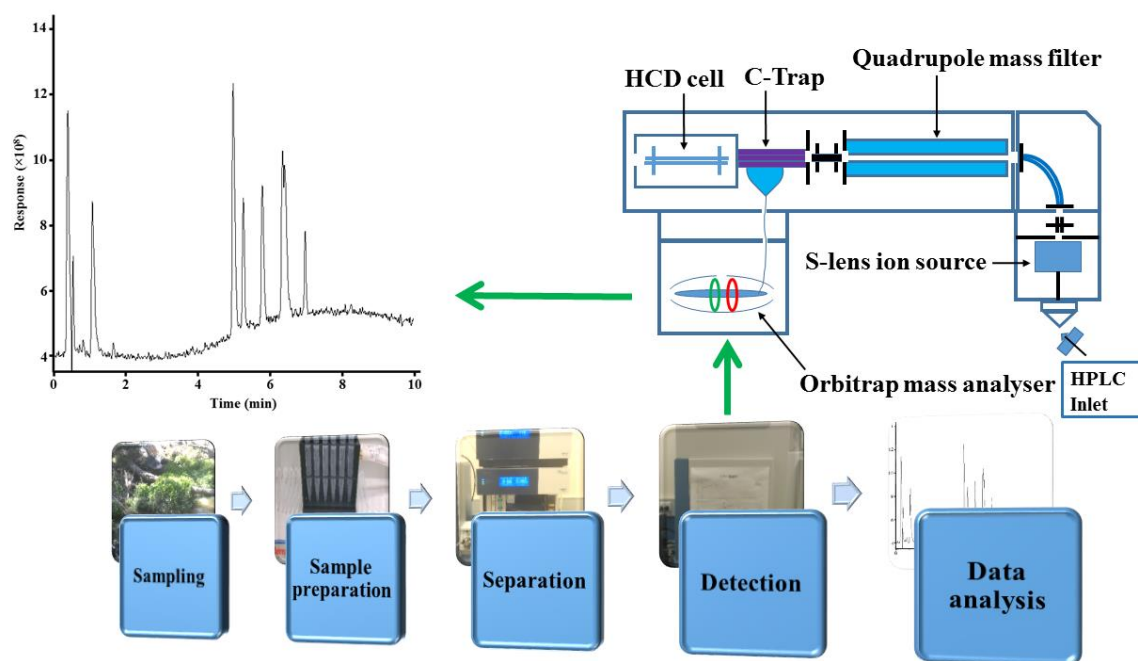
Figure 2 Typical Orbitrap mass analyser system

1.3.5 Quadrupole time-of-flight mass spectrometer

An important tool for trace analysis is the quadrupole time-of-flight mass spectrometer (QTOFMS), which can be used to perform full scan analysis (using total transfer of ions through the QMS) or MS/MS analysis where the mass scan capabilities of the first quadrupole is now invoked. This system is also widely used for identification and quantification analysis because of the capability of achieving high resolution accurate mass (HRAM) analysis. Although QTOFMS provides both reduced resolution and mass accuracy compared to other HRAM mass analysers such as Orbitrap, it has faster data acquisition rates which is preferable for fast elution chromatography applications [52]. This system can also be coupled to ultra-high performance liquid chromatography (UHPLC) which makes it a robust technology that can be effectively used to identify pharmaceuticals especially when co-elution issues need to be addressed [3]. The UHPLC when coupled with QTOFMS can be used for screening or qualification of thousands of contaminants in some environmental samples without needing to have standards because of its accurate mass and MS/MS capabilities [63]. As an example, this

system can also be coupled with two dimensional LC to perform an identification of lipids [64].

Figure 3 shows a schematic diagram of the QTOFMS system.
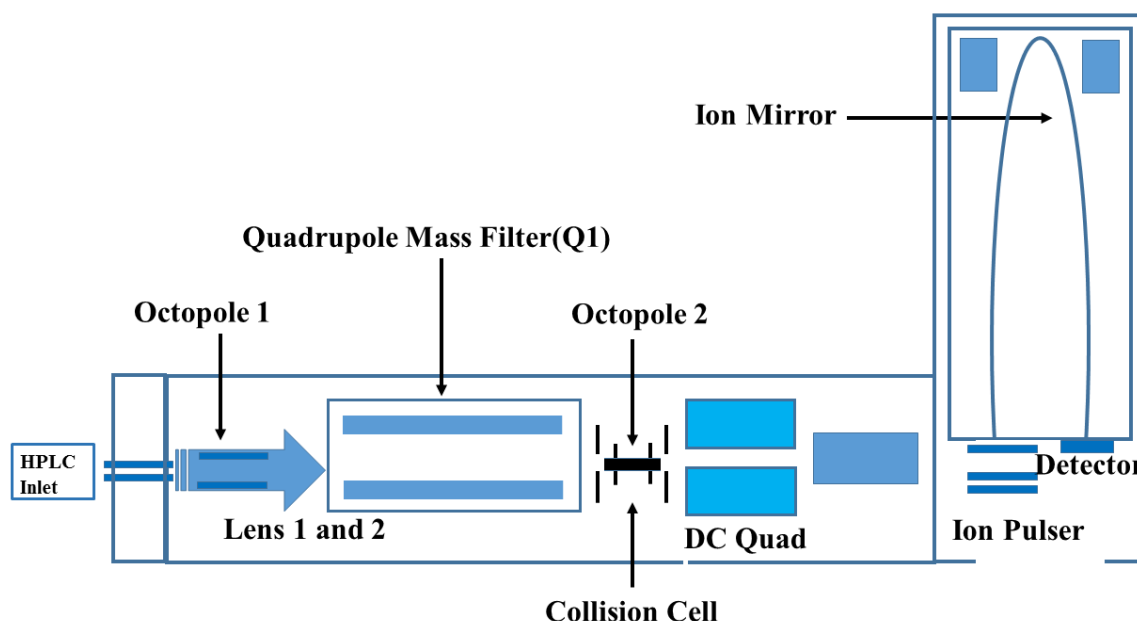
Figure 3 QTOFMS mass analyser system

1.3.6 Triple quadrupole mass spectrometer

The LC–QQQMS instrument plays an important role in quantitative analysis for all manner of analytes and in all manner of sample types, and so is one of the most common instruments for routine analysis. The QQQMS mass spectrometer is preferable compared to QTOFMS when it comes to stability, reliability of quantification, and linear dynamic range, in addition to reducing interferences from sample matrix [65, 66]. One of the advantages of this detector is that using more than one transition for quantification and confirmation lead to achieving higher quality results especially for environmental analysis. Multiple reaction monitoring (MRM) is a common QQQMS technique used to generate MS/MS data by selecting a precursor ion to perform a fragmentation process, and then produce product ion(s) that can be used for quantification and confirmation. Although this step can be done manually, manufacturers have developed software solutions to automatically optimise the collision

energy and fragmentor voltage for each precursor ion, reducing the need for manual intervention. The automated process establishes a database for compounds before running MRM mode. Other examples of MRM modes include dynamic multiple reaction monitoring (dMRM) and triggered multiple reaction monitoring (tMRM) which can be employed to perform even more sensitive analysis especially with many transitions from different compounds. **Figure 4** illustrates the QQQMS mass analyser system.

MRM mode sensitivity can be improved to handle large numbers of compounds and their transitions at the same time. This can be done by using dMRM mode which can quantify compounds at trace level by only focussing on the eluted compounds from the column [18]. tMRM can also be used in this case, which focusses on the most abundant transition for quantification purposes. When the system detect that transition, it triggers more cycles to include other transitions of the same analyte; therefore, an MRM spectrum can be generated to confirm the identity of the analyte. Moreover, sensitivity of the tMRM mode can be increased by optimising the collision energies of all product ions of the same compound [20]. One of the applications of tMRM mode is the study presented in Chapter 4. This mode was effectively employed to detect 10 PPCPs and their transitions. More details can be found in that chapter in this thesis.
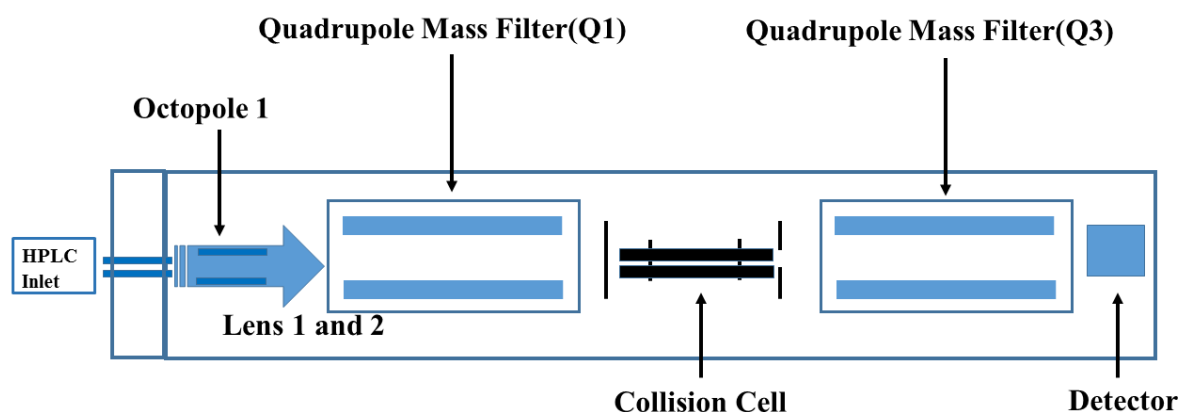
Figure 4 QQQMS mass analyser system.

Although all these analytical technologies have been effectively used to analyse PPCPs in different matrices, much effort is still required in order to tackle this environmental issue. For instance, one of the possible environmental problems of PPCPs is their metabolites and product ions which can be generated by degradation processes of the primary PPCP compound which might occur during the chemical water treatment. Additional analytical studies and/or further methods to detect these metabolites are needed because most analytical approaches have been focussed on the primary compound detection [67]. This can be done using cutting-edge instruments such as the LC–MS systems which have been discussed above, indicating the importance of using other MS modes which have the ability to track multiple product ions of each compound. Optimising MS/MS modes such as PRM, MRM, dMRM and tMRM are necessary for each PPCP; therefore, the metabolites and transformation products of PPCPs can be easily identified and quantified in environmental samples. Moreover, simple and fast analytical methods are important which lead to detection of contaminants at trace levels [68].

1.4 Sample preparation techniques

Although some analytical instruments are very sensitive, and are able to detect analytes even at very low concentrations, it is normally necessary to include additional step(s) before introducing the sample to the instrument, which involves cleaning up and/or removing the matrix, or concentrating the sample. This is important because water, and WWTP and sewage samples for example may have high levels of other impurities [69]. Several sample preparation techniques can be used to prepare a variety of environmental samples such as LLE, various micro-extraction methods, solid phase extraction (SPE) and quick, easy, cheap, effective, rugged and safe (QuEChERS) methods.

1.4.1 Liquid/liquid extraction (LLE)

LLE or solvent extraction is an extraction method that can transfer compounds from one phase to another based on the differential solubility of the compound in two solvents. The LLE extraction method must have three steps. First, adding the extractant, which is the second solvent required to dissolve and extract the analyte of interest, to the first solvent (diluent) that should be immiscible with the extractant. The second step is mixing the two solvents following which process an interface should form between them; additives such as salts may be added to encourage extraction. The analyte of interest should choose to be dissolved in the extractant based on the solubility factor which leads to its separation from other components in the diluent. The last step is separating the extractant and diluent from each other resulting in having the compound of interest in the extractant layer and then this compound can be isolated or recovered by other physical processes such as evaporation. Many factors can play an important role in order to have a successful LLE process. For instance, the compound needs to be easily dissolved in the extractant, therefore it can move readily from the diluent to the extractant. Moreover, the compound needs to be easily separated from the extractant after the extraction.

The disadvantages of this method are the formation of emulsions and the large volumes of solvents that may need to be used to effectively implement quantitative extraction [70].

In this section, SPE and QuEChERS methods are discussed specifically as they have been considered two of the most common extraction methods for environmental samples. These sample preparation techniques are available, affordable and also are very well established in the literature, therefore they can be used here with some modifications and optimisations. There are many different SPE cartridge chemistries that can extract a variety of compounds based on the physical and chemical properties of the compound and the cartridge. Oasis HLB cartridges (Waters Corp., Milford, MA) are commonly used to extract pharmaceuticals from water and they are suitable to extract acidic, basic and neutral PPCPs. HLB (Hydrophilic-lipophilic balanced sorbent) is a reversed phase cartridge which is made of two monomers, the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene. This type of cartridges provides stability at different pH range and in different solvents which makes the retention of polar analytes for example are higher than other conventional silica based cartridges [71]. Although these sample preparation techniques are common, they still need to be optimised in order to develop a method in terms of number of steps, solvent to elute the compounds, and the necessary volume to elute the compounds of interest.

1.4.2 Solid phase extraction (SPE)

The conceptual use of SPE involves separating analytes between solid (a sorptive layer on the solid support) and liquid (sample) phases, in a cartridge-dimension format. The task of the solid phase is to sorb and concentrate compounds from a liquid solution normally with exclusion of a bulk of the matrix from the solid phase [69]. Typical SPE methods have four steps. First, a conditioning step employing two solvents, an organic and another solvent similar to the sample. This is to ensure that SPE cartridges are clean and wet, ready for the extraction

process. The second step includes loading sample through the cartridge which involves a given sample volume and constant flow rate. The third optional step is washing the solid phase used for extraction using a suitable solvent that does not elute analyte. The last step is eluting the analytes from the cartridges by using a solvent which can only elute the compounds of interest from the cartridges and this step must be done preferably using adjusted flow rate and minimum solvent. This type of extraction method can be automated using commercial systems [69, 72, 73].

Figure 5 is an example of an automated SPE system, used in the present study (Thermo Fisher Scientific, Scoresby, Australia). This system is an offline system which has 6 separate pumps suitable to extract 6 samples simultaneously. Depending on the required extraction method, this system can apply many steps to extract a number of samples involving several steps of conditioning, rinsing, eluting and drying. The system can save time and cost by processing many samples in a short time. Accuracy is improved compared to a manual extraction process. It can be used to extract different sample volumes, from 10 mL to 2000 mL which is ideal for water and wastewater samples. Different cartridges sizes can also be used such as 1 mL, 3 mL and 6 mL SPE cartridges which are commercially available. Using HLB cartridges is common to extract PPCPs and other organic pollutants due to its hydrophilic/lipophilic properties. This type of cartridge can be used to extract acidic, basic and neutral compounds from water samples. [56]. These cartridges can perform much better than other cartridges as the recoveries can be higher even with dry cartridges [74]. HLB cartridges are considered reversed- phase sorbents and they are stable at different pH. Hence, this type of cartridge can be used for different applications rather than just PPCPs, such as pesticides.

Figure 5 Dionex AutoTrace 280 (Automated Solid Phase extraction - Thermo Fisher Scientific)

1.4.3 Quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction method

QuEChERS was first developed by Michelangelo Anastassiades et al. in 2002 [75] to extract pesticides from vegetables and fruits samples [76]. This extraction method has been widely used to extract herbicide residues [77], pesticides [78], PAHs [79], and drugs [80] in different matrices. QuEChERS has also been extended to analyse different environmental contaminants in different matrices such as water and soil [81]. This extraction method is simple and requires few steps when applied in food analysis in different matrices [82]. Compared to other extraction methods such as SPE, QuEChERS uses less solvent and time [83]. In general, QuEChERS consists of steps which can be optimised according to the type of analytes of interest, and sample matrix. These steps involve weighing the sample, adding ACN, shaking for a short time, adding QuEChERS salt solution, centrifuging all components and finally taking the upper layer of ACN to be analysed by a suitable instrument. Additional clean up steps such as dispersive SPE can also be added and optimised.

QuEChERS approach can be explained for example with ACN being used to enhance the extraction process, whereas other salts such as magnesium sulphate and sodium chloride are used to help with the separation of water and organic phases. The extract can then be transferred into a vial which contains primary secondary amine (PSA) sorbent that can strongly interact with some compounds such as fatty acids to remove them from the ACN layer. This is based on the interaction between the weak anion exchanger (PSA) and acidic compounds such as fatty acids. Adding magnesium sulphate can also remove the water residue from the extract [81]. Alternative solvents can also be used to reduce sample matrix such as hexane but optimising and testing the extraction method are required in order to ensure this does not affect the recovery of the target analytes.

1.5 Research problem and the importance of this research

1.5.1 Scope

1) To develop appropriate methods of analysis based on LC approaches, to permit quality data to be derived, at the levels of relevance to wastewater streams.

2) To implement sound sampling procedures to ensure sample viability.

3) To perform validated sample preparation procedures to isolate PPCP fractions.

4) To evaluate environmental levels in water streams across Melbourne, and in various biota such as invertebrates, to permit a status report on PPCP occurrence.

5) To provide supporting data for an eco-system evaluation on the effects of PPCPs.

1.5.2 The importance of this research

1) Evaluation of PPCPs has been considered as a new environmental research area in recent years [84].

2) These substances are very important in daily life because they are commonly used by the population; therefore, the aim of this research is to determine the quantity or presence of PPCPs that are discharged to the environment.

3) This research will be based on PPCPs that are used widely by Australians and the occurrence of these substances in the aquatic environment.

4) There needs to be more investigation of these substances since by their nature they often have biological activity, and to understand the environmental impacts of PPCPs is an integral part of the assessment [85].

5) The majority of PPCPs and their transformation products studies have been done in Germany and Spain. USA and Canada had some investigations regarding these contaminants. However, more research is still needed regarding this type of contaminants in other countries such as Australia and China [40].

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Chapter 2

Liquid Chromatography – Quadrupole Orbitrap Mass Spectrometry Method for Selected Pharmaceuticals in Water Samples

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2.1 Abstract

This study developed an analytical approach for sub-ppb level detection and confirmation of 13 pharmaceuticals and personal care products (PPCPs) in water samples using ultra high pressure liquid chromatography hyphenated with a quadrupole Orbitrap mass spectrometer (UHPLC- Q-Orbitrap-MS). Sample preparation was performed by using solid phase extraction (SPE) employing hydrophilic-lipophilic balance cartridges, with elution of sorbed analytes using methanol. Acceptable automatic gain control (AGC) target and maximum injection time (IT) were 1×10^6 and 200 ms, respectively, resulting in a mass accuracy <2 ppm. High response signals with sufficient data points per peaks (20–30) were obtained whilst maintaining high resolution of approximately 70,000 full width at half maximum. Extracted ion chromatograms provided quantitative analysis with linearity (R^2) ranging from 0.9875 to 0.9993 and method detection limits ranging from 0.01–0.61 ng mL^{-1}. Compounds were further analysed by MS/MS analysis, with the MS operated in parallel reaction monitoring (PRM) mode under precursor ion analysis intervals and collision energies chosen for the different PPCPs. The developed method was applied to analyse water samples obtained from sources in Victoria, Australia.

2.2 Article

Liquid chromatography – quadrupole Orbitrap mass spectrometry method for selected pharmaceuticals in water samples

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ABSTRACT

This study developed an analytical approach for sub-ppb level detection and confirmation of 13 pharmaceuticals and personal care products (PPCPs) in water samples using ultra high pressure liquid chromatography hyphenated with a quadrupole Orbitrap mass spectrometer (UHPLC- Q-Orbitrap-MS). Sample preparation was performed by using solid phase extraction (SPE) employing hydrophilic-lipophilic balance cartridges, with elution of sorbed analytes using methanol. Acceptable automatic gain control (AGC) target and maximum injection time (IT) were 1×10^6 and 200 ms, respectively, resulting in a mass accuracy <2 ppm. High response signals with sufficient data points per peaks (20–30) were obtained whilst maintaining high resolution of approximately 70,000 full width at half maximum. Extracted ion chromatograms provided quantitative analysis with linearity (R^2) ranging from 0.9875 to 0.9993 and method detection limits ranging from 0.01–0.61 ng mL^{-1}. Compounds were further analysed by MS/MS analysis, with the MS operated in parallel reaction monitoring (PRM) mode under precursor ion analysis intervals and collision energies chosen for the different PPCPs. The developed method was applied to analyse water samples obtained from sources in Victoria, Australia.

1. Introduction

Thousands of chemical substances may be considered PPCP products, such as prescription medicines, perfumes, cosmetics, pet care preparations, and many compounds in common daily use [1]. Amongst PPCPs, chemicals such as bacteriocides and antifungals [2] can have adverse environment effects following discharge of effluent which contain PPCPs into water streams, or by application of sludge (e.g. from waste water/sewage treatment facilities) as fertiliser [3]. Wastewater treatment plants (WWTPs) have been considered as one of the main inputs of PPCPs to the environment subsequent to water treatment [4,5], largely due to the inadequacies for removal of PPCPs from the WWTP input. Consequently it is important to monitor the effectiveness of treatment plants, and to enhance conventional technologies applied in WWTPs in order to reduce PPCPs in the environment [4]. PPCPs have received increased attention in the last decade as emerging contaminants comprising chemicals which need to be taken into account when released to the environment. There is concern regarding the effect

of PPCPs on the environment and the ecosystem [6] arising from the interaction between PPCPs and organisms in the environment [7]. Therefore, profiling these chemicals is a priority, and understanding their impacts on the environment in order to ascertain safe environmental limits, and to establish records of the occurrence of PPCPs in the ecosystem. Harmonised methodologies and reporting precise and accurate analytical measurements across environmental sinks is of concern. Evaluation of PPCPs is now considered an emerging environmental research area in recent years [8] and an essential measure of environmental risk management.

Due to the molecular heterogeneity of the samples, appropriate sample preparation and separation techniques are usually performed prior to detection by MS. Both gas chromatography (GC) and liquid chromatography (LC) are commonly used for separation of PPCPs. Both techniques show excellent capabilities for detection of pollutants in the environment, especially when integrated with MS. LC is preferred to GC for direct analysis of aqueous samples, and especially for solutes which are thermally unstable. GC usually requires extraction of samples of volatile and thermally stable analytes [9] and use of a GC-compatible solvent. Typical recently reported studies on PPCPs in various matrices using selected analytical methodologies such as GC and LC have been reported [10–17].

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The Orbitrap MS has been commercially available since 2005, and since then a variety of different analysers based on this concept have been introduced [18,19]. The Q-Exactive Plus Hybrid Quadrupole (Q)–Orbitrap MS is one such example. Of relevance to the present discussion, the Q-Exactive Plus offers resolving power up to 280,000 full width at half maximum (FWHM), with mass accuracy <3 ppm as well as polarity switching between positive and negative modes as one full cycle in <1 s, with acquisition speed up to 12 Hz. Functionally, the quadrupole filter can select or transfer target ions, to the Orbitrap, for differentiation and accurate mass measurement. Precursor ions can be selected in parallel reaction monitoring mode (PRM) in order to conduct MS/MS analysis, according to analyte fragmentation and in a multi-component sample selected based on analyte chromatographic retention time. This mass analyser has been considered a cutting edge technology in terms of high resolution and accuracy [20].

Considerations of separation and mass analysis dimensionality for hyphenated analysers have been proposed [21]. Orbitrap technology has been applied to a range of complex matrices [22] such as environmental and biological samples. Ultra-high performance liquid chromatography (UHPLC) provides advantages of high efficiency and low flow by using small particle size, and is suited to hyphenation with the Orbitrap MS, for identification and quantification of many compounds in samples [23].

In this study, 13 PPCPs were chosen to be investigated. The selected compounds represent a wide range of PPCPs which include different types of drugs and at least one personal care product (triclosan). Another reason is that several environmental studies tend to focus on some of these classes of PPCPs such as antidepressants (citalopram, paroxetine, fluoxetine and sertraline) which were included in this study. Other PPCPs such as caffeine can be used as an organic tracer for domestic inputs and it is likely to be detected in many water ways. Other PPCPs used here such as metformin, cimetidine, ranitidine, ciprofloxacin, amoxicillin, diphenhydramine and cetirizine are common prescription drugs.

A UHPLC-Q-Orbitrap MS method for identification and quantification of 13 PPCPs was developed. The method was evaluated according to mass accuracy, linearity of calibration curves and reproducibility of chromatographic results, including peak area and number of points per peak, of the studied compounds. The developed approach was then applied to analyse PPCPs in wastewaters from different sources.

2. Experimental

2.1. Chemicals and reagents

Metformin (1,1-dimethylbiguanide hydrochloride) (97% purity), ranitidine hydrochloride, diphenhydramine hydrochloride (≥98%), cimetidine, caffeine (reagent plus), ciprofloxacin (≥98% HPLC grade), and triclosan (≥97% HPLC grade) were purchased from Sigma-Aldrich (Castle Hill, Australia). Amoxicillin trihydrate (98% purity), citalopram hydrobromide (98% HPLC grade), paroxetine HCl (98% HPLC grade), fluoxetine hydrochloride (98% HPLC grade), sertraline HCl (98% HPLC grade), cetirizine 2HCl (98% purity) were obtained from A. K. Scientific (Union City, CA). Acetonitrile (HPLC grade), methanol (HPLC grade) and acetic acid were purchased from Merck KGaA (Darmstadt, Germany). Milli-Q water was used for cleaning and sample preparation purposes. Supplementary Information Table S1 summaries molar mass, molecular formula and structure of the PPCPs.

2.2. Sample preparation

A stock solution of each standard (10,000 ppm) was prepared in methanol. The individual solutions of 13 standards were mixed and serially diluted in water to appropriate concentrations. All solutions were stored at (<−15 °C) until required for analysis. Water samples S1, S2, S3 and S4 were collected from different sites in Victoria, Australia. These are treated wastewater samples and they were collected from the effluent of two WWTP discharge streams, one of which leads to a small creek. All samples are grab samples and they were collected in 1 L glass bottles wrapped with aluminium foil and kept at 4 °C until extraction. All were extracted within 48 h.

2.3. Extraction procedure

A procedure for preparing an extract of PPCPs from water [12,24] was used with some modifications of the extraction process and the final analytical technique used for analysing samples. Solid phase extraction was used for clean-up and to extract analytes of interest. OASIS® HLB 3cc (60 mg) extraction cartridges (Waters Corp., Milford, MA) were used. Cartridges were conditioned with 4 mL methanol and 6 mL of Milli-Q water. Then, 30 mL of each water sample was applied using vacuum (Buchi Labortechnik AG, Flawil, Switzerland). The extracted components were eluted with 5 mL of methanol. 300 µL of Milli-Q water was then added to the extracted MeOH solution before evaporating under nitrogen gas to approximately 1.0 mL. The extract test tube was vortexed to dissolve analyte on the wall of the test tube. A 2 mL vial was weighed before and after transferring the extract, for quantitative purposes. An identical extraction method was applied for extraction of solution containing 29.5 mL of water and 0.5 mL of standard solutions (1 ppm) as a recovery test.

2.4. UHPLC-Q-OrbitrapMS

Standards and samples were analysed by ultra-high performance liquid chromatography (UHPLC) hyphenated with a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Scoresby, Australia) which was equipped with a heated electrospray ionisation source (HESI), binary pump, and autosampler. An Agilent Extend-C18 column (1.8 µm particle size, 2.1 × 50 mm) was purchased from Agilent Technologies (Santa Clara, CA). Samples were separated at 25 °C, with 0.1% v/v acetic acid in water, and acetonitrile, used as mobile phases A and B, respectively. The elution gradient started at 10% v/v of mobile phase B for 2 min then was linearly increased to 27% v/v B at 5 min, from 27 to 50% v/v B at 10 min and from 50 to 100% v/v B at 14 min. The mobile phase content was held at 100% v/v B for 1 min and was then decreased to 10% v/v B, and equilibrated at 10% v/v B for 4 min. Total analysis time was 20 min. The flow rate was 0.30 mL min^{-1} with injection volume of 15 µL. The HESI source was separately operated in either positive or negative modes. The source conditions were sheath gas 35, auxillary gas 10, sweep gas 0, spray voltage 3.0 kV, capillary temperature 320 °C and auxillary gas heater temperature 300 °C. The MS was operated in both either positive and negative full scan modes (from 50 to 400 m/z), applied with varied MS parameters: resolution (from 17,500 to 280,000 FWHM), AGC target, which regulates the number of ions to be injected into the Orbitrap analyser, (from 2×10^4 to 5×10^6) and maximum injection time or IT, which is the highest required injection time in order to obtain the AGC target value, of 20, 200 and 2000 ms. MS/MS analysis using PRM was also performed. The chromatographic and general MS conditions were the same as that employed in full scan analysis with various MS parameters altered: resolution (17,500 FWHM), AGC target (2.0×10^5) and maximum IT (100 ms). The protonated molecule was selected as a precursor ion for each PPCP, with different product ions selected for precursor scan analysis at different time intervals around the retention time of each PPCP peak. The system was calibrated daily before analysis for both positive and negative modes.
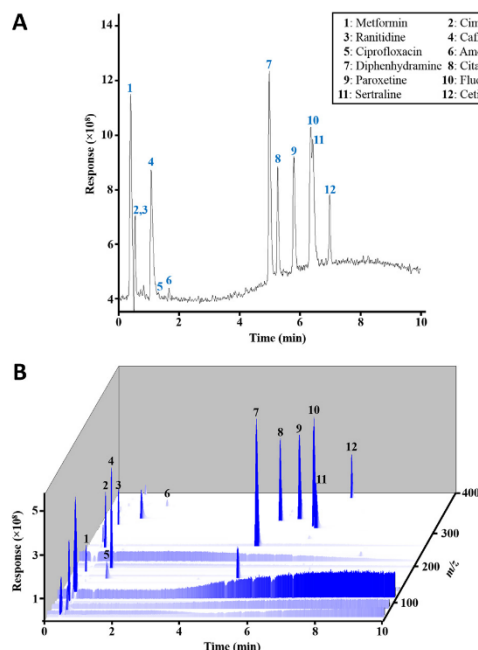
Fig. 1. UHPLC-Orbitrap MS result for a standard containing 13 PPCPs detected in the positive ion mode: (A) Total ion chromatogram and (B) the corresponding 3D plot (intensity, time and ion mass m/z). The peaks were labelled according to the retention time and accurate m/z values of the protonated molecular masses of the PPCPs, as shown in Table 1.

2.5. Data analysis

Xcalibur 3.0.63 (Thermo Fisher) software was used to control the instrument and for data analysis. TraceFinder 3.0 software (Thermo Fisher) was applied for identification, confirmation and quantification analysis with Microsoft Excel 2010.

3. Results and discussion

Reversed-phase columns packed with 1.8 μm particles were used for the separation of all the samples in this study. The mixture of 13 PPCPs was eluted from the chromatographic column and detected by Orbitrap MS in full scan mode with either positive or negative ionisation mode resulting in total ion chromatograms (TIC) with acceptable chromatographic resolution where all targeted peaks were eluted within <10 min as shown in Fig. 1A except triclosan which was eluted after 10 min in the negative mode. A total analysis time of 20 min was required for each analysis, including column re-equilibration time prior to the next injection.

3.1. Selection of MS parameter

Since MS parameters can influence the chromatographic outcome (e.g. signal intensity, sampling frequency or number of points per peak in a chromatogram), selection of these parameters should thus take into account the compromise (e.g. to optimise MS resolution and at the same time obtain sufficient data points per peak and obtain improved signal intensity) between MS and chromatographic performances where good chromatographic and MS results were obtained.

This study focussed on maximising the peak area and obtaining an adequate number of data points per peak, as an indication of the capability to detect lower quantities with acceptable quantification of compounds. Too few data points for fast-eluting peaks risks reduced quantitative reproducibility. Obtaining a high MS resolution is also desirable, in order to provide specificity of detection for applications where potentially many hundreds of co-extractable analytes and interferences may arise. Resolution for the Orbitrap MS is governed by acquisition time. Although a resolution of up to 280,000 FWHM can be employed, this requires long residence time for ions in the analyser, resulting in reduction of signal intensity and the number of points that can be acquired over the elution time of peaks issuing from the column. The average peak width at half height for caffeine was ±0.056 min (for a 1 ppm solution). Effects of different MS parameters on chromatographic results of selected PPCPs are shown in Fig. 2. The AGC target, IT and resolution of 10^6, 200 ms and 70,000 FWHM respectively were found to be suitable for analysis, resulting in high peak area and number of data points across peaks, as well as high resolution as indicated by the red marker spots located in Fig. 2A–H.

Data for retention times, accurate mass (experimentally measured protonated ion mass) and mass accuracy for each PPCP are shown in Table 1. Note that mass accuracy is expressed in parts per million (ppm) being $10^6 \times$ [(exact mass – accurate mass)/exact mass]. Confirmation of each analyte relied upon constancy of retention time, and according to the accurate mass agreement to the exact mass; a narrow mass tolerance window (5 ppm) indicated good selectivity of the method towards each compound. This also displayed good resolving power, as the ability of the mass spectrometer to separate ions with two different but close m/z values to ensure that only target ions contributed to the desired measurement, with minimisation of false detection. Generally good peak shapes for each analyte was attained by hyphenation with the Orbitrap MS, operated with a mass resolution of 70,000 FWHM and a mass tolerance window of 5 ppm. Extracted ion chromatograms (EICs) of all target ions were obtained by using a 5 ppm mass window around the protonated exact mass [M+H]$^+$ (as well as the [M−H]$^-$ of triclosan) with mass accuracy being <2 ppm (averaged from several injections; Table 1). Reconstruction of all EICs along the m/z axis resulted in a three-dimensional presentation (according to response, time and m/z) which allows facile identification and confirmation, e.g. of 12 PPCPs in a single analysis in positive mode as illustrated in Fig. 1B.

3.2. Validity of the method

Calibration curves were prepared for every compound using Milli-Q water. The correlation coefficient (R^2) was at least 0.99 for every analyte except for citalopram (0.9896) and fluoxetine (0.9875) (Table 2). The calibration range was 0.1–100 ppb with 7 data points in the calibration curve. Mass accuracy was below 2 ppm for all 13 PPCPs (Table 1). The detection limit for the instrument was 10–1000 pg mL^{-1}. The LOD was identified by reducing concentration of injected compounds in Milli-Q water until peak heights were less than three times noise level in EIC analysis. For matrix interference, finding a water sample without all PPCPs of interest was difficult. Matrix effects were studied using two compounds, cimetidine and ranitidine. Having small retention times, they so could suffer more interference, but showed good recoveries of 101% (±3) and 95% (±9) respectively when spiked in a water sample. However, it should be noted that quantification with calibration curves prepared in water can lead to errors in the results for wastewater analysis. Recovery data of standards spiked in a water sample (relative to that in Milli Q water) are presented in Table
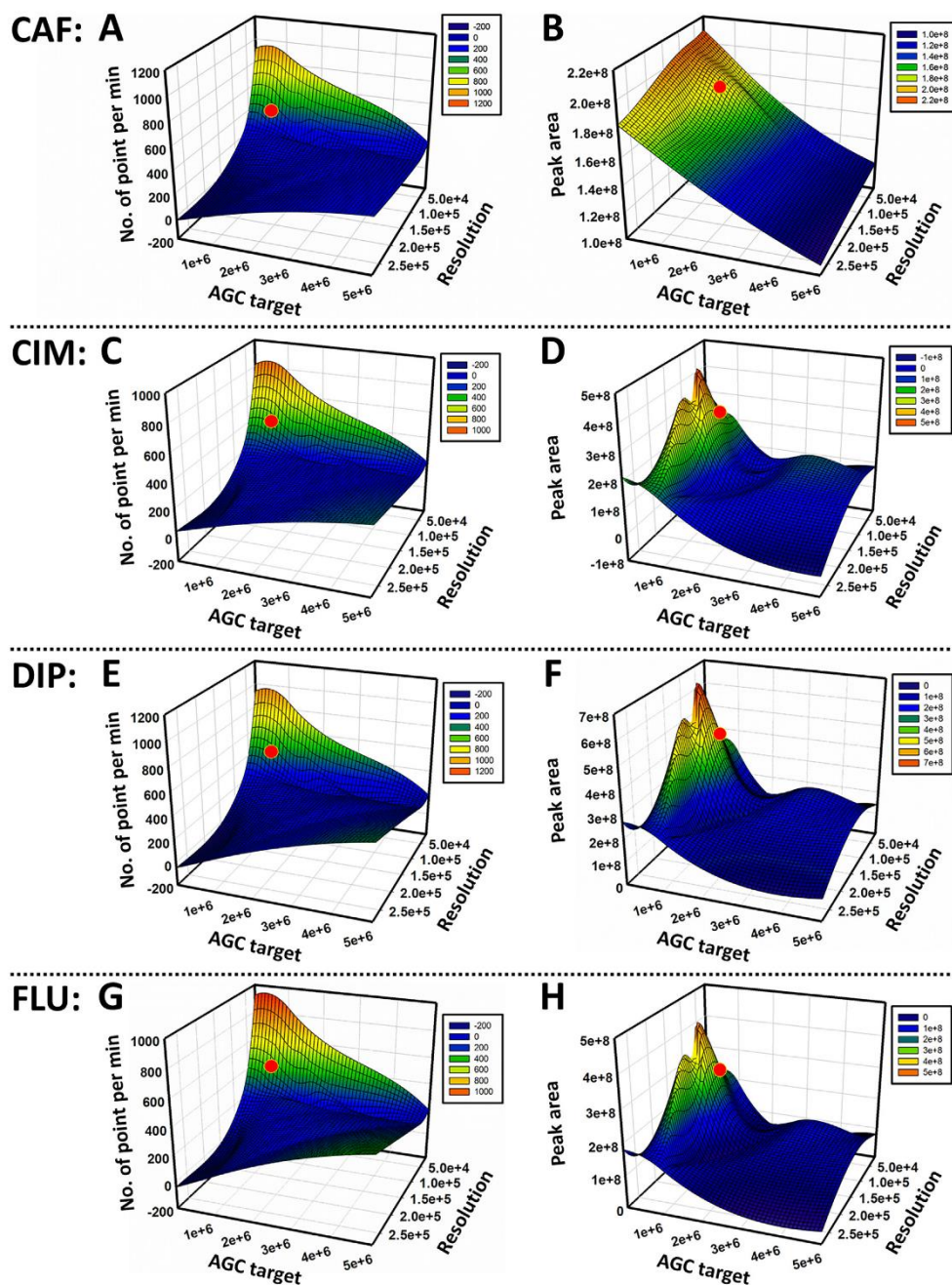
Fig. 2. Effect of MS parameters: AGC target and resolution, operated with fixed maximum IT being 200 ms and $m/z = 50$–400, on chromatographic performance: (A) number of data point per peak and (B) peak area of caffeine, CAF. The corresponding plots were also provided for (C and D) cimetidine, CIM, (E and F) diphenhydramine, DIP, and (G and H) fluoxetine, FLU.

Table 1
Retention time, exact protonated or deprotonated mass and mass accuracy of each PPCP in standard and sample (1).

No.	Compound	Retention time (min)	*m/z* value of [M+H][a]	Mass accuracy (ppm)	
				Standard	Sample (1)
1	Metformin	0.41	130.1087	−0.3	−0.5
2	Cimetidine	0.53	253.1230	0.9	−0.4
3	Ranitidine	0.54	315.1485	0.6	−0.3
4	Caffeine	1.07	195.0877	0.7	0.2
5	Ciprofloxacin	1.36	332.1405	0.8	0.1
6	Amoxicillin	1.66	366.1118	0.3	NA
7	Diphenhydramine	5.09	256.1696	0.8	0.3
8	Citalopram	5.26	325.1711	0.9	−0.4
9	Paroxetine	5.80	330.1500	0.7	0.0
10	Fluoxetine	6.50	310.1413	0.5	0.0
11	Sertraline	6.51	306.0811	0.3	1.1
12	Cetirizine	6.98	389.1627	0.5	0.0
13	Triclosan	12.30	286.9439[a]	0.0	−0.1

[a] Triclosan was detected in negative ion mode [M−H]⁻.

Table 2
Calibration curve information (intercept, slope and R^2) for each compound (calibration range is 0.1–100 ppb using 7 data points).

Compound	Intercept	Slope	R^2
Metformin	−5.84E+06	2.46E+03	0.9980
Cimetidine	2.94E+06	1.41E+03	0.9934
Ranitidine	−1.54E+06	5.22E+02	0.9955
Caffeine	3.94E+06	2.61E+03	0.9993
Ciprofloxacin	−2.32E+06	6.54E+02	0.9969
Amoxicillin	5.84E+04	9.15E+01	0.9972
Diphenhydramine	−6.41E+06	2.38E+03	0.9965
Citalopram	−3.62E+06	7.03E+02	0.9896
Paroxetine	−8.32E+06	1.63E+03	0.9900
Fluoxetine	−1.33E+07	2.40E+03	0.9875
Sertraline	−5.07E+06	1.05E+03	0.9908
Cetirizine	−1.88E+06	7.95E+02	0.9949
Triclosan	−2.80 E+05	2.52 E+02	0.9983

S4 for some compounds not detected in the water sample without spiking.

3.3. Optimisation of precursor ion analysis intervals and collision energies for further confirmation of PPCPs in MS/MS analysis

Under the same chromatographic conditions, MS/MS analysis with the MS operated in PRM mode was performed by selecting 13 precursor ions at different intervals according to accurate molecular mass and retention time values of the target PPCPs. The number of data points assigned to any given peak in a chromatogram will be shared when several precursor ions are analysed within the same window interval. For example, instead of 20 data points per peak, the number of points will be reduced to about 2 data points per peak if 10 precursor ions were analysed in the same interval. In order to increase the number of points for an analyte, the analysis of precursor ions was divided into a number of retention time windows so as to minimise intersection of several precursor ion analysis at the same time. The window tolerance chosen had a margin ±0.5 min around the retention time of each PPCP for most of the compounds, corresponding to a maximum of 3 precursor ions within the suite of analyte peaks. In addition, MS resolution was also reduced allowing faster data collection over the separation. As a result, AGC target, IT and resolution of 2.0×10^{-5}, 100 ms and 17,500 FWHM were applied, allowing MS/MS analysis with acceptable MS resolution as well as providing sufficient number of data points (~10 points per peak, being acceptable for qualitative analysis) in chromatograms. Furthermore, collision energy was optimised for MS/MS analysis of each PPCP in order to maximise confirmation ion intensities for each compound. In order to investigate the effect of collision energy for each MS/MS transition, the number of data points in a TIC was alternately divided into 3–4 groups. This means variation of 3–4 collision energies can be performed within a single chromatographic run, and any 3–4 adjacent data points in the TIC were collected under MS/MS modes applying different collision energy values. For example in a case when three collision energies (20, 30 and 40 eV) are varied within a single TIC analysis, the 1st, 4th, 7th, 10th etc. data points in the TIC can be studied at 20 eV; whilst the 2nd, 5th, 8th, 11th etc. data points can be studied at 30 eV and the 3rd, 6th, 9th, 12th etc. data points are studied at 40 eV. Note that >4 collision energy variations could not be performed due to insufficient data points per peak. To this end, two chromatographic runs were performed in order to cover the collision energies ranging from 10 to 70 eV. The first and the second runs examined 10, 20 and 30 eV, and 40, 50, 60 and 70 eV, respectively. The example for caffeine, with

Table 3
The optimised collision energy, retention windows, and MS/MS transitions applied in analysis of each PPCP.

No	Compound	Collision energy (eV)	Ret. time window (min)	Confirmation ion (1) (*m/z*)	Confirmation ion (2) (*m/z*)	Precursor ion (*m/z*)
1	Metformin	60	0.10–1.50	60.0562 (1.7)[a]	113.0822 (−0.9)	130.1087
2	Cimetidine	30	0.10–1.00	159.0699 (1.3)	117.0481 (−1.7)	253.1230
3	Ranitidine	30	0.10–1.50	176.0488 (0.6)	224.0978 (0)	315.1485
4	Caffeine	50	1.00–2.00	138.0663 (2.2)	110.0713 (−2.7)	195.0877
5	Ciprofloxacin	30	1.20–4.50	288.1506 (1.0)	245.1084 (0)	332.1405
6	Amoxicillin	20	0.00–3.00	160.0427 (1.2)	207.0765 (0)	366.1118
7	Diphenhydramine	30	5.00–7.50	167.0856 (1.2)	152.0621 (3.3)	256.1696
8	Citalopram	40	5.00–7.50	109.0451 (0.9)	262.1027 (0.8)	325.1711
9	Paroxetine	60	5.50–8.50	70.0657 (2.9)	192.1183 (0)	330.1500
10	Fluoxetine	10	6.50–9.00	148.1121 (0.7)	277.0359 (0.7)	310.1413
11	Sertraline	20	6.50–9.50	275.0388 (1.1)	158.9763 (1.9)	306.0811
12	Cetirizine	30	6.50–8.50	201.0466 (1.0)	187.1078 (0)	389.1626
13	Triclosan[a]	20	10.00–20.00	250.9673 (0.4)	136.5424 (0)	286.9439

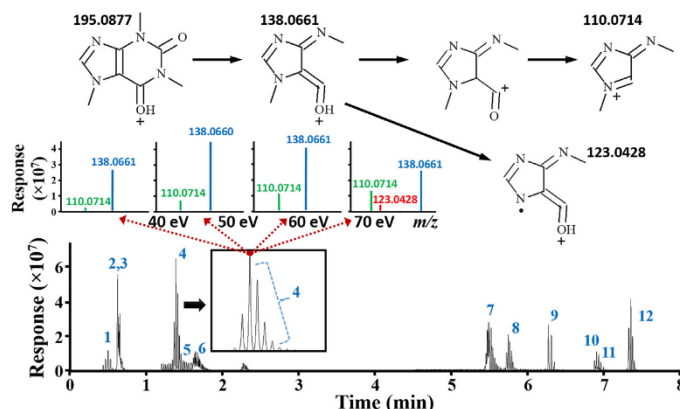[a] Mass accuracy (ppm) of confirmation ion.

Fig. 3. UHPLC–Orbitrap MS results for the standard containing 13 PPCPs with MS, 12 of them operated in positive targeted PRM mode for different precursor ions as shown in Table 4 within the time interval around each analyte retention time and different collision energy (CE) from 40 to 70 eV varied along each chromatographic data point. Example optimisation of CE was illustrated for caffeine including the fragmentation mechanism of this compound.

collision energies varied from 40 to 70 eV, is presented in Fig. 3. The collision energy of 50 eV was selected, due to the highest intensity of fragment ion that results, with m/z 138.0663 selected as the confirmation ion. The optimised collision energies for all the studied compounds are shown in Table 3.

3.4. Application of the methodology for analysis of water samples

All relevant information on retention times, mass accuracy and concentration of each compound in various samples can be found in Tables 1 and 4. An example full scan chromatogram for the PPCPs of interest is shown in Fig. 1. Fig. 4A shows the TIC for water sample (S1). Clearly under these conditions and concentration, the levels are far too low to provide clarity in the TIC chromatogram.

Taking cetirizine as an example, this compound in both standard and sample (1) eluted at the same retention time (Fig. 5A & C) using full scan mode. While the EIC of this compound in full scan mode shows a clear signal of the precursor ion in the standard (Fig. 5B), it shows a low signal for the same precursor ion (m/z 389.1626) in the mass spectrum in sample (S1) due to other low mass signals from interfering noise (Fig. 5D). When cetirizine is extracted in PRM mode, it can also be seen in both standard and sample (1) (Fig. 5E & G) with the latter some 500-fold less abundant. Using PRM mode also produces a fragment ion of m/z 201.0467 which can be reliably used to confirm cetirizine in sample (1) by providing a clean signal in the mass spectrum in sample (1) (Fig. 5H) which matched with the standard as illustrated in (Fig. 5F). The collision energy was optimised for this compound which resulted in a CE of 30 eV. All compounds were detected by positive ion mode except triclosan which was detected by negative ion mode, and they all were separated either by the difference in retention time and the accurate mass in full scan mode or by generating different fragment ions in PRM mode. The recovery for most of the studied PPCPs was higher than 80%, however; some of the analytes (Supplementary Information Table S2) showed poor recoveries. Although low recovery was observed, low concentration of PPCPs could still be detected indicating the capability of this method to analyse trace levels of PPCPs in environmental samples. The advanced instrumental approach applied here provided detection of all the studied compounds. However, since excessively low or high values were obtained for some compounds, and which is normally considered to be unacceptable, further development of the sample preparation method is warranted to clarify the sources of error which might
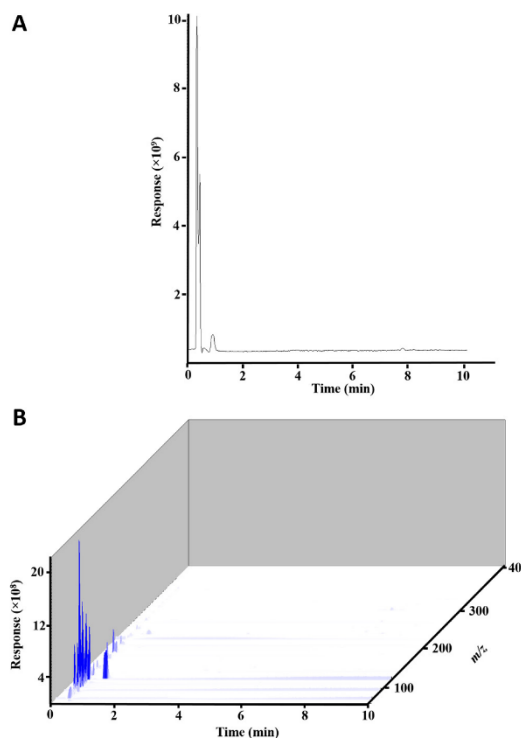
Fig. 4. UHPLC–Orbitrap MS result for a water sample (S1) detected in the positive mode: (A) Total ion chromatogram, and (B) The corresponding 3D plot (intensity, time and ion mass m/z).

account for these recoveries (such as incorrectly chosen sorbent material), when analysis employs a very sensitive instrument.

The HLB cartridge is expected to have hydrophilic/lipophilic properties and can be considered as a reversed-phase sorbent that can be applied to extract acid, basic and neutral analytes from water. However, due to the absence of chargeable functional groups

Table 4
Concentration and (standard deviation) of each PPCP in the real samples calculated from the concentrations of the extracted solution using full scan mode.

| Compound	Concentration (standard deviation) in real samples ($\mu g\,L^{-1}$)			
	S1	S2	S3	S4
Metformin	2.11 (0.22[a])	2.24 (0.29)	7.57 (0.75)	9.08 (0.84)
Cimetidine	ND[b]	<LOQ[c]	<LOQ	ND
Ranitidine	<LOQ	<LOQ	<LOQ	<LOQ
Caffeine	0.99 (0.21)	0.31 (0.08)	1.25 (0.23)	<LOQ
Ciprofloxacin	ND	ND	ND	ND
Amoxicillin	ND	ND	ND	ND
Diphenhydramine	0.12 (0.04)	0.17 (0.03)	0.12 (0.04)	0.11 (0.04)
Citalopram	0.20 (0.06)	0.20 (0.06)	0.40 (0.13)	0.14 (0.05)
Paroxetine	0.23 (0.01)	ND	0.24 (0.01)	0.25 (0.01)
Fluoxetine	0.23 (0.03)	0.23 (0.03)	0.24 (0.03)	0.23 (0.03)
Sertraline	0.21 (0.01)	0.21 (0.01)	0.28 (0.01)	0.22 (0.01)
Cetirizine	0.29 (0.32)	0.32 (0.31)	1.13 (1.13)	<LOQ
Triclosan[d]	0.35 (0.09)	0.45 (0.03)	0.87 (0.11)	<LOQ

[a] = Standard deviation obtained with n = 3.
[b] = Not detected.
[c] = Detected with lower concentration than limit of quantification.
[d] = Detected and quantified using full scan mode only.
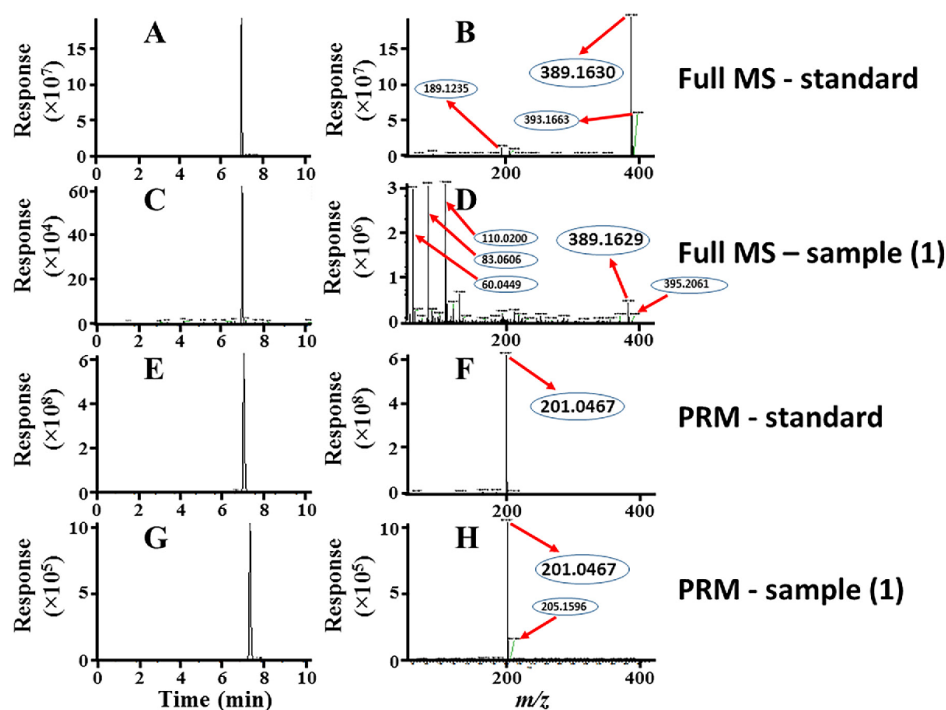
Fig. 5. Comparison of UHPLC-Orbitrap MS results for cetirizine in standard and sample S1. (A) and (C): extracted ion chromatograms (EIC; m/z = 389.1626) with MS operated in full scan mode. (E) and (G): corresponding EIC with MS operated in PRM mode with optimised collision energy of 30 eV for precursor ion of m/z = 389.1626 and product ion of m/z = 201.0467. The spectra for cetirizine peaks in A, C, E, and G are shown in B, D, F and H, respectively.

and negligible silanol activities, this cartridge performance can be expected to be less effective with either positively and/or negatively charged molecules. With the studied waste water samples (as the medium for extraction), several molecules can be protonated (see compounds with basic functionalities with high pK_a, e.g. >9 in Table S2) and/or deprotonated (see compounds with acid functionalities with low pK_a, e.g. <6 in Table S2) in equilibrium with uncharged species. In addition, it is less effective in extraction of charged compounds with good solubility in water (e.g. those with

log K_{ow} <1). Thus, the recovery of these compounds, such as metformin, amoxicillin and cimetidine, from the HLB cartridge can be <20% (see Table S2). Thus low recovery values may arise for the water sample analysis in this study.

The limit of detection (LOD) and limit of quantification (LOQ) can also be found in the Supplementary Information, Table S3. The LOD was identified by injection of compounds with reduced concentration in Milli-Q water until their peak heights were less than three times of the noise level in EIC analysis. In order to calculate

the LOQ, the signal to noise should be 10 times lower than peak heights.

The results of water sample analysis are summarised in Table 4. These results show that most of PPCPs of interests can be reliably quantified in these samples although most concentrations in the water samples near the WWTP discharge are at low levels. Some solutes (ciprofloxacin and amoxicillin) were below detection limits, and ranitidine and cimetidine were generally below LOQ. However most of the PPCPs can be detected in the samples (above the LOD and LOQ) using the Orbitrap system. Metformin was generally the highest analyte with highest concentrations in samples S3 and S4.

4. Conclusion

Application of UHPLC-Q-Orbitrap MS for analysis of PPCPs with a high performance separation in reversed-phase mode and high resolution MS analysis was demonstrated. High resolution MS and MS/MS analysis produces high selectivity and sensitivity based on the exact mass measurement of protonated and fragment ions using a narrow mass extraction window. Optimisation approaches were performed to develop an adequate method that allows high resolution analysis of a range of PPCPs in water samples with reliable quantification and confirmation of compound identities.

Acknowledgements

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.2017.08.003.

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2.3 Supplementary information

Supplementary Information

Journal of Chromatography A

Liquid Chromatography – Quadrupole Orbitrap Mass Spectrometry Method for Selected Pharmaceuticals in Water Samples

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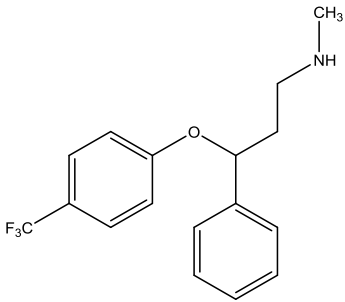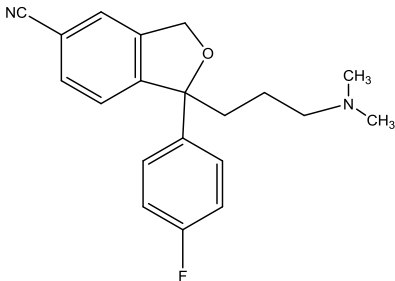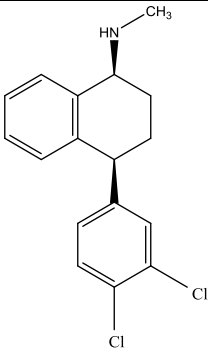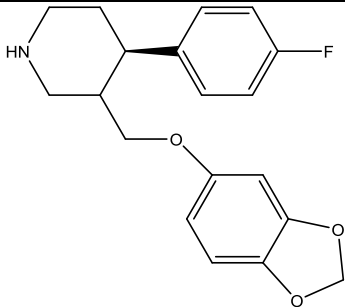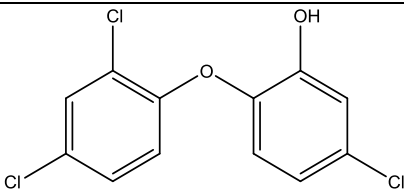
Table S1 Molecular weight, molecular formula and structure of each PPCP.

Table S2 Recovery for all 13 compounds
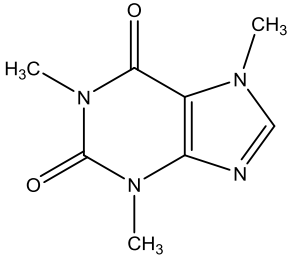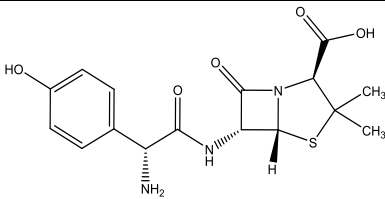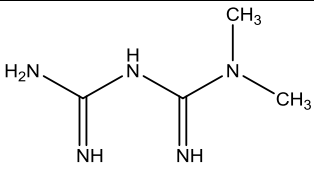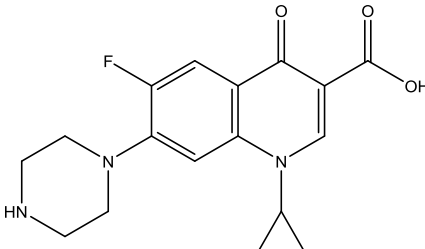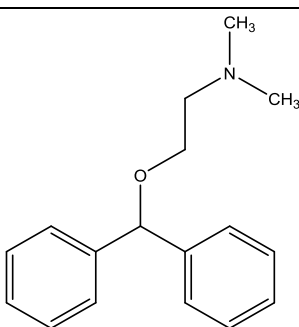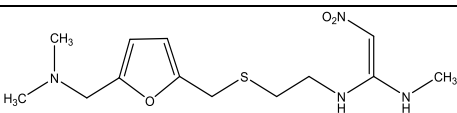
Table S3 LOD and LOQ for all 13 compounds

Table S4 Recovery data (and standard deviation) of standards spiked in a water sample

(relative to that in Milli Q water) for some compounds not detected in the water sample

without spiking.

Table S1 Molecular weight, molecular formula and structure of each PPCP.

Compound	Accurate mass of [M+H$^+$]	Molecular formula	Structure
Fluoxetine	310.1413	$C_{17}H_{18}F_3NO$	
Citalopram	325.1711	$C_{20}H_{21}FN_2O$	
Sertraline	306.0811	$C_{17}H_{17}NCl_2$	
Paroxetine	330.1500	$C_{19}H_{21}ClFNO_3$	
Triclosan	286.9439	$C_{12}H_7Cl_3O_2$	

Caffeine	195.0877	$C_8H_{10}N_4O_2$	
Amoxicillin	366.1118	$C_{16}H_{19}N_3O_5S$	
Metformin	130.1087	$C_4H_{11}N_5$	
Cimetidine	253.1230	$C_{10}H_{16}N_6S$	
Ciprofloxacin	332.1405	$C_{17}H_{18}FN_3O_3$	
Diphenhydramine	256.1696	$C_{17}H_{21}NO$	
Ranitidine	315.1485	$C_{13}H_{22}N_4O_3S$	

Cetirizine	389.1627	$C_{21}H_{25}ClN_2O_3$	

Table S2 Recovery for all 13 compounds. p*Ka* and log *K*ow values are provided as listed in the literature reference. The standards were spiked in water at 16.67 ppb concentration.

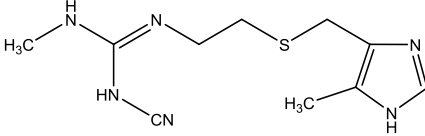| Compound (p*Ka* // log K_{ow})	Recovery (%)	RSD (%)[*]
Caffeine (-0.92 // -0.1) [https://www.drugbank.ca/drugs/DB00201]	96.06	6.05
Ranitidine (8.1 // 0.3) [https://www.drugbank.ca/drugs/DB00863]	60.22	103.58
Paroxetine (9.8 // 3.6) [https://www.drugbank.ca/drugs/DB00715]	88.08	0.96
Metformin (12.3 // -0.5) [https://www.drugbank.ca/drugs/DB00331]	15.20	8.08
Fluoxetine (9.8 // 4.1) [https://www.drugbank.ca/drugs/DB00472]	103.60	0.48
Diphenhydramine (9.9 // 5.1) [https://www.drugbank.ca/drugs/DB01104]	132.47	5.48
Citalopram (9.8 // 3.5) [https://www.drugbank.ca/drugs/DB00215]	188.44	14.10
Ciprofloxacin (5.8 and 8.7 // 0.3) [https://www.drugbank.ca/drugs/DB00537]	13.32	48.45
Cimetidine (6.9 // 0.4) [https://www.drugbank.ca/drugs/DB00501]	28.98	119.93
Cetirizine (2.7, 3.6 and 7.6 // 2.8) [https://pubchem.ncbi.nlm.nih.gov/compound/55182#section=Top] // [https://www.drugbank.ca/drugs/DB00341]	77.12	67.35
Amoxicillin (2.7, 7.1 and 9.6 // 0.9) [http://www.ijppsjournal.com/Vol3Issue3/2249.pdf] // [https://www.drugbank.ca/drugs/DB01060]	11.95	173.21
Sertraline (9.9 // 5.1) [https://www.drugbank.ca/drugs/DB01104]	103.76	1.45
Triclosan (7.9 // 5.0) [https://www.drugbank.ca/drugs/DB08604]	46.17	4.86

*n = 3

Table S3. LOD and LOQ for all 13 compounds

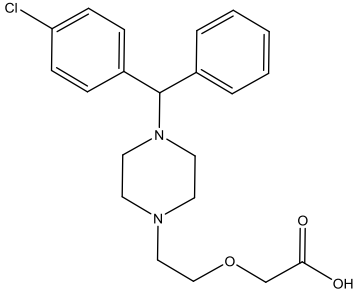| Compound	Instrument LOD (ppb)	Instrument LOQ (ppb)	Method LOD (ppb)	Method LOQ (ppb)
Metformin	0.1	0.3	0.03	0.09
Cimetidine	0.4	1.3	0.02	0.08
Ranitidine	2.3	7.8	0.08	0.26
Caffeine	1.0	3.3	0.05	0.15
Ciprofloxacin	2.3	7.8	0.61	2.05
Amoxicillin	1.0	3.3	0.12	0.38
Diphenhydramine	0.4	1.3	0.01	0.04
Citalopram	1.0	3.3	0.02	0.07
Paroxetine	1.0	3.3	0.05	0.15
Fluoxetine	1.0	3.3	0.04	0.13
Sertraline	1.0	3.3	0.04	0.13
Cetirizine	0.7	2.3	0.06	0.20
Triclosan	0.4	1.3	0.04	0.12

Table S4. Recovery data (and standard deviation) of standards spiked in a water sample (relative to that in Milli Q water) for some compounds not detected in the water sample without spiking.

Compound	Recovery (%)						
	100 ppt	1 ppb	10 ppb	25 ppb	50 ppb	75 ppb	100 ppb
Cimetidine	3.1 (5.4)	48.3 (27.3)	51.2 (14.8)	57.5 (13.7)	67.6 (15.6)	65.9 (12.1)	78.4 (30.5)
Ranitidine	0 (0)	220.2 (206)	138.4 (35.4)	138 (27)	122 (29.1)	106.7 (12.2)	124 (53.1)
Triclosan	ND	59.5 (23.8)	70.4 (4.1)	75.4 (0.2)	120.9 (40.7)	85.6 (1.7)	123.5 (5.7)

Additional information related to Chapter 2, (peak areas of all replicates for each PPCP), not included in the original published Supplementary Information.

Concentration (ppb)	Ranitidine			Paroxetine		
	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.10	NA	NA	NA	NA	NA	NA
1.00	1.41E+04	NA	2.24E+04	1.45E+05	3.67E+05	9.19E+04

5.00	4.43E+05	1.13E+06	3.15E+05	2.54E+06	3.64E+06	2.16E+06
10.00	1.77E+06	2.97E+06	1.84E+06	7.54E+06	9.17E+06	6.26E+06
25.00	1.21E+07	1.07E+07	1.34E+07	2.90E+07	2.56E+07	2.51E+07
50.00	2.62E+07	2.48E+07	2.72E+07	6.03E+07	5.39E+07	5.48E+07
100.00	4.99E+07	6.03E+07	4.92E+07	1.62E+08	1.56E+08	1.46E+08

	Metformin			Fluoxetine		
Concentration (ppb)	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.10	7.43E+04	1.86E+04	2.31E+04	NA	NA	NA
1.00	5.89E+05	5.44E+05	5.89E+05	3.46E+04	4.21E+05	6.05E+04
5.00	4.57E+06	4.75E+06	3.67E+06	2.69E+06	5.61E+06	2.33E+06
10.00	1.34E+07	1.24E+07	1.29E+07	8.38E+06	1.36E+07	6.54E+06
25.00	5.38E+07	4.20E+07	5.25E+07	4.04E+07	3.89E+07	3.60E+07
50.00	1.14E+08	8.90E+07	1.11E+08	9.06E+07	8.42E+07	8.20E+07
100.00	2.43E+08	2.00E+08	2.29E+08	2.36E+08	2.34E+08	2.27E+08

	Diphenhydramine			Citalopram		
Concentration (ppb)	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.10	7.40E+03	NA	6.63E+03	NA	NA	NA
1.00	4.79E+05	6.68E+05	4.77E+05	2.58E+04	1.93E+05	1.84E+04
5.00	4.29E+06	6.98E+06	4.09E+06	9.54E+05	2.33E+06	9.49E+05
10.00	1.29E+07	1.52E+07	1.20E+07	2.82E+06	5.06E+06	2.92E+06
25.00	5.08E+07	4.21E+07	5.01E+07	1.23E+07	1.13E+07	1.22E+07
50.00	1.05E+08	9.42E+07	1.04E+08	2.73E+07	2.65E+07	2.78E+07
100.00	2.37E+08	2.39E+08	2.22E+08	6.92E+07	8.52E+07	6.89E+07

	Ciprofloxacin			Cimetidine		
Concentration (ppb)	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.10	NA	NA	NA	1.09E+05	NA	1.04E+05
1.00	7.52E+03	1.72E+05	NA	1.54E+06	1.50E+06	1.43E+06

5.00	7.27E+05	2.19E+06	1.15E+05	8.29E+06	8.84E+06	7.74E+06
10.00	3.76E+06	5.75E+06	2.49E+06	1.70E+07	1.91E+07	1.88E+07
25.00	1.42E+07	1.60E+07	1.19E+07	4.46E+07	4.93E+07	4.84E+07
50.00	2.81E+07	3.28E+07	2.47E+07	7.91E+07	9.62E+07	8.24E+07
100.00	6.42E+07	7.46E+07	5.04E+07	1.40E+08	1.96E+08	1.47E+08

Concentration (ppb)	Cetirizine			Caffeine		
	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.10	1.08E+04	NA	NA	NA	NA	NA
1.00	2.41E+05	5.58E+04	2.92E+05	3.25E+06	2.44E+06	2.90E+06
5.00	2.15E+06	5.57E+05	2.26E+06	1.61E+07	1.17E+07	1.45E+07
10.00	5.30E+06	1.46E+06	5.72E+06	3.07E+07	2.43E+07	2.96E+07
25.00	1.73E+07	5.09E+06	1.85E+07	7.34E+07	6.04E+07	7.01E+07
50.00	3.40E+07	9.62E+06	3.53E+07	1.35E+08	1.14E+08	1.26E+08
100.00	7.98E+07	2.23E+07	8.50E+07	2.63E+08	2.29E+08	2.48E+08

Concentration (ppb)	Amoxicillin			Sertraline		
	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.10	NA	NA	NA	NA	NA	NA
1.00	5.94E+03	3.16E+04	5.69E+03	8.78E+03	8.79E+04	1.69E+04
5.00	3.99E+05	5.88E+05	3.91E+05	1.49E+06	2.28E+06	1.32E+06
10.00	9.43E+05	1.32E+06	9.97E+05	4.73E+06	5.53E+06	4.39E+06
25.00	2.55E+06	3.53E+06	2.80E+06	1.94E+07	1.66E+07	1.81E+07
50.00	4.88E+06	6.45E+06	4.58E+06	4.14E+07	3.55E+07	3.92E+07
100.00	9.04E+06	1.44E+07	9.37E+06	1.04E+08	1.04E+08	9.92E+07

Concentration (ppb)	Triclosan		
	Peak area 1	Peak area 2	Peak area 3
0.10	1.47E+04	NA	1.40E+04
1.00	6.26E+04	1.28E+05	8.42E+04

5.00	9.07E+05	9.57E+05	1.21E+06
10.00	2.50E+06	2.24E+06	2.28E+06
25.00	5.80E+06	5.54E+06	6.24E+06
50.00	1.16E+07	1.06E+07	1.15E+07
100.00	2.53E+07	2.34E+07	2.51E+07

Chapter 3

Determination of selected emerging contaminants in freshwater invertebrates using a universal extraction technique and liquid chromatography accurate mass spectrometry

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3.1 Abstract

A simple sample preparation method based on a modified liquid-phase extraction approach to extract selected pharmaceuticals and personal care products from freshwater organisms is described. Extracted samples were analysed using liquid chromatography with Q-Exactive plus hybrid quadrupole Orbitrap mass spectrometry, using 2.6 μm C18 media. A 0.1% v/v acetic acid/acetonitrile mobile phase was applied over a 20 min gradient. Method detection limits in full scan mode were ca. 0.04–2.38 ng of analyte per g of sample. Linearity ranged from 0.9750 to 0.9996 over the calibration range of 0.01–100 μg/L; MS mass accuracy was <2 ppm for most analytes. This method was applied to quantify six pharmaceuticals and personal care products in seven invertebrate samples. For tandem mass spectrometry analysis, selection of precursor ions was performed for each pharmaceutical, with Mass Frontier software illustrating the fragmentation mechanism. Effects of collision energy on intensities of ions was further investigated. The tandem mass spectrometry condition resulting in the highest signal of respective selected product ion was selected to confirm each pharmaceutical, which was initially observed in the full scan mode. Results indicate that pharmaceuticals and personal care products found to be present in water-ways, may be incorporated into organisms that live in the environment of affected water streams.

3.2 Article

RESEARCH ARTICLE

Determination of selected emerging contaminants in freshwater invertebrates using a universal extraction technique and liquid chromatography accurate mass spectrometry

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A simple sample preparation method based on a modified liquid-phase extraction approach to extract selected pharmaceuticals and personal care products from freshwater organisms is described. Extracted samples were analysed using liquid chromatography with Q-Exactive plus hybrid quadrupole Orbitrap mass spectrometry, using 2.6 μm C18 media. A 0.1% v/v acetic acid/acetonitrile mobile phase was applied over a 20 min gradient. Method detection limits in full scan mode were ca. 0.04–2.38 ng of analyte per g of sample. Linearity ranged from 0.9750 to 0.9996 over the calibration range of 0.01–100 μg/L; MS mass accuracy was <2 ppm for most analytes. This method was applied to quantify six pharmaceuticals and personal care products in seven invertebrate samples. For tandem mass spectrometry analysis, selection of precursor ions was performed for each pharmaceutical, with Mass Frontier software illustrating the fragmentation mechanism. Effects of collision energy on intensities of ions was further investigated. The tandem mass spectrometry condition resulting in the highest signal of respective selected product ion was selected to confirm each pharmaceutical, which was initially observed in the full scan mode. Results indicate that pharmaceuticals and personal care products found to be present in water-ways, may be incorporated into organisms that live in the environment of affected water streams.

KEYWORDS
freshwater invertebrates, liquid chromatography, personal care products, pharmaceuticals

1 | INTRODUCTION

Emerging chemical contaminants are of increasing importance, and so are the focus of a greater number of studies in recent years. In this category of pollutants found in waterways are pharmaceuticals and personal care products (PPCPs), which can affect aquatic life and the ecosystem. These chemicals may accumulate in the environment, threaten water quality, and the habitats of fish and freshwater invertebrates may be affected with damage to biota inferred. To evaluate environmental effects of these contaminants, a variety of compartments including water and biota (plants, fish, animals, and freshwater invertebrates) need to be investigated, for their occurrence in the environment. PPCPs constitute a wide range of chemicals which can affect the environment in different ways when discharged to water streams.

Adequate data regarding potential environmental effects, and bioaccumulation of such chemicals in invertebrates, are still lacking [1]. Concerns about the environmental effects of emerging contaminants have increased in recent years [2, 3] although in general these chemicals are often found at low concentrations [3]. A broader spectrum of personal care

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Article Related Abbreviations: AGC, automatic gain control; ACN, acetonitrile; EIC, extracted ion chromatogram; FWHM, full-width-at-half-maximum; HXN, hexane; IT, injection time; PPCP, pharmaceuticals and personal care product; PRM, parallel reaction monitoring mode; TIC, total ion chromatogram

products, monitored using an unbiased sampling method, need to be investigated across different compartments since to date few of these chemicals have been analysed in different relevant matrices [4]. PPCPs as found in the treated wastewater, may affect fish behavior and their activities [5]. Interestingly, these contaminants may act as chemosensitisers in addition to affecting multixenobiotic resistance in zebrafish [6].

Pharmaceuticals may reach water-ways as unaltered or as a metabolite form [7], and these may have different effects on aquatic organisms such as freshwater invertebrates, which are considered an essential part in the food chain. Environmental monitoring programs may be interested in invertebrates as bioindicators or sentinel organisms, since they have the ability to accumulate pollutants in the environment [8]. Some invertebrates such as snails and shrimps have been selected to monitor PPCPs in the aquatic ecosystem [9]. A number of studies report the toxicological impacts of PPCPs such as fluoxetine, citalopram, cetirizine, sertraline, diclofenac, propranolol, simvastatin, carbamazepine, and triclosan on invertebrates such as freshwater and Mediterranean mussels, sea urchin, Ephemeroptera, Baetis sp., Decapoda, and M. lanchesteri [10–16]. While pharmaceuticals such as fluoxetine and clofibric acid can affect the development of some crustaceans, others (e.g. antibiotics) can lead to changes in the population sex ratio [17]. A study by Luna et al. [18] showed that 17α-ethynylestradiol and fluoxetine reduced the population growth of some invertebrates such as pulmonate snails. Another study found that some pharmaceuticals such as carbamazepine, diclofenac, fluoxetine, and orlistat, in addition to soil characteristics, could affect earthworm species such as *Eisenia fetida* by modifying internal pH of these invertebrates [19].

A variety of sensitive and precise analytical instruments have been employed in order to identify emerging contaminants in waterways, and to investigate a variety of parameters such as different treatment technologies [3]. One reported instrument is the Orbitrap MS, proposed by Makarov in 2000 [20], having high mass accuracy and excellent sensitivity, and thus is relevant to analysing emerging contaminants at trace levels [21]. UHPLC offers fast and good resolution chemical separation. When hyphenated with a Q-Orbitrap-MS short analysis times with mass resolution of up to 280 000 full-width-at-half-maximum (FWHM), mass accuracy of <3 ppm, and the capability to perform polarity switching between positive and negative modes with acquisition speed up to 12 Hz, can be realised [22]. Full scan MS analysis with all ions in a given m/z window range monitored, complements use of the quadrupole filter to select specific ions for transfer to the Orbitrap mass analyser for accurate mass measurement. This supports MS/MS analysis, with several precursor ions selected and fragmented, with product ions monitored in parallel reaction monitoring mode (PRM). This is performed within a single analysis, according to analyte

chromatographic retention time. This can be applied for analysis of a wide range of compounds in different samples, and is suited to PPCPs in water and invertebrates [22–25]. This emphasises the importance of emerging MS technologies, to provide improved analytical capability to perform complex mixture analysis [26].

Various sample preparation processes have been employed to extract pharmaceuticals and metabolites from invertebrates, such as liquid-liquid extraction, pulverised liquid extraction, and sonication [25,27–29]. Although these methods can successfully extract PPCPs from the samples, they need to be carefully optimised in order to achieve adequate recovery. Some of the above preparation techniques may have poor recoveries, depending on the compound polarity. On the other hand, a QuEChERS approach has been successfully used to extract PPCPs from invertebrates [1,30,31]. This method has been increasingly popular in recent years to extract pharmaceuticals from some environmental matrices. Moreover, it was demonstrated that QuEChERS provides acceptable recoveries compared to other methods for environmental analysis, in addition to flexibility and simplicity of this extraction method [32].

This work reports a simple 'universal' sample preparation method based on the QuEChERS approach, which has been broadly applied elsewhere for extraction of a wide range of compounds, e.g. PPCPs, pesticides, PCBs, and PAHs in different matrices [30,32,33]; here it is used to extract six PPCPs (most of which are drugs that can be used as medications for both humans and/or animals) from selected freshwater invertebrates. Extracted samples were analysed using a UHPLC−Q-Orbitrap-MS instrument in both full scan and MS/MS modes.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Metformin (1,1-dimethylbiguanide hydrochloride) (97% purity), ranitidine hydrochloride, caffeine (reagent plus), ciprofloxacin (≥ 98% HPLC grade), and triclosan (≥97% HPLC grade) were purchased from Sigma-Aldrich (Castle Hill, Australia). Cetirizine 2HCl (98% purity) was obtained from A K Scientific (Union City, CA). ACN (HPLC grade), methanol (HPLC grade), and acetic acid were purchased from Merck (Darmstadt, Germany). Ultra-pure water was provided by a Milli-Q system (Millipore, Merck). Further information about the selected PPCPs can be found in Supporting Information Table S1.

Analyte stock solutions were prepared in methanol or water (for ciprofloxacin, a few drops of 0.1 M HCl were added to help this PPCP to be dissolved) at a concentration of 10 000 mg/L. Different solvents were applied in order to

improve solubility of each compound. All stock and working solutions were stored at –18°C.

2.2 | Sample collection and extraction

Seven invertebrate samples were collected from different locations in Victoria, Australia as shown in Supporting Information Table S2. A Q-sep QuEChERS extraction salt packet (Q110 – EN Method) from RESTEK (Bellefonte, PA) was dissolved in 23.4 mL of Milli-Q water. Each sample of insect was crushed in an Eppendorf tube using a glass rod. After 4 min of grinding, 600 μL of acetonitrile (ACN) was added into the tube, and grinding was continued for a further 4 min. Then, 600 μL of QuEChERS solution was added into the tube, which was manually shaken for 2 min. Centrifugation was performed using a refrigerated centrifuge (Sigma 3–16KL, Sigma Laborzentrifugen, Germany) at 4 500 rpm for 2 min. Two hundred microlitre of the top layer of ACN was transferred into a vial insert and carefully dried under N_2 gas. The contents were reconstituted using 200 μL of Milli-Q water and the aqueous phase was transferred into another vial insert to avoid injection of undissolved material of the insect which was retained in the former vial. The same process was performed with a control sample (200 μL of 1 mg/L of a mixture of standards). Supporting Information Figure S1 shows all steps in a flowchart.

2.3 | Instrumental analysis

An ultra-high performance liquid chromatography quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Scoresby, Australia) was used to analyse both standards and samples. An Accucore-C18 column (2.6 μm particle size, 50 mm × 2.1 mm) was purchased from Thermo Fisher Scientific. The column temperature was held at 25°C, with an injection volume of 15 μL. The mobile phases were 0.1% v/v acetic acid in water (A) and ACN (B). The chromatographic method, modified from reference [34], used a flow rate of 0.30 mL/min. In this method, gradient elution began with 10% v/v of mobile phase B for 2 min followed by a linear gradient to 27% v/v B at 5 min, then to 50% v/v B at 10 min, and finally to 100% v/v B at 14 min. The mobile phase content was held at 100% v/v B for 1 min before decreasing it to 10% v/v B (held for 4 min). Total analysis time was 20 min.

The heated electrospray ionisation source was operated in both positive and negative modes as required, using the polarity switch option. The source conditions were sweep gas 0, sheath gas 35, auxillary gas 10, spray voltage 3.0 kV, auxillary gas heater temperature 300°C, and capillary temperature 320°C. The MS was firstly operated using full scan modes (50 to 400 m/z), applied with varied MS parameters to examine different settings. General settings were: resolution (70 000 FWHM); automatic gain control (AGC) target, which

controls the number of ions injected into the Orbitrap analyser, (1×10^6); maximum IT, which is the maximum injection time in order to reach the AGC target value (200 ms). MS/MS analysis using PRM was also performed. The chromatographic and general MS conditions were the same as that employed in full scan analysis however, various MS parameters were altered as follows: resolution (17 500 FWHM); AGC target (2.0×10^5); maximum IT (100 ms). The protonated molecule, which is generally of acceptable abundance in the full scan mode with positive heated electrospray ionisation was chosen as precursor ion for each analyte. Different product ions were also selected for precursor scan analysis at different time intervals centred on the retention time of each PPCP chromatographic peak. The system was calibrated daily before analysis for both positive and negative modes.

Xcalibur 3.0.63 and HighChem Mass Frontier 7.0 (Thermo Fisher) software used to were process the data in full scan MS and MS/MS modes. TraceFinder 3.0 software (Thermo Fisher) and Microsoft Excel 2010 were applied for quantification of each PPCP.

3 | RESULTS AND DISCUSSION

3.1 | Optimisation of the liquid phase extraction method

A QuEChERS method was selected according to extraction performance for the six PPCPs. The method was tested using spiked Milli-Q water samples with six PPCP standards which were extracted using the proposed QuEChERS method. The total recovery (summation of the recovery values of all six PPCPs with a concentration of 1 mg/L) was acceptable, ranging from 54.6 to 98.3% for most of the analytes (Supporting Information Table S3). This level of recovery is sufficient for analysis with the Q Exactive Plus. However, the recovery values in this study are clearly low for some of the compounds, especially for those analytes with low K_{ow} values (Supporting Information). Further method variation and optimisation in order to investigate improving the recoveries is recommended for routine analysis in the future. External standards were used in this study. The most appropriate IS will be isotopically labelled standards, but these are not available in all cases, so it was decided to rely on external standards. Using an IS will not improve the recovery for compounds, regardless of whether a labelled IS is available. Using external standards and accurate mass with MS/MS confirmation was used for quantification, with sample replicates to monitor the reproducibility of the method. Lower recoveries were established to correct for the reported analyte levels in the invertebrates. Use of labelled IS, if available, is recommended if specific reduced recovery needs to be confirmed.
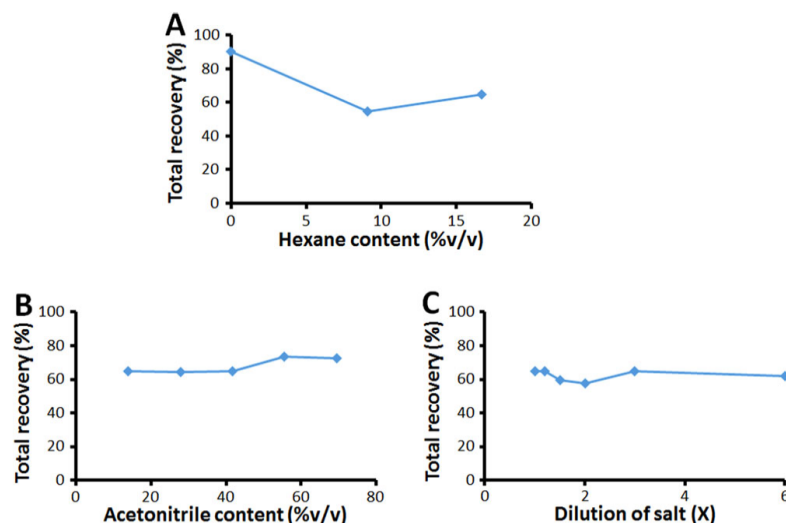
FIGURE 1 Effect of different parameters on the QuEChERS performance: HXN content (A), ACN content (B) and dilution of salt (C) on the total recovery of the 6 PPCPs

The effects of hexane (HXN), ACN, and QuEChERS salt solution (4 g magnesium sulfate, 1 g sodium chloride, 1 g trisodium citrate dihydrate, and 0.5 g disodium hydrogen citrate sesquihydrate) were studied by evaluating analyte recoveries in QuEChERS solutions containing different composition of each solvent. A suitable method based on these results was selected and applied to extract seven freshwater invertebrate samples. In order to reduce sample matrices, HXN can be added into the QuEChERS solution. Different volumes of HXN were tested which revealed that the total recovery reduced from approximately 90 to 60% when adding a significant amount of this solvent into the solution (Figure 1A). For both ACN (Figure 1B) and QuEChERS salt solution (Figure 1C), there were small differences when applying different volumes of these two solvents, and the recovery remained in the recommended range from 60 to 80%. However, a sufficient amount of ACN should be added in order to provide a clearly visible separated phase after the extraction. Note that the ACN phase is the final medium containing target PPCPs to be transferred to UHPLC−Q-Orbitrap-MS analysis. In addition, the QuEChERS solution should contain sufficiently high concentration of salt in order to maintain separate aqueous and ACN phases. As a result, the final method was chosen as: 600 mL of ACN (50% v/v) and 600 mL of the salt solution, without adding HXN to the extraction solution.

3.2 | Validation of the method

Mixtures of all six analytes of different concentrations were prepared in Milli-Q water, and calibration curves were plotted for all compounds as shown in Supporting Information Table S4. The method detection limit in full scan mode was in the range 0.04–2.38 ng/g (Supporting Information Table S5). The linearity, which ranged from 0.98 to 1.00 over the calibration range of 0.01–100 µg/L included nine data points and mass accuracy (< 2 ppm for most of the analytes – Table 1) were both acceptable. The instrument detection limit was 10–1000 ng/L for all the studied compounds. The LOD was determined by injecting progressively lower concentrations until peak heights were approximately three times the noise level in the extracted ion chromatogram (EIC).

3.2.1 | Fragmentation patterns for product ions selected in MS/MS analysis

By using Mass Frontier software, different fragmentation patterns of the studied PPCPs were generated, as shown in Figure 2. This study selects the protonated molecular ions of PPCPs as the precursor ions with their m/z values shown in Figure 2 and Supporting Information Table S6. The protonated molecular ion of PPCPs, fragmented in the collision cell, results in different product ions as shown in Figure 2. For example, protonation of the metformin molecule followed by loss of –NH_3 can lead to a product ion of m/z 113.0822 (see ion 2 in Figure 2A). For the same analyte, addition of a proton followed by charge site rearrangement (α,β) mechanism resulted in products of m/z 60.0562 (3) and 88.0869 (4). For ranitidine, protonation of the molecular mass $[M+H]^+$ and charge site rearrangement (γ) leads to the product ion m/z 176.0488 (see ion 6 in Figure 2B). This compound can rearrange to form a product ion of m/z 124.0757 (7). Additionally, this compound can also be further fragmented by
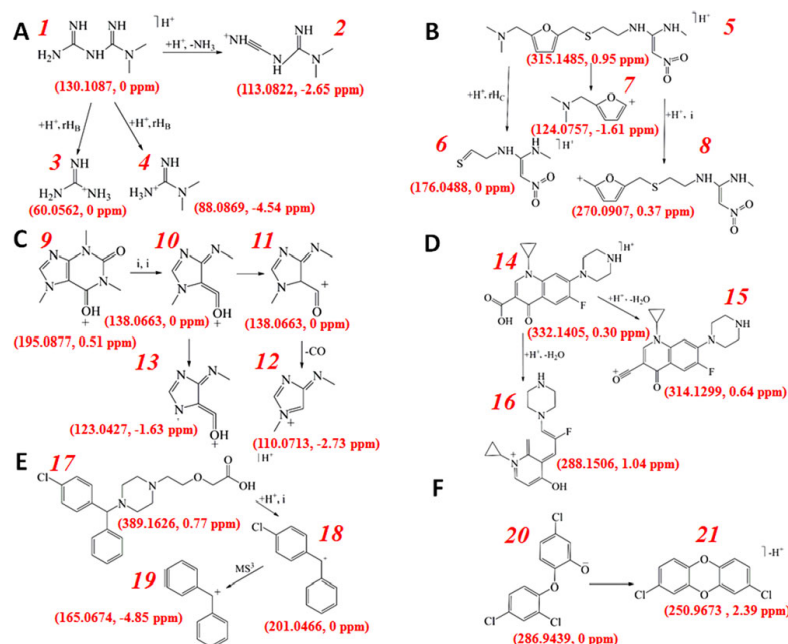
FIGURE 2 Fragmentation patterns for all analytes in the positive mode. +H$^+$ = protonation, rH$_b$ = charge site rearrangement (α,β), rH$_c$ = charge site rearrangement (γ) and i = inductive cleavage

adding a proton followed by inductive cleavage which resulted in a product ion of m/z 270.0907 (8). Inductive cleavage of the two amide bonds of the protonated molecular ion of caffeine resulted in the product ion of m/z 138.0663 (see 10 in Figure 2C). This product can be either directly fragmented by losing the methyl group into a less abundant form (13 with m/z 123.0427 or rearranged into a more abundant form (11). The ion 11 can be further fragmented by using significantly high collision energy (>50 eV, see the reduced area trend of the purple line plotted in Figure 3C) was applied leading to the loss of –CO and resulting in the product (12) with m/z 110.0713. Losing a –H$_2$O molecule in addition to protonation of ciprofloxacin can produce a product ion m/z 314.1299 (see 15 in Figure 2D). This compound can also be rearranged to produce a product ion of m/z 288.1506 (16). The product ion m/z 201.0466 (ion 18 in Figure 2E) can be formed by inductive cleavage of the precursor ion of cetirizine, m/z 389.1626 (17). However, this precursor ion can be further fragmented to form a product ion of m/z 165.0674 (19).

3.2.2 | Optimisation of collision energy

Sufficient collision energy applied for effective ion fragmentation (here, of [M+H]$^+$) is different depending on stabilities of the molecular and product ions, which are mainly governed by the collisional cross-section of the parent ion and vibrational frequency of chemical bonds related to each

fragmentation pathway [35]. In general, sufficient energy should be applied to generate acceptable fragment ion signal abundance of target product ions. However, due to ion loss during the collision process [36], total ion intensity decreases when higher energy collision was applied, e.g. as evidenced by the reduced TIC peak response of all the studied compounds at higher collision energies, Figure 3A-F. As a result, the applied collision energies should be optimised to result in maximum signals of target product ions (e.g. maximum peak areas in EIC plots of the ions). In this study, molecular ions were selected as the parent ions for all the studied compounds, and fragment 1 ions (in Figure 3) were selected as the target product ions. The studied compounds necessitated different optimised collision energies. An energy of 60 eV was selected for fragmentation of molecular ions of metformin although other energies are also possible and provided a similar result. This is to ensure that high peak areas can be obtained in EIC of its target fragment 1 of m/z 60.0562 (see product ion 3 in Figure 2). Application of significantly higher collision energies for triclosan resulted in reduced signals of either molecular or the product ions (Figure 3F), therefore 20 eV was chosen in this case. On the other hand, an enhanced peak area of caffeine in EIC of the target fragment 1 (m/z 138.0663, see product ion 11 in Figure 2) required higher collision energy up to 50 eV (Figure 3C), whilst an intermediate collision energy of 30 eV was required to enhance the target fragment 1 ion signal for
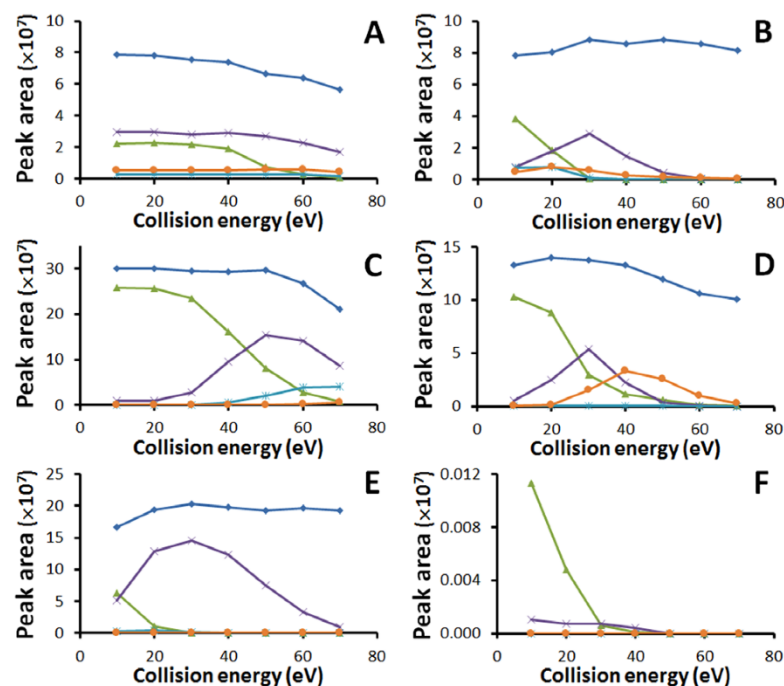
FIGURE 3 Plots of peak areas in TIC (blue) and EIC of molecular ions (green) and the product ions 1, 2, and 3 (purple, cyan, and orange, respectively) for: (A) metformin, (B) ranitidine, (C) caffeine, (D) ciprofloxacin, (E) cetirizine, and (F) triclosan. The peak area in TIC of triclosan cannot be quantified due to the low signal to noise ratios (S/N < 3). See also the molecular ions and product ions 1, 2, and 3 for each compound in Supporting Information Table S6

ranitidine, ciprofloxacin and cetirizine. Interestingly, higher collision energy can be applied to generate smaller and stable product ions of caffeine and ciprofloxacin (with m/z 110.0713 (12) and m/z 245.1081, respectively). Their enhanced signals could be observed at higher collision energies of 70 and 40 eV, respectively, Figure 3C and D, which can be useful transitions to confirm these compound identities especially in the case when analysis of fragment 1 is not possible (*e.g.* with high interference signals from non-target ions).

3.3 | Analysis of invertebrate samples

A C18 column was used to separate analytes in the extracts from 7 invertebrate samples collected from different locations in Victoria, Australia (see Supporting Information Table S2). The mobile phase comprised 0.1% acetic acid in water and 100% ACN starting from 10 to 100% v/v ACN, over a 20 min gradient analysis time, according to Section 2.3. This method was successfully applied to quantify six PPCPs in seven invertebrate samples, which was confirmed by MS/MS analysis. Data were processed using quantification software (TraceFinder 3.1) and Microsoft Excel. A typical EIC for all the studied PPCPs at a concentration of 100 µg/L in full scan
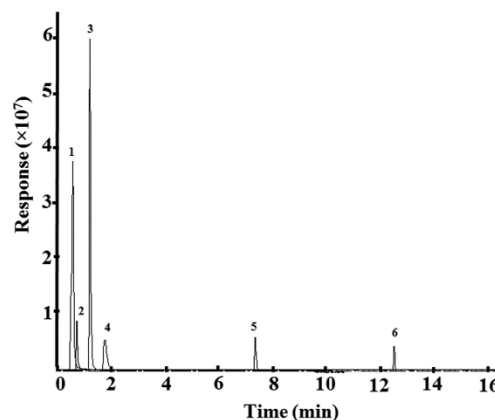
FIGURE 4 EIC of 6 standards (see Table 1) in full scan mode using switch polarity mode. These peaks are labelled according to Table 1

MS mode is shown in Figure 4. Information on retention time, [M+H]$^+$ mass, and mass accuracy (full scan) for each PPCP in the standard solution can be found in Table 1. The major-

TABLE 1 Retention time, exact protonated or deprotonated mass and mass accuracy of each PPCP in standard and sample (1)

No.	Compound	Retention time (min)	m/z value of $[M+H]^+$	Mass accuracy (ppm)	
				Standard	Sample
1	Metformin	0.57	130.1087	0.00[a]	0.00[a]
2	Ranitidine	0.69	315.1485	0.95	ND
3	Caffeine	1.20	195.0877	0.51	1.03
4	Ciprofloxacin	1.70	332.1405	0.30	ND
5	Cetirizine	7.28	389.1627	0.77	ND
6	Triclosan	12.42	286.9439*	0.00	5.58

*Triclosan was detected in negative ion mode $[M-H]^-$
[a]Metformin had identical masses in the standard and sample with the calculated mass

TABLE 2 Concentrations found in invertebrate samples

Compound	Concentration in invertebrate samples (ng/g)						
	S1	S2	S3	S4	S5	S6	S7
Metformin	312	28.4	40.2	ND[a]	ND	ND	ND
Ranitidine	ND	ND	ND	ND	ND	ND	ND
Caffeine	164	212	217	97.8	54.5	32.9	27.5
Ciprofloxacin	ND	ND	ND	ND	ND	ND	ND
Cetirizine	ND	ND	ND	ND	ND	ND	ND
Triclosan	24.1	4.38	3.03	1.40	1.53	0.241	0.771

[a]= Not detected

ity of the analytes were not detected in most of the samples, possibly due to the large dilution factor in the water streams and insufficient bioaccumulation in the invertebrate (Table 2). Results indicate that PPCPs may be found in water-ways, and also in organisms which live in impacted water streams.

UHPLC–MS/MS analysis was also performed in order to confirm the presence of PPCPs in each sample. The applied effective collision energies were 60, 30, 50, 30, 30, and 20 eV for analysis of metformin, ranitidine, caffeine, ciprofloxacin, cetirizine, and triclosan, respectively which were previously optimised [22]. These conditions resulted in enhanced signals of the product ions m/z 60.0562, 176.0488, 138.0663, 288.1506, 201.0466, and 250.9673, respectively. The resulting EIC for caffeine and metformin are shown in Figure 5 illustrating chromatograms for caffeine and metformin in both standard and sample using PRM mode.

Information on retention times, mass accuracies, and concentrations found in invertebrate samples are provided in Tables 1 and 2. Taking caffeine and metformin as an example, applying MS/MS analysis (PRM mode), each PPCP eluted at its characteristic retention time for both standard and sample (Figures 5A and C for caffeine, Figures 5E and G for metformin) using PRM mode. Mass spectra are also similar for both standard and sample with acceptable mass accuracy of the major fragments of each compound. Caffeine has major fragment ions of m/z 138.0663 and 110.0713, which can be used to confirm this compound in the sample by comparing MS/MS patterns between standard and sample (Figure 5B

and D). Another example is metformin with fragment ions of m/z 60.0562 and 113.0822. These ions can be observed in both standard and sample (Figure 5F and H).

The LOD and LOQ (Supporting Information Table S5) were calculated for the instrument and the method. For the instrument, LOD was determined using the EIC peak by reducing the concentrations using Milli-Q water until peak heights were less than three times the noise level. LOQ was calculated by considering peak heights ten times higher than S/N. After calculating LOD and LOQ for the instrument, the method LOD and LOQ can then be calculated by considering the enrichment factor of each PPCP in the QuEChERS step, based on a water matrix, and dividing the LOD and LOQ of the instrument by the respective enrichment factor.

4 | CONCLUDING REMARKS

A suitable method to extract freshwater invertebrate samples using a modified QuEChERS approach, and to analyse them using HPLC with quadrupole Orbitrap mass spectrometry was developed. This extraction method is simple and low cost, as the extraction can be conducted without resorting to complex or expensive instrumental tools. However, the analytical instrumental step is favoured by use of very sensitive technology. The extraction showed acceptable recoveries for most of the analytes, although their wide polarity range does lead to some low recoveries. It is anticipated that this technique can be used for other PPCPs in different invertebrate sample matrices. Full scan analysis was used to detect analytes in samples, and MS/MS analysis was investigated using PRM mode to confirm the compound identity. Switching polarity mode was applied in this analysis, demonstrating that the Orbitrap system has the ability to achieve adequate sensitivity to detect selected target compounds in the studied samples when this mode is implemented. A number of PPCP were shown to bio-accumulate in the target invertebrates, and illustrates that the effects of this route to environmental impact of PPCPs is worth further investigation.
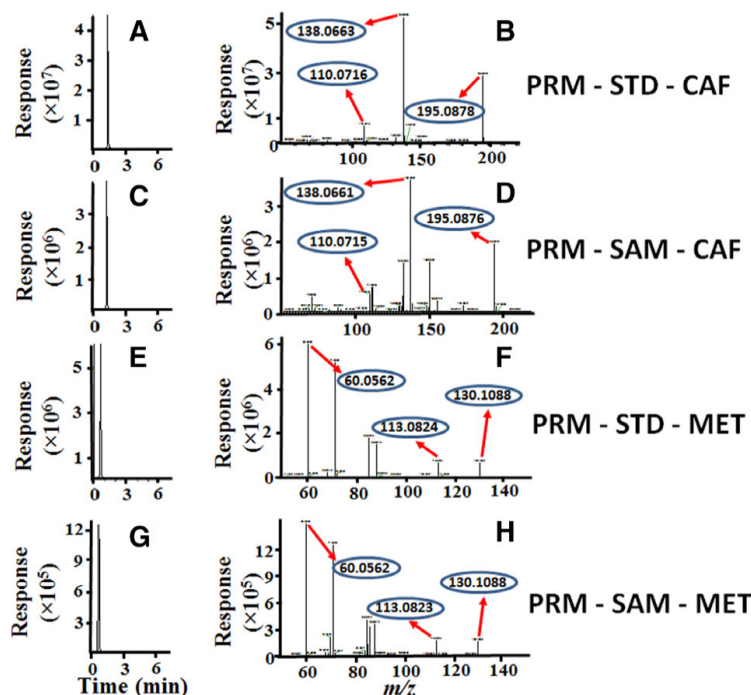
FIGURE 5 EIC of caffeine and metformin in the PRM mode for both standard and sample S1. (A) and (C): EIC ($m/z = 138.0663$) with MS operated in PRM mode. (E) and (G): EIC ($m/z = 60.0562$) with MS operated in PRM mode for the product ion. The spectra for caffeine and metformin peaks in A, C, E, and G are shown in B, D, F, and H, respectively

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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3.3 Supplementary information

Supplementary Information
Journal of Separation Science

Determination of selected emerging contaminants in freshwater invertebrates using a universal extraction technique and liquid chromatography accurate mass spectrometry

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Table S1. Information and properties of selected PPCPs
Table S2. Invertebrate samples information
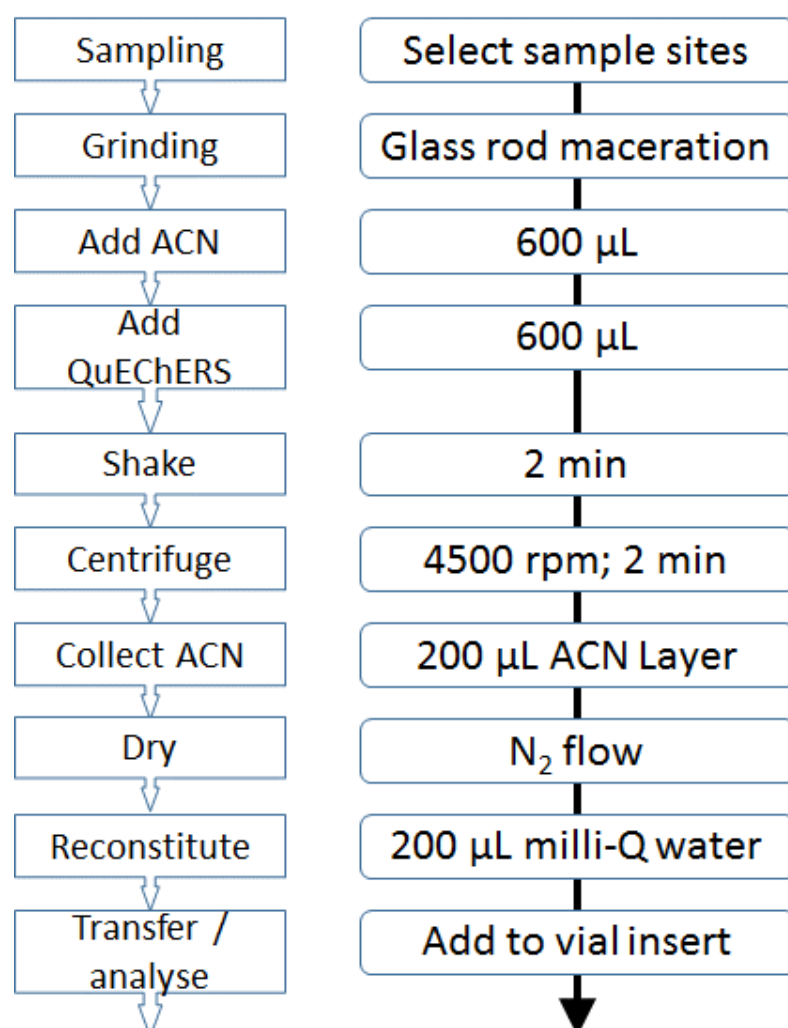**Table S3.** Recovery for the studied PPCPs (1 mg L^{-1}) in QuEChERS experiment
Table S4. Calibration curve data (intercept, slope and R^2) for each compound (Calibration range 0.01 – 100 µg L^{-1} using 9 data points)
Table S5. LOD and LOQ for all 6 compounds
Table S6. Product ions of the studied PPCPs with respective protonated molecular ions $[M+H]^+$ selected as the precursor ions
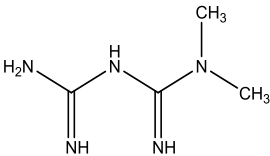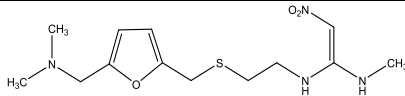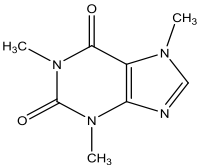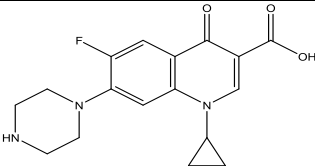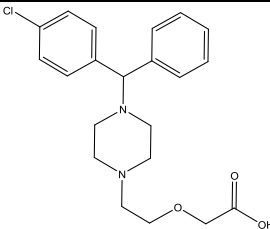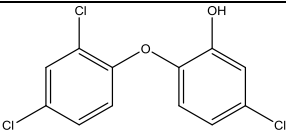
Figure S1. Flow chart of analytical method

Sampling	Select sample sites
Grinding	Glass rod maceration
Add ACN	600 µL
Add QuEChERS	600 µL
Shake	2 min
Centrifuge	4500 rpm; 2 min
Collect ACN	200 µL ACN Layer
Dry	N_2 flow
Reconstitute	200 µL milli-Q water
Transfer / analyse	Add to vial insert

Table S1. Information and properties of selected PPCPs

Compound	Therapeutic class	CAS no.	Log K_{ow}*	Molar Mass (g mol^{-1})	Molecular formula	Structure
Metformin	Antidiabetic	1115-70-4	-2.64	129	$C_4H_{11}N_5$	
Ranitidine	Decreases stomach acid production	66357-59-3	-1.22	350.86	$C_{13}H_{22}N_4O_3S$	
Caffeine	Stimulant	58-08-2	0.16	194.19	$C_8H_{10}N_4O_2$	
Ciprofloxacin	Antibiotic	85721-33-1	-0.00	331.34	$C_{17}H_{18}FN_3O_3$	
Cetirizine	Antihistamine	83881-52-1	NA	461.81	$C_{21}H_{25}ClN_2O_3$	
Triclosan	Antibacterial	3380-34-5	4.66	289.54	$C_{12}H_7Cl_3O_2$	

* Log K_{ow} (source: KOWWIN v. 1.68 database, USEPA EPI suite 4.11 software)

Table S2. Invertebrate samples information

Sample ID	Site	Species	Common name	Weight (mg)
S1	Sassafras Creek	Leptophlebidae	May fly	32.36
S2	Sassafras Creek	Economidae	Caddis fly	21.091
S3	Sassafras Creek	Leptophlebidae	May fly	23.09
S4	Ferny Creek	Atytidae	Shrimp	48.523
S5	Ferny Creek	Notonectidae	Beetle	96.861
S6	Mullum Creek	Notonectidae	Beetle	136.504
S7	Lyrebird Creek	Gyrinidae	Beetle	154.101

Table S3. Recovery for the studied PPCPs (1 ppm) in QuEChERS experiment.

Compound	Recovery (%)	Std. Dev.
Metformin	22.4	2.3
Ranitidine	54.6	5.2
Caffeine	98.3	12.4
Ciprofloxacin	88.5	11.7
Cetirizine	29.4	6.3
Triclosan	63.2	14.4

Table S4. Calibration curve data (intercept, slope and R^2) for each compound (Calibration range 0.01 – 100 ppb using 9 data points).

Compound	Intercept	Slope	R^2
Metformin	-5.84E+06	2.46E+03	0.9821
Ranitidine	-1.54E+06	5.22E+02	0.9750
Caffeine	3.94E+06	2.61E+03	0.9996
Ciprofloxacin	-2.32E+06	6.54E+02	0.9949
Cetirizine	-1.88E+06	7.95E+02	0.9894
Triclosan	-2.80 E+05	2.52 E+02	0.9914

Table S5. LOD and LOQ for all 6 compounds

Compound	Instrument LOD (ppb)	Instrument LOQ (ppb)	Method LOD (ppb)	Method LOQ (ppb)
Metformin	0.01	0.03	0.04	0.13
Ranitidine	1.00	3.30	1.83	6.05
Caffeine	0.40	1.30	0.41	1.32
Ciprofloxacin	1.00	3.30	1.13	3.73
Cetirizine	0.70	2.30	2.38	7.82
Triclosan	0.04	0.10	0.06	0.16

Table S6. Product ions of the studied PPCPs with respective protonated molecular ions $[M+H]^+$ selected as the precursor ions

Compound	*m/z* value (mass accuracy, ppm)			
	Molecular ion	Product ion 1	Product ion 2	Product ion 3
Metformin	130.1087 (0.00)	60.0562 (0.00)	113.0822 (-2.65)	88.0869 (-4.54)
Ranitidine	315.1485 (0.95)	176.0488 (0.00)	270.0907 (0.37)	124.0757 (-1.61)
Caffeine	195.0877 (0.51)	138.0663 (0.00)	110.0713 (-2.73)	123.0427 (-1.63)
Ciprofloxacin	332.1405 (0.30)	288.1506 (1.04)	314.1299 (0.64)	245.1081 (-1.63)
Cetirizine	389.1626 (0.77)	201.0466 (0.00)	187.1076 (0.00)	165.0674 (-4.85)
Triclosan	286.9439 (0.00)	250.9673 (2.39)		

Additional information related to Chapter 3, (peak areas of all replicates for each PPCP), not included in the original published Supplementary Information.

Concentration (ppb)	Metformin			Ranitidine		
	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.01	4.65E+05	4.87E+05	5.02E+05	ND	ND	ND
0.10	8.53E+05	7.19E+05	7.80E+05	ND	ND	ND
1.00	1.82E+06	1.68E+06	2.07E+06	4.03E+04	2.65E+04	2.83E+04
5.00	4.48E+06	3.82E+06	4.88E+06	8.56E+05	7.58E+05	9.35E+05
10.00	9.90E+06	9.58E+06	1.06E+07	1.21E+06	1.21E+06	1.16E+06
25.00	7.53E+07	6.64E+07	8.56E+07	9.39E+06	8.14E+06	1.05E+07
50.00	9.26E+07	8.48E+07	1.05E+08	2.25E+07	2.01E+07	2.51E+07
75.00	1.80E+08	1.63E+08	1.91E+08	4.39E+07	3.94E+07	4.88E+07
100.00	2.29E+08	2.23E+08	2.33E+08	3.41E+07	3.56E+07	3.02E+07

	Caffeine			Ciprofloxacin		
Concentration (ppb)	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.01	ND	ND	6.15E+04	ND	ND	ND
0.10	ND	ND	3.36E+05	ND	ND	ND
1.00	2.40E+06	2.40E+06	3.00E+06	4.51E+04	1.59E+04	ND
5.00	1.07E+07	1.07E+07	1.41E+07	1.86E+06	1.74E+06	2.35E+06
10.00	2.33E+07	2.33E+07	2.97E+07	3.77E+06	3.79E+06	7.25E+06
25.00	5.78E+07	5.78E+07	7.54E+07	1.31E+07	1.51E+07	1.98E+07
50.00	1.14E+08	1.14E+08	1.39E+08	2.78E+07	3.11E+07	4.42E+07
75.00	1.74E+08	1.74E+08	2.11E+08	4.65E+07	5.11E+07	7.31E+07
100.00	2.31E+08	2.31E+08	2.71E+08	6.17E+07	5.06E+07	1.18E+08

	Cetirizine			Triclosan		
Concentration (ppb)	Peak area 1	Peak area 2	Peak area 3	Peak area 1	Peak area 2	Peak area 3
0.01	ND	ND	ND	1.41E+04	1.78E+04	ND
0.10	ND	ND	ND	1.94E+04	ND	3.43E+04
1.00	6.68E+04	1.73E+04	3.59E+04	6.73E+04	4.37E+04	5.68E+04
5.00	1.55E+06	1.08E+06	1.94E+06	3.66E+05	4.69E+05	4.59E+05
10.00	2.31E+06	1.48E+06	3.02E+06	9.39E+05	1.06E+06	1.41E+06
25.00	1.08E+07	7.69E+06	1.36E+07	2.33E+06	2.97E+06	2.68E+06
50.00	2.16E+07	1.48E+07	2.70E+07	6.05E+06	6.75E+06	5.87E+06
75.00	3.52E+07	2.49E+07	4.12E+07	1.07E+07	9.83E+06	1.03E+07
100.00	4.00E+07	2.71E+07	4.92E+07	1.36E+07	1.42E+07	1.29E+07

Chapter 4

Investigation of carryover effects on quantification of pharmaceuticals and personal care products in environmental samples using silica hydride stationary phase and ultra high performance liquid chromatography hyphenated with triple quadrupole mass spectrometer

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4.1 Abstract

In this research, an optimised mass spectrometry method for ultra high performance liquid chromatography triple quadrupole mass spectrometry (UHPLC−QqQMS) was developed. The implemented sample preparation technique was solid phase extraction. The extracted samples were then separated under aqueous normal phase (ANP) mode applying Cogent Diamond Hydride column (100 mm×2.1 mm) with 2.2 µm particle size prior to analysis with QqQMS. Carryover which frequently happens during the analysis of pharmaceuticals and personal care products (PPCPs) in environmental samples was also investigated. The detection limit was between 0.01 and 1.00 ppb whereas the limit of quantification was between 0.03 and 3.33 ppb with R^2 of the calibration curves ranging from 0.97 to 0.99. The mobile phase was acetonitrile and milli-Q water, both acidified with 0.1% formic acid. The gradient method time was 20 min and the system was operated using positive mode. The method was successfully applied to quantify 10 PPCPs in 3 water samples. The collision energy and fragmentation pathway were both optimised for each compound using the software Optimizer supplied by Agilent Technologies. A carryover problem or memory effect apparently arising from insufficient flushing of the sample introduction step, was minimised according to an improved washing process, achieved by washing the injection needle with an acidified washing solution for an extended period than normally recommended. Three injections of methanol blank was required between sample injections in order to reduce carryover of sample. The developed approach was applied to improve trace analysis of PPCPs with reduced carryover effects.

4.2 Manuscript

Investigation of carryover effects on quantification of pharmaceuticals and personal care products in environmental samples using silica hydride stationary phase and ultra high performance liquid chromatography hyphenated with triple quadrupole mass spectrometer

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Abstract

In this research, an optimised mass spectrometry method for ultra high performance liquid chromatography triple quadrupole mass spectrometry (UHPLC–QqQMS) was developed. The implemented sample preparation technique was solid phase extraction. The extracted samples were then separated under aqueous normal phase (ANP) mode applying Cogent Diamond Hydride column (100 mm×2.1 mm) with 2.2 µm particle size prior to analysis with QqQMS. Carryover which frequently happens during the analysis of pharmaceuticals and personal care products (PPCPs) in environmental samples was also investigated. The detection limit was between 0.01 and 1.00 ppb whereas the limit of quantification was between 0.03 and 3.33 ppb with R^2 of the calibration curves ranging from 0.97 to 0.99. The mobile phase was acetonitrile and milli-Q water, both acidified with 0.1% formic acid. The gradient method time was 20 min and the system was operated using positive mode. The method was successfully applied to quantify 10 PPCPs in 3 water samples. The collision energy and fragmentation pathway were both optimised for each compound using the software Optimizer supplied by Agilent Technologies. A carryover problem or memory effect apparently arising from insufficient flushing of the sample introduction step, was minimised according to an improved washing process, achieved by washing the injection needle with an acidified washing solution for an extended period than normally recommended. Three injections of methanol blank was required between sample injections in order to reduce carryover of sample. The developed approach was applied to improve trace analysis of PPCPs with reduced carryover effects.

Keywords:

Triple quadrupole; PPCPs; pharmaceuticals; diamond hydride; silica; carryover

Introduction

Environmental assessment of pharmaceuticals and personal care products (PPCPs) is one of the hot topics these days, in terms of their environmental prevalence and bioaccumulation effects. Various scientific disciplines such as chemistry, biology, microbiology, and marine, life,

pharmaceutical and biomedical sciences intensively study these chemicals for a range of purposes such as studying the effect of some PPCPs on animal behaviour or monitoring the bioaccumulation of PPCPs in some invertebrates. In order to understand how these emerging contaminants might affect wildlife and marine life, monitoring programmes in different matrices and sinks such as water streams, sediments and biota are required. One critical environmental issue related to PPCPs is their environmental impact upon the ecosystem, and reliable determination of their concentrations in different matrices to produce data regarding their presence, concentration / biomagnification across the environment [1]. Several factors are expected to contribute to the environmental impacts of PPCPs such as their release to and concentration in receiving waters, and their bioaccumulation and stability, therefore these contaminants need to be investigated in order to understand their impacts on water resources [2]. PPCPs cannot be easily removed from water ways, although some studies report the reduction of some PPCPs in water by chlorination and ozonation processes [3]. A study by Archer et al. [4] mentioned that some PPCPs can be detected at high levels in wastewater treatment plant effluent, suggesting that these contaminants persist after the treatment process.

High performance liquid chromatography (HPLC) is a standard method for assessing the concentration and speciation of PPCPs. A variety of LC modes, but usually reversed-phase (RP) HPLC using C18 material, is commonly used. Hydrophilic interaction liquid chromatography (HILIC) can also be used to analyse polar compounds. Novel stationary phases have emerged in recent years, and their application to PPCPs is of interest. Amongst recently developed stationary phase materials, silanisation of conventional type-B silica with triethoxysilane under aqueous conditions results in silica hydride stationary phases with <5% silanol groups remaining on the surface (**Figure S1**). Some silica hydride stationary phases (such as unmodified, diamond hydride, perfluorinated C8 or undecanoic acid modified hydride [5, 6] can be used in either RP or aqueous normal phase (ANP) since they can retain not only hydrophobic but also hydrophilic analytes [7]. A number of studies on application of silica hydride for analysis of PPCPs, metabolites, amino acids and peptides using silica hydride as stationary phase have been published in recent years [8-13].

Diamond hydride was found to be an excellent choice of column to analyse different types of pharmaceuticals [14] since it can be used to analyse a wide variety of polar and non-polar analytes due to its unique selectivity which can be operated in RP, ANP, ion-exchange mode or mixed modes of separation, depending on the applied mobile phases [15, 16]. According to a developed method by USEPA [17] to analyse PPCPs in different matrices, cimetidine, metformin and ranitidine may be analysed using a HILIC column in ANP mode, whereas other PPCPs such as fluoxetine and diltiazem can be analysed using a RP C18 column. Therefore, use of the novel diamond hydride media with its unique selectivity can provide an analytical method to analyse both groups of PPCPs.

Both ANP and HILIC modes can be used to separate highly polar pharmaceuticals because the retention of polar analytes is increased in this case as long as the organic content in mobile phase is high. While both are favourable approaches for polar compounds, HILIC has poor reproducibility compared to ANP using silica hydride. In addition, HILIC needs longer time in order to equilibrate the system between samples. Silica hydride stationary phase proved to be an excellent approach to monitor some drugs such as cycloserine and their metabolites [18]. Moreover, silica hydride can also be used for the analysis of basic pharmaceuticals which provides excellent peak shape compared to conventional type-B silica columns [19].

Liquid chromatography hyphenated with advanced mass spectrometry is a preferred method when it comes to quantify different types of analytes such as pharmaceuticals [20-25]. The added selectivity arising from multiple reaction monitoring (MRM) mode, which is able to target individual compounds in complex analyte mixtures, is a preferred best choice for quantification and qualification analysis [26]. Liquid chromatography hyphenated with triple quadrupole mass spectrometry (LC−QqQMS) is the most common instrument for quantification, exploiting more advanced modes of MRM such as dynamic multiple reaction monitoring (dMRM), and triggered multiple reaction monitoring (tMRM). These modes can be used to increase sensitivity especially with a large number of MRM transitions. For instance, dMRM can be effectively used to quantify low levels of

compounds in complex samples due to the selectivity of this mode, permitting focus on transitions after elution from the column. Therefore, MS is fully dedicated to only monitor the eluted analytes [26]. Another example is using tMRM to increase sensitivity by focussing on the main transition that is used for quantification. After detecting the main transition − usually the most abundant transition − the system triggers additional cycles to involve other MRM transitions for the same compound and generate an MRM 'spectrum' which can be used for confirmation. Optimisation of collision energy for each product ion further improves sensitivity with this MRM mode [27].

In this study, a method to analyse and quantify 10 PPCPs in water samples was optimised. Off-line automated solid phase extraction (auto-SPE) was used for sample preparation and UHPLC−QqQMS with diamond hydride stationary phase was chosen to separate, detect, quantify and confirm the studied PPCPs in standard solutions, and then applied to water samples. Carryover effects associated with the injection step were also investigated in detail. The optimised method was applied to quantify 10 pharmaceuticals in 3 water samples collected from locations in Victoria.

Experimental
Chemicals

Metformin (1,1-dimethylbiguanide hydrochloride) (97% purity), ranitidine hydrochloride, cimetidine, were purchased from Sigma-Aldrich (Castle Hill, Australia). Citalopram hydrobromide (98% HPLC grade), paroxetine HCl (98% HPLC grade), fluoxetine hydrochloride (98% HPLC grade), sertraline HCl (98% HPLC grade), cetirizine 2HCl (98% purity), trimethoprim (99% HPLC), diltiazem HCl (98% HPLC) were obtained from A. K. Scientific (Union City, CA). Acetonitrile (LC-MS grade), methanol (LC-MS grade) were purchased from Honeywell International Inc. (Muskegon, MI, USA) and acetic acid were purchased from Merck KGaA (Darmstadt, Germany). Milli-Q water was used for vessel cleaning and sample preparation purposes. All stock standards and samples were kept at − 16 °C until the time of analysis. Supplementary Information **Table S1** summaries molar mass, molecular formula, structure of all PPCPs and other information.

Sample preparation procedure

Water samples were collected from locations in Victoria, Australia. The samples were extracted using Oasis HLB cartridges (200 mg, 6 mL) purchased from Waters (Waters Corp., Milford, MA). Extraction was accomplished on an automatic Dionex AutoTrace 280 SPE Instrument (Thermo Fisher Scientific Inc., Scoresby, Australia). Cartridges were conditioned with 5 mL each of two solvents (LC−MS-grade methanol and Milli-Q water). Then, 1 L of water sample was applied through the automated SPE system. The extracted sample was eluted with 10 mL methanol. A 0.5 mL volume of Milli-Q water was added before drying under nitrogen to ca. 0.5 mL. The content was vortexed in a 10 mL test tube before transferring it to a vial for analysis by LC−MS/MS system. Some water extracts were spiked with different concentrations (0.1, 1.0, 5.0, 10, 25, 50, 75 and 100 ppb) of the 10 PPCPs and processed using the same extraction technique in order to evaluate recovery and matrix effects of the method.

UHPLC−QqQMS

Ultra-high performance liquid chromatography (UHPLC) hyphenated with a model 6470 triple quadrupole mass spectrometer (Agilent Technologies, Mulgrave, Australia) equipped with a Jet Stream electrospray ionisation source (ESI), binary pump and autosampler was used to analyse all standards and samples. This system is reportedly 5 times more sensitive than normal ESI according to Agilent Technologies.

Samples were separated at 30 °C, with 0.1% v/v formic acid in water and acetonitrile, used as mobile phases A and B, respectively. The aqueous normal phase mode using diamond hydride involves initial use of low aqueous concentration mobile phase, followed by higher amounts of aqueous mobile phase. Thus, gradient elution commences at 90% v/v mobile phase B for 2 min, decreased to 73% v/v at 5 min, then the gradient was linearly increased to 50% v/v B at 10 min, held at 50% v/v B for 5 min to clean the column. The gradient time was 20 min. Column equilibration for

4 min after returning to 90% v/v B readied the system for the next injection. The flow rate was 0.30 mL min^{-1} with injection volume of 5 μL. Similar gradients with different total analysis times (to test carryover) were applied as follows: a 10 min gradient, commencing at 90% v/v B for 1 min, decreased to 73% v/v B at 2.5 min, then linearly increased to 50% v/v B at 5 min (to vary gradient time), held at 50% v/v B for 2.5 min, and from 50 to 90% v/v B for 2 min prior to the next injection. A 5 min gradient, starting at 90% v/v B for 0.5 min, decreased to 73% v/v at 1.25 min, then linearly increased to 50% v/v B at 2.5 min, held at 50% v/v B for 1.25 min, and from 50 to 90% v/v B for 1 min prior to the next injection. In this study, the lowest content of acetonitrile (ACN) in the mobile phase was limited to 50% v/v in order to avoid the reverse phase behaviour of the diamond hydride at the lower ACN content [6] leading to much stronger retentions of (which is harder to elute) more hydrophobic compounds. The Jet Stream ESI was operated in positive ion analysis mode using gas temperature 300 $^{\circ}$C, gas flow 10 L/min, nebuliser pressure 45 psi, sheath gas temperature 380 $^{\circ}$C, capillary voltage 3500 V and nozzle voltage 500 V. The LC-MS/MS system was calibrated before starting the analysis in the positive ion mode. All MS parameters related to enhancing the detection of each PPCP such as collision energy and fragmentation pathway were optimised.

Data analysis

MassHunter (Agilent Technologies) qualitative and quantitative software were used for both identification, quantification and confirmation of the analysis in addition to Microsoft Excel 2013.

Results and discussion

Optimising LC-MS method

A Cogent diamond hydride column (100 mm × 2.1 mm) with 2.2 μm particle size was used to analyse 10 PPCPs in 3 water samples. This column technology is similar to a HILIC column but slightly different in terms of mobile phase used and the type of particles. The A and B mobile phases comprise of Milli-Q water (MQW) and acetonitrile (ACN) both of which were acidified with 0.1% formic acid. For most of the analytical studies, the flow rate was 0.3 mL/min and the column temperature was 30 $^{\circ}$C, with the following gradient: the gradient started with 90% v/v of ACN for 2

min before being reduced to 73% at 5 min. Then, the ACN content was decreased to 50% v/v at 10 min, continued for a further 5 min. Finally, the ACN content was returned to 90% v/v at 16 min and allowed to equilibrate the column for 4 min. The total analysis time was 20 min. The mass range was *m/z* 100 to 500 while the method was only operated in the positive mode. **Figure S2** shows the extracted ion chromatogram of the target compounds in MRM mode.

The mass spectrometry method was optimised by injecting standards to initially choose the most abundant ions that can be used to quantify and confirm the PPCPs in water samples. This was accomplished by directly injecting a 1 ppm standard solution to the MS. After checking the mass for each PPCP, an MRM method was conducted in order to optimise the collision energy and fragmentor voltage for relevant transitions of each PPCP. Then, one quantifier and two qualifiers were chosen for each PPCP. Each compound resulted in 3 product ions with a specific optimised collision energy for each product ion (see **Table 1**). This process was completed using Optimizer software (Agilent Technologies, Mulgrave, Australia). The quantifier was chosen as the highest abundance product ion whereas the qualifier ions were selected based on the second and third highest abundance ions. All the information relating to quantifier, qualifiers and retention time can be found in **Table 2**. The *m/z* values of the quantifiers (*i.e.* the most abundant product ions), for cetirizine, fluoxetine, paroxetine, sertraline, citalopram, diltiazem, trimethoprim, cimetidine, metformin and ranitidine were 201.0, 148.0, 70.1, 158.9, 109.0, 177.9, 230.0, 159.0, 60.1 and 176.0 respectively.

Table 1 Collision energy and abundance for each PPCP product ion

Compound	Product Ion	Collision Energy	Peak area abundance
Cetirizine	201	16	7.17E+06
	165	86	5.82E+06
	165.6	52	1.81E+06
Cimetidine	159	12	7.24E+06
	95	28	6.70E+06
	117	16	3.58E+06
Citalopram	109	28	6.55E+06
	262	20	2.93E+06

	234	28	1.22E+06
Fluoxetine	148	4	7.25E+05
	91	100	3.74E+04
	65.1	104	3.58E+04
Metformin	60.1	12	9.87E+06
	71.1	24	8.55E+06
	85.1	12	1.71E+06
Paroxetine	70.1	32	2.13E+06
	192	20	1.47E+06
	123	28	3.39E+05
Ranitidine	176	16	7.69E+06
	130	24	3.91E+06
	102	36	3.31E+06
Sertraline	158.9	32	9.61E+06
	274.9	8	7.32E+06
	122.9	64	2.34E+06
Trimethoprim	230	24	9.10E+06
	123	24	6.59E+06
	261	24	6.01E+06
Diltiazem	177.9	24	1.51E+07
	150	52	1.21E+07
	108.9	74	1.21E+07

Table 2 Retention time, quantifier and qualifiers ion masses (*m/z*) for each PPCP

Compound	Quantifier ion (*m/z*)	Qualifier 1 ion (*m/z*)	Qualifier 2 ion (*m/z*)	Precursor ion (*m/z*)	RT (min)
Cetirizine	201.0	165.0	165.6	389.2	6.28
Fluoxetine	148.0	91.0	65.1	310.1	6.40
Paroxetine	70.1	192.0	123.0	330.2	6.50
Sertraline	158.9	274.9	122.9	306.1	6.51
Citalopram	109.0	262.0	234.0	325.2	6.69
Diltiazem	177.9	150.0	108.9	415.2	6.83

Trimethoprim	230.0	123.0	261.0	291.2	7.08
Cimetidine	159.0	95.0	117.0	253.1	7.59
Metformin	60.1	71.1	85.1	130.1	8.47
Ranitidine	176.0	130.0	102.0	315.2	8.74

**Carryover (memory effect) of PPCPs using diamond hydride column**

This problem may be considered a significant challenges when developing LC-MS methods, especially for PPCPs at low concentration. If this issue is ignored, it can affect the accuracy and precision of all results which makes the developed method inapplicable. There are different sources which are responsible for this problem which may be separately attributable to the column, mobile phase, autosampler system (*i.e.* injection needle) and detector. In this study, one of the most common sources of contamination, which is the injection needle of the autosampler, was deemed to be a likely cause of carryover, and so was investigated in terms of using different percentages of MeOH + water to wash the needle, applying different port flush times and comparing different analysis times using different gradients.

To study the effect of different washing solvent ratios, (methanol: water here), 4 mixing solutions were compared. Methanol and water are popular wash solvents in LC/MS analysis. A combination of these solvents can be used to wash the injection needle directly after taking the exact amount of sample volume. These are suitable solvents for the vast majority of drugs and PPCPs, and hence they can be used to clean the needle after immersing it in the sample vial. Using 100% methanol was found the best solvent to wash the needle (**Figure 1**). Other combinations (25%, 50% and 75% MeOH/water) can also be used as the total average peak areas for all three blanks of the 3 PPCPs was less than 0.06% of the original standard, which is considered very low compared to the original higher concentration standard (100 ppb in this case).
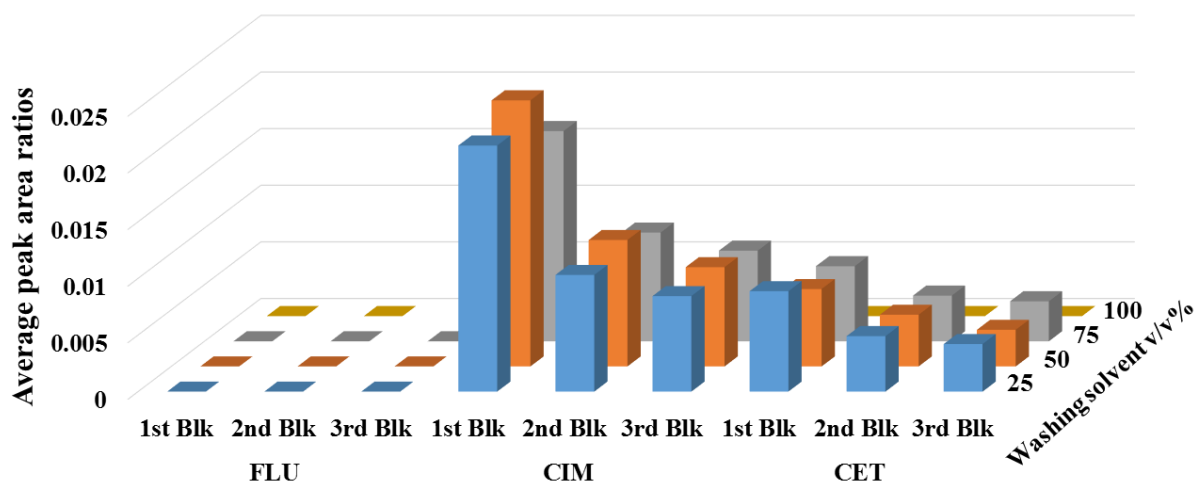
Figure 1 The effect of different washing solvents (%v/v of MeOH), using a 20 min gradient method and 20 s of port flushing time. The maximum error caused by the carryover with washing solvent effect can be approximately 0, 10 and 18 ng L^{-1} for fluoxetine (FLU), cimetidine (CIM) and cetirizine (CET), respectively, in the next analysis.

On the other hand, using different flushing port times was found not to have a large difference although it can be noticed that using 10 s had the lowest average peak area compared to other flushing times (**Figure 2**). Although this clearly showed there is no effect of using longer time to wash the needle, it is highly recommended to have enough time to wash the needle after injection in order to ensure there is minimal contamination between samples (**Figure 2**). In this figure, it can be noted that fluoxetine showed no carryover, and the other compounds (cimetidine and cetirizine) had low carryover in the third blank, so performing sufficient number of blanks is important to remove residual PPCPs from the previous standard or sample. Although the maximum error depends on the tested concentration, the maximum error value already reported in each figure is the absolute maximum taken from the concentration resulting in highest error values.
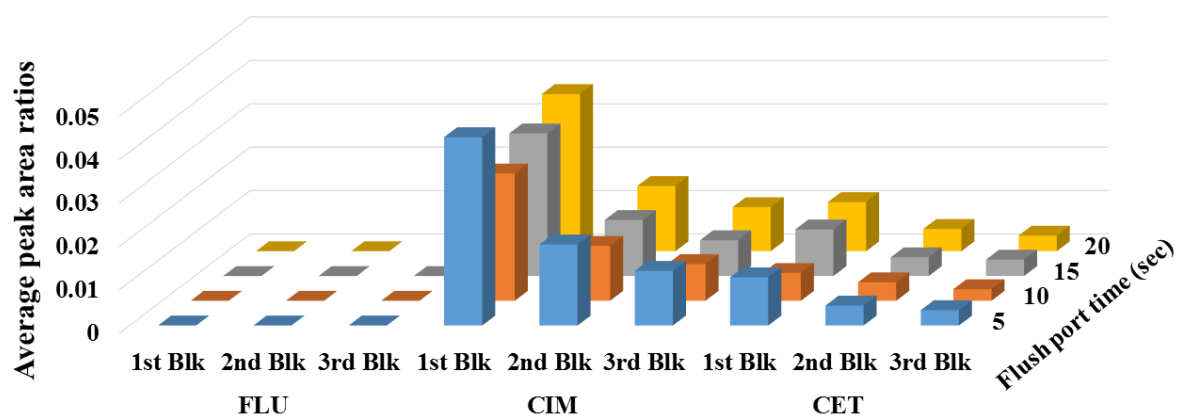
Figure 2 The effect of different flushing port time, using a 20 min gradient method and 25 % v/v of MeOH as the washing solvent. The maximum error caused by the carryover with flushing port time effect can be approximately 0, 28 and 34 ng L^{-1} for FLU, CIM and CET, respectively, in the next analysis.

When carryover is encountered, a useful tool to ensure the column is suitably cleaned before subsequent sample analysis is to use a suitable accelerated gradient method to check for residual PPCPs and to clean the column, especially after injecting higher standard solution concentrations. In **Figure 3** and **Figure 4**, the effect of using different port flushing times was tested again but with faster gradients. A 10 min total time analysis gave larger amount of carryover for all the three blanks and also fluoxetine, which had no carryover with the original 20 min gradient (**Figure 3**), again emphasising the importance of investigating carryover for trace analysis with fast chromatographic methods that may exacerbate memory effects. Similar results can be noticed when using a 5 min total analysis time (**Figure 4**). Although column elution should have no effects on the amount of carryover on the sample injection needle, increase in carryover arising from the injection needle by accelerated gradient elution was observed. This can be explained in the way that the carryover already existed in all the cases showing significantly high peak area ratios (e.g. >0.01). This is a why a faster gradient

could not effectively remove the carryover from the column and it appeared in the next run. Preventing carryover can commence with initial method development to ensure minimising this analytical artefact.
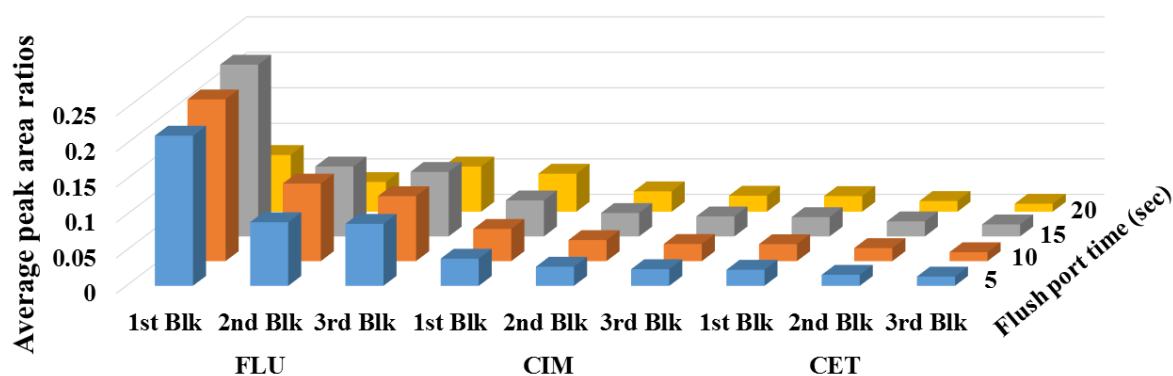
Figure 3 The effect of different port flushing time using a 10 min gradient method and 25 % v/v of MeOH as the washing solvent. The maximum error caused by the carryover with flushing port time effect can be approximately 2407, 39 and 88 ng L^{-1} for FLU, CIM and CET, respectively, in the next analysis.
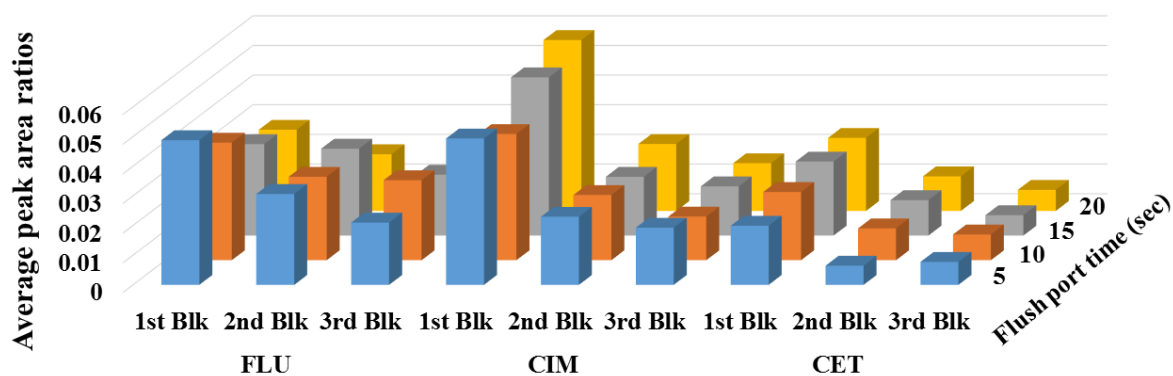
Figure 4 The effect of different flushing port time using a 5 min gradient method and 25 % v/v of MeOH as the washing solvent. The maximum error caused by the carryover with flushing port time effect can be approximately 2222, 43 and 85 ng L^{-1} for FLU, CIM and CET, respectively, in the next analysis.

Method validity

The correlation coefficient (R^2) was between 0.9669 and 0.9993 (**Table S2**) over the calibration range was 0.01 – 100 ppb for different PPCPs. The instrument detection limit was 0.01 – 1.00 ng mL^{-1} whereas the method detection limit was 0.08 – 14.15 pg mL^{-1} (**Table S2**). The recovery was between 70.7% (± 3.9) and 105.0% (± 2.2) (see **Table S3**). The matrix effect was investigated by spiking different concentrations of standards (0.1, 1.0, 5.0, 10, 25, 50, 75 and 100 ppb) in real water sample extracts which showed an acceptable recovery for all PPCPs. The recovery data of spiked standards in water samples relative to that spiked in MQW are shown in **Table S3**.

Application on real samples

The optimised method was applied to analyse 3 water samples collected from different regions in Victoria, Australia. The results are shown in **Table 3** which indicates that no PPCPs of interest was detected except for the possible presence of ranitidine which was below the method detection limit. Although PPCPs were not detected in all the investigated water samples (**Table 3**), this indicated there may be some PPCP present in each sample but with the amount below LOD reported in this study. However, it should be noted that the LOD can be further reduced by improvement of the solid phase extraction approach to result in higher recovery values (to be >80%) than that reported in this work (**Table S3**). Although there were no pharmaceuticals detected positively in these samples, the reported LC–MS/MS proved to be a very sensitive approach to their quantification, with some DLs at ppq levels for standards (**Table S2**), which makes this suitable to detect PPCPs in environmental samples in aqueous matrices. Using 3 transitions is recommended in order to quantify emerging

contaminants in environmental samples with confidence. This proves that UHPLC−QqQMS is a robust and reliable system to analyse PPCPs in different matrices.

Table 3 Concentrations of PPCPs in water samples collected from different locations in Victoria, Australia

Compound	Concentrations in real samples (ng L^{-1})		
	1.S.R	2.S.R	3.S.R
Cetirizine	ND	ND	ND
Fluoxetine	ND	ND	ND
Paroxetine	ND	ND	ND
Sertraline	ND	ND	ND
Citalopram	ND	ND	ND
Diltiazem	ND	ND	ND
Trimethoprim	ND	ND	ND
Cimetidine	ND	ND	ND
Metformin	ND	ND	ND
Ranitidine	< LOD	< LOD	< LOD

Conclusion

A method to analyse some PPCPs in water samples using UHPLC−QqQMS was developed. A different type of column technology was investigated in this study. In addition, the carryover which is common problem in routine analysis was investigated to reduce this problem when performing trace analysis in different environmental samples. This can lead to a reliable quantification method as demonstrated here for the quantification of PPCPs in water samples with good repeatability, good linearity range and low LOD and LOQ. MS/MS condition was optimised for each PPCP lead to selection of the best collision energy and fragmentation pathways for each analyte. According to the observation in this study, it is highly recommended that special attention should be paid on the method development when analysing PPCPs in environmental samples in terms of applying enough time to remove all the carryover analytes from the UHPLC−QqQMS system before injection of the next sample. Running more blanks is also recommended in addition to start the analysis with low level concentrations, which is common practice in analytical chemistry in order to reduce or prevent the carryover effects.

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4.3 Supplementary information

Supplementary Information
Journal of Separation Science

Investigation of carryover effects on quantification of pharmaceuticals and personal care products in environmental samples using silica hydride stationary phase and ultra high performance liquid chromatography hyphenated with triple quadrupole mass spectrometer

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CONTENTS:

Figure S1 The differences between stationary phases with silica hydride and type-B fused silica
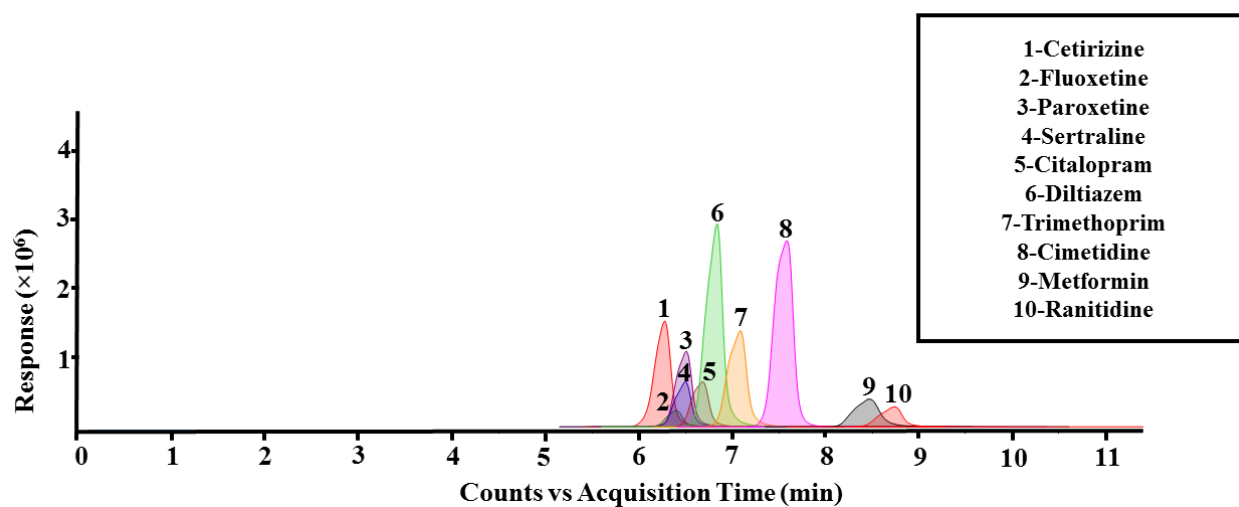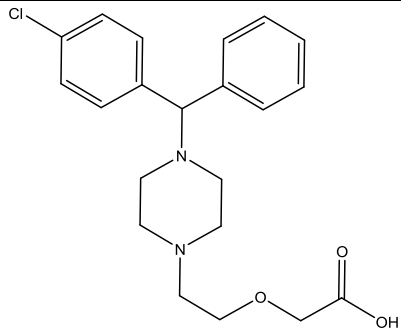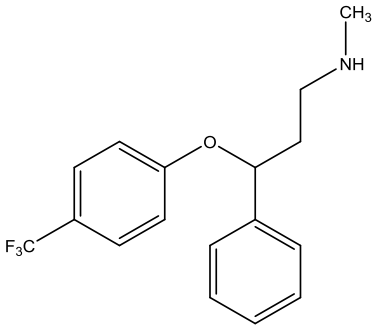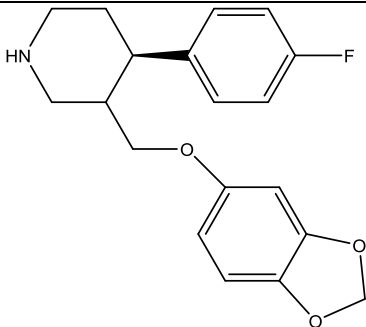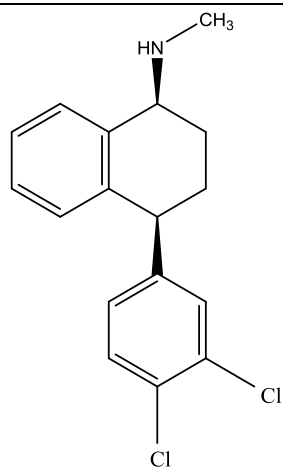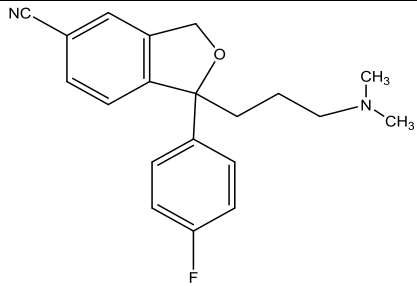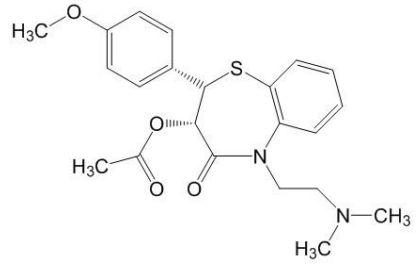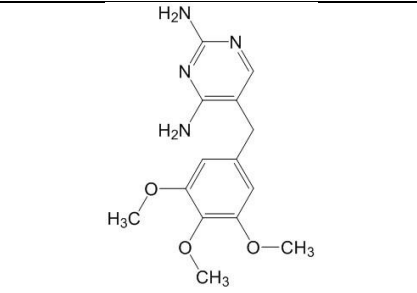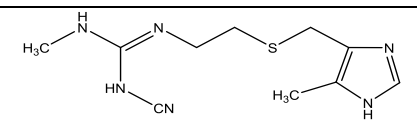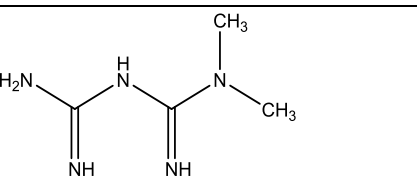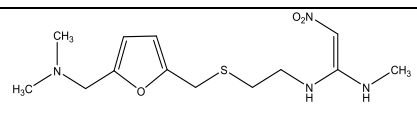

Figure S2 Extracted ion chromatogram of the target compounds in MRM mode

Table S1 Characteristics of PPCPs

Compound	Therapeutic class	CAS no.	Log K_{ow} *	Molar Mass (g mol^{-1})	Molecular formula	Structure
Cetirizine	Antihistamine	83881-52-1	-0.61	388.90	$C_{21}H_{25}ClN_2O_3$	
Fluoxetine	Antidepressant	56296-78-7	4.65	309.33	$C_{17}H_{18}F_3NO$	
Paroxetine	Antidepressant	78246-49-8	3.95	329.37	$C_{19}H_{20}FNO_3$	
Sertraline	Antidepressant	79559-97-0	5.29	306.24	$C_{17}H_{17}NCl_2$	

Citalopram	Antidepressant	59729-32-7	3.74	324.40	$C_{20}H_{21}FN_2O$	
Diltiazem	Nondihydropyridine	33286-22-5	2.79	414.52	$C_{22}H_{26}N_2O_4S$	
Trimethoprim	Antibiotic	738-70-5	0.73	290.32	$C_{14}H_{18}N_4O_3$	
Cimetidine	Histamine	51481-61-9	0.57	252.34	$C_{10}H_{16}N_6S$	
Metformin	Antidiabetic	1115-70-4	-2.64	129.17	$C_4H_{11}N_5$	
Ranitidine	Histamine-2 blockers	66357-59-3	0.29	314.41	$C_{13}H_{22}N_4O_3S$	

* Log K_{ow} (source: KOWWIN v. 1.68 database, USEPA EPI suite 4.11 software)

Table S2 The correlation coefficient (R^2), LOD and LOQ for both instrument and method

Compound	R^2	Instrument LOD (ng mL^{-1})	Instrument LOQ (ng mL^{-1})	Method LOD (pg mL^{-1})	Method LOQ (pg mL^{-1})
Cetirizine	0.9916	0.02	0.05	0.18	0.61
Fluoxetine	0.9831	0.70	2.33	8.36	27.85
Paroxetine	0.9864	0.39	1.31	4.46	14.86
Sertraline	0.9801	0.70	2.33	8.06	26.88
Citalopram	0.9725	1.00	3.33	12.55	41.84
Diltiazem	0.9887	0.05	0.17	0.64	2.12
Trimethoprim	0.9970	0.01	0.03	0.10	0.34
Cimetidine	0.9993	0.01	0.03	0.08	0.26
Metformin	0.9749	1.00	3.33	14.15	47.18
Ranitidine	0.9669	1.00	3.33	12.81	42.70

Table S3 Recovery (%) and relative standard deviation (RSD) of standards spiked in water sample

Compound	Recovery (%)	RSD
Cetirizine	82.1	6.3
Fluoxetine	83.8	1.8
Paroxetine	87.9	0.7
Sertraline	86.8	0.6
Citalopram	79.7	0.7
Diltiazem	78.5	0.8
Trimethoprim	97.4	3.3
Cimetidine	105.0	2.2
Metformin	70.7	3.9
Ranitidine	78.1	2.1

Chapter 5

High resolution liquid chromatography mass spectrometry for pharmaceuticals and personal care products analysis in freshwater invertebrates: a comparison of quadrupole time of flight and quadrupole Orbitrap mass spectrometers

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5.1 Abstract

High performance liquid chromatography hyphenated with mass spectrometry is a powerful technique that can be reliably used to identify and quantify PPCPs in water samples and other matrices such as freshwater invertebrates. This type of technology can be even more important based on a number of factors such as mass accuracy and resolution. Other factors such as scan speed and MS/MS analysis are also important especially for analysing environmental samples as PPCPs are likely to be detected at trace levels. The purpose of this study was to compare the capabilities of both UHPLC–Q-Orbitrap MS and HPLC-QTOFMS, which are both high resolution mass spectrometers, hyphenated with liquid chromatography. The two advanced systems were compared using the same chromatographic method to analyse selected PPCPs in freshwater invertebrates using full scan mode. Both systems showed an excellent performance to detect these contaminants at low concentration levels. However, UHPLC-Q-Orbitrap MS proved that full scan mode can only be sufficient to analyse PPCPs in invertebrate samples. The lowest instrument detection limit found here amongst the PPCPs for UHPLC-Q-Orbitrap MS was 0.01 ng mL^{-1} using full scan mode and it was 0.1 ng mL^{-1} for HPLC-QTOFMS. Although UHPLC-Q-Orbitrap MS proved to be more sensitive and accurate than the other system, both LC-MS systems showed acceptable sensitivity and mass accuracy which was < 5 ppm for all the studied PPCPs.

5.2 Manuscript

High resolution liquid chromatography mass spectrometry for pharmaceuticals and personal care products analysis in freshwater invertebrates: a comparison of quadrupole time of flight and quadrupole Orbitrap mass spectrometers

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High performance liquid chromatography hyphenated with mass spectrometry is a powerful technique that can be reliably used to identify and quantify PPCPs in water samples and other matrices such as freshwater invertebrates. This type of technology can be even more important based on a number of factors such as mass accuracy and resolution. Other factors such as scan speed and MS/MS analysis are also important especially for analysing environmental samples as PPCPs are likely to be detected at trace levels. The purpose of this study was to compare the capabilities of both UHPLC– Q-Orbitrap MS and HPLC-QTOFMS, which are both high resolution mass spectrometers, hyphenated with liquid chromatography. The two advanced systems were compared using the same chromatographic method to analyse selected PPCPs in freshwater invertebrates using full scan mode. Both systems showed an excellent performance to detect these contaminants at low concentration levels. However, UHPLC-Q-Orbitrap MS proved that full scan mode can only be sufficient to analyse PPCPs in invertebrate samples. The lowest instrument detection limit found here amongst the PPCPs for UHPLC-Q-Orbitrap MS was 0.01 ng mL^{-1} using full scan mode and it was 0.1 ng mL^{-1} for HPLC-QTOFMS. Although UHPLC-Q-Orbitrap MS proved to be more sensitive and accurate than the other system, both LC-MS systems showed acceptable sensitivity and mass accuracy which was < 5 ppm for all the studied PPCPs.

Keywords:

Orbitrap; QTOF; PPCPs; pharmaceuticals; QuEChERS; freshwater invertebrates

1.0 Introduction

The trend towards using cutting edge instruments is increasingly exploited to analyse emerging contaminants in environmental matrices, usually with an aim to both reduce the measureable levels at which contaminants are reported, and to improve selectivity of the method. Widespread availability of the systems might be a limiting factor, and especially having a number of alternative technologies in the one laboratory, plus the expertise required to take full advantage of instrumental capability and operational complexity, a baseline comparative study can serve the purpose of providing basic analytical figures of merit on which to contrast competing claims. Additional interest will be in acquisition speed of the mass analyser, that can determine the overall throughput when considering development of fast HPLC analysis, and the potential need to deconvolute in the mass dimension, multiple overlapping components from the separation dimension. As an example of MS capability, full scan mode, operated in both positive and/or negative modes, can be used to identify and quantify analytes in different matrices. Moreover, accurate mass capabilities and the possibility of detecting compounds at trace levels using a high resolution MS instruments can add confidence to the analytical method. The interest here is application of high resolution MS.

A number of comparisons have focussed of use of high resolution MS systems. A study by Glausera et al. [1] compared two advanced HPLC-MS systems, the QTOFMS and Exactive Plus MS analysers, and found that both systems demonstrated good performance to analyse untargeted plant metabolomics. The Exactive Plus MS (Orbitrap mass analyser) provided lower detection limit for some of the compounds, but both systems were essentially equivalent in terms of overall sensitivity and mass accuracy. Another study by Henry et al. [2] compared triple quadrupole MS (TQ-MS) and Exactive MS capabilities for quantifying drugs in some plasma samples, in this instance using full scan mode for the Orbitrap MS, and selected reaction monitoring (SRM) mode for the TQ-MS. Results were found to be comparable in terms of sensitivity, linearity and accuracy, and generally supported the role of full scan mode of the Orbitrap MS for quantitative analysis. Other comparisons were performed to contrast the performance of different high resolution MS systems to analyse

pesticides, drug metabolites, proteins and peptides in different matrices. Some of these MS systems are TQ-MS, TOFMS, QTOFMS and Orbitrap MS [3-12].

In previous studies by the present authors, PPCPs were reported in various water samples [13] and some freshwater invertebrates such as *Leptophlebidae* and *Atytidae* [14]. In addition to other studies, [15-22] this demonstrated that PPCPs can be detected in environmental matrices and that potential bioaccumulation might occur in some species, although this has to be comprehensively investigated. Possible bioaccumulation might also result in the food chain of the aquatic ecosystem. For instance, some PPCPs may be transferred from one species to another up the food chain [16] which should be considered a major route in the aquatic ecosystem. This field of research still needs further investigations because it will add another route by which PPCPs to reach organisms in the environment.

In this general study, a QuEChERS extraction method was developed to extract some PPCPs in a spider species (*Tetragnatha*). These samples were then analysed by a variety of LC-MS systems, according to available facilities, which allowed comparison of their capabilities. Of specific interest here was full scan accurate mass analysis using QTOFMS and Q-Orbitrap MS, and switching polarity mode. The study compared LOD and LOQ of each instrument, and demonstrated the suitability of full scan mode and accurate mass to perform qualification and quantification studies. To our knowledge, this is the first study to compare full scan mode in both systems for PPCP analysis in invertebrates.

2.0 Materials and methods
2.1 Chemicals and reagents

Metformin (1,1-dimethylbiguanide hydrochloride) (97%purity), ranitidine hydrochloride, cimetidine, were purchased from Sigma-Aldrich (Castle Hill, Australia). Citalopram hydrobromide (98% HPLC grade), sertraline HCl (98% HPLC grade), were obtained from A. K. Scientific (Union

City, CA). Caffeine (reagent plus) and triclosan ($\geq$ 97% HPLC grade) were purchased from Sigma-Aldrich (Castle Hill, Australia). Acetonitrile (LC-MS grade), methanol (LC-MS grade) were purchased from Honeywell International Inc. (Muskegon, MI, USA) and acetic acid were purchased from Merck KGaA (Darmstadt, Germany). Milli-Q water was used for cleaning and sample preparation. All stock standards and samples were kept at -16 $^{\circ}$C until the time of analysis. Further information about the selected PPCPs can be found in **Table S1**.

2.2 Sample preparation procedure

Five invertebrate (spider) samples (*Tetragnatha*) were collected from a creek located in Victoria, Australia as shown in **Table S2**. A QuEChERS extraction salt (Q110 – EN Method – RESTEK, Bellefonte, PA) solution was prepared using 23.4 mL Milli-Q water. Each spider was crushed using a glass rod for about 4 min before adding 600 µL of ACN to a glass test tube. Then, the grinding continued for another 4 min. 600 µL of QuEChERS solution was then added into the tube. The glass tube was manually shaken for about 2 min. 120 µL of hexane (HXN) and 40 µL of internal standard, carbamazepine-d10 (10 mg L^{-1}), were added to the mixture before centrifuging the contents at 4500 rpm for 2 min. The ACN layer (200 µL) was then transferred into a vial to be dried under a gentle stream of N_2. 200 µL of Milli-Q water was added to the vial to reconstitute the contents after drying. The same steps were applied for a control sample (200 µL of 1 mg L^{-1} of standard mixture).

2.3 Chromatographic method

The same HPLC chromatographic method [13, 14] was followed to analyse PPCPs in spider samples using both systems UHPLC-Q-Orbitrap MS and HPLC-QTOFMS. This method was only used in full scan mode in both systems. An Accucore-C18 column (2.6 µm particle size, 50 mm × 2.1 mm - Thermo Fisher Scientific) was used to separate the analytes. The column temperature was 25 °C and the injection volume was 15 µL. The flow rate was 0.30 mL min^{-1} whereas total analysis time

was 20 min. The mobile phases were 0.1% v/v acetic acid in water (A) and ACN (B). The gradient method started with 10% v/v of mobile phase B for 2 min followed by 27 % v/v B at 5 min, then increased to 50 % v/v B at 10 min, and finally to 100 % v/v B at 14 min. The gradient was then held at 100% v/v for 1 min before equilibrating the column again with 10% v/v B, which was held for 4 min.

2.4 HPLC-QTOFMS

An HPLC-QTOFMS (Agilent Technologies, Mulgrave, Australia) was used to analyse the invertebrate samples. This system employed an Agilent 1290 Infinity LC system with two binary pumps that has a maximum pressure limit of 600 bar. The ion source of this system is a dual jet stream electrospray ionisation source (Dual AJS ESI). This technology increases the sensitivity by using superheated nitrogen to improve the ionisation process. The Dual AJS ESI ion source was operated separately either in positive or negative mode. The parameters of this source were nebuliser pressure 45 psig, drying gas flow rate 10 L/min, drying gas temperature 250 °C, sheath gas flow rate 11 L/min, sheath gas temperature 350 °C, fragmentor voltage 190 V, capillary voltage 3500 V and skimmer voltage 65 V. The resolution of the system was 35,000 ± 500 FWHM. The mass range was *m/z* 50 to 400. Calibration was performed for the system before each set of analyses.

2.5 UHPLC-Q-Orbitrap MS

An UHPLC−Q-Orbitrap-MS (Thermo Fisher Scientific, Scoresby, Australia) was also available to perform the analysis of spider samples. This MS analyser was coupled to the UHPLC system with maximum pressure limit of 1200 bar. The heated electrospray ionisation (HESI) source was operated in both positive and negative modes. The conditions of the source were sweep gas 0, sheath gas 35, auxillary gas 10, spray voltage 3.0 kV, auxiliary gas heater temperature 300 °C and capillary temperature 320 °C. For the present analysis, the MS was only operated using full scan mode and the mass range was *m/z* 50 to 400. Other MS parameters which were previously optimised [13] were resolution (70,000 FWHM); AGC target (1×10^6) and maximum IT (200 ms). The mass

analyser was calibrated before analysis for performing a series of analyses, in both positive and negative modes.

3.0 Results and discussion

3.1 Optimisation of the QuEChERS extraction method

A QuEChERS method was previously developed and discussed elsewhere [14] to extract some PPCPs in freshwater invertebrates. However, in this study, two more steps were added to the QuEChERS method, by including 120 µL hexane (HXN) and 40 µL internal standard (carbamazepine-d10). This was because extracting spider samples presented some difficulties in adequately crushing some parts of the insect (*i.e.* spider legs) using the glass rod. In this case, HXN was used in order to reduce sample matrices by isolating parts of the solid residue, although HXN might reduce the recovery [14]. The internal standard was added to correct for extraction quantification, and analysis using the instrument.

3.2 Comparison of the performance of QTOFMS and Q-Orbitrap MS

The two LC-MS systems are high resolution mass spectrometers, and are reportedly comparable in terms of sensitivity and accuracy. However, the mass accuracy for the Q-Orbitrap was generally < 2ppm whereas it was < 5 ppm for the QTOF (**Table 1**). Although some compounds showed better mass accuracy using QTOFMS such as metformin (0.8 ppm) and ranitidine (-0.6 ppm), other compounds such as cimetidine (0.4 ppm), caffeine (-0.5 ppm), sertraline (0.0 ppm) and triclosan (0.3 ppm) showed better mass accuracy using the Q-Orbitrap system. Only citalopram (0.3 ppm) showed the same mass accuracy in both systems.

Table 1 Retention time, exact protonated or deprotonated mass and mass accuracy of each PPCP in standard for both Q-Orbitrap and QTOF LC/MS systems

Compound	[M+H]$^+$	Q-Orbitrap MS		QTOFMS	
		Retention time (min)	Mass accuracy (ppm)	Retention time (min)	Mass accuracy (ppm)
Metformin	130.1087	0.48	-1.5	0.55	0.8
Cimetidine	253.1230	0.57	0.4	0.66	-3.6
Ranitidine	315.1485	0.61	1.0	0.7	-0.6
Caffeine	195.0877	1.14	-0.5	1.27	2.6
Citalopram	325.1711	6.36	0.3	6.83	0.3
Sertraline	306.0811	7.57	0.0	8.08	2.6
Triclosan	286.9439	12.58	0.3	13.17	3.5

Unlike HPLC-QTOFMS, the HESI source in the UHPLC-Q-Orbitrap-MS was operated in both positive and negative modes. Although a switching polarity option is also available in the QTOFMS system, it was not fast enough to switch between the two modes at the desired rate. This lead to lowering the sensitivity of the system which resulting in an inability to adequately detect some PPCPs in the standard mixture. In this case, analysis using QTOFMS system had to be repeated using two sequential methods, one method operated in positive mode and another operated in negative mode. On the other hand, the switching polarity option was successfully applied in the Q-Orbitrap MS system resulting in detection of caffeine in all samples. See (**Figure 1**) and (**Table 2**). However, QTOFMS could not detect caffeine in the spider samples even though switching polarity was not applied in this case. See (**Figure 2**) and (**Table 2**). **Table 3** summaries key differences between Q-Orbitrap MS and QTOFMS mass analysers. Note that it may be expected to detect caffeine in Sample 4 (of the highest concentration) by using QTOFMS. However, this is not detected. The possible explanation can be matrix interference of Sample 4 suppressing the caffeine signal with QTOFMS analysis. In addition, possible contamination such as analyte carryover in the Q-Orbitrap MS measurement could also occur in this case. It is clear that Q-Orbitrap MS has a number of features that improve its applicability for the present application using full scan mode.
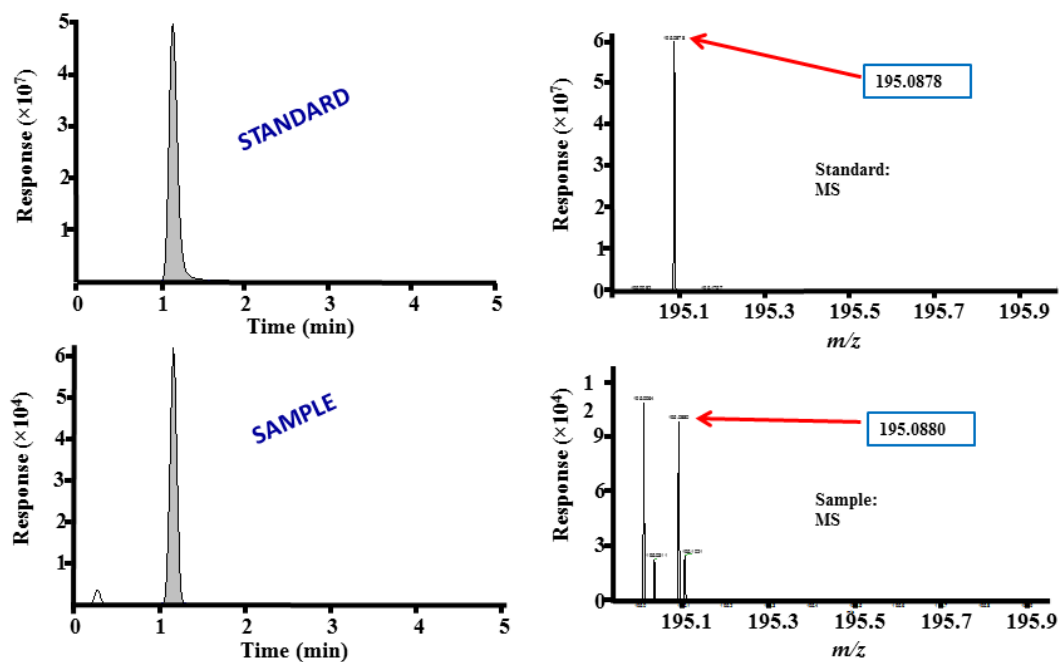
Figure 1 EIC and spectra of caffeine in full scan mode for both standard and sample using Q-Orbitrap system
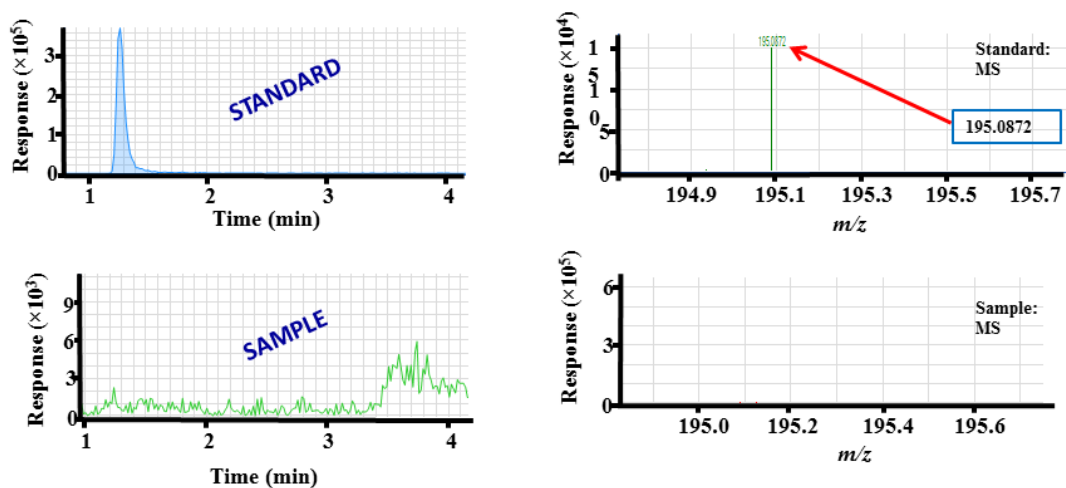
Figure 2 EIC and spectra of caffeine in full scan mode for both standard and sample using QTOFMS system

Table 2 PPCPs concentrations in invertebrate samples (ng.g^{-1}).

Compound	Q-Orbitrap MS					QTOFMS				
	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5
Metformin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cimetidine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ranitidine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Caffeine	9.56	9.67	3.45	21.91	20.67	ND	ND	ND	ND	ND
Citalopram	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sertraline	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Triclosan	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 3 Main differences between Q-Orbitrap and QTOF mass analysers

	Q-Orbitrap MS	**QTOFMS**
Resolution	Up to 280,000	Up to 35,000 ± 500
Mass accuracy	Better than 2 ppm (in this case)	Better than 4 ppm (in this case)
Polarity switching	faster	fast
Mass range	Up to 6000 *m/z*	Up to 3200 *m/z*
MS/MS	YES	YES
Detection limit	0.01 ng mL^{-1} (Full scan mode)	0.1 ng mL^{-1} (Full scan mode)

3.3 Method validity

Different concentrations of all standards were prepared in Milli-Q water. The method detection limit was in the range $1 - 10$ ng g^{-1} (**Table S3**). The instrument detection limit for Q-Orbitrap MS in full scan mode was $0.01 - 1$ ng mL^{-1} whereas it was $0.1 - 10$ ng mL^{-1} for the QTOFMS (**Table S4**). So in general, about a 10-fold decrease in DL is offered by the former system. The LOD was determined by injecting decreasing concentrations until peak heights were approximately three

times the noise level. The linearity exceeded 0.98 for all compounds over the calibration range of $0.01 - 100 \, \mu g \, L^{-1}$ which included 8 data points. The mass accuracy (< 2 ppm for the Q-Orbitrap and < 5 ppm for the QTOF – **Table 1**) was deemed acceptable for the required analysis. Although not a metric related to the MS system, the recovery was acceptable and it was in the range 52.2 to 147.5% except for sertraline and triclosan which were 30.5 and 40.3% respectively (**Table S5**). The sample preparation method and recovery tests were performed by QTOFMS system. The performance of QuEChERS depends on solubility of compounds in ACN phase relative to the others (water and salt). This is governed by several factors of the analytes such as hydrophobicity, acid-base equilibrium which can be described by log K_{OW} and pK_a of the analytes. Since the investigated compounds in this study have a wide range of log K_{OW} and pK_a, their recovery range is thus expected to be relatively large.

3.4 Application to spider samples

The analytical method which was previously developed by the present authors [13, 14] was applied to quantify 7 PPCPs in 5 invertebrate (spider) samples. **Figure 1** shows the extracted ion chromatogram (EIC) of caffeine in both standard and sample, which was confirmed by the mass spectrum of the analyte. Retention time, exact protonated or deprotonated ion mass, and mass accuracy for both systems can be found in **Table 1**. Caffeine was the only analyte detected in these samples using the Q-Orbitrap MS system (**Table 2**). The other PPCPs were not detected by both systems in all samples. With negative results for most of the PPCPs, it is difficult to validate the extraction and LC-MS methods. In addition, detection of caffeine does not mean that this compound is bioaccumulated. log Kow of 0.16 (Table S1) does not suggest strong bioaccumulation. Caffeine also does not seem to bioaccumulate in the human body. Note that this experiment was undertaken in the field, on field collected samples. The evidence is therefore only circumstantial, but consumption of insects that contain PPCPs, by spiders, seems a likely mechanism for PPCP incorporation in the spider. Spiders do not drink the water. Moreover, more experiments need to be done in order to

investigate the transfer process from species to another. Metformin with log K_{ow} of -2.64 was selected as the example of compound that is not expected to be bio-concentrated (due to its high polarity) in order to broaden the analyte range of the analysis in this study. These results highlight the importance of exploring this field of research in order to provide answers regarding the effects and impact of PPCPs in the environmental ecosystem.

4.0 Conclusion

In this study, an extraction method based on QuEChERS was developed to extract some spider samples. Two instruments were compared using their full scan mode capability. By using HPLC-QTOFMS it was not possible to detect the studied analytes using full scan mode, whereas the improved detection limits of UHPLC-Q-Orbitrap MS enabled detection of caffeine in all 5 invertebrate samples using full scan mode. In general, the LOD and LOQ of the Q-Orbitrap MS system were lower than that of QTOFMS. Although Q-Orbitrap MS has improved sensitivity and mass accuracy, the QTOFMS system, in most cases, can be beneficial in environmental analysis because it has sufficient sensitivity and accurate mass which is suitable for many applications. In Unlike Q-Oribitrap MS system, the QTOFMS does not have UHPLC unit although the applied chromatographic method was the same. It is recommended to do further investigations which can be done to compare the capabilities of MS/MS analysis of both systems to analyse PPCPs in different environmental matrices.

Acknowledgements

Conflict of interest statement

The authors report no conflict of interest

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5.3 Supplementary information

Supplementary Information

High resolution liquid chromatography mass spectrometry for pharmaceuticals and personal care products analysis in freshwater invertebrates: a comparison of quadrupole time of flight and quadrupole Orbitrap mass spectrometers

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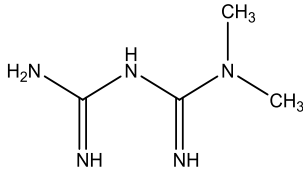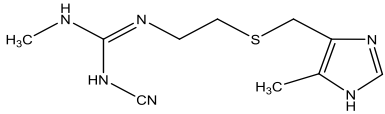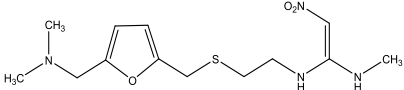
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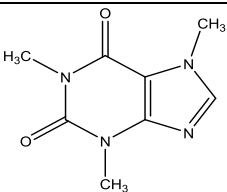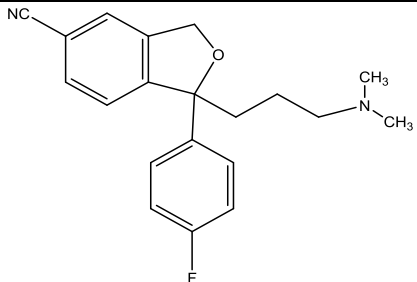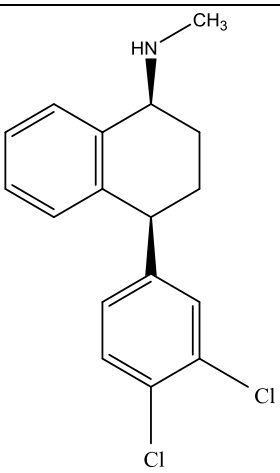
Table S1 Characteristics of PPCPs

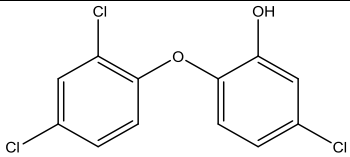**Table S2** Invertebrate samples information

Table S3 LOD and LOQ of the method

Table S4 LOD and LOQ for both Q-Orbitrap and Q-TOF instruments

Table S5 Recovery for the studied PPCPs (1 ppm) spiked in some invertebrates

Table S1 Characteristics of PPCPs

Compound	Therapeutic class	CAS no.	Log K_{ow}*	Molar Mass (g mol^{-1})	Molecular formula	Structure
Metformin	Antidiabetic	1115-70-4	-2.64	129.17	$C_4H_{11}N_5$	
Cimetidine	Histamine	51481-61-9	0.57	252.34	$C_{10}H_{16}N_6S$	
Ranitidine	Histamine-2 blockers	66357-59-3	0.29	314.41	$C_{13}H_{22}N_4O_3S$	
Caffeine	Stimulant	58-08-2	0.16	194.19	C8H10N4O2	
Citalopram	Antidepressant	59729-32-7	3.74	324.40	$C_{20}H_{21}FN_2O$	
Sertraline	Antidepressant	79559-97-0	5.29	306.24	$C_{17}H_{17}NCl_2$	

Triclosan	Antibacterial	3380-34-5	4.66	289.54	C12H7Cl3O2	

* Log K_{ow} (source: KOWWIN v. 1.68 database, USEPA EPI suite 4.11 software)

Table S2. Invertebrate samples information

Sample ID	Site	Species	Common name	Weight (mg)
S1	Brushy Creek	*Tetragnatha*	Spider	22.137
S2	Brushy Creek	*Tetragnatha*	Spider	20.822
S3	Brushy Creek	*Tetragnatha*	Spider	32.476
S4	Brushy Creek	*Tetragnatha*	Spider	5.474
S5	Brushy Creek	*Tetragnatha*	Spider	6.704

Table S3 LOD and LOQ of the method

Compound	Method LOD (ng g^{-1})	Method LOQ (ng g^{-1})
Metformin	5.0	16.7
Cimetidine	1.0	3.3
Ranitidine	5.0	16.7
Caffeine	1.0	3.3
Citalopram	1.0	3.3
Sertraline	10.0	33.3
Triclosan	5.0	16.7

Table S4 LOD and LOQ for both Q-Orbitrap and Q-TOF

	Q-Orbitrap		Q-TOF	
Compound	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Metformin	0.01	0.03	10.0	33.3
Cimetidine	0.1	0.33	1.0	3.3
Ranitidine	1	3.33	5.0	16.7
Caffeine	0.01	0.03	0.1	0.3
Citalopram	1	3.33	1.0	3.3
Sertraline	0.01	0.03	5.00	16.7
Triclosan	1	3.33	5.0	16.7

Table S5 Recovery for the studied PPCPs (1 ppm) spiked in some invertebrates

Compound	Recovery (%)	SD
Metformin	140.0	13.3
Cimetidine	67.8	14.1
Ranitidine	147.5	6.4
Caffeine	52.2	7.3
Citalopram	124.3	5.4
Sertraline	30.5	6.0
Triclosan	40.3	5.7

Chapter 6

Concluding remarks and future perspectives

Chapter 6: Conclusion and future works

6.1 Concluding remarks

Use of PPCPs has been increasingly popular in recent years, and PPCPs have now been recognised as an emerging class of contaminants that can be found extensively distributed across different environmental matrices. These contaminants comprise a large group of chemicals with widely differing physical and chemical properties. They are also used in daily life, whether for personal use, in agriculture, and for animal and human medications. Many of these contaminants find their way to the environment, largely through transport in water systems, which poses a major challenge to address their distribution and environmental impacts. Hence, it is critical to analyse PPCPs in a wide range of different matrices to provide information regarding their occurrence and concentrations. This is best performed using state-of-the-art analytical instruments, and best practice in sample extraction, to ensure accurate qualitative and quantitative results are produced especially in environmental samples which are likely to be have target compounds at trace levels. In addition, sample preparation techniques are needed that introduce environmental samples with suitable recovery to the analytical instrument, knowing that a very advanced instrumental systems is no surety of valid sampling, and cannot compensate for sampling errors.

This thesis focusses on the development of sample preparation techniques and analytical methods used for the analysis of PPCPs in different environmental matrices. Several advanced LC-MS systems have been employed for this purpose which are used to identify and quantify some PPCPs in environmental samples. Reliable and simple sample preparation methods based on SPE and QuEChERS approaches are optimised and used to extract PPCPs from different environmental samples in order to reduce the interferences which can affect the accuracy of the analysis. Different MS/MS modes such as PRM, MRM and tMRM are used to optimise the collision energies of different product ions which can be used to improve confirmation of the identity of the compound.

The analytical method was developed using UHPLC–Q-Orbitrap MS (Chapter 2). This instrument proved to be well suited to analyse PPCPs in different matrices. This system has the ability

to detect PPCPs at very low concentrations, at which they are likely to be found in environmental samples. It also demonstrated high resolution mass spectrometry accuracy which can be reliably used to quantify PPCPs in the environmental samples using full scan mode in both positive and negative modes. MS/MS fragmentation was also optimised in this study in order to confirm different pharmaceuticals in water samples. Different MS parameters were tested to choose an appropriate resolution, automatic gain control (AGC) target and maximum injection time. In can be noted that resolution needs to be chosen carefully as this might affect the data points per peak either in full scan or MS/MS modes.

A QuEChERS extraction procedure was developed to extract selected PPCPs from invertebrates, and to analyse them using UHPLC–Q-Orbitrap MS (Chapter 3). This extraction method is simple, low cost, available and applicable to many environmental applications. The mass spectrometer was operated in MS/MS mode in order to investigate the PRM mode to confirm compound identity. This included testing different collision energies for each PPCPs. Switching polarity mode was used, which allows analysis of PPCPs in positive and negative modes simultaneously. The results indicate that PPCPs can also be found in freshwater invertebrates which live near the affected water ways.

A method to quantify PPCPs using UHPLC–QQQ/MS was optimised and developed (Chapter 4). A number of transitions for each analyte were chosen as quantifier and qualifier ions to confirm the compound in the sample. Collision energy was optimised for each product ion. A different type of column (diamond hydride) was tested and a new LC gradient method was developed. Using this column, a particular carryover problem was investigated, that caused concern for successive sample analysis protocols; it was found that using several blanks between samples and applying a longer time to flush the port for the injection needle is recommended in order to minimise this problem. It also showed that reasonable analysis time is required in order to reduce the carryover. The analytical method was employed to quantify selected PPCPs in some water samples.

A comparison between UHPLC–Q-Orbitrap MS and HPLC–QTOFMS was conducted (Chapter 5). A QuEChERS extraction method was applied to extract some PPCPs in invertebrate (spider) samples. The extraction method showed an acceptable recovery and it was successfully applied to real samples. The Q-Orbitrap MS system showed improved sensitivity compared to QTOFMS; the former has the ability to detect down to sub-ppb level analytes in full scan mode only. The Q-Orbitrap MS also could be operated at higher resolution, which is useful especially for confirming compound identity in the selected matrices. Using the polarity switching option, Q-Orbitrap MS was faster than QTOFMS to switch between positive and negative modes, resulting in detection of caffeine in all spider samples. However, mass accuracy for both instruments was less than 5 ppm for most of the compounds, even though in general the Q-Orbitrap MS was more favourable. Mass accuracy < 5 ppm is usually well suited to most environmental samples.

6.2 Future works

6.2.1 Study of the relation between water and invertebrate samples collected from different wastewater treatment plants outlets

In order to evaluate how PPCPs can affect the environment and importantly biota that depend on the water stream, more water samples should be collected from affected areas such as the Brushy Creek Treatment Plant, and more widely across the urban area. This will support connection and comparison of the results between 'sources and sinks' and evaluate the effects of polluted water with PPCPs on freshwater invertebrate species. It may further prove the contention that PPCPs can transfer from water streams and accumulate in invertebrate tissue, especially across the ecosystem food chain. It is recognised that formal linkage between the environmental levels of PPCP and the bioaccumulation that is proposed in various biota such as biofilm and invertebrates needs to be confirmed. Various studies are underway elsewhere to examine this, but confirming this will be an important study to confirm bioaccumulation processes that might arise in some species. Preliminary

results were obtained during this PhD project for environmental samples collected from the same location, showing that PPCPs can be found in water, some invertebrates species (*Leptophlebidae*) and spider species (*Tetragnatha*). This is evidence that PPCPs might transfer from one species to another in the food chain.

6.2.2 Analysing different environmental samples using GC and GC×GC

In a further study, both GC and GC×GC methodology should be tested for applicability to appropriate PPCPs. Once successful GC with various MS instruments is demonstrated, the higher resolution of GC×GC should be tested, for instance optimised using a new modulator (J&X Solid State Modulator) available in this laboratory, to analyse PPCPs in standards and extracted water samples. The technology of GC×GC is not commonly used to analyse these compounds, but if suitable for selected PPCPs, can provide useful data especially for untargeted analysis, with all the attendant benefits of improved sensitivity, very high resolution, and removal of matrix interferences. In particular, the increase in resolution of compounds means that co-elution problems can be solved, and in GC–MS the reported problems of matrix suppression in LC–MS is expected to be significantly reduced. Depending of compound properties, this method may include additional sample preparation processes such as derivatisation. GC×GC can also be coupled to QTOFMS and QQQMS and other mass analysers, which can be beneficial in many other environmental applications.

6.2.3 Developing a database for PPCPs and their metabolites

Although some databases can be found from some manufacturers for selected pharmaceuticals and drugs, it is limited to a number of compounds in association with known fragmentation patterns. Therefore, a database for PPCPs, their metabolites and transition products can be useful and then deployed for the identification and quantification of these contaminants in different matrices. Moreover, the availability of advanced MS systems such as Orbitrap MS, QQQMS and QTOFMS can be very useful tools to develop and validate a database for PPCPs which can be used by different

authorities to monitor these chemicals in the environment. In this proposed expanded database, a single compound can be tested in both positive and negative modes to understand the fragmentation mechanism and select the mode which provides the highest intensity in the spectrum. Different MS analysers can also be used in order to establish a comprehensive database with high resolution mass spectrometric data.