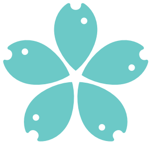

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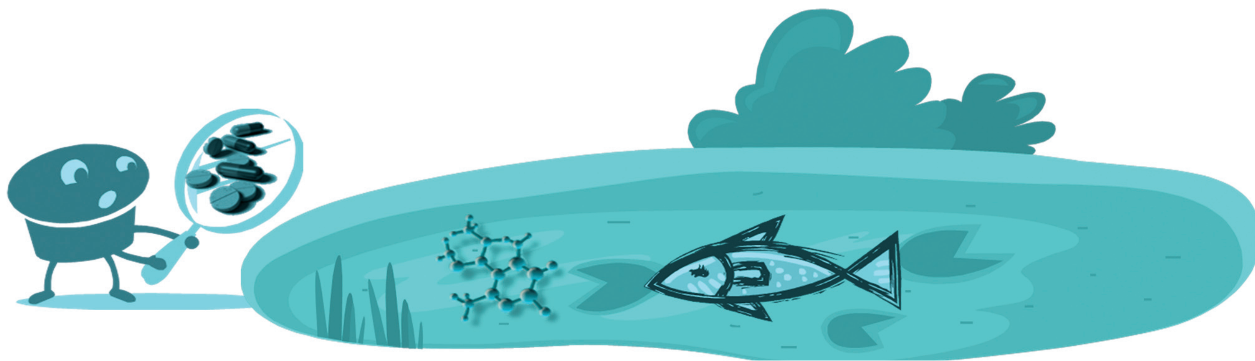
Fate of polar organic pollutants in aquatic environment

Osud polárních organických polutantů ve vodním prostředí



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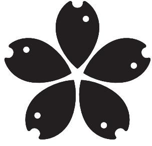

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Ph.D. thesis, USB FFPW, RIFCH, Vodňany, 2013, 112 pages, with the summary in English and Czech.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. EMERGING POLLUTANTS IN AQUATIC ENVIRONMENT

Analysis of polar organic contaminants (POCs) in aqueous ecosystems has lately become an issue of great interest in the field of environmental chemistry. Traditionally hydrophobic persistent organic pollutants were studied extensively. Until the middle of 1990-s, environmental water analysis was mainly focused on persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and furans (PCDD/Fs) and organochlorine pesticides (OCPs), as well as other lipophilic pollutants as polycyclic aromatic hydrocarbons (PAHs) (Quintana and Rodriguez, 2006). They are easily bioaccumulated and biomagnified through the trophic chain in the aquatic system. Meanwhile the awareness developed that polar contaminants may also be a significant problem. Pharmaceuticals and personal care products (PPCPs), some pesticides, perfluorinated compounds and synthetic musks are considered to be the representatives of so-called emerging pollutants. Most of these compounds are not bioaccumulative but highly water soluble. Thus, many of them are ubiquitously distributed in the water bodies, which can be considered to be the most susceptible environmental matrices for contamination by POCs (Pal et al., 2012). They are introduced to the aquatic ecosystem by anthropogenic inputs, and are not effectively removed by wastewater treatment plants (WWTP), which are considered to be the main source of POCs in aquatic environment. Consequently, above mentioned contaminants are detected in surface waters (Kolpin et al., 2002; Roberts and Thomas, 2006; Zhao et al., 2009), as well as in drinking water (Arnold et al., 2013; Kuch and Ballschmiter, 2001; Vulliet et al., 2011; Webb et al., 2003) and groundwater (Devier et al., 2013; Hass et al., 2012).

There is a growing interest in the occurrence and fate of emerging pollutants in the aquatic environment. PPCPs are one of the important aspects of current environmental research. Nowadays, there are more than 4000 pharmaceuticals for human and veterinary use. Although most of PPCPs are present in water at trace concentrations (ng-µg L^{-1}), there is a concern that they can have possible adverse effects on sensitive non-target aquatic species (Corcoran et al., 2010; Daughton and Ternes, 1999). Pharmaceuticals are designed to be biologically active. Most of them are excreted unchanged by human body after being used, while some compounds are metabolized to more polar molecules. Therefore, most pharmaceuticals are released to the environment as active compounds. Considering their high polarity, PPCPs will end up in aqueous compartment and will be dispersed along the waterways. Moreover, strong sorption affinity to particles has been reported for some compounds, as for example antibiotics from fluoroquinolone group, which lead to their binding to the sediments and thus prolong their stay in the environment (Uslu et al., 2008).

Up to date, there is a lack of information about the ecotoxicological effects of POCs and the consequences of their presence in the environment are largely unknown. The fact that they are present in water in complex mixtures brings complications into the risk assessment as we cannot exclude interactions of hundreds of different chemicals. Endocrine disruption, effects on reproductive function, and toxicity to aquatic organisms were documented (Crofton et al., 2007; Kvarnryd et al., 2011; Ricart et al., 2010). Several studies have already shown negative effects of some POCs in environmentally relevant concentrations. For example, one of the recent studies of Brodin and co-authors showed that anxiolytic drugs in surface waters alter animal behavior, and thus can have ecological and evolutionary consequences

(Brodin et al., 2013). Juvenile fish of European perch was exposed to oxazepam at the concentrations found in the aquatic environment, and the effect on their behavior and feeding rate was shown. Moreover, bioaccumulation of oxazepam in fish muscle was reported. Environmental concentrations of anti-inflammatory drug diclofenac can be dangerous for the development of sensitive individuals in aquatic and riparian ecosystems (Feito et al., 2012). In the next study Feito and co-authors suggest acute lethal and chronic sublethal toxicity in vascular plants exposed to environmental concentrations of antidepressant venlafaxine (Feito et al., 2013). Also, antibiotics are of great concern due to their contribution to the development of antibiotic-resistant bacteria (Heuer et al., 2009; Rhodes et al., 2000). All those findings trigger further examining the fate and the effects of „new" pollutants and indicate that their environmental impact was underestimated.

1.2. MAIN SOURCE OF POCs IN WATER

POCs are released to the environment during different stages of their life cycle: production, usage and after-use discharges. They can enter aquatic ecosystem by the number of various routes, including wastewater effluent, agricultural runoff, aquaculture facilities, emissions from manufacturing, etc. (Boxall et al., 2012). Among all above mentioned pathways, wastewater effluent is considered to be the main one (Daughton and Ternes, 1999). Wastewater treatment facilities remove bulk organic matter from the influent wastewater as organic carbon and inorganic and organic nitrogen and phosphorus. On the other hand, WWTPs were not designed to eliminate hundreds of trace pollutants, which are coming with the municipal wastewater. WWTPs play an important role in the life cycle of PPCPs in aquatic environment. After being used, pharmaceuticals reach WWTP influent with urine and feces. Improper disposal of expired and unused medication can also contribute to this contamination (Zuccato et al., 2000). Domestic and industrial wastewaters are also assumed to be the main source of perfluorinated chemicals (PFCs) in aquatic environments (Murakami et al., 2008). Most PFCs are resistant to hydrolysis, photolysis, microbial degradation, and metabolism (Jahnke and Berger, 2009), hence their removal presents significant challenges. As wastewater effluents are the main point discharges of POCs, their elimination within WWTP is of great interest.

In general, POCs are only partially eliminated during wastewater treatment. Sometimes, dilution factor is the main way of reducing the concentrations of this kind of compounds (Boxall, 2004). The removal efficiency of WWTP depends on the type of the treatment process used, the nature of particular compound and climatic conditions (Kasprzyk-Hordern et al., 2009; Pal et al., 2012). Ability to interact with solid particles and biodegradation are considered to be the main ways of elimination of pollutants during the common treatment process (Carballa et al., 2004). Highly polar small molecules, with low sorption affinity, are not retained during the treatment process and thus can pass through WWTP and consequently enter surface and drinking waters. For instance, antiepileptic drug carbamazepin is poorly removed during biological process, using trickling filter beds, as well as during treatment process using a biological activated sludge (Kasprzyk-Hordern et al., 2009). Moreover, an increase of concentrations in the effluent water can be observed. This situation is typical for the group of compounds which are present in untreated wastewater as conjugates, from which the free parent drug can later be released, leading to the negative removal efficiency (Boxall et al., 2012; Kasprzyk-Hordern et al., 2009).

For anti-inflammatory drug diclofenac removal efficiences varied strongly (0–70%) depending on the process of treatment (Clara et al., 2005). Consequently, a lot of PPCPs are detected in wastewater effluent at rather high levels (Bueno et al., 2012; Ghosh et al., 2009), which contributes to their occurrence in surface water. Although these concentrations are much lower than therapeutic levels, we should bear in mind that aquatic organisms are exposed to the coctail of pharmacologically active compounds continuously for a very long time. Taking into account all mentioned above, the importance of wastewater monitoring should be stressed.

Manufacturing facilities represent another potential route of PPCPs release (Larsson et al., 2007; Zuccato et al., 2000). Control of pharmaceutical production by the regulation and high price of produced drugs suppose to assure that minor releases of pharmaceutical substances will occur (Larsson et al., 2007). Nevertheless, production facilities can be a significant source of water contamination. Extremely high concentrations of some fluoroquinolone antibiotics were found in the effluent of the biggest pharmaceutical industry in Hyderabad, India. The effluent from bulk drug manufacturing contained ciprofloxacin at the level 14 mg L^{-1}, and the concentration of this compound in the surface lake water was reported at 6.5 mg L^{-1} (Fick et al., 2009). The estimated amount of ciprofloxacin released during one day was equal to 45000 daily doses (Larsson and Fick, 2009). Environmental release of such big amounts of antibiotics is an issue of global concern due to the risk of development of antibiotic resistant bacteria.

Aquaculture facilities can also contribute to the environmental load of antibiotics. Heavy amounts of antibiotics are commonly used in intensive aquaculture in fish feed both prophylactically (disease prevention) and to treat disease (Sapkota et al., 2008). The accelerated growth of aquaculture has resulted in a series of harmful environmental effects. Using of antibiotics can create antibiotic resistance in non-pathogenic bacteria, the resistance genes of which can be transferred to disease-causing bacteria, resulting in antibiotic-resistant infections for humans (Greenlees, 2003). Another problem created by the excessive use of antibiotics in industrial aquaculture is the presence of residual antibiotics in commercialized fish and shellfish products (Cabello, 2006).

1.3. ANALYSIS OF POCs

Monitoring of emerging pollutants is the first step in the following of their fate in the environment. Information on their occurrence and measured concentrations of certain pollutants in different environmental matrices is the basis for further risk assessment of those pollutants for aquatic ecosystem. Determination of chemical substances in environmental samples is generally a multistep process. These steps include sample preservation, extraction, pre-concentration and analysis. Sample preparation is often considered to be a fundamental step in analytical procedures. It is carried out in order to remove potential interferences from the sample matrix and to enrich the analytes of interest, which are present at trace concentrations. Sample preservation and storage is also of great importance, because if it is not performed properly the original sample composition may be seriously changed.

As most of the emerging contaminants are highly polar and water-soluble, liquid chromatography coupled to mass spectrometry (LC/MS) is the method of choice for their determination and quantification. It has become a routinely applicable and robust method which allows wide coverage of hydrophilic and amphiphilic

contaminants, as pharmaceuticals, pesticides, and perfluorinated compounds. LC/MS can provide high sensitivity, which is crucial issue for the environmental analysis when most of the target pollutants are present at trace concentrations.

1.3.1. Sample collection and preservation

Sampling is the initial step of any environmental monitoring program. It is constitutive part of the analytical process and its importancy should not be underestimated. In some cases samples collection and handling can be the main source of error of the whole process of analysis, especially when trace concentrations are being measured (Madrid and Zayas, 2007).

Grab sampling

Among water sampling methods, grab sampling is the most commonly used one. The main disadvantage is that the information obtained from the sample is unique to the place and the time selected. The main drawback of using this approach is that concentrations of pollutants in the environment can vary. Another limitation is large volume of water which must be collected and extracted for the detection of trace levels of organic contaminants. To obtain more representative data on the pollution situation automatic samplers can be used. In this case, sub-samples are collected at regular pre-set time intervals. Disadvantage is that this sampling method is rather expensive and labor-intensive (Madrid and Zayas, 2007). Additional shortcoming of active sampling of water is the storage issue. Although unreasonably very little attention is paid to this issue, handling and storage of the samples is of great importance, as the initial composition of the sample should be maintained to reflect the original situation. Especially, it could be a matter of concern, when dealing with wastewater samples, as that majority of PPCPs are bioactive and hence likely susceptible to breakdown by bacteria or other transformation reactions.

Another sampling approach which is gaining popularity is passive sampling (PS). It is less sensitive to accidental, extreme variations of the pollutant concentration and gives time-weighted average (TWA) concentrations.

Principles of passive sampling

The main principle of passive sampling was defined in the review of Gorecki and Namiesnik: „The uptake of target compounds in passive samplers is based on free flow of analyte molecules from the sampled medium to a collecting medium as a result of a difference in chemical potentials“ (Gorecki and Namiesnik, 2002). Most PS devices represent a receiving phase and a diffusion-limiting barrier.

Among the most widely-used samplers there are semi-permeable membrane devices (SPMDs) for hydrophobic organic pollutants, polar organic chemical integrative samplers (POCIS) for hydrophilic pollutants and the diffusion-gradient in thin-films (DGTs) for metals and inorganic compounds (Gorecki and Namiesnik, 2002). SPMD, presented by Huckins et al., was the first PS device, which was applied for the sampling of hydrophobic pollutants (Huckins et al., 1990). SPMD consists of a tubular polyethylene membrane containing lipid triolein (Huckins et al., 1993). In comparison to traditional water sampling, SPMD can be deployed over extended

time periods and can detect low levels of non-polar and moderately polar organic contaminants. Moreover, it mimics the absorption of compounds through cell membranes and therefore can be used as a bioindicator of pollutants' bioavailability and helps to assess their bioconcentration in aquatic organisms (Esteve-Turrillas et al., 2008). Performance reference compounds (PRC) approach is used for the back-calculation of the amount of pollutants in the sampler to water concentrations.

During last several decades interest in environmental analysis shifted towards hydrophilic pollutants, therefore POCIS gained more attention (Soderstrom et al., 2009). POCIS is an effective sampling tool for many water soluble compounds. It comprises a sequestration phase (sorbent) sandwiched between two polyethersulfone (PES) membranes (Alvarez et al., 2004). Unlike SPMDs, PRC approach for the back-calculations of the pollutants' concentrations is not working effectively in case of POCIS. Thus, POCIS should be calibrated in order to obtain sampling rate values. Sampling rate is calculated according to the following equation:

$$Rs = M_{POCIS} C_W^{-1} t^{-1} \qquad (1)$$

Where Rs is sampling rate (L day^{-1}), M_{POCIS} is the amount of target compound in the POCIS (ng), C_W = average water concentration (ng L^{-1}), and t = exposure period (days).

Rs value can be affected by the following environmental conditions: water temperature, turbulence, biofouling. These factors should be taken into account when carrying out the calibration of POCIS.

Currently, two configurations of POCIS are commercially available : "pharmaceutical" configuration, which contains Oasis HLB sorbent, and "pesticide" configuration with a triphasic mixture of a hydroxylated polystyrene-divinylbenzene resin (Isolute ENV+) and a carbonaceous adsorbent (Ambersorb 1500) dispersed on a styrene divinylbenzene copolymer (S-X3 Bio Beads). Those two configurations are often exposed simultaneously for the effective sampling of a broad range of compounds.

Application of POCIS for water analysis: advantages and limitations

PS can overcome limitations of conventional sampling and provide data on time-integrated concentrations (Bueno et al., 2009). It can successfully combine sampling and pre-concentration of target analytes into a single step, and therefore reduce time and costs for the further sample preparation. A further advantage of passive sampling is the ability to mimic biological uptake, therefore avoiding use of aquatic organisms for biomonitoring (Alvarez et al., 2005; Kot et al., 2000). The sampler enables estimation of the cumulative exposure to bioavailable hydrophilic organic chemicals (Arditsoglou and Voutsa, 2008). POCIS has been used for the sampling of polar organic compounds such as pharmaceuticals, pesticides, and illicit drugs, as well as hormones and UV-blockers (Harman et al., 2011; Lissalde et al., 2011; Zenker et al., 2008; Zhang et al., 2008). Unfortunately, the limitation of this approach is lack of the calibration data. Available sampling rate values for polar organic compounds are limited to a small number of pesticides and pharmaceuticals (Bartelt-Hunt et al., 2011; Mazzella et al., 2008). Another complication is that most of the calibration experiments are carried out under the laboratory conditions, and thus are not always applicable under the real conditions, which can be quite different from the

lab ones. Thus, extensive research is needed to overcome those limitations and to get accurate sampling rate values for wide range of emerging contaminants.

1.3.2. Sample pre-treatment

The determination of polar contaminants in environmental samples is normally preceded by an extraction step in order to enrich the target analytes (Zwiener and Frimmel, 2004). This extraction should be as selective as possible in order to minimize the co-extraction of matrix that may interfere with analyte detection. Especially this is relevant for the analysis of wastewater samples and also biological matrices, as matrix components can cause ion suppression or enhancement in the ion source (Salvador et al., 2007). For this purpose, solid phase extraction (SPE) is the most widely used preconcentration procedure (Rodriguez-Mozaz et al., 2007). SPE is typically carried out manually. Manual (off-line) SPE has some significant disadvantages: it is time-consuming as well as labor-intensive and costly; safety issues due to the exposure to hazardous or infectious matrices such as sewage; reproducibility problems (Fatta et al., 2007). These problems can be solved by automation of the process and using in-line-SPE. Thus, following benefits can be reached: time saving; direct injections of untreated samples; improved precision and accuracy; reduction of health risk; samples can be run unattended (Rossi and Zhang, 2000).

1.3.3. Instrumental analysis

LC/MS presents various advantages, such as reduced sample pre-treatment and the capability to determine very polar compounds and transformation products without derivatization (Ibanez et al., 2005). Two atmospheric pressure ionization interfaces are currently available for the ionization of target analytes: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), which can be operated in positive and negative ion modes (de Alda et al., 2003). ESI and APCI are applicable for the analysis of a broad range of compounds, including non-volatile, thermally labile and polar species. In addition, high enough sensitivity can be reached, which is compulsory for environmental analysis where contaminants are often found at trace (ng L^{-1} or µg L^{-1}) levels.

The majority of POCs analyses are carried out using LC coupled to triple quadrupole tandem mass spectrometry (QqQ-MS/MS). QqQ mass spectrometer is the instrument of choice for the analysis of complex matrices. This configuration has sensitivity and selectivity advantages (Karolak et al., 2010; Terzic et al., 2010). Limit of detection for analysis of human and veterinary drugs in different types of water samples was reported to be at the range of 8–20 ng L^{-1} (de Alda et al., 2003). A new trend in the environmental water analysis is in-line SPE-LC/MS/MS analysis, which allows pre-concentration and analysis of the sample in a single run, making large volume samples and time-consuming off-line SPE extraction procedures unnecessary. In comparison with the commonly used off-line SPE, this procedure allows significant reduction of time and costs for the sample preparation step. Several studies have already reported successful application of in-line SPE-LC/MS/MS for the analysis of some PPCPs and illicit drugs (Khan et al., 2012; Salvador et al., 2007; Segura et al., 2007). They are focused mainly on one or two pharmaceutical classes, thus some improvements should be introduced towards the multi-residual analysis.

In spite of the fact that these methods based on QqQ detection are highly selective and precise, this approach has some limitations and disadvantages. It is time-consuming, number of compounds is limited in one instrumental method and definition of time windows is required for each analyte (Kellmann et al., 2009). Moreover, the mass resolution of quadrupole MS is not usually high enough to allow the determination of the molecular formula of an unknown compound. Thus, the techniques used for residue analysis are moving from target-orientated methods (mainly based on liquid chromatography in combination with QqQ MS detection) towards high resolution MS (HRMS) techniques.

Full scan HRMS approaches enable retrospective analysis (i.e. without re-injecting the sample). This method gives an advantage of the analyzing of unlimited number of compounds simultaneously (van der Heeft et al., 2009). Advanced HRMS instruments combine crucial features such as improved selectivity and improved mass resolution, lower cost, and relatively easy maintenance. Accurate mass full scan analysis using time-of-flight (ToF) and Orbitrap mass spectrometers proved to be an appropriate alternative to triple quadrupole instruments for both targeted and non-targeted analysis (Bijlsma et al., 2012; Calza et al., 2012; Thomas et al., 2010; Wille et al., 2011).

1.4. DIRECTION OF THE PRESENT STUDY

The main objective of the study was monitoring of emerging polar organic pollutants in aquatic environment with the focus on pharmaceuticals and personal care products, illicit drugs and perfluorinated compounds. Special attention was paid to the analytical method development for the analysis of POCs in different environmental samples (Chapter 2). Although a lot of work has been already done in this field, POC analysis at trace levels still remains a challenge. Most of the existing methods do not cover a wide range of analytes and use costly and time-consuming extraction procedures, which are considered to be the main limitation of the analysis. Taking into account the number of pollutants which are currently gaining interest, the trend in analytical method development is shifting towards the multi-residue methods with minimal sample pre-treatment, which enable us significantly reduce time and costs of the analysis. Different environmental matrices (surface water, wastewater, fish tissues) were considered in the study, as monitoring of different compartments of aquatic ecosystem is crucial for understanding of pollutants' fate.

Passive sampling for the monitoring of emerging pollutants was studied. Application of POCIS for wastewater sampling was investigated. Up to date, significant shortcoming of this novel approach is lack of the calibration data. Sampling rate values are available only for limited number of compounds. Moreover, reported sampling rates were usually obtained under the laboratory conditions, which are significantly different from those ones in the field. The experiment in WWTP effluent was carried out to calibrate POCIS for selected perfluorinated compounds (Chapter 3).

Analysis of wastewater is of great importance first of all, because it is the main source of POCs in aquatic environment. Monitoring of WWTP effluent can provide relevant information for further risk assessment of aquatic organisms' exposure. On the other hand, data on the concentrations in urban WWTP influent can reflect the usage patterns in the studied area (Chapter 4). For instance, it can be the only way to get more realistic data on the illicit drugs consumption in the local community (Chapter 2).

Another important issue of wastewater analysis is storage and handling of the samples. Unreasonably low attention is paid to this factor. Nevertheless, storage conditions can significantly affect initial concentrations of target compounds in the sample and thus give inaccurate information on their concentrations. Shor-term as well as long-term storage of wastewater samples was studied in order to assess the effect of different conditions on the concentrations of more than 100 of PPCPs (Chapter 4).

The aims of the study were:

- Analytical method development for the multi-residue analysis of pharmaceuticals in water samples.
- Analytical method development for the multi-residue analysis of antibiotics in fish samples.
- Analytical method development for the analysis of illicit drugs in wastewater influent using novel accurate mass high resolution mass spectrometry.
- Calibration of POCIS passive samplers in WWTP effluent.
- Assessment of storage effect of wastewater samples on the concentrations of PPCPs.
- Estimation of Tamiflu use and compliance from measured concentrations in influent wastewater.

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CHAPTER 2

NEW ANALYTICAL METHODS FOR THE MONITORING OF EMERGING POLLUTANTS IN AQUATIC ENVIRONMENT

2.1. Grabic, R., Fick, J., Lindberg, R.H., Fedorova, G., Tysklind, M., 2012. Multi-residue method for trace level determination of pharmaceuticals in environmental samples using liquid chromatography coupled to triple quadrupole mass spectrometry. Talanta 100, 183–195.

2.2. Fedorova, G., Nebesky, V., Randak, T., Grabic, R., 2013. Simultaneous determination of 32 antibiotics in aquaculture products using LC-MS/MS. Chemical Papers. (under review)

2.3. Fedorova, G., Randak, T., Lindberg, R.H., Grabic, R., 2013. Comparison of the quantitative performance of Q-Exactive high resolution mass spectrometer with that of triple quadrupole MS/MS for the analysis of illicit drugs in wastewater. Rapid Communications in mass spectrometry. (submitted)

It was allowed by publisher on 6th April / 2nd July, 2013 to include the papers in this Ph.D. thesis.

Talanta 100 (2012) 183–195

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Multi-residue method for trace level determination of pharmaceuticals in environmental samples using liquid chromatography coupled to triple quadrupole mass spectrometry

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ARTICLE INFO

Article history:
Received 4 May 2012
Received in revised form
18 August 2012
Accepted 22 August 2012
Available online 30 August 2012

Keywords:
LC–MS/MS
Pharmaceuticals analysis
SPE extraction
Waste water

ABSTRACT

A multi-residue method for the simultaneous determination of more than 90 pharmaceuticals in water samples was developed and validated. The developed method utilizes a single liquid chromatography–tandem mass spectrometry (LC–MS/MS) run after sample enrichment using solid-phase extraction (SPE). The pharmaceuticals included in this method were chosen based on their potency (effect/concentration ratio) and potential to bioaccumulate in fish. Because the selection was based on ecotoxicological criteria and not on ease of detection, the pharmaceuticals have a wide range of physico-chemical properties and represent 27 distinct classes. No method for surface, waste water or similar matrices was previously described for 52 of the 100 target analytes. Four chromatographic columns were tested to optimize the separation prior to detection by mass spectrometry (MS). The resulting method utilizes a Hypersil Gold aQ column. Three different water matrices were tested during method validation: Milli-Q water, surface water (river water from the Umea River) and effluent from the Umea waste water treatment plant (WWTP). Four of the selected pharmaceuticals exhibited poor method efficiency in all matrices. Amiodarone, Dihydroergotamine, Perphenazine and Terbutalin were omitted from the final analytical method. In addition, five compounds were excluded from the method for surface water (Atorvastatin, Chloropromazin, Dipyridamol, Furosemid and Ranitidin) and three other pharmaceuticals (Glibenclamid, Glimepirid and Meclozine) from waste water method respectively. Absolute recoveries were above 70% for Milli-Q water, surface water, and sewage effluent for most pharmaceuticals. The limits of quantification (LOQs) ranged from 0.05 to 50 ng L^{-1} (median 5 ng L^{-1}). The use of matrix-matched standards led to the elimination of ionization enhancement or suppression. The recoveries of the method for real matrices were in the range of 23–134% for surface water (only three compounds were outside of the range of 40–130%) and in the range of 47–162% for waste water (five compounds were outside of the range of 40–130% at lower validated concentration).

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1. Introduction

A wide range of pharmaceuticals have been detected in surface waters globally, raising concerns about the potential adverse environmental effects [1,2]. Pharmaceuticals are widely used and seldom fully metabolized, which results in their discharge into the aquatic environment via municipal and hospital sewage water [3,4]. It has recently been shown that pharmaceutical development and production facilities in Asia, Europe, and USA elevate surface water concentrations of antibiotics, antifungals, antidepressants, opioids and muscle relaxants considerably [5,6]. Numerous laboratory studies on aquatic organisms have illustrated that certain pharmaceuticals have negative effects on growth, development, and reproduction [1,7–9]. Consequently, there is a growing need to develop reliable analytical methods that enable rapid, sensitive, and selective determination of these emerging pollutants at trace levels in environmental samples. Multi-residue analytical methods are becoming essential tools that provide reliable information about the occurrence and fate of pharmaceuticals in the environment. Analytical methods are available for the detection of particular classes of these compounds in surface and wastewaters, including several multi-residue methods [10–18]. Current methods cover a relatively narrow range of pharmaceuticals that correspond to requests

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http://dx.doi.org/10.1016/j.talanta.2012.08.032

from regulatory bodies, e.g., priority compounds listed by the US EPA or European Water Framework Directive [11,14]. The need for relatively simple multi-residue methods for heretofore uninvestigated pharmaceutical compounds is growing.

Currently, there are more than 6000 pharmaceuticals listed in the Martindale database [19]. Therefore, prioritization approaches must be used to select those pharmaceuticals that should be included in monitoring schemes. Various strategies have been applied, including the use of sales statistics, as well as more rational strategies, such as mode-of-action-based tests [20–22]. One useful approach, suggested by Huggett [20], has been designated as the "fish plasma model." This model is based on the assumption that if two species possess the same drug target, the pharmaceuticals will activate this target at roughly the same plasma concentration. The fish plasma model generates a concentration ratio (CR) between the human therapeutic plasma concentrations (H_TPC) and a measured, or theoretically predicted, fish steady state plasma concentration ($F_{SS}PC$). If the concentration ratio is ≤ 1, then the plasma concentration in the exposed fish is equal to or higher than the plasma concentration that is known to cause a pharmacological response in humans. A lower ratio thus reflects a higher risk. A major benefit of this model is that it enables the calculation of the theoretical risks for the great majority of pharmaceuticals, because human therapeutic plasma concentrations are readily available in the literature. However, data for the measured plasma levels of pharmaceuticals in fish following exposure via water are scarce [23–26]; thus, risk calculations still largely rely on theoretically predicted $F_{SS}PCs$.

Fick et al. [27] recently calculated the surface water concentration for 500 pharmaceuticals that theoretically would result in a pharmacologically relevant fish steady state plasma concentration. This surface water concentration was described as the "critical environmental concentration" (CEC) and was derived from the theoretically predicted $F_{SS}PCs$ and the published human therapeutic plasma concentrations. By combining the predicted environmental concentrations (PECs), which are based on sales, with the CEC values for these 500 pharmaceuticals, it is possible to calculate the CRs in a specific region.

The aim of this study was to develop a sensitive, multi-residue, single chromatographic run method based on a single SPE protocol followed by LC-ESI-MS/MS for the simultaneous analysis of pharmaceuticals in surface water and sewage effluent. The selection of the pharmaceuticals included in this method was based on relevant ecotoxicological criteria and also based on a request to include pharmaceuticals from as many different classes as possible.

2. Materials and methods

2.1. Selection of pharmaceuticals

The selection of the pharmaceuticals to be included in the study was based on the CEC values for 500 pharmaceuticals [27] and the calculated PECs based on the amounts sold in Sweden in 2005 (statistics from Apoteket AB, Sweden). A full description of how the CEC values were calculated can be found in Fick et al. [27]. The PEC values were calculated according to the following equation:

$$PE,,--1.. = ,A \, x \, (100-R)-365 \, x \, P \, x \, V \, x \, D \, x \, 100. \quad (1)$$

where A is the total pharmaceutical sales (μg year^{-1}); R is the removal rate due to loss by adsorption to sludge particles, volatilization, hydrolysis, and biodegradation (%); P is the human population (number of individuals); V is the volume of waste water per capita per day (L day^{-1}); and D is a factor for the dilution of waste water by surface water. In order to study a worst case scenario, no removal was assumed ($R=0$). The additional parameters used were $P=9\,047\,752$, $V=200$ (default), and $D=10$ (default). The concentration ratio was calculated by dividing the CEC value by the PEC value for each pharmaceutical. The final selection of pharmaceuticals to be included was made using the criteria of low CRs and commercially available reference standards. Efforts were made to include as many therapeutic classes as possible, and several antibiotics were included to complete the selection. Fifty-two pharmaceuticals that previously lacked an analytical protocol for their determination in environmental samples are included in this selection. No LC/MS method was reported for four of the selected pharmaceuticals. An LC/MS method for pharmacokinetic or similar studies at the therapeutic concentration level in human blood and tissue was previously reported in the literature for 48 of the selected pharmaceuticals. Only 48 of the 100 compounds have a validated method for a water matrix described in the literature (based on Web of Knowledge in November 2011).

2.2. Chemicals

All of the pharmaceutical reference standards were classified as analytical grade (> 98%). Sulfuric acid (99.999%) was purchased from Sigma-Aldrich (Steinheim, Germany) and ethyl acetate (Analytical reagent, 99.8%) was purchased from Labscan Ltd. (Dublin, Ireland). $^{2}H_6$-amitriptyline, $^{2}H_{10}$-carbamazepine, $^{13}C_3^{15}N$-ciprofloxacin, $^{2}H_5$-fluoxetine, $^{13}C_6$-sulfamethoxazole, $^{13}C^{2}H_3$-tramadol and $^{13}C_3$-trimethoprim were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). $^{2}H_5$-oxazepam, $^{2}H_7$-promethazine, $^{2}H_4$-risperidone, and $^{13}C_2^{15}N$-tamoxifen were bought from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile were purchased in LC/MS grade quality (Lichrosolv – hypergrade, Merck, Darmstadt, Germany). The purified water was prepared by a Milli-Q Gradient ultrapure water system (Millipore, Billerica, USA), equipped with a UV radiation source. Acidification of the mobile phases was performed by addition of 1 mL of formic acid (Sigma-Aldrich, Steinheim, Germany) to 1 L of solvent.

A triple-stage quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used as the analytical system. Xcalibur (Thermo Fisher Scientific, San Jose, CA, USA) software was used for the creation of instrument methods, the running of samples, and subsequent work with the collected chromatograms.

2.3. Mass spectrometry

Heated electrospray (HESI) in positive or negative ion mode was used for ionization. The fused-silica capillary (standard set up) was replaced with a metal capillary. The key parameters were set as follows: ionization voltage 3.5 kV; sheath gas 50, and auxiliary gas 35 arbitrary units; vaporizer temperature 200 °C; capillary temperature 325 °C; and collision gas (argon) flow 1.5 mL min^{-1}. Both the first and third quadrupoles were operated at a resolution of 0.7 FMWH. The above-mentioned ionization conditions were set as tuning conditions for the single reaction monitoring SRM of individual compounds. The tuning was performed with an infusion of 1 μg mL^{-1} solution of each analyte into the stream of the mobile phase (250 μL min^{-1} of water/MeOH/ACN 50/30/20 all solvents acidified by 0.1% of formic acid). The tube lens voltage and collision energy of the three most abundant transitions were optimized.

2.4. Liquid chromatography

Due to the wide range of physico-chemical properties of the studied pharmaceuticals, four different reversed chromatography stationary phases were used for the LC/MS/MS method development.

Fully endcapped C18 phase Hypersil GOLD aQ (50 mm × 2.1 mm ID × 5 μm particles, Thermo Fisher Scientific, San Jose, CA, USA) was tested as an alternative to the conventional C18 phase Hypersil GOLD (50 mm × 2.1 mm ID × 3 μm particles, Thermo Fisher Scientific, San Jose, CA, USA). Less hydrophobic C-phenyl phase Hypersil GOLD Phenyl (50 mm × 2.1 mm ID × 3 μm particles, Thermo Fisher Scientific, San Jose, CA, USA) and porous graphite column Hypercarb (50 mm × 2.1 mm ID × 5 μm particles, Thermo Fisher Scientific, San Jose, CA, USA) were tested with the expectation of different separation properties compared with the C18. All columns were preceded by a guard column (2 mm × 2.1 mm i.d., 3 or 5 μm particles) of the same packing material from the same manufacturer. The separation of the pharmaceuticals was performed under the same or very similar conditions. Generally, a gradient of MeOH and ACN in water (all solvents were acidified by 0.1% formic acid) was used for the elution of analytes. The elution conditions were programmed as follows: 200 μL min^{-1} 5% methanol in water for 1 min, isocratically followed by a gradient change to 20/20/60 water/ACN/MeOH at a flow of 250 μL min^{-1} in 8 min and a final gradient change to ACN/MeOH 40/60 at a flow of 300 μL min^{-1} in 11 min. These parameters were held for 1 min and then changed to the starting conditions and held for 4 min to equilibrate the column for the next run. The only difference among the columns was in the initial mobile phase composition. Pure water was used for the Hypersil Gold aQ and Hypercarb columns due to their ability to work under this condition. All of the experiments were performed at 22 °C ambient temperature.

A 20 μL injection loop was used for injection of the standards and samples. The injected volume ranged from 5 to 20 μL depending on the content of MeOH or ACN in the sample and the expected concentration of target analytes.

2.5. Pre-treatment of water samples and solid phase extraction

All samples (100 mL of Milli-Q, surface water, and sewage effluent from the Umea WWTP) were filtered through a 0.45 μm membrane filter (MF, Millipore, Sundbyberg, Sweden) and acidified to pH 3 using sulfuric acid. Five nanograms of each surrogate standard were added to each sample. The solid phase extraction of samples was carried out with a Visiprep SPE manifold (Supelco, Bellefonte, PA, USA). Oasis HLB (200 mg) cartridges were sequentially conditioned with 5 mL MeOH and 5 mL pure water. Samples were applied to the SPE cartridges at a flow rate of 5 mL min^{-1}. Water with 5% methanol was used to wash the SPE column before elution with 5 mL methanol and 3 mL ethyl acetate. The eluate was collected in 10 mL vials, evaporated to 20 μL under a gentle air stream, and dissolved in 5% acetonitrile in water with 0.1% formic acid to a final volume of 1.0 mL. The most diverse pretreatment protocol among the multi-residue methods was selected and validated [28,29] due to the problems associated with the simultaneous optimization of the extraction efficiencies and matrix effects of 97 different analytes.

2.6. Quality assurance/quality control

Stock solutions of each of the pharmaceuticals were prepared in methanol and stored at −18 °C. Calibration standards were prepared in the mobile phase. Any pharmaceutical that lacked a labeled internal standard was matched with a suitable surrogate standard based on the physico-chemical properties, retention time, negative or positive ionization.

Possible memory effects were evaluated by a blank injection of Milli-Q water following the injection of the standard samples at varying concentrations.

A seven-point calibration curve was constructed with a broad concentration range (0.005–500 ng mL^{-1} with the same concentration of IS). The calibration curve was used for evaluating the linearity. The instrumental limits of quantification (LOQs) were estimated from the calibration curve. The LOQs for the different matrices were calculated based on the instrument LOQs and relative response ratios in real samples. The recoveries were determined by spiking the standard solution of the matrices at the following concentration levels: Milli-Q water at 100 ng L^{-1}; surface water at 100 ng L^{-1} and 500 ngL^{-1}; and sewage effluent at 1000 ng L^{-1} and 2500 ng L^{-1}. To distinguish between ionization suppression or enhancement and recovery efficiency on SPE, the matrix-matched standards were prepared as follows: The same aliquots of surface or waste water were extracted, and the native compounds/internal standards were added at a level of 500/5 ng for surface water and 2500/5 ng for waste water to 1 mL of mobile phase reconstituted extract.

3. Results and discussion

3.1. HESI –MS/MS

The quantification and qualification of the SRM transitions for each target compound and internal standard are listed in Table 1. The time window for each SRM is defined by the retention time (RT) and by a window factor, a number that multiplies the default time window (1 min). This procedure is possible only in the EZ

Table 1
MS transitions used in this method.

Analyte	Group	Mod	Precursor	Product	CE (V)	Tube lens (V)	Type	Ratio quan/qual	tR (min)
Alfuzosin	Urological	+	390.1	156.2	26	111	Quan	1.4490	6.85
		+	390.1	235.1	26	111	Qual		
Alprazolam	Psycholeptics	+	309.0	205.1	39	103	Quan	1.3020	8.18
		+	309.0	281.0	25	103	Qual		
Amiodarone	Antiarrhythmics	+	645.9	100.2	30	134	Quan	1.3670	12.00
		+	645.9	58.2	46	134	Qual		
Amitryptyline*	IS	+	284.1	191.1	27	99	Quan		
		+	284.1	233.2	15	99	Qual		
Amytriptyline	Antidepressant	+	278.1	233.2	15	99	Quan	1.6143	8.80
		+	278.1	117.2	23	99	Qual		
Atenolol	Hypertension drug	+	267.0	145.1	26	98	Quan	1.1501	4.49
		+	267.0	190.1	19	98	Qual		
Atorvastatin	Statin	+	559.2	250.0	43	120	Quan	1.7372	9.04
		+	559.2	440.4	20	120	Qual		
Atracurium	Muscle relaxant	+	358.1	151.2	29	108	Quan	4.2569	6.50
		+	358.1	206.1	18	108	Qual		
Azelastine	Anti-histamine	+	382.1	112.2	25	110	Quan	14.8620	8.63

Table 1 (*continued*)

Analyte	Group	Mod	Precursor	Product	CE (V)	Tube lens (V)	Type	Ratio quan/qual	tR (min)
		+	382.1	58.4	50	110	Qual		
Azithromycine	ATB	+	749.4	158.2	37	148	Quan	3.7330	7.90
		+	749.4	591.6	28	148	Qual		
Beclomethasone	Antiinflammatory corticoide	−	453.2	297.2	27	92	Quan	1.3709	8.17
		−	453.2	377.3	19	92	Qual		
Bezafibrate	Cholesterol statin	−	360.0	154.1	30	101	Quan	3.4903	8.57
		−	360.0	274.1	21	101	Qual		
Biperiden	Anti-Parkinson	+	312.1	294.3	15	103	Quan	9.8316	8.78
		+	312.1	98.3	23	103	Qual		
Bisoprolol	Hypertension drug	+	326.1	116.2	17	105	Quan	6.0582	7.28
		+	326.1	74.4	26	105	Qual		
Bromocriptine	Anti-Parkinson	+	654.1	301.0	35	124	Quan	1.8565	8.66
		+	654.1	346.0	26	124	Qual		
Budesonide	Antiinflammatory corticoide	+	431.2	413.4	7	116	Quan	1.0374	8.90
		+	431.2	323.1	12	116	Qual		
Buprenorphine	Analgesic	+	468.2	468.2	25	126	Quan	118.0852	7.90
		+	468.2	55.4	54	126	Qual		
Bupropion	Antidepressant	+	240.0	131.2	25	77	Quan	1.6599	7.21
		+	240.0	184.1	12	77	Qual		
Carbamazepin	Psycholeptics	+	237.0	193.2	35	118	Quan	3.3815	7.57
		+	237.0	194.2	19	118	Qual		
Carbamazepin*	IS	+	247.0	204.0	19	118	Quan		
Chlorpromazine	Psycholeptics	+	319.0	214	35	76	Quan	9.8304	9.23
		+	319.0	86.3	19	76	Qual		
Chlorprothixene	Psycholeptics	+	316.0	231	29	103	Quan	1.0738	9.41
		+	316.0	271	18	103	Qual		
Cilazapril	Hypertension drug	+	418.1	114.2	34	92	Quan	27.7288	8.50
		+	418.1	211.1	19	92	Qual		
Ciprofloxacin	ATB	+	332.0	288.2	16	117	Quan	4.8062	6.40
		+	332.0	231.1	35	117	Qual		
Ciprofloxacin*	IS	+	336.0	318	20	106	Quan		
Citalopram	Antidepressant	+	325.1	109.2	27	104	Quan	1.6882	7.90
		+	325.1	262.1	18	104	Qual		
Clarithromycine	ATB	+	748.4	158.1	27	156	Quan	1.5751	9.30
		+	748.4	590.5	17	156	Qual		
Clemastine	Antidepressant	+	344.0	180	31	73	Quan	4.4051	9.65
		+	344.0	215.1	18	73	Qual		
Clindamycine	ATB	+	425.1	126.2	31	110	Quan	8.3053	7.69
		+	425.1	377.3	18	110	Qual		
Clomipramine	Antidepressant	+	315.0	242.1	26	77	Quan	10.0845	9.33
		+	315.0	86.3	17	77	Qual		
Clonazepam	Psycholeptics	−	313.9	278	17	94	Quan	2.0840	7.84
		−	313.9	286.1	18	94	Qual		
Clotrimazol	Antimycotic	+	277.0	165.1	26	81	Quan	2.2601	8.90
		+	277.0	241.1	26	81	Qual		
Codeine	Analgesic	+	300.1	215.1	23	102	Quan	1.0014	4.86
		+	300.1	165.2	41	102	Qual		
Cyproheptadine	Anti-histamine	+	288.1	191.1	28	100	Quan	1.1406	8.55
		+	288.1	96.2	24	100	Qual		
Desloratidin	Anti-histamine	+	311.0	259.1	20	81	Quan	3.6887	7.40
		+	311.0	294	16	81	Qual		
Diclofenac	NSAID	−	294.0	250	15	96	Quan	1.7302	9.22
		−	296.0	252	16	84	Qual		
Dicycloverine	Drug for gastrointestinal disorders	+	310.1	109.2	19	103	Quan	1.0246	10.10
		+	310.1	237.2	26	103	Qual		
Dihydroergotamine	Analgesic	+	584.2	253.1	31	134	Quan	2.9101	8.10
		+	584.2	270.1	28	134	Qual		
Diltiazem	Hypertension drug	+	415.1	150.1	37	95	Quan	3.3427	8.15
		+	415.1	178.1	23	95	Qual		
Diphenhydramine	Anti-histamine	+	256.1	165.1	37	73	Quan	2.6942	7.82
		+	256.1	167.1	13	73	Qual		
Dipyridamole	Antithrombotic agent	+	505.3	385.3	40	127	Quan	1.3469	8.29
		+	505.3	429.5	39	127	Qual		
Duloxetine	Antidepressant	+	298.1	123.5	50	74	Quan	56.8644	8.70
		+	298.1	44.3	12	74	Qual		
Eprosartan	Hypertension drug	+	425.1	207.1	23	117	Quan	1.9458	7.42
		+	425.1	107.1	47	117	Qual		
Etonogestrel	Hormonal contraceptive	+	325.1	91.2	50	110	Quan	1.4819	9.62
		+	325.1	257.2	18	110	Qual		
Ezetimibe	Cholesterol statin	−	408.1	119.3	59	132	Quan	35.5912	8.77
		−	408.1	271.2	19	132	Qual		
Fentanyl	Analgesic	+	337.1	105.2	35	106	Quan	1.8395	7.60
		+	337.1	188.2	22	106	Qual		
Fenofibrate	Cholesterol statin	+	361.0	139.0	27	99	Quan	1.6734	10.23
		+	361.0	233.1	16	99	Qual		
Fexofenadine	Anti-histamine	+	502.2	171.1	35	101	Quan	2.1410	8.60

Table 1 (*continued*)

Analyte	Group	Mod	Precursor	Product	CE (V)	Tube lens (V)	Type	Ratio quan/qual	tR (min)
		+	502.2	466.5	26	101	Qual		
Finasteride	Urological	+	373.2	305.2	29	110	Quan	15.6825	8.98
		+	373.2	95.4	36	110	Qual		
Flecainide	Antiarrhythmics	+	415.1	301.1	31	114	Quan	3.1111	7.94
		+	415.1	398.2	22	114	Qual		
Fluconazole	Antimycotic	+	307.1	238.1	16	91	Quan	1.0401	6.06
		+	307.1	220.1	17	91	Qual		
Fluoxetine	Antidepressant	+	310.1	44.3	13	83.6	Quan	one SRM	9.00
Fluoxetine*	IS	+	315.1	44.0	13	83.6	Quan		
Flupentixol	Psycholeptics	+	435.1	265.0	34	116	Quan	1.1536	10.10
		+	435.1	305.1	28	116	Qual		
Fluphenazine	Psycholeptics	+	438.1	143.2	28	93	Quan	1.1992	9.90
		+	438.1	171.2	22	93	Qual		
Flutamide	Antiandrogen	–	275.0	202.0	25	90	Quan	1.2387	8.72
		–	275.0	205.0	23	90	Qual		
Furosemide	Diuretic	–	328.9	205.0	23	81	Quan	1.1965	7.28
		–	328.9	285.0	18	81	Qual		
Glibenclamide	Anti diabetic	–	492.1	369.2	11	137	Quan	4.1732	9.57
		+	494.1	170.0	31	105	Qual		
Glimepiride	Anti diabetic	–	489.2	352.2	11	121	Quan	3.1344	9.27
		+	491.2	225.0	36	122	Quan		
Haloperidol	Psycholeptics	+	376.0	123.1	36	88	Quan	1.1849	7.81
		+	376.0	165.1	22	88	Qual		
Hydroxyzine	Psycholeptics	+	375.1	166.1	35	97	Quan	2.0692	8.70
		+	375.1	201.1	18	97	Qual		
Irbesartan	Hypertension drug	+	429.2	180.1	38	110	Quan	5.3791	8.39
		+	429.2	207.1	22	110	Qual		
Ketoconazole	Antimycotic	+	531.1	254.9	34	134	Quan	1.1539	9.27
		+	531.1	244.0	33	134	Qual		
Levonorgestrel	Hormonal contraceptive	+	313.0	109.2	29	103	Quan	1.2621	9.51
		+	313.0	245.2	17	103	Qual		
Loperamide	Antipropulsive	+	477.2	210.2	45	121	Quan	2.2773	9.23
		+	477.2	266.2	24	121	Qual		
Maprotiline	Antidepressant	+	278.1	219.2	24	99.3	Quan	1.6236	8.80
		+	278.1	250.2	17	99.3	Qual		
Meclozine	Anti-histamine	+	391.1	200.1	16	100	Quan	376.4917	10.03
		+	391.1	166.1	36	100	Qual		
Medroxyprogesterone	Hormonal contraceptive	+	345.1	123.3	24	107	Quan	3.6353	9.68
		+	345.1	97.2	34	107	Qual		
Megestrol	Cancer treatment	+	385.1	267.2	18	98	Quan	1.2117	9.91
		+	385.1	325.2	15	98	Qual		
Memantine	Psycholeptics	+	180.1	107.2	24	77	Quan	2.9151	7.87
		+	180.1	163.2	16	77	Qual		
Metoprolol	Hypertension drug	+	268.1	191.1	16	98	Quan	1.1511	6.44
		+	268.1	159.1	20	98	Qual		
Mianserin	Antidepressant	+	265.0	118.2	30	98	Quan	7.5547	7.90
		+	265.0	208.1	20	98	Qual		
Miconazole	Antimycotic	+	414.9	159.0	29	114	Quan	1.5514	10.70
		+	416.9	161.0	31	114	Qual		
Mirtazapine	Antidepressant	+	266.1	194.1	40	98.2	Quan	2.9146	6.39
		+	266.1	195.1	25	98.2	Qual		
Morphine	Analgesic	+	286.1	201.1	24	110	Quan	1.1019	3.63
		+	286.1	165.1	35	110	Qual		
Naloxone	Opoid overdose drug	+	328.0	212.0	36	119	Quan	5.3991	4.88
		+	328.0	310.2	18	119	Qual		
Nefazodone	Antidepressant	+	470.1	246.2	32	120	Quan	3.5340	9.06
		+	470.1	274.2	27	120	Qual		
Norfloxacin	ATB	+	320.0	233.1	23	114	Quan	4.6996	6.00
		+	320.0	302.1	20	114	Qual		
Ofloxacin	ATB	+	362.1	261.1	25	138	Quan	1.1384	6.04
		+	362.1	318.2	17	138	Qual		
Orphenadrine	Anti-histamine	+	270.1	165.1	44	70	Quan	1.9599	8.35
		+	270.1	181.1	13	70	Qual		
Oxazepam	Psycholeptics	+	287.0	241.1	22	84	Quan	1.4517	7.98
		+	287.0	269.1	15	84	Qual		
Oxazepam*	IS	+	292.0	246.1	22	84	Quan		
Paroxetine	Antidepressant	+	330.0	192.1	20	105	Quan	2.5801	8.60
		+	330.0	70.4	30	105	Qual		
Perphenazine	Psycholeptics	+	404.1	143.2	27	113	Quan	1.5155	9.60
		+	404.1	171.2	21	113	Qual		
Pizotifen	Analgesic	+	296.0	199.1	26	101	Quan	11.8679	8.60
		+	296.0	96.3	21	101	Qual		
Progesterone	Hormon therapy	+	315.0	109.2	26	103	Quan	1.0684	8.90
		+	315.0	97.2	24	103	Qual		
Promethazine	Anti-histamine	+	285.1	86.3	16	65	Qual	2.1383	8.40
		+	285.1	198.0	26	65	Quan		

Table 1 (*continued*)

Analyte	Group	Mod	Precursor	Product	CE (V)	Tube lens (V)	Type	Ratio quan/qual	tR (min)
Promethazine*	IS	+	292.1	89.3	16	65	Quan		
Ranitidine	Drug for peptic ulcer	+	315.0	176.1	17	82	Qual	4.1880	4.62
		+	315.0	125.1	26	82	Quan		
Repaglinide	Anti diabetic	+	453.2	162.2	19	118	Quan	2.5285	8.76
		+	453.2	230.2	25	118	Qual		
Risperidone	Psycholeptics	+	411.1	110.2	44	94	Quan	21.1823	7.20
		+	411.1	191.1	27	94.0	Qual		
Risperidone*	IS	+	415.1	195.1	27	95	Quan		
Rosuvastatin	Statin	+	482.1	258.1	32	118.0	Quan	2.1341	8.21
		+	482.1	272.2	33	118.0	Qual		
Roxithromycine	ATB	+	837.4	158.1	33	148.0	Quan	1.6586	9.41
		+	837.4	679.6	20	148.0	Qual		
Sertraline	Antidepressant	+	306.0	159.0	27	100	Quan	1.0710	9.55
		+	306.0	275	12	100	Qual		
Sotalol	Hypertension drug	+	273.0	213.1	17	86.3	Quan	1.6099	4.38
		+	273.0	255	11	86.3	Qual		
Sulfamethoxazol	ATB	+	254.0	156	15	92	Quan	1.4587	5.92
		+	254.0	108.2	22	92	Qual		
Sulfamethoxazol*	IS	+	260.0	162.1	15	97	Quan		
		+	260.0	114.2	23	97	Qual		
Tamoxifen	Anticancer	+	372.2	129.1	26	113	Quan	12.0177	10.90
		+	372.2	72.4	22	113	Qual		
Tamoxifen*	IS	+	375.2	75.2	22	110	Quan		
Telmisartan	Hypertension drug	+	515.2	276.1	43	114.6	Quan	6.3890	9.13
		+	515.2	305.1	41	114.6	Qual		
Terbutaline	Adrenergic	+	226.1	107.2	29	94	Quan	3.2882	4.37
		+	226.1	152.1	15	94	Qual		
Tramadol	Analgesic	+	264.1	246.2	10	82	Quan	54.2862	6.37
		+	264.1	58.4	16	82	Qual		
Tramadol*	IS	+	268.1	58.4	16		Quan		
Trihexyphenidyl	Anti-Parkinson	+	302.2	70.3	39	102	Quan	18.7338	8.70
		+	302.2	98.3	20	102	Qual		
Trimethoprim	ATB	+	291.0	123.2	25	106	Quan	1.7931	5.61
		+	291.0	230.1	23	106	Qual		
Trimethoprim*	IS	+	294.1	233.2	22	101	Quan		
		+	294.1	126.2	24	101	Qual		
Venlafaxine	Antidepressant	+	278.1	121.2	29	99.3	Quan	1.6165	7.40
		+	278.1	260.2	10	99.3	Qual		
Verapamil	Hypertension drug	+	455.2	165.1	28	118	Quan	3.5710	8.26
		+	455.2	303.3	23	118	Qual		
Zolpidem	Psycholeptics	+	308.1	235.2	32	103	Quan	2.4769	6.89
		+	308.1	263.1	24	103	Qual		
Zuclopenthixol	Psycholeptics	+	401.0	231	35	112	Quan	1.2926	9.80
		+	401.0	271.1	25	112	Qual		

mode of the MS/MS method. The algorithm of the measurement is as follows: The system is switched to the negative mode and all of the transitions in this mode are measured. The mode is then changed to the positive mode, and all of the SRM transitions in this mode are measured. The EZ method contains some simplifications: only a resolution of 0.7 or 0.2 FWHM is allowed, and there is a fixed cycle time that is divided equally among all of the SRMs at a defined time. However, it is the only method for easily handling hundreds of SRMs.

As shown in Table 1, most of the compounds showed at least two MS/MS transitions (with the exception of Fluoxetin, which had only one SRM found by tuning) from a protonized (positive mode) or deprotonized (negative mode) molecule. Unfortunately, the intensities of the second (qualifying) MS/MS transition varied from the intensity obtained for the quantifying SRM to values of less than 1% of the quantifying SRM. The histogram of the intensity ratios is shown in Fig. 1. The separation to four fractions was done in accordance with the requirements of the Commission Decision 2002/657/EC on the performance of analytical methods and the interpretation of the results (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32002D0657:EN:HTML). There are different tolerances for mass ratios in different groups; 20%, 25%, 30%, and 50%, tolerances of the qualification/quantification ratio are allowed for intensities of qualifying mass $>50\%$, 20–50%, 10–20%, and $<10\%$, respectively. It is obvious from the histogram that most (78 of 100) qualifying ions show intensities higher than 20% of the quantifying mass transition.

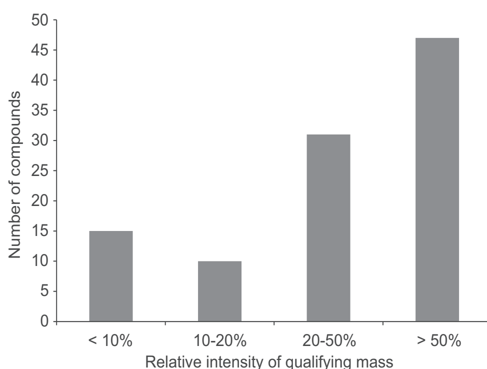

Fig. 1. Histogram of quantification/qualification transition ratios.

3.2. Liquid chromatography

Because the selection of the pharmaceuticals in this study was based solely on the potential environmental effects, the pharmaceuticals consisted of a heterogeneous set with a wide range of physicochemical properties. It was difficult to find conditions providing acceptable chromatographic behavior for the suitable quantification of this selection in a single run. The four above-mentioned columns were used to achieve, as much as possible, quantifiable compounds in a single run. The tRs for all of the pharmaceuticals in the four separation systems are given in Supplementary Table S1.

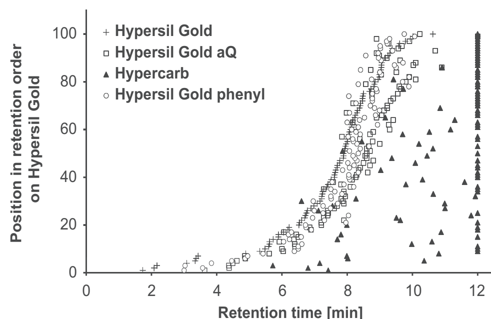

Fig. 2. Retention time shift compared to Hypersil Gold RT for all studied compounds.

The differences among the columns were visualized on a graph, with the *x*-axis representing the retention time and the *y*-axis representing the position in retention order in the Hypersil Gold column. Fig. 2 shows the shift of the tR for the individual compounds compared to Hypersil Gold. The porous graphite column is not a good choice for multi-residue analysis due to the irreversible retention of 58 of the 100 tested pharmaceuticals. Even prolonging the time at 100% organic phase to 5 min did not lead to the elution of these compounds. The best peak shape was obtained in Hypercarb column at least for the early eluting compounds (see Fig. 3). The peak shape of the first few eluted compounds on the Hypersil Gold column is not acceptable. In addition, the peaks are eluted too close to the dead volume. Both of the remaining columns show acceptable peak shapes for early eluting compounds but only one compound is eluted earlier then in 4 min in aQ column (four in Gold Phenyl). Target compounds are less affected with ionic compounds eluted close to dead volume in Hypersil Gold aQ column. At the given chromatographic conditions, the Hypersil Gold Phenyl column has a steeper elution profile of pharmaceutical mixtures than the Hypersil Gold aQ column. The method characteristics for all four columns are given in Table 2. The Hypersil Gold Phenyl has the shortest elution window of target compounds, which results in higher target analytes overlapping. Median number of SRM in cycle is comparable for both columns (51 for aQ and 53 for Phenyl) but maximal values are quite different (76 for aQ and 100 for Phenyl). Insufficient separation can be most likely resolved with a slower gradient, but this will prolong analysis time. The Hypersil Gold aQ was chosen for the single run analyses based on the best separation of the analytes, on good peak shapes at chromatogram

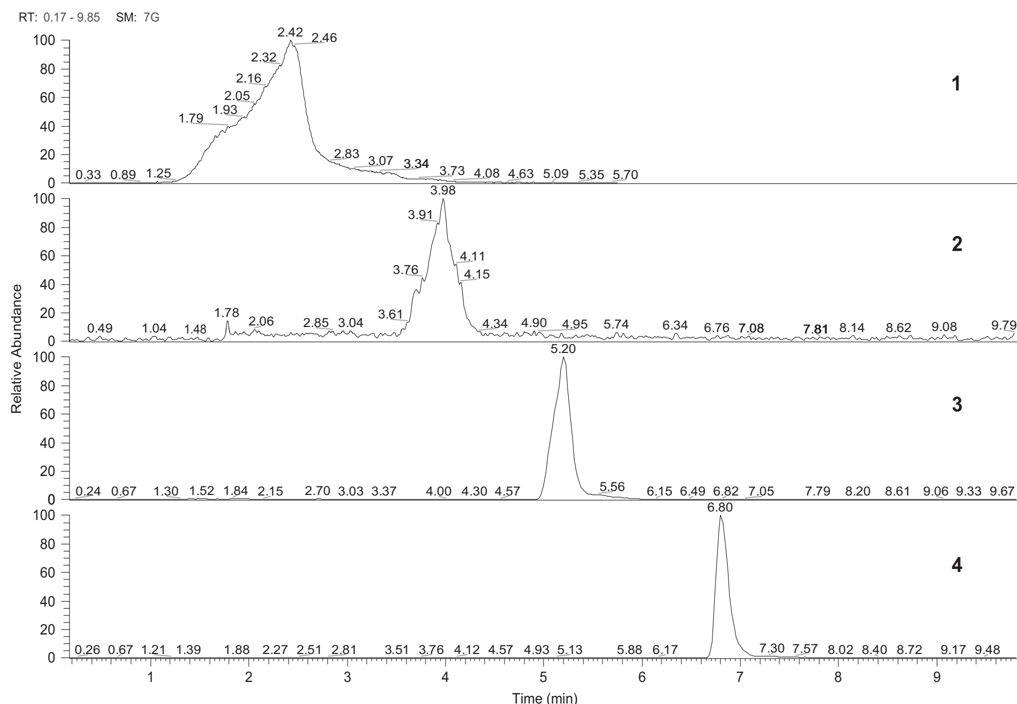

Fig. 3. Chromatogram of Sotalol on four columns under conditions as described in Section 2.4. (1) Hypersil Gold (50 × 2, 1 mm, 3 μm particles), (2) Hypersil Gold Phenyl (50 × 2, 1 mm, 3 μm particles), (3) Hypersil Gold aQ (50 × 2, 1 mm, 5 μm particles), (4) HyperCarb (50 × 2, 1)

Table 2
Comparison of method parameters for four analytical columns under the constant parameter settings of peak width, cycle time, points per peak.

Method parameters	Hypercarb	Gold Phenyl	Gold aQ	Hypersil Gold
Total No. of analytes and IS	114	114	114	114
Cycle time (s)	1	1	1	1
SRM per analyte	2	2	2	2
Retention time of first compound (min)	5.72	3.00	3.63	1.73
Retention time of final compound (min)	12.00	10.07	12.00	10.74
Ret. Window (min)	6.28	7.07	8.37	9.00
Median No. of SRM in cycle	134	53	51	46
Maximal No. of SRM in cycle	138	100	76	84
Median time per SRM (ms)	7.0	19.0	20.0	22.0
Minimal time per SRM (ms)	7.0	10.0	13.2	11.9

beginning and on compatibility of the column with 100% water in mobile phase, This column shows somewhat slow equilibration in 100% water. When the column is equilibrated for approximately 4 min, some of the early eluted compounds have different retention times compared to the same run following 20 min equilibration in water. However, the repeatability of the retention time with a 4 min equilibration is excellent; it is only necessary to run the first sample in the sequence twice to get the same tR in the second and following runs.

3.3. Method performance

3.3.1. Linearity

The quantification of the target compounds was based on the internal standard calibration. The isotope-labeled pharmaceuticals that were selected as the surrogates for each analyte are shown in Table 3, together with the other method parameters,

Table 3
IS used, LOQ and linearity.

Analyte	Surrogate standard used	LOQ (ng L^{-1})[a]				R^2 from LOQ to 5000 (ng L^{-1})[a]
		Instrumental	Milli-Q water[b]	Surface water[b]	Waste water[b]	
Alfuzosin	Tramadol	0.1	0.09	0.11	0.15	0.998
Alprazolam	Tramadol	10	9.9	13	8.9	0.993
Amiodarone	Tramadol	50	119	185	61	0.981
Amytriptyline	Amitryptiline	5	5.9	4.9	5.1	1.000
Atenolol	Tramadol	5	8.6	8.8	5.2	1.000
Atorvastatin	Amitryptiline	50	245	196	59	0.996
Atracurium	Tramadol	0.5	0.46	0.53	0.49	1.000
Azelastine	Tramadol	5	4.5	5.9	5.9	1.000
Azithromycine	Carbamazepin	5	6.9	6.1	4.6	1.000
Beclomethasone	Oxazepam	50	47	44	47	0.999
Bezafibrate	Oxazepam	1	1.3	0.96	1.6	1.000
Biperiden	Amitryptiline	0.1	0.11	0.09	0.09	1.000
Bisoprolol	Tramadol	0.1	0.10	0.11	0.12	1.000
Bromocriptin	Tramadol	5	6.0	6.8	6.1	0.982
Budesonide	Fluoxetine	5	5.8	4.2	5.1	0.998
Buprenorphin	Tramadol	10	10	12	12	1.000
Bupropion	Tramadol	0.1	0.09	0.12	0.11	1.000
Carbamazepin	Carbamazepin	1	0.93	1.2	0.94	1.000
Chlorpromazine	Amitryptiline	10	12	11	11	1.000
Chlorprothixen	Amitryptiline	5	8.7	14	6.1	0.999
Cilazapril	Tramadol	1	0.79	1.20	0.98	0.996
Ciprofloxacin	Ciprofloxacin	10	12	8.1	11	0.997
Citaprolam	Tramadol	5	4.2	6.2	6.3	1.000
Clarithromycin	Amitryptiline	1	1.3	1.4	0.62	1.000
Clemastine	Oxazepam	0.5	0.50	0.46	0.56	0.997
Clindamycine	Tramadol	1	1.0	1.9	0.98	1.000
Clomipramine	Amitryptiline	0.5	0.54	0.50	0.51	1.000
Clonazepame	Tramadol	5	4.2	4.9	6.1	0.959
Clotrimazol	Amitryptiline	1	1.2	0.97	1.2	1.000
Codeine	Tramadol	0.5	0.61	0.52	0.46	0.997
Cyproheptadine	Tramadol	5	4.3	5.3	5.8	0.996
Desloratidine	Risperidone	0.5	0.49	0.46	0.43	0.999
Diclofenac	Tramadol	10	15	11	12	0.999
Dicycloverin	Oxazepam	5	4.0	4.7	4.2	0.999
Dihydroergotamine	Tramadol	50	88	168	78	0.576
Diltiazem	Tramadol	0.5	0.47	0.61	0.53	1.000
Diphenhydramine	Tramadol	0.05	0.04	0.06	0.07	1.000
Dipyridamol	Tramadol	50	61	56	50	0.997
Duloxetin	Tramadol	1	1.0	1.5	1.1	1.000
Eprosartan	Carbamazepin	5	6.2	5.5	4.9	0.999
Etonogestrel	Amitryptiline	0.5	0.65	0.43	0.60	1.000
Ezetimibe	Amitryptiline	50	88	44	46	1.000
Fentanyl	Fluoxetin	50	56	51	54	0.998
Fenofibrate	Carbamazepin	0.5	1.3	1.0	0.4	0.999
Fexofenadine	Amitryptiline	5	7.0	5.9	4.5	1.000
Finasteride	Oxazepam	10	11	7.5	8.5	1.000
Flecainide	Tramadol	0.1	0.08	0.09	0.12	1.000
Fluconazole	Trimetoprim	0.5	0.43	0.46	0.36	0.999
Fluoxetine	Fluoxetine	5	5.3	5.6	4.7	1.000
Flupentixol	Oxazepam	5	5.5	5.9	3.2	1.000
Fluphenazine	Oxazepam	10	12	19	7.2	1.000

Table 3 (*continued*)

Analyte	Surrogate standard used	LOQ (ng L^{-1})[a]				R^2 from LOQ to 5000 (ng L^{-1})[a]
		Instrumental	Milli-Q water[b]	Surface water[b]	Waste water[b]	
Flutamide	Amitryptiline	5	5.6	4.6	5.1	0.998
Furosemid	Carbamazepin	50	46	37	53	1.000
Glibenclamide	Oxazepam	5	6.5	5.4	3.2	1.000
Glimepiride	Oxazepam	5	6.1	4.7	3.3	1.000
Haloperidol	Tramadol	0.1	0.09	0.12	0.17	0.999
Hydroxyzine	Amitryptiline	0.5	0.54	0.49	0.48	1.000
Irbesartan	Amitryptiline	0.5	0.61	0.57	0.49	0.999
Ketoconazole	Amitryptiline	50	80	80	50	1.000
Levonorgestrel	Oxazepam	50	71	48	52	1.000
Loperamide	Amitryptiline	0.5	0.53	0.51	0.50	1.000
Maprotiline	Amitryptiline	5	5.9	4.8	4.7	1.000
Meclozine	Oxazepam	5	4.3	4.0	3.7	0.998
Medroxyprogesterone	Oxazepam	50	74	52	55	1.000
Megestrol	Oxazepam	50	57	53	55	1.000
Memantin	Tramadol	0.5	0.40	0.49	0.51	1.000
Metoprolol	Tramadol	5	4.6	4.6	4.0	1.000
Mianserin	Tramadol	1	0.88	1.2	1.4	1.000
Miconazole	Tramadol	5	6.6	7.3	7.1	0.998
Mirtazapine	Tramadol	10	8.9	8.4	9.6	1.000
Morphine	Trimetoprim	10	17	20	24	0.998
Naloxon	Tramadol	1	1.3	0.89	0.97	1.000
Nefazodon	Amitryptiline	0.5	0.70	0.57	0.52	1.000
Norfloxacin	Ciprofloxacin	10	11	9.3	9.2	0.998
Ofloxacin	Ciprofloxacin	10	12	7.9	14	0.997
Orphenadrine	Amitryptiline	0.1	0.12	0.09	0.07	1.000
Oxazepam	Oxazepam	5	5.3	5.3	3.8	1.000
Paroxetin	Amitryptiline	10	14	10	11	1.000
Perphenazine	Oxazepam	10	15	65	12	1.000
Pizotifen	Amitryptiline	0.5	0.66	0.60	0.54	1.000
Progesterone	Oxazepam	10	13	12	11	1.000
Promethazine	Amitryptiline	10	14	18	10	1.000
Ranitidine	Amitryptiline	0.5	0.97	1.5	0.69	1.000
Repaglinide	Amitryptiline	0.5	0.56	0.52	0.48	1.000
Risperidone	Risperidone	0.1	0.10	0.11	0.09	1.000
Rosuvastatin	Tramadol	10	10	18	10	1.000
Roxithromycin	Amitryptiline	50	76	73	47	0.999
Sertraline	Amitryptiline	10	11	9.0	11	0.964
Sotalol	Tramadol	0.5	0.63	0.51	0.54	1.000
Sulfamethoxazole	Sulfamethoxazol	5	5.3	4.9	5.1	1.000
Tamoxifen	Tamoxifen	5	5.2	4.8	5.0	1.000
Telmisartan	Amitryptiline	50	67	61	72	0.982
Terbutaline	Tramadol	0.5	1.9	1.6	0.71	1.000
Tramadol	Tramadol	0.5	0.50	0.51	0.43	1.000
Trihexyphenidyl	Amitryptiline	0.1	0.11	0.09	0.10	0.999
Trimethoprim	Trimetoprim	0.1	0.10	0.11	0.10	1.000
Venlafaxine	Tramadol	0.5	0.40	0.48	0.43	1.000
Verapamil	Tramadol	10	8.6	12	8.5	1.000
Zolpidem	Tramadol	0.5	0.43	0.60	0.55	1.000
Zuclopenthixol	Oxazepam	5	7.6	9.5	7.2	0.998
Median		5	5.3	4.9	4.6	
Min		0.05	0.04	0.06	0.07	
Max		50	245	196	78	

[a] Based on Instrumental LOQ and SPE enrichment 100 times (100 mL of samples and 1 mL final volume).
[b] Median of LOQs calculated in validation data set (calculated to matrix matched standard and recovery of native compounds).

including the linear range, R^2, coefficient of concentration versus ratio peak area analyte to peak area IS, and LOQs for the different matrices. The developed LC–MS/MS chromatographic procedure exhibits excellent linearity ($R^2 > 0.980$) except for three compounds – Clonazepam, Dihydroergotamine and Sertraline. Dihydroergotamine exhibited poor precision and recovery efficiency, and in the end, we decided to omit this compound from the other experiments. However, both the precision and accuracy of the method for the Clonazepam and Sertraline were acceptable.

3.3.2. Limits of quantification

Quantification in the method is based on the ratio of the target analyte peak area to the internal standard peak area. For highly selective detection method such as MS/MS, the S/N ratio is an auxiliary parameter for the LOQ estimation. Stability of the above mentioned analyte/IS ratio is the determining factor for the LOQ estimation. Relative response factor was used in calculation (ratio of peak area native/IS normalized to concentration of both native compounds and IS in calibration standard(s) used) as it is defined in EPA methods. The second point in the linear range of the calibration curve (i.e., the concentration range where the relative response factor is constant) was set as the instrumental LOQ with an auxiliary criterion of $S/N > 10$ in the real sample. The recalculated LOQs for 100 mL sample aliquots with a final sample volume of 1 mL ranged from 0.05 to 50 ng L^{-1} (median 5 ng L^{-1}) (Table 3). Sixty-eight of the 100 compounds showed LOQs $\leq$ 5 ng L^{-1}, 18 had an LOQ of 10 ng L^{-1}, and 14 analytes had an LOQ of 50 ng L^{-1}. In the real

samples, the LOQs are calculated the same way as concentration of analytes but peak area corresponding to instrument LOQ is taken for the calculation instead of peak area of native compound. It means that all compounds in each sample have individual LOQs. Medians of LOQs obtained in validation data set (triplicates of no fortified matrix waters) are given in Table 3. These data were calculated using matrix matched standards and recovery. The highest LOQ values, i.e., the worst sensitivities, were found for Milli-Q and surface water due to the low recoveries of some compounds which were omitted from the method (see later). The variation in the LOQ was expected due to the wide selection of pharmaceuticals and was satisfactory for the main target to produce a simple single-run method. In addition, the LOQ value is a confusing parameter due to its relevancy. An LOQ of 50 ng L^{-1} is acceptable for antibiotic determination in waste water, whereas 5 ng L^{-1} is inadequate for hormones in surface water.

3.3.3. *Recovery and matrix effect*

The absolute recoveries in Milli-Q water, surface water, and sewage effluent are given in Table 4. The recoveries ranged from 5.2% for Perphenazine in surface water to 246% for Furosemide in effluent. There are greater differences among the matrices than between concentration levels of the same matrix. Atorvastatin and Terbutalin exhibited absolute recovery lower than 40% in Milli-Q water (20 and 27%). In the case of Atorvastatin, it can be addressed to ion suppression effect of coeluting pharmaceuticals. The ratio Atorvastatin to IS measured for individual compound is about 40% higher than the same ration measured in mixture of all pharmaceuticals. This effect is eliminated in the presence of waste water matrix. However, Atorvastatin was removed from the method for surface water due to both low recovery and high variability of results. Terbutalin as the second eluted compound cannot be affected the same way. Its recoveries are low in all matrices even using matrix matched standard and this compound was removed from the method.

The group of compounds with much lower recoveries in surface water than in other two matrices was found (Amiodarone, Dihydroergotamin, Dipyridamol, Chlorpromazine, Fluphenazine, Flupetixol, Perphenazine, Ranitidine). The data were recalculated to relative recoveries using the recovery of the native analogues of internal standards (IS) as a calculation factor. A summary of the recalculated data is given in Supplementary Table S2. Some of the lowest recoveries were enhanced but do not represent a real improvement. To distinguish between ion suppression or enhancement and other changes in the recovery, matrix-matched standards were used. The recoveries that were obtained with matrix-matched standards are given in Supplementary Table S3. The comparison of both data sets for all matrices is shown in Fig. 4. Box graphs show that variation in the recoveries is significantly lower for the matrix matched standard calculated data with some outlaying extremes. The recoveries of above mentioned compounds did not improve in surface water but it was satisfactory in waste water. It can be concluded that ion suppression in waste water is eliminated with using matrix matched standards. Low recoveries in surface water are obviously caused by different effects (ionization suppression from other pharmaceuticals as in the case of Atorvastatin was not confirmed by single compounds/mixture measurements). Amiodarone and Perphenazine showed high uncertainty of determination so they were removed from the final method for both matrices. Dihydroergotamin was omitted from the method too due to both low recovery and nonlinear response. Chlorpromazine, Dipyridamol

Table 4
Recoveries and recovery uncertainties of pharmaceuticals from different matrices

Analyte	Milli-Q water		Surface water		Surface water		Effluent		Effluent	
	100 ng L^{-1} Recovery	$n=10$ RSD (%)	100 ng L^{-1} Recovery	$n=10$ RSD (%)	500 ng L^{-1} Recovery	$n=10$ RSD (%)	1000 ng L^{-1} Recovery	$n=10$ RSD (%)	2500 ng L^{-1} Recovery	$n=10$ RSD (%)
Alfuzosin	111	10	92	4.2	103	3.1	70	11	81	9.7
Alprazolam	101	12	82	6.7	101	4.5	68	5.5	67	8.8
Amiodarone	42	36	22	27	11	23	38	37	40	59
Amytriptyline	85	2.4	98	9.7	100	4.9	102	3.8	91	7.1
Atenolol	58	15	56	8.5	65	10	60	13	35	13
Atorvastatin	20	62	26	48	34	12	39	25	51	17
Atracurium	108	11	91	3.8	83	5.6	90	5.8	79	7.2
Azelastine	112	16	71	4.6	79	1.0	87	15	103	13
Azithromycine	72	19	63	26	60	11	105	7.9	103	6.5
Beclomethasone	106	13	119	25	105	8.6	151	31	132	8.3
Bezafibrate	80	5.0	117	17	105	15	58	23	55	17
Biperiden	91	6.0	110	5.7	104	4.5	104	4.1	97	4.1
Bisoprolol	99	18	97	7.4	107	6.5	126	3.4	164	8.7
Bromocriptin	84	11	47	29	38	12	59	28	80	33
Budesonide	86	14	132	25	104	14	90	8.4	101	15
Buprenorphin	99	14	82	8.8	88	4.6	57	16	70	15
Bupropion	115	11	99	4.6	105	6.3	89	9.6	107	9.2
Carbamazepin	107	10	88	5.4	110	3.2	112	6.9	109	7.7
Chlorpromazine	58	16	21	49	24	56	85	9.0	73	8.7
Chlorprothixen	85	18	63	6.3	62	11	75	12	74	12
Cilazapril	127	15	102	6.5	138	4.5	120	2.7	158	9.2
Ciprofloxacin	87	8.9	68	14	36	35	81	20	88	21
Citaprolam	119	12	84	4.9	100	2.3	60	17	70	11
Clarithromycin	74	38	73	26	89	11	112	11	67	18
Clemastine	100	23	60	21	56	11	85	33	66	11
Clindamycine	98	19	58	18	111	5.3	128	4.7	139	9.6
Clomipramine	93	12	95	5.4	92	5.0	89	9.2	86	9.2
Clonazepame	119	16	96	11	90	5.5	73	11	91	10
Clotrimazol	84	3.4	106	3.5	99	5.5	82	9.5	82	7.3
Codeine	82	15	104	10	96	5.1	92	7.8	89	9.7
Cyproheptadine	115	14	86	7.5	98	8.2	80	14	124	13
Desloratidine	102	6.8	81	8.3	79	6.2	108	5.2	95	3.2
Diclofenac	67	19	104	13	93	20	78	16	82	27
Dicycloverin	125	16	81	5.8	76	1.2	125	27	99	6.5

Table 4 (*continued*)

Analyte	Milli-Q water		Surface water		Surface water		Effluent		Effluent	
	100 ng L^{-1} Recovery	$n=10$ RSD (%)	100 ng L^{-1} Recovery	$n=10$ RSD (%)	500 ng L^{-1} Recovery	$n=10$ RSD (%)	1000 ng L^{-1} Recovery	$n=10$ RSD (%)	2500 ng L^{-1} Recovery	$n=10$ RSD (%)
Dihydroergotamine	57	21	18	35	13	11	54	26	69	18
Diltiazem	107	7.2	88	3.4	99	3.5	84	18	117	9.9
Diphenhydramine	114	21	87	6.2	95	6.0	74	12	95	9.9
Dipyridamol	82	14	45	47	20	55	150	17	126	14
Duloxetin	101	22	62	15	90	5.2	96	15	132	17
Eprosartan	80	7.2	81	6.4	92	8.2	113	17	98	9.3
Etonogestrel	77	20	111	11	88	8.8	88	15	83	8.3
Ezetimibe	57	12	113	26	40	20	50	27	35	28
Fentanyl	90	5.0	74	7.2	75	10	82	12	81	9.3
Fenofibrate	40	40	52	16	24	37	62	27	41	38
Fexofenadine	71	7.9	100	3.8	106	6.2	113	8.4	99	7.9
Finasteride	95	13	90	9.4	75	4.3	90	37	64	9.1
Flecainide	121	12	98	5.8	95	4.1	85	9.7	112	8.2
Fluconazole	115	10	128	5.4	122	4.4	157	6.5	107	5.4
Fluoxetine	94	4.7	107	8.1	85	22	110	5.3	109	9.5
Flupentixol	90	12	26	22	18	12	77	37	41	26
Fluphenazine	85	10	16	20	18	11	72	36	43	24
Flutamide	90	6.9	100	13	110	5.5	93	8.5	81	9.1
Furosemid	108	12	64	83	69	49	246	28	252	24
Glibenclamide	77	14	70	19	52	28	62	50	41	36
Glimepiride	82	18	65	14	54	19	62	48	41	27
Haloperidol	106	7.3	80	4.7	85	2.9	54	24	106	17
Hydroxyzine	93	4.4	100	3.9	102	5.6	115	4.5	97	3.4
Irbesartan	82	5.4	103	5.5	124	6.0	109	4.0	96	6.1
Ketoconazole	63	11	60	24	53	8.1	105	8.1	88	13
Levonorgestrel	70	15	121	25	106	6.7	107	29	104	6.5
Loperamide	95	12	94	6.3	77	12	71	18	65	16
Maprotiline	85	2.5	103	4.2	100	4.7	102	3.9	91	7.3
Meclozine	117	12	43	19	32	19	62	44	34	38
Medroxyprogesterone	68	14	102	9.8	104	7.3	102	30	98	16
Megestrol	87	17	103	9.6	103	6.4	85	32	88	12
Memantin	124	13	106	6.9	95	3.4	116	7.7	122	9.2
Metoprolol	108	7.6	104	6.3	99	8.2	151	5.8	127	35
Mianserin	114	11	73	3.6	82	2.2	71	12	119	11
Miconazole	75	23	46	14	34	19	45	28	76	34
Mirtazapine	112	11	86	4.0	42	12	104	2.7	100	6.6
Morphine	60	13	37	7.9	40	13	42	14	54	12
Naloxon	80	20	117	7.9	107	7.3	101	4.1	76	11
Nefazodon	71	13	68	6.0	72	8.7	67	18	78	16
Norfloxacin	92	8.4	68	18	50	32	104	21	92	27
Ofloxacin	81	4.9	57	11	45	33	47	20	31	20
Orphenadrine	82	15	113	9.0	102	6.4	105	8.0	83	4.1
Oxazepam	94	3.3	96	7.0	102	3.9	126	17	82	9.2
Paroxetin	71	14	80	8.1	81	5.1	95	7.7	78	7.3
Perphenazine	69	14	5.2	46	6.1	44	77	35	65	24
Pizotifen	76	14	92	4.2	98	4.9	96	4.0	88	5.5
Progesterone	80	12	93	6.9	98	6.4	82	31	90	26
Promethazine	72	10	41	27	63	11	88	4.2	74	8.0
Ranitidine	52	20	16	42	16	7.2	61	9.7	55	32
Repaglinide	89	5.5	113	3.5	117	7.0	112	2.8	96	4.1
Risperidone	98	2.2	95	4.1	100	3.7	103	2.9	89	3.0
Rosuvastatin	100	12	103	11	170	4.0	102	14	101	9.8
Roxithromycin	66	32	75	25	81	10	98	8.6	72	15
Sertraline	92	15	101	6.2	89	12	84	11	83	14
Sotalol	80	10	88	6.2	81	11	106	6.9	104	13
Sulfamethoxazole	95	2.9	97	8.4	101	5.6	103	5.9	90	7.5
Tamoxifen	96	3.6	109	4.0	121	5.6	133	6.3	111	13
Telmisartan	74	12	63	11	75	14	66	19	92	16
Terbutaline	27	20	29	12	38	16	35	7.9	19	13
Tramadol	100	10	100	16	102	4.3	116	9.7	86	13
Trihexyphenidyl	94	6.5	120	4.5	102	4.8	100	6.9	93	4.3
Trimethoprim	96	6.4	100	3.6	103	2.7	106	2.9	93	4.9
Venlafaxine	124	17	96	4.6	102	7.7	102	5.5	100	7.6
Verapamil	116	10	101	5.7	99	4.2	110	13	122	12
Zolpidem	115	11	103	4.0	109	3.6	91	4.8	116	10
Zuclopenthixol	66	15	30	19	45	15	69	19	76	12
Average	89	14	81	14	80	11	91	15	88	14
Median	90	12	88	8.4	90	7.1	90	12	88	11
Max. value	127	62	132	83	170	56	246	50	252	59
Min. value	20	2.2	5.0	3.4	6.0	1.0	35	2.7	19	3.0

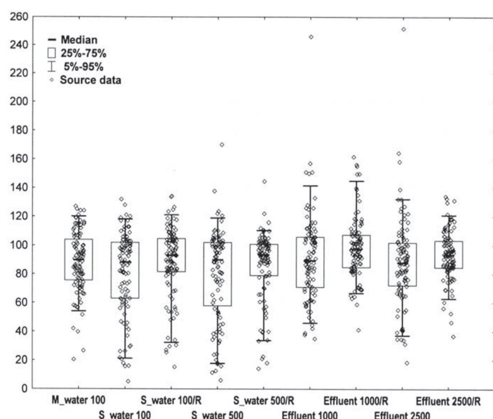

Fig. 4. The comparison of absolute recoveries and recoveries recalculated to matrix matched standard. Recalculated data are indicated with *R* in the name. The line in the box represents the median of the data set; the box is the 50th percentile; the range between bottom and upper line segments represents 90th percentile; and individual points are projected as empty rhombi.

and Ranitidine were removed from the surface water method for the same reasons as Amiodarone and Perphenazine but the results for waste water are acceptable. Low recovery for Atorvastatin and Ranitidine in surface water (40% and 50%, respectively) was also found by Gross [10]. Ionization suppression was not identified as the reason of low recoveries for above mentioned pharmaceuticals group. The sorption on glass surface during sample handling can be excluded too because of good recoveries in Milli-Q water. Humic acid mediated degradation (sorption) of some pharmaceuticals was described recently [30,31]. Low extraction efficiency in Swedish river water could be related to sorption or binding the pharmaceuticals to humic acids.

Excessively high recoveries of Furosemide in waste water were caused by ionization enhancement in this matrix; recoveries using the matrix-matched standard were 94.1% and 96% instead of 246% and 252%. Furosemide in surface water and Glibenclamide, Meclozine, and Glimepiride in waste water showed high variability in the results despite the fact that they had good recoveries (RSD $>$ 30% at both concentration levels). Those pharmaceuticals have not been included to the method for corresponding matrices too. Finally, 91 compounds in surface water and 93 compounds in waste water showed acceptable performance. Cut off criterions were set as follows: 40–140% recovery at least at one of the concentration levels analyzed and RSD lower than 30%. Fifty-two of 100 studied pharmaceuticals were lacking analytical protocol for environmental matrices (water or soil), 49 of them remains in surface water method and 47 in waste water method.

The method was successfully applied for the screening of pharmaceuticals presence in effluent from small WWTPs in Sweden. The results of this screening are given in Supplementary Table S4. Forty-seven to 66 target compounds were found above LOQ (45–64 compounds when omitted pharmaceuticals are not included) in six WWTPs effluent analyzed.

4. Conclusions

An efficient LC/MS/MS method based on one injection, one pre-treatment protocol and a 15 min retention time was developed. The method measures multiple ecotoxicologically relevant pharmaceuticals. Due to the selection approach based on the potential of the pharmaceuticals to bioconcentrate in fish, the included compounds represent 27 different classes with a wide variety of physico-chemical properties. Forty-nine pharmaceuticals in surface water (47 pharmaceuticals in waste water respectively) previously lacking an analytical protocol for their determination in environmental samples are included in this method. The use of internal standard addition combined with matrix-matched standards resolves most of the challenges with ionization suppression or enhancement. Both of the above mentioned approaches must be included in the quantification method, i.e. preparation of matrix matched standard for each series of samples and each analyzed matrix.

Acknowledgments

This study was supported by the Foundation for Strategic Environmental Research (MISTRA) (within the research project MISTRAPHARMA), CENAKVA No. CZ.1.05/2.1.00/01.0024, the GAJU No. 047/2010/Z and the project of GA CR P503/11/1130.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.08.032.

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Chemical Papers
DOI: 10.2478/s11696-013-0428-3

ORIGINAL PAPER

Simultaneous determination of 32 antibiotics in aquaculture products using LC-MS/MS

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Received 22 January 2013; Revised 8 March 2013; Accepted 27 April 2013

An analytical multiclass, multi-residue method for the determination of antibiotics in aquaculture products was developed and validated. A fast, cheap, and straightforward extraction procedure followed by liquid chromatography–tandem mass spectrometry analysis was proposed. This method covers 32 antibiotics of different classes, which are frequently used in aquaculture. Three different extraction procedures were compared, and the extraction with acetonitrile (0.1 vol. % formic acid) showed the best results. The selected extraction procedure was validated at four different fortification levels (10 μg kg^{-1}, 25 μg kg^{-1}, 50 μg kg^{-1}, and 100 μg kg^{-1}). Recoveries of the tested antibiotics ranged from 70 % to 120 %, with the relative standard deviation (RSD) of triplicates lower than 20 %. The limits of quantification (LOQ) ranged from 0.062 μg kg^{-1} to 4.6 μg kg^{-1}, allowing for the analysis of trace levels of these antibiotics in aquaculture products. The method was applied to the analysis of selected antibiotics in fish and shrimp meat available in the Czech market.
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Keywords: antibiotic residues, aquaculture products, LC-MS/MS detection, validation

Introduction

Aquaculture is a fast-growing food production sector. The increasing demand for fish products has promoted the intensification of aquaculture production in many countries (Cañada-Cañada et al., 2009) which has led to the wide application of antibiotics, used for both prevention and treatment of bacterial diseases. Excessive use of antibiotics in industrial aquaculture results in the presence of residual antibiotics in commercial fish and shellfish products (Cabello, 2006). Unintentional consumption of antibiotics can lead to the development of antibiotic resistance in bacteria that are pathogenic to humans (Greenlees, 2003). The maximum residue limits (MRLs) for antibiotics in different tissues are set in the annexes of the European Union (EU) directive No 37/2010 (European Commision, 2010).

To monitor the occurrence of these undesirable residues in edible tissues, a simple and reliable analytical method is required. Currently, liquid chromatography (LC) techniques coupled with mass spectrometric (MS) detection are used (Li et al., 2011; Peters et al., 2009; Romero-González et al., 2007; Yu et al., 2011). Most of the published methods are focused on one or two antibiotic groups (Dasenaki & Thomaidis, 2010; Jo et al., 2011; Johnston et al., 2002; Nakazawa et al., 1999). However, the current analytical strategy is shifting towards multi-residue and multiclass methods, which save time because all the target antibiotics are analyzed in one run. At present, such methods are uncommon. The extraction process is the limiting factor of any multi-residual method, since it should provide acceptable recoveries of analytes with a broad range of physicochemical properties. Moreover, it should be simple, fast and cheap, to increase the sample throughput.

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There are few methods covering all the antibiotic classes that are widely used in aquaculture. Among the published analytical multiclass methods applicable to foodstuffs, there are many dealing with matrices which differ from fish muscle (Bilandžić et al., 2011; De Alwis & Heller, 2010; Martínez Vidal et al., 2009). Some methods that have been validated for meat samples include tedious clean-up procedures (e.g. solid-phase extraction, SPE) (Kaufmann et al., 2008; Peters et al., 2009). Methods applicable to fish products still lack a complete coverage of antibiotic classes, as well as the validation based on the analysis of real samples from various fish species (Villar-Pulido et al., 2011; Romero-González et al., 2007).

The aim of this study was to develop a fast and simple sample pre-treatment, suitable for extracting a wide range of antibiotic residues from various aquaculture products. The most popular groups of antibiotics used for the treatment of fish diseases, as well as banned antibiotics, were selected based on the legislation and their use in aquaculture (Heuer et al., 2009). In total, 32 antibiotics were analyzed, including eight fluoroquinolones, thirteen sulfonamides, two tetracyclines, three macrolides, two β-lactams, two amphenicols, one lincosamide, penicillin, one bacteriostatic antibiotic, and one antiviral. All the selected antibiotics were analyzed in one run. The method was validated determining its linearity, accuracy, repeatability, and limits of quantification (LOQ). Subsequently, the method was applied to the analysis of antibiotic residues in fish and shrimp samples available in the Czech market.

Experimental

LC-MS grade methanol and acetonitrile (LiChrosolv Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid (FA), used to acidify mobile phases, was purchased from Labicom (Olomouc, Czech Republic). Ultrapure water was obtained from Aqua-MAX-Ultra System (Younglin, Kyounggido, Korea). All pharmaceuticals used were obtained as solids of analytical standard purity or > 98 % content of the target compound. Florfenicol (FF) was obtained from Sigma–Aldrich (St. Louis, MO, USA), and oxolinic acid (OXO) from Chem Service (West Chester, PA, USA). Ciprofloxacin (CIP), norfloxacin (NOR), enoxacin (ENO), enrofloxacin (ENR), levofloxacin (LEV), difloxacin (DIF), flumequine (FLU), tetracycline (TC), oxytetracycline (OTC), chloramphenicol (CP), erythromycin (ERY), roxithromycin (ROX), clindamycin (CLI), clarithromycin (CLA), penicillin V (PEN), oseltamivir (OS), trimethoprim (TRI), amoxicillin (AMO), ampicillin (AMP), sulfadiazine (SDZ), sulfathiazole (STZ), sulfamerazine (SMR), sulfapyridine (SPD), sulfamoxol (SMX), sulfamethazine (SMT), sulfamethizole (SMTZ), sulfamethoxypyridazine (SMP), sulfamethoxazole (SMTX), sulfadimethoxine (SDM), sulfaquinoxaline (SQX), sulfaphenazole (SPZ), and sulfasalazine (SSZ) were kindly donated by the Laboratory of Environmental Chemistry, Umea University (Umea, Sweden). Four mass-labeled compounds were used as internal standards: trimethoprim, sulfamethoxazole, carbamazepine, and amitriptyline. Trimethoprim (13C3) and sulfamethoxazole (13C6) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA); carbamazepine (D10) and amitriptyline (D6) were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Stock solutions of all antibiotics were prepared in methanol at the concentration of 1 mg mL^{-1}, and stored at –20 ℃. A spiking mixture was prepared by diluting the stocks in methanol to the final concentration of 1 µg mL^{-1} for each compound, and it was stored at –20 ℃.

HybridSPE®-Phospholipid columns for the clean-up procedure were purchased from Supelco Analytical (Bellefonte, PA, USA).

A triple stage quadrupole MS/MS TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics, Zwingen, Switzerland) was used. The system was fitted with a Hypersil GOLD Phenyl column (50 mm × 2.1 mm i.d., 3-µm particle size) and a Hypersil GOLD Phenyl guard column (10 mm × 2.1 mm i.d., 3-µm particle size), both from Thermo Fisher Scientific.

The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). Both phases were acidified with 0.1 vol. % formic acid. The gradient is presented in Table 1.

Heated electrospray in positive and negative (amphenicoles only) ion modes was used for the ionization of the target compounds. The key parameters were set as follows: ionization voltage at 3.5 kV in the positive and at 2.7 kV in the negative mode, sheath gas at 35 arbitrary units and auxiliary gas at 15 arbitrary units, vaporizer temperature at 250 ℃, and capillary temperature at 350 ℃. Both first and third quadrupoles were operated at the resolution of 0.7 FMWH. Two SRM transitions were monitored for each analyte. All details of the analytical method (mass transitions – m/z, collision energies, and retention times) are presented in Table S1 (ESM). Chromatograms for the target compounds are presented in Figs. S1 and S2 (ESM).

Sample preparation and analysis

Fish muscle (0.5 g) was weighed in an Eppendorf tube, and a certain amount of surrogate standard was added. Quality assurance/quality control and method development samples were fortified with native target compounds. Fortification was done at the level of 100 µg kg^{-1}. Blank samples and fortified blank sam-

Table 1. LC gradient for the elution of target compounds

Time	Mobile phase composition/vol. %[a]		Flow rate
min	water	acetonitrile	µL min^{-1}
0.00	95	5	300
1.00	95	5	300
8.00	60	40	350
10.00	20	80	400
13.00	0	100	400
13.01	95	5	300
16.00	95	5	300

a) Both constituents acidified with 0.1 vol. % of FA.

ples for method development were prepared in triplicate.

After adding 1 mL of the extraction mixture, the samples were homogenized at 30000 min^{-1} (TissueLyser II, Qiagen, Hilden, Germany) for 5 min. Then, they were centrifuged (Hettich Zentrifugen MIKRO 200, Tuttlingen, Germany) at 10000 min^{-1} and 4 ℃ for 10 min; and afterwards the supernatants were filtered through 0.45 µm regenerated cellulose syringe filters (Labicom, Olomouc, Czech Republic). The extracts were evaporated to approximately 50 µL and 5 µL of each extract were analyzed by LC-MS/MS.

To get proper recoveries, various extraction solvents (methanol, water, acetonitrile) and different formic acid concentrations were tested. The composition of the extraction mixtures is described in detail in the Results and discussion section.

The method was validated determining its linearity, accuracy, repeatability, and limits of quantification (LOQ) at the level of interest. The fortification levels were 10 µg kg^{-1}, 25 µg kg^{-1}, 50 µg kg^{-1}, and 100 µg kg^{-1}.

The validation was accomplished using blank fish muscle from carp (*Cyprinus carpio*), tilapia (*Oreochromis niloticus*), and shrimp (*Metapenaeus ensis*).

To check the applicability of the proposed method, 97 real fish and shrimp samples bought at local supermarkets were tested for the presence of antibiotics.

Results and discussion

Optimization of the extraction procedure

Different combinations of extraction solvents were tested: water/methanol 50/50 ($\varphi_r = 50 : 50$), water/methanol 30/70 ($\varphi_r = 30 : 70$), and acetonitrile. Using water/methanol 50/50 as the extraction solvent resulted in turbid extracts which were not clean enough to be analyzed by LC-MS/MS.

Two-step extraction with the water/methanol 30/70 mixture and acetonitrile was carried out as follows. First, 1 mL of water/methanol 30/70 was added to the sample. The mixture was centrifuged and the supernatant was carefully decanted. Then, 1 mL of acetonitrile (ACN) acidified with 0.1 vol. % formic acid (FA) was added to the residue, and the procedure was repeated. The resulting supernatant was added to the supernatant of the previous step. The fish extracts obtained with the two-step extraction method were sufficiently clean for the LC-MS/MS analysis.

Finally, acidified acetonitrile was used as the extraction solvent. Two concentrations of formic acid were tested: 0.1 vol. % and 1 vol. %.

Co-extraction of bulk matrix components like fats and proteins is undesirable because it can cause problems with ionization, and thus lead to ion suppression or enhancement (Niessen et al., 2006). To get rid of phospholipids, supernatants were cleaned using HybridSPE®-Phospholipid columns based on zirconia-coated silica. When applying vacuum, small target molecules pass through, while precipitated proteins and phospholipids are retained in the HybridSPE phase. This procedure was described in detail by Monko (Monko, 2011).

Among the extraction solvents tested, acetonitrile showed the best results. Extraction with acetonitrile (0.1 vol. % FA) followed by a clean-up procedure with HybridSPE-Phospholipid columns gave satisfactory recoveries of most of the target analytes. Unacceptably low recoveries were obtained for seven antibiotics from the fluoroquinolone group (ciprofloxacin, enrofloxacin, levofloxacin, norfloxacin, difloxacin, enoxacin, oxolinic acid) and for two tetracyclines (tetracycline and oxytetracycline). These antibiotics are of great interest for the analysis of aquaculture products. The use of tetracycline is permitted in aquaculture. Concerning fluoroquinolones, some of them are authorized for use in veterinary medicine while others are used for human treatment only. Moreover, because of their high persistence, fluoroquinolones (except for flumequine and oxolinic acid) are banned in aquaculture in the United States, Australia, and Canada (Johnston et al., 2002).

The above mentioned compounds were found in sample extracts that did not undergo the clean-up procedure. This suggests that some antibiotics were trapped in the zirconia-coated silica phase of the HybridSPE columns. When the clean-up step was skipped, recoveries of all antibiotics were in the range

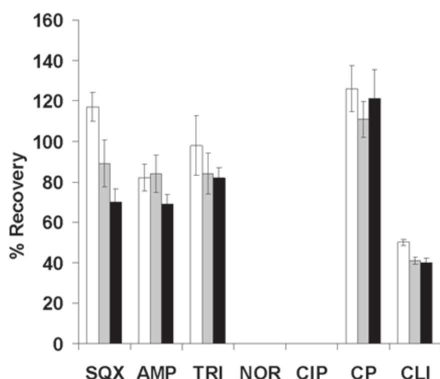

Fig. 1. Recoveries (%) of representative antibiotics from fish tissue using different extraction solvents: water/methanol–ACN (□), ACN with 0.1 vol. % FA (▒), and ACN with 1 vol. % FA (■), with a clean-up step.

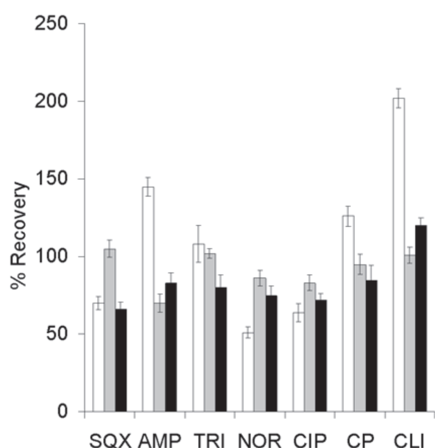

Fig. 2. Recoveries (%) of representative antibiotics from fish tissue using different extraction solvents: water/methanol–ACN (□), CAN with 0.1 vol. % FA (▒), and ACN with 1 vol. % FA (■), without a clean-up step.

of 70–120 %, except for amoxicillin, which had the recovery of 50 %. Results of the optimization of the extraction procedure for the most interesting representatives of antibiotics are shown in Figs. 1 and 2. The comparison of different extraction procedures was performed at a relatively high antibiotic concentration (100 μg kg^{-1}). To examine the suitability of the selected extraction method, validation was performed at lower antibiotic concentrations (see the Method validation section).

Thus, the extraction procedure with acetonitrile acidified with 0.1 vol. % FA was chosen for the analysis. The clean-up step was omitted because it caused loss of some antibiotics which are of high interest. Moreover, omitting this step made the procedure simple and fast, with the workflow of about 100 samples in one day.

Co-extraction of the matrix and signal suppression/enchancement effects

On one hand, a wide range of antibiotics needs to be extracted from the fish muscle as efficiently as possible. On the other hand, the co-extraction of matrix components such as fats and proteins is undesirable. The matrix can reduce the lifetime of the analytical column, and it often causes ionization suppression or enhancement during the MS/MS analysis.

To examine the possible matrix effect, the response factors of the calibration standards in the solvent were compared with those of the target compounds spiked in the blank fish extracts (matrix-matched standards). The observed matrix effects are summarized in Table S2 (ESM). In spite of the low injection volume (5 μL), significant matrix effects (more than 20 %, marked with bold in the table) were observed for most of the analytes. Only 12 of the 32 target compounds did not seem to be affected by the matrix. Equal numbers of analytes were suppressed or enhanced, but greater differences from the solvent standard were observed with the enhancements, especially for some antibiotics from the fluoroquinolone group.

Evidently, matrix-matched standards must be used for correct quantification of target compounds in real samples.

Method validation

Method validation was based on the evaluation of linearity, accuracy, repeatability, and limits of quantification.

A five point calibration curve was prepared by spiking blank fish muscle extract with target compounds in the concentration range from 1 μg kg^{-1} to 250 μg kg^{-1}, plus a constant concentration of the internal standard (50 μg kg^{-1}). Matrix-matched standards underwent the same extraction procedure as the other samples. One more calibration point (600 μg kg^{-1}) was prepared especially for flumequine, as this is the only compound with such a high MRL. All analytes showed good linearity in the investigated interval, with R^2 values higher than 0.995 for most of the analytes. Parameters of the calibration curve are presented in Table S3 (ESM).

The accuracy of the method was tested by evaluating the recovery of the target compounds from the matrix. Blank fish samples were spiked with the mixture of target antibiotics at different levels. According to the European Directive 96/23/EC (European Council, 1996), the level of interest should correspond to

Table 2. Validation parameters for the developed LC-MS/MS method

Antibiotic	Recovery/%[a]				LOQ	MRL/(µg kg^{-1})[b]
	10 µg kg^{-1}	25 µg kg^{-1}	50 µg kg^{-1}	100 µg kg^{-1}	µg kg^{-1}	
Enoxacin	105 (7)	105 (16)	87 (12)	87 (7)	0.25	–
Norfloxacin	107 (3)	108 (7)	112 (16)	86 (4)	0.43	–
Levofloxacin	119 (20)	114 (12)	120 (3)	96 (3)	0.39	–
Ciprofloxacin	88 (11)	120 (10)	113 (3)	83 (2)	0.37	100 (sum of ciprofloxacin
Enrofloxacin	107 (8)	113 (7)	103 (5)	101 (5)	0.52	and enrofloxacin)
Difloxacin	111 (10)	111 (9)	101 (3)	93 (3)	0.46	300
Oxolinic acid	116 (11)	116 (1)	118 (5)	97 (3)	0.52	100
Flumequine[c]	113 (1)	111 (8)	97 (7)	96 (12)	0.58	600
Sulfadiazine	99 (5)	90 (15)	86 (1)	97 (10)	0.11	
Sulfapyridine	107 (9)	100 (16)	84 (8)	100 (8)	0.091	
Sulfamerazine	111 (2)	98 (11)	103 (4)	97 (9)	0.083	
Sulfathiazole	119 (7)	107 (7)	80 (6)	94 (8)	0.12	
Sulfamoxol	80 (20)	94 (18)	103 (7)	96 (8)	0.13	
Sulfamethazine	109 (15)	108 (11)	120 (15)	98 (7)	4.6	
Sulfamethizole	115 (1)	102 (1)	112 (2)	93 (13)	0.10	100 (sum of all
Sulfamethoxypyridazine	81 (12)	87 (3)	82 (6)	96 (8)	0.091	sulfonamides)
Sulfamethoxazole	103 (1)	91 (4)	92 (5)	95 (10)	0.082	
Sulfadimethoxine	100 (9)	91 (2)	81 (13)	98 (12)	0.10	
Sulfaquinoxaline	113 (1)	95 (4)	89 (7)	105 (9)	0.083	
Sulfaphenazole	106 (5)	84 (5)	85 (7)	97 (12)	0.10	
Sulfasalazine	119 (20)	119 (11)	108 (10)	92 (12)	0.071	
Oxytetracycline	70 (14)	79 (12)	78 (4)	70 (2)	0.062	100
Tetracycline	88 (19)	82 (10)	80 (13)	102 (8)	0.39	100
Erythromycin	117 (4)	107 (5)	88 (2)	98 (3)	0.11	200
Clarithromycin	119 (12)	108 (7)	90 (1)	98 (2)	0.12	–
Roxithromycin	116 (2)	115 (12	99 (2)	95 (1)	0.12	–
Amoxicillin	42 (7)	45 (6)	52 (7)	50 (5)	1.0	50
Ampicillin	75 (7)	76 (19)	71 (9)	80 (12)	0.35	50
Florfenicol	120 (3)	117 (12)	111 (13)	105 (7)	2.1	100
Chloramphenicol	80 (20)	102 (8)	85 (0)	95 (7)	2.8	banned
Clindamycin	115 (11)	115 (5)	120 (4)	101 (1)	0.092	–
Penicillin V	82 (10)	107 (8)	102 (5)	109 (7)	0.92	–
Trimethoprim	103 (19)	90 (6)	89 (3)	102 (2)	0.094	50
Oseltamivir	116 (1)	116 (6)	101 (3)	92 (2)	0.082	–

a) RSD values are given in brackets, $n = 3$; *b*) according to directive EC 37/2010 (European Commision, 2010); *c*) recovery of flumequine at the level 600 µg kg^{-1} was 105 % with the RSD value of triplicate samples estimation of 15 %.

the MRL value. Among the selected antibiotics with set MRLs, trimethoprim, amoxicillin, and ampicillin have the lowest MRL (50 µg kg^{-1}). Chloramphenicol has no MRL, as it is a banned compound. To validate the recovery, the following fortification levels were chosen: 10 µg kg^{-1}, 25 µg kg^{-1}, 50 µg kg^{-1}, and 100 µg kg^{-1} (one more point, 600 µg kg^{-1}, was included for flumequine). The established rule of spiking at 0.5-, 1-, and 1.5-times the MRL value was not strictly followed. The main aim was not only to compare the measured concentrations with the set limits to assess the human health risk, but also to reach the lowest possible levels of detection for further bioaccumulation studies of pharmacologically active compounds, as they are currently recognized as emerging pollutants in the aquatic environment.

Triplicate samples were prepared for each fortification level. The recoveries of all the target compounds, at different fortification levels and limits of quantification (LOQs), are presented in Table 2. The obtained recoveries were in the range of 70–120 % for all the target analytes, except amoxicillin, which had the recovery of 42–52 %. For this reason, amoxicillin was excluded from the analytical method.

Repeatability was assessed at four concentration levels. Triplicate samples were prepared for each fortification level. The relative standard deviation (RSD) of the triplicate experiment results is given in Table 2. RSD was lower than 20 % for all target antibiotics. Data on the intra-day and inter-day precision for the average response factors and retention times are given in Table S4 (ESM).

LOQs were calculated by analyzing blank samples spiked at low concentrations (0.5–10 µg kg^{-1}). LOQs ranged from 0.062 µg kg^{-1} for oxytetracycline to 4.6 µg kg^{-1} for sulfamethazine, allowing for the analysis of these antibiotics in aquaculture products at trace levels.

The only limitation of the method concerns chloramphenicol. As the use of this substance is banned

Table 3. Fish samples from the Czech market analyzed in the study

Cultured fish		Wild fish	
Species	Number of samples	Species	Number of samples
Salmon	4	Squid	3
Sea bream	3	Salmon	3
Mussels	1[a]	Sea bream	3
Pangasius	23	Mackerel	3
Seawolf	3	Sprat	1[a]
Carp	8	Tuna fish	1
Rainbow trout	16	Herring	6
American char	2	Cod	7
Tilapia	3	Shark	1
Crayfish	1[a]	Nile perch	1
Shrimp	2[a]	Shrimp	2[a]

a) Pooled sample of ten items was analyzed.

Table 4. Antibiotic concentrations in positive samples and MRLs set in EU

Antibiotic	Fish species, country of origin/catching area	Concentration in sample/(μg kg^{-1})	MRL/(μg kg^{-1})[a]
Norfloxacin	Squid (S1), FAO 77	14	not supposed to be used in aquaculture
	Squid (S2), FAO 77	12	
Ciprofloxacin	Squid (S1), FAO 77	13	100 (sum of ciprofloxacin and enrofloxacin)
	Mussels (S4), Italy	7.3	
Enoxacin	Squid (S1), FAO 77	9.3	not supposed to be used in aquaculture
	Squid (S2), FAO 77	10	
	Atlantic salmon (S5), Norway	3.9	
	Atlantic salmon (S6), Norway	4.5	
Flumequine	Squid (S1), FAO 77	16	600
	Squid (S2), FAO 77	19	
	Squid (S3), FAO 77	12	
	Herring (S7), FAO 027	5.5	
	Herring (S8), FAO 027	25	
	Pangassius (S9), Vietnam	3.8	
	Pangassius (S10), Vietnam	3.2	
	Shark (S12), FAO 34	2.9	
	Pangassius (S11), Vietnam	9	
Ampicillin	Squid (S2), FAO 77	10	50
	Squid (S3), FAO 77	4.9	
Sulfadiazine	Squid (S2), FAO 77	4.4	100 (sum for all sulfonamides)
	Squid (S3), FAO 77	3.0	
	Mackerel (S13), FAO 27	10	
	Rainbow trout (S14), Spain	20	
	Rainbow trout (S15), Spain	19	
Sulfasalazine	Squid (S2), FAO 77	3.1	100 (sum for all sulfonamides)
Trimethoprim	Rainbow trout (S14), Spain	15	50
	Rainbow trout (S15), Spain	13	
Erythromycin	Rainbow trout (S14), Spain	10	200

a) According to Directive No 37/2010 (European Commision, 2010).

in aquaculture, the guidance sets minimum required performance limits (MRPL) for its analysis. The MRLP for chloramphenicol in aquaculture products is 0.3 μg kg^{-1} (European Commision, 2003). Unfor-

tunately, the analytical system is not sensitive enough for this compound, so chloramphenicol cannot be detected at such low levels. The LOQ of chloramphenicol is 2.8 μg kg^{-1} (Table 2). Due to insufficient sensitivity, chloramphenicol was excluded from the analytical method.

Application of the method in the analysis of real samples

The proposed method was used to analyze 97 fish and shrimp samples commercially available in the Czech Republic (CR). Exact numbers of samples for each fish species are presented in Table 3.

The fish and shrimp samples were bought from the three biggest supermarkets in České Budějovice, which belong to a network of shops covering the whole CR. Samples of wild and farmed fish (carp, salmon, trout, tilapia, pangasius, etc.), and shrimp of foreign (European, Asian, African) and Czech origin were obtained. This is the first study of this kind in CR covering a broad range of compounds, not only those with set MRLs, and focusing both on farmed and wild fish.

Among the 97 samples analyzed, 15 were positive for at least one antibiotic tested (Table 4). The most frequently found antibiotic was flumequine, which was detected in nine of the 15 positive samples. Other antibiotics of the quinolone family were also prevalent, being present in 12 of the 15 samples. Concentrations of all the antibiotics detected are shown in Table 4. All the values were lower than the MRLs established by the EU.

Conclusions

This paper describes an LC-MS/MS method for simultaneous determination of 32 antibiotics of different classes in aquaculture products. The method is simple, fast and it does not require any clean-up procedure, allowing thus high sample throughput. The use of matrix-matched standards is recommended to eliminate the matrix effect. The method demonstrated excellent performance parameters. Recoveries of all antibiotics, at the level of fortification of 10 μg kg^{-1}, ranged between 70 % and 120 %. LOQs ranged from 0.062 μg kg^{-1} to 4.6 μg kg^{-1}. The method allows for the determination of trace levels of selected antibiotics in aquaculture products.

Acknowledgements. This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic CENAKVA No. CZ.1.05/2.1.00/01.0024, Czech Science Foundation GACR P503/11/113, the Grant Agency of the University of South Bohemia No. 038/2011/Z and the Grant Agency of the University of South Bohemia No. 087/2013/Z. We thank Edanz editing agency for the English language corrections.

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Research Article

Rapid Communications in Mass Spectrometry

Received: 8 March 2013 Revised: 10 May 2013 Accepted: 13 May 2013 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2013**, *27*, 1751–1762
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6628

Comparison of the quantitative performance of a Q-Exactive high-resolution mass spectrometer with that of a triple quadrupole tandem mass spectrometer for the analysis of illicit drugs in wastewater

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RATIONALE: Analysis of drugs in wastewater is gaining more interest, as new approaches to estimate drug consumption from the amount of drug residues in wastewater have been proposed. The aim of this study was to compare the quantitative performance of high-resolution mass spectrometry with that of triple quadrupole mass spectrometry.
METHODS: A Q-Exactive mass spectrometer was operated in full scan (HRFS) (70 000 FWHM) and product scan (HRPS) (17 500 FWHM) modes. The first and third quadrupoles of the QqQ MS/MS instrument were operated at 0.7 FWHM. A mass-extracted window of 5 ppm around the theoretical *m/z* of each analyte was used to construct chromatograms. An HESI-II ion source was used for the ionization of target compounds. In-line-SPE-LC configuration was used for the extraction and separation of target analytes.
RESULTS: All three methods showed good linearity and repeatability. High-resolution detection of product ions exhibited better sensitivity and selectivity for some compounds. For most of the tested compounds, LOQs ranged from 0.46 to 20 ng L^{-1}. Good agreement between measured and nominal concentrations was observed for most of the compounds at different levels of fortification. Both MS/MS methods showed good selectivity, while HRFS gave some false positive results.
CONCLUSIONS: The Q-Exactive mass spectrometer proved to be suitable for trace detection and quantification of most of the tested drugs in wastewater, with performance comparable to that of the commonly used MS/MS triple quadrupole, but with better selectivity. Copyright © 2013 John Wiley & Sons, Ltd.

Illicit drugs are emerging pollutants of the aquatic environment. Drug consumption is increasing worldwide. Consequently, several studies have reported the occurrence of drugs in waste and surface water.[1–4] As with pharmaceuticals and personal care products, illicit drugs enter waterways through urban wastewater after being excreted.[5] The analysis of drug residues in wastewater can provide reliable information on their usage pattern in the local community. Zuccato *et al*. proposed an approach to estimate the amount of consumed drugs from the concentration of drug residues in influent wastewater.[6] In addition, the presence of drugs in the aquatic ecosystem is gaining attention because of their possible adverse effects on aquatic organisms.[7] Thus, not only forensic, but also environmental analysis of illicit drugs is important.

Most of the methods dealing with the analysis of illicit drugs in wastewater are based on liquid chromatography/tandem mass spectrometry (LC/MS/MS).[8,9] The triple quadrupole (QqQ) mass spectrometer is the instrument of choice for the analysis of complex matrices. This configuration has sensitivity and selectivity advantages. Nevertheless, analysis of drug traces in aqueous environmental samples remains a challenge. A significant disadvantage of using triple quadrupoles for detection purposes is their inability to detect non-target compounds, as the analysis methodology should be optimized regarding the *m/z* values of precursor and product ions, as well as the collision energy and electrospray ionization source conditions. Thus, high-resolution mass spectrometry (HRMS) is becoming widespread for the analysis of illicit drugs.[10] Advanced HRMS instruments combine crucial features such as improved selectivity and improved mass resolution, lower cost, and relatively easy maintenance. Accurate mass full scan analysis using time-of-flight (ToF) and Orbitrap mass spectrometers proved to be an appropriate alternative to triple quadrupole instruments for both targeted and non-targeted drug detection in clinical toxicology.[11–14] Routine ToF instruments have a resolution of 30 000–40 000 full width at half maximum (FWHM) and an accuracy <5 ppm, while the resolving power and accuracy of Orbitrap instruments are significantly better, being up to 70 000 FWHM and <2 ppm.[15] Higher mass accuracy could

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be helpful for environmental analyses, where many co-extracted components from the matrix are present. The application of these techniques for the detection of illicit drugs is shifting from mere qualitative analysis towards obtaining quantitative data.[14,16–18]

A new generation Orbitrap instrument (the Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer) combines high-performance quadrupole precursor selection with high-resolution, accurate-mass (HR/AM) Orbitrap detection. It allows the combination of non-targeted screening in full scan (HRFS) with targeted MS/MS (HRPS) analysis, thus representing an excellent tool for fast and easy environmental screening. Several reports on the application of the Q-Exactive for drug analysis in doping control have been published.[19–21] To the best of our knowledge, there are no publications on the use of in-line-SPE-LC/HRPS for the environmental analysis of drug residues. The objective of our study was to investigate the advantages of a new bench-top mass spectrometer, which combines a quadrupole mass filter, a higher energy collisional dissociation (HCD) cell and an Orbitrap analyzer, for the analysis of 27 drugs of abuse in influent wastewater. The list of selected drugs includes widely consumed drugs (or metabolites), such as methamphetamine, MDMA, cocaine, THC,[22] as well as compounds which are used for human treatment purposes and their nonmedical use is prohibited, e.g. ketamine, tramadol. Moreover, some compounds such as oxazepam represent high interest in the environmental risk assessment due to their adverse effects on non-target species.[7] We also aimed to compare the quantitative performance of HRMS-based detection (Q-Exactive mass spectrometer) versus unit mass resolution-based MS/MS (triple quadrupole) detection. The Q-Exactive was operated both in full scan and targeted MS/MS modes.

EXPERIMENTAL

Materials

LC-MS grade methanol and acetonitrile (LiChrosolv Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid, used to acidify mobile phases, was purchased from Labicom (Olomouc, Czech Republic). Ultrapure water was obtained from an Aqua-MAX-Ultra system (Younglin, Kyounggi-do, Korea). Amphetamine, benzoylecgonine, cathinone HCl, cocaine, ketamine HCl, MBDB HCl, MDA, MDEA, MDMA, methamphetamine, methylphenidate, midazolam, norketamine HCl, 2-oxo-3-hydroxy-LSD, LSD oxycodone, EDDP perchlorate, methadone, norbuprenorphine glucuronide, THC-COOH, amphetamine-D5, benzoylecgonine-D8, cocaine-D3, MDA-D5, MDMA-D5, methadone-D9, methamphetamine-D5, and THC-COOH-D9 were bought from Cerilliant (Round Rock, Texas, USA) as 0.1 or 1 mg L^{-1} standards in either methanol or acetonitrile. Codeine and oxazepam were purchased from Sigma Aldrich (St. Louis, MO, USA). Mephedrone was obtained from the National Measurement Institute (Sydney, Australia). Tramadol was obtained from the Council of European Pharmacopoeia (Strasbourg, France). All substances were classified as >99% pure, except for 2-oxo-3-hydroxy-LSD (>97.7%) and LSD (>98.9%). All substances were stored according to the supplier's instructions. A spiking mixture was prepared by diluting stock solutions in methanol to a final concentration of 1 μg mL^{-1} for each compound, and it was stored at –20 °C.

Wastewater samples

A composite sample (collected during 24 h, with a 15 min sampling interval) of wastewater influent to a treatment plant (WWTP) was obtained in Petrzalka, Bratislava, Slovak Republic. Ten replicates (10 mL aliquots) were filtered through 0.45 μm regenerated cellulose filters (Labicom, Olomouc, Czech Republic), and spiked with the mixture of mass-labeled compounds to achieve an internal standard concentration of 500 ng L^{-1}. Other sets of samples (ten replicates each) were spiked with the target analytes at three concentration levels: 20, 200 and 1000 ng L^{-1}, to check recovery of the analytes. The fortification corresponds to limit of quantification (LOQ), ten times LOQ and 50 times LOQ. All sets of samples were analyzed by three different methods: in-line-SPE-LC/MS/MS, in-line-SPE-LC/HRFS, and in-line-SPE-LC/HRPS.

Liquid chromatography

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 600 LC pump (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used. A Cogent bidentate (50 mm × 2.1 mm i.d., 3 μm particles; Thermo Fisher Scientific) column, preceded by a guard column (10 mm × 2.1 mm i.d, 3 μm particles) of the same packing material and from the same manufacturer, was used for chromatographic separation of target compounds.

Filtered and spiked water samples were analyzed using an in-line-SPE-LC system fitted with a C18 column (Hypersil Gold, 20 mm × 2.1 mm i.d, 12 μm particles; Thermo Fisher Scientific). In-line-SPE allows the pre-concentration and analysis of samples in a single run, making large volume samples and time-consuming SPE extraction unnecessary. The extraction and analytical process required 15 min and used only 1 mL of sample. Details of the method are described elsewhere.[23,24] Briefly, a high-flow Accela 600 LC pump was used for sample loading, and a low-flow Accela 1250 LC pump was used for liquid chromatography at the analytical column. Analytes retained in the extraction column were flushed with a reversed flow of the gradient of acetonitrile in water and introduced into the analytical column, where they were separated and detected by MS/MS, HRFS or HRPS. The LC gradients are given in Supplementary Table S1 (see Supporting Information).

Mass spectrometry

MS/MS parameters

A triple-stage quadrupole MS/MS TSQ Quantum Ultra (Thermo Fisher Scientific) was used for the detection of target analytes. A heated electrospray ionization (HESI-II) source in positive ion mode was used for the ionization of target compounds. The key parameters were as follows: ionization voltage, 3.5 kV; sheath gas, 35 arbitrary units; auxiliary gas, 15 arbitrary units; vaporizer temperature, 200 °C; capillary

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temperature, 325 °C; and collision gas (argon) pressure at 1.5 mTorr min^{-1}. Both first and third quadrupoles were operated at a resolution of 0.7 FWHM. Two selected reaction monitoring (SRM) transitions were monitored for all analytes. Details of the analytical method (mass transitions – *m/z*, collision energies, and retention times) are presented in Table 1.

HRMS parameters

A Q-Exactive mass spectrometer (Thermo Fisher Scientific) was used for the detection of target compounds. Instrument calibration was performed daily by infusing a calibration mixture (caffeine, MRFA and Ultramark® 1621).

An HESI-II interface was used with the above-mentioned parameters. Full scan data in positive mode was acquired at a resolving power of 70 000 FWHM. Extracted ion chromatograms (XIC) of target analytes were constructed with an optimized mass extraction window (MEW) 5 ppm. Spectra were collected in lock mass (391.28429 – $C_{24}H_{38}O_4$) mode, allowing better precision and accuracy. The maximum target capacity of the C-trap (AGC target) was defined as 3 000 000 ions and the maximum injection time was set to 100 ms. For the compounds of interest, a scan range of *m/z* 100–800 was chosen. For the confirmation purposes a second abundant ion of isotopic pattern was used. The drawback of this approach is much lower intensity for qualification mass (approximately 10% of intensity for the quantification ion).

Mass scale stability was assessed throughout full scan acquisition, covering different concentrations of target compounds. For each positive peak exact mass in the beginning, top and end of the peak was taken. Average experimental mass and its RSD are given in Table 2.

In addition to the full scan acquisition method, targeted MS/MS analysis was performed using the mass inclusion list and expected retention times of the target analytes, with a 1 min time window. In this case, the first quadrupole

Table 1. MS/MS parameters for triple quadrupole detection

Compound	Mode	Quantification transition[a]	Confirmation transition[a]	Tube lens voltage (V)	RT (min)
2-Oxy-3-hydroxy-LSD	+	356.2 > 236.8 (20)	356.2 > 221.7 (29)	111	5.60
Amphetamine	+	136.1 > 118.8 (5)	136.1 > 90.9 (17)	85	5.28
Amphetamine D5	+	141.1 > 93.2 (16)		85	5.28
Benzoylecgonine	+	290.0 > 167.8 (17)	290.0 > 76.8 (43)	98	6.18
Benzoylecognine D8	+	298.2 > 170.7 (17)		99	6.18
Cathinone	+	150.1 > 117.2 (21)	150.1 > 131.8 (8)	73	4.78
Citalopram	+	325.2 > 108.8 (27)	325.2 > 261.7 (16)	102	8.36
Cocaine	+	304.1 > 181.7 (17)	304.1 > 104.7 (30)	100	7.35
Cocaine D3	+	307.1 > 184.6 (17)		100	7.35
Codeine	+	300.1 > 214.8 (22)	300.1 > 164.8 (40)	99	5.03
EDDP	+	278.2 > 233.9 (29)	278.2 > 218.8 (40)	102	9.93
Ketamine	+	238.1 > 125.2 (28)	238.1 > 88.9 (49)	92	5.97
LSD	+	324.2 > 207.4 (30)	324.2 > 222.7 (21)	96	7.45
MBDB	+	208.1 > 134.6 (17)	208.1 > 77.0 (38)	74	6.24
MDA	+	180.1 > 134.9 (18)	180.1 > 105.2 (19)	70	5.43
MDA D5	+	185.1 > 137.6 (17)		80	5.43
MDEA	+	208.1 > 162.8 (10)	208.1 > 104.9 (24)	78	6.06
MDMA	+	194.0 > 163.2 (11)	194.0 > 134.8 (20)	87	5.70
MDMA D5	+	199.1 > 165.2 (11)		80	5.70
Mephedrone	+	178.1 > 159.8 (8)	178.1 > 143.8 (28)	73	5.89
Metamphetamine	+	150.1 > 90.9 (17)	150.1 > 65.2 (36)	71	5.60
Metamfetamine D5	+	155.1 > 91.6 (20)		75	5.60
Methadone	+	310.2 > 264.9 (11)	310.2 > 105.1 (27)	83	10.07
Methadone D9	+	319.3 > 267.8 (12)		87	10.07
Methylphenidate	+	234.1 > 84.0 (18)	234.1 > 90.9 (40)	91	6.61
Midazolam	+	326.1 > 290.7 (24)	326.1 > 248.8 (33)	112	8.35
Norbuprenorphine glucuronide	+	590.3 > 413.8 (30)	590.3 > 589.5 (7)	176	5.66
Norketamine	+	224.1 > 124.9 (25)	224.1 > 114.9 (50)	90	5.73
Oxazepam	+	287.0 > 240.8 (21)	287.0 > 268.7 (12)	101	8.25
Oxycodone	+	316.1 > 297.7 (17)	316.1 > 255.9 (23)	101	5.34
Risperidone	+	411.2 > 190.9 (26)	411.2 > 109.8 943)	113	7.50
THC-COOH	+	345.2 > 298.8 (18)	345.2 > 192.7 (23)	105	10.52
THC-COOH D9	+	354.2 > 307.5 (16)		106	10.52
Tramadol	+	264.1 > 58.1 (15)	264.1 > 43.2 (80)	81	5.76
Venlafaxine	+	278.2 > 120.7 (29)	278.2 > 58.1 (18)	97	6.38

[a]Collision energies are given in brackets (eV).

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Table 2. Parameters for HRFS detection

Compound	Chemical formula	Theoretical exact mass	Average experimental exact mass[a] (RSD, ppm)	Confirmation ion[b]
2-Oxy-3-hydroxy-LSD	C20H25N3O3	356.1969	356.197 (0.31)	357.2002
Amphetamine	C9H13N	136.1126	136.1123 (028)	137.1154
Amphetamine D5		141.1435	141.1437 (0.34)	142.1468
Benzoylecgonine	C16H19NO4	290.1387	290.1387 (0.50)	291.142
Benzoylecgonine D8		298.1889	298.1891 (0.23)	299.1923
Cathinone	C9H11NO	150.0913	150.0916 (0.33)	151.0947
Citalopram	C20H21FN2O	325.1711	325.1713 (0.34)	326.1744
Cocaine	C17H21NO4	304.1543	304.1544 (0.32)	305.1577
Cocaine D3		307.1732	307.1734 (0.16)	308.1765
Codeine	C18H21NO3	300.1594	300.1598 (0.46)	301.1628
EDDP	C20H23N	278.1903	278.1905 (0.35)	279.1937
Ketamine	C13H16ClNO	238.0993	238.0994 (0.37)	240.0964
LSD	C20H25N3O	324.207	324.2072 (0.25)	325.2104
MBDB	C12H17NO2	208.1332	208.1336 (0.35)	209.1366
MDA	C10H13NO2	180.1019	180.1021 (0.33)	181.1053
MDA D5		185.1333	185.1333 (0.34)	186.1366
MDEA		208.1332	208.1333 (0.37)	209.1366
MDMA	C11H15NO2	194.1176	194.1179 (0.44)	195.1209
MDMA D5		199.1489	199.149 (0.32)	200.1523
Mephedrone	C11H15NO	178.1226	178.1227 (0.60)	179.126
Methamphetamine	C10H15N	150.1277	150.1279 (0.29)	151.1311
Metamphetamine D5		155.1591	155.1592 (0.32)	156.1625
Methadone	C21H27NO	310.2165	310.2165 (0.22)	311.2199
Methadone D9		319.273	319.2736 (0.59)	320.2764
Methylphenidate	C14H19NO2	234.1489	234.149 (0.49)	235.1522
Midazolam	C18H13ClFN3	326.0855	326.0854 (0.60)	328.0825
Norbuprenorphine glucuronide	C31H43NO10	590.296	590.2947 (0.26)	591.2993
Norketamine	C12H14ClNO	224.0837	224.0839 (0.51)	226.0807
Oxazepam	C15H11ClN2O2	287.0582	287.0583 (0.37)	289.0552
Oxycodone	C18H21NO4	316.1543	316.1547 (0.37)	317.1577
Risperidone	C23H27FN4O2	411.2191	411.2193 (0.46)	412.2224
THC-COOH	C21H28O4	345.206	345.206 (0.36)	346.2094
THC-COOH D9		354.2625	354.2626 (0.28)	355.2659
Tramadol	C16H25NO2	264.1958	264.1961 (0.27)	265.1992
Venlafaxine	C17H27NO2	278.2115	278.2117 (0.23)	279.2148

[a]Calculated as average value for all positive peaks in the sequence (HRFS acquisition) – at the beginning, top and end of the peak.
[b]Second abundant ion in isotopic pattern.

was operated at 0.7 FWHM and the Orbitrap spectrometer was operated at 17 500 FWHM. The AGC target was set to 1 000 000, with maximum injection time of 50 ms. Collision energy values were optimized for all the compounds of interest. Other possible scan types such as data-dependent acquisition or all ion fragmentation were not considered in this study. The reason for that is their poor selectivity in comparison with targeted MS/MS scan regarding complexity of wastewater matrix. Parameters for both HRMS detections are presented in Tables 2 and 3.

Method performance

The performance of the methods (MS/MS, HRFS and HRPS detection) was assessed regarding linearity, limits of quantification (LOQs), trueness, precision and selectivity.

The linearity of the calibration curves was tested in the range relevant for wastewater analysis, from 10 ng L^{-1} to 2000 ng L^{-1}. Calibration curves were measured at the beginning and the end of the sequence to check instrument stability.

LOQs were defined as one-quarter of the lowest point of the calibration curve section where the relative standard deviation (RSD) of the average response factor was <30%. LOQs were corrected relative to the internal standard (IS), and the recovery for each individual analyte and sample was obtained.

Trueness was evaluated from the ratio of the concentration of target analytes in wastewater spiked at three concentration levels: 20, 200 and 1000 ng L^{-1}. Ten replicates were analyzed for each concentration. Matrix effect was assessed for each compound; corrections of ion suppression or enhancement were performed by using matrix-matched standards. Matrix-matched standards were prepared from wastewater spiked with both IS and native compounds at concentration levels of 500 ng L^{-1} and 2000 ng L^{-1}, respectively. The peak

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Table 3. Parameters for HRPS detection

Compound	Mode	Quantification transition (*m/z*)	Confirmation transition (*m/z*)	NCE (%)	RT (min)
2-Oxy-3-hydroxy-LSD	+	237.102	222.0549	35	5.57
Amphetamine	+	119.0856	91.0546	45	5.25
Amphetamine D5	+	93.0672		45	5.25
Benzoylecgonine	+	168.1017	105.0338	50	6.20
Benzoylecognine D8	+	171.1207		50	6.20
Cathinone	+	132.0809	117.0575	45	4.79
Citalopram	+	262.1022	109.0450	40	8.32
Cocaine	+	182.1174	150.0912	45	7.14
Cocaine D3	+	185.1365		45	7.14
Codeine	+	215.1065	243.1014	35	5.10
EDDP	+	234.1274	249.1508	50	9.36
Ketamine	+	179.062	220.0884	40	5.94
LSD	+	223.1225	281.1644	40	7.28
MBDB	+	177.0905	147.0801	35	6.17
MDA	+	163.0751	133.0648	40	5.39
MDA D5	+	168.1067		40	5.39
MDEA	+	163.0752	105.0702	35	5.97
MDMA	+	163.075	133.0647	40	5.65
MDMA D5	+	165.0879		40	5.65
Mephedrone	+	160.1108	145.0884	40	5.84
Metamphetamine	+	119.0855	91.0545	50	5.57
Metamfetamine D5	+	92.0607		50	5.57
Methadone	+	265.1583	105.0338	40	9.54
Methadone D9	+	268.1775		40	9.54
Methylphenidate	+	84.0812	234.1485	40	6.55
Midazolam	+	291.1162	244.0321	50	8.11
Norbuprenorphine glucuronide	+	414.2639	396.2532	35	5.66
Norketamine	+	207.0568	125.0152	30	5.76
Oxazepam	+	241.0524	269.0472	35	8.28
Oxycodone	+	298.1434	256.1328	35	5.32
Risperidone	+	191.1177	110.0603	50	7.40
THC-COOH	+	299.2006	327.1953	30	10.61
THC-COOH D9	+	308.2567		30	10.61
Tramadol	+	58.0658	*	30	6.43
Venlafaxine	+	121.0648	58.0658	35	7.30

*No confirmation ion.

area/IS ratio determined in non-spiked samples was subtracted from the peak area/IS ratio in matrix-matched standards to achieve the matrix-affected response factor.

Precision (repeatability) was assessed by the analysis of ten replicate samples of influent wastewater ('blank' samples) and also samples spiked with target analytes at different concentration levels.

The selectivity of the selected methods was studied. Optimization of mass extraction window (MEW) was carried out for HRMS detection.

RESULTS AND DISCUSSION

Method performance

The quantitative performance of the three mass spectrometric methods was assessed regarding linearity, repeatability, matrix effects, LOQs, and selectivity (obvious interference at quan or qual masses). The parameters of method performance for the three different detection approaches are presented in Tables 4, 5 and 6. The complete data set with the measured concentrations of target analytes using different detection methods is presented in Supplementary Tables S2–S4 (see Supporting Information).

Linearity

In the concentration range from 10 to 2000 ng L^{-1}, all target compounds exhibited good linearity in the three detection methods. R-squared values ranged from 0.996 to 1.

Mass stability

Mass scale stability was demonstrated in HRMS full scan acquisition. Experimental mass was investigated for each analyte throughout the whole sequence for positive peaks (beginning, top and end of the peak). Average experimental masses and RSD values were calculated (Table 2).

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Table 4. Quantitative performance of three MS methods for the tested drugs (repeatability and recovery)

Analyte	Repeatability non-spiked sample (RSD, %)			Repeatability spiked sample (RSD, %)									Ratio determined to spiked concentration (%)[a]								
	MS/MS[b]	HRFS[b]	HRPS[b]	MS/MS[b]			HRFS[b]			HRPS[b]			MS/MS[b]			HRFS[b]			HRPS[b]		
				1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2-Oxy-3-hydroxy-LSD	nd	nd	nd	16	7	20	31	5	3	19	4	4	100	102	113	9	28	105	111	118	100
Amphetamine	nd	nd	4	22	12	8	nd	7	5	3	8	3	113	124	93	348	239	111	na	119	105
Benzoylecgonine	9	15	4	8	8	6	14	7	5	6	9	3	na	125	112	na	99	109	na	134	105
Cathinone	24	nd	nd	29	12	8	30	13	23	8	6	19	34	68	108	0	263	122	139	114	134
Citalopram	22	16	19	6	7	6	3	7	5	5	6	4	na	132	102	na	120	105	na	135	98
Cocaine	9	35	4	7	7	4	9	7	4	4	4	3	na	114	105	na	70	107	na	124	99
Codeine	12	3	17	13	15	10	5	6	10	12	14	5	na	89	118	na	110	106	na	134	107
EDDP	5	nd	4	4	5	16	4	4	3	3	4	3	na	93	100	13	14	103	na	122	104
Ketamine	nd	nd	nd	15	10	5	8	5	4	5	8	3	128	131	105	41	47	104	128	121	104
LSD	nd	nd	nd	16	5	5	10	7	6	7	6	4	107	108	107	58	77	110	132	122	103
MBDB	nd	24	nd	14	6	3	8	6	5	6	9	2	134	131	106	102	102	109	116	119	102
MDA	nd	5	nd	nd	13	5	8	7	3	4	8	4	57	121	93	81	112	107	121	120	105
MDEA	nd	nd	nd	24	8	4	17	7	5	7	5	2	120	123	102	105	100	107	132	119	103
MDMA	23	nd	4	15	6	4	15	7	4	7	4	3	112	106	104	55	41	108	143	124	105
Mephedrone	nd	nd	nd	20	14	5	14	7	3	5	3	3	87	76	100	33	33	104	146	117	102
Methamphetamine	14	5	10	5	4	23	4	3	4	3	6	3	na	na	114	na	na	108	na	na	109
Methadone	6	8	3	5	9	3	5	4	3	5	4	2	82	89	100	11	14	104	122	121	99
Methylphenidate	nd	nd	nd	29	28	4	15	20	6	8	13	2	85	84	102	69	66	101	146	93	98
Midazolam	nd	nd	nd	15	8	4	9	4	6	9	4	3	96	109	104	73	85	109	126	119	101
Norbuprenorphine glucuronide	nd	nd	nd	30	26	27	20	10	8	20	10	7	36	98	123	0	10	105	19	100	105
Norketamine	nd	nd	nd	11	7	3	10	3	4	14	5	3	111	109	105	33	44	106	109	121	106
Oxazepam	21	9	18	8	9	5	3	7	5	4	7	4	na	139	106	na	116	107	na	130	101
Oxycodone	33	nd	nd	36	9	4	13	6	5	6	9	4	78	114	102	218	187	111	107	112	105
Risperidone	nd	nd	nd	10	10	6	17	6	3	8	6	6	117	132	100	62	95	102	134	134	97
THC-COOH	18	61	21	14	6	9	11	6	7	8	6	3	na	98	85	na	132	90	na	107	105
Tramadol	12	6	11	5	8	6	4	6	6	4	6	3	na	na	105	na	na	110	na	na	100
Venlafaxine	16	13	17	6	9	7	3	6	3	5	5	5	na	na	110	na	na	108	na	na	99

[a]Concentration found in non-spiked sample was subtracted, matrix-matched standard quantification was used.
[b]Quantification transition.
1 addition of target compounds at concentration 20 ng L^{-1}. **2** addition of target compounds at concentration 20 ng L^{-1}. **3** addition of target compounds at concentration 20 ng L^{-1}.
na: not applicable – when spiked concentration of target compound was higher than the initial concentration in the sample.

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Table 5. Matrix effects for target analytes

	Matrix effect (%)		
Analyte	**MS/MS**[a]	**HRFS**[a]	**HRPS**[a]
2-Oxy-3-hydroxy-LSD	0	**-38**	**-23**
Amphetamine	7	**45**	16
Benzoylecgonine	–9	**–33**	–9
Cathinone	**28**	**–28**	**–24**
Citalopram	**–42**	**–51**	–19
Cocaine	9	5	14
Codeine	**54**	**41**	**33**
EDDP	**–90**	–9	–6
Ketamine	–9	–17	–12
LSD	**–30**	**–71**	–14
MBDB	0	0	8
MDA	–4	**–39**	10
MDEA	10	–2	16
MDMA	17	8	15
Mephedrone	1	10	17
Metamphetamine	10	17	**29**
Methadone	**–82**	19	13
Methylphenidate	**23**	**52**	**49**
Midazolam	**–40**	**–88**	**–48**
Norbuprenorphine glucuronide	**–80**	**58**	–7
Norketamine	**–64**	**–57**	**–42**
Oxazepam	**–103**	**–185**	**–86**
Oxycodone	**23**	–8	–7
Risperidone	**–38**	**–24**	**–28**
THC-COOH	19	5	**–168**
Tramadol	15	16	19
Venlafaxine	–9	**–64**	–12

[a]Quantification ion. Positive value corresponds to ion suppression, negative to ion enhancement.

Repeatability

Method repeatability was tested for both spiked and non-spiked samples (Table 4). For all methods, the RSD of samples spiked at three different concentration levels was lower than 20% for most of the target analytes. In general, higher RSD values were obtained for the lowest level of fortification: amphetamine (22%), cathinone (29%), MDEA (24%), methylphenidate (29%), oxycodone (36%) in MS/MS detection and 2-oxy-3hydroxy-LSD (31%), cathinone (30%) in HRFS detection. Such compounds as MDA (MS/MS detection) and amphetamine (HRFS detection) were found < LOQ at the spiked level of 20 ng L^{-1}.

Matrix effect and trueness

When the extraction and pre-concentration of the sample is carried out in-line, it is difficult to define recovery of the target analyte separately from the matrix effect, which often causes ionization suppression or enhancement. The observed matrix effects are summarized in Table 5. The matrix effects were assessed by comparing differences between the responses obtained for spiked matrices (matrix-matched standard) and calibration standards prepared in tap water. Significant matrix effects (above 20%, in bold in Table 5) were observed for most of the analytes. In comparison, ion enhancement was prevalent for most compounds and it was of greater magnitude. Only five of all the analyzed drugs did not seem to be affected by the matrix.

Signal suppression or enhancement can be separated into three different groups. Significant ionization related changes can be found for codeine, norketamine and risperidone, where the effect is about the same for both instruments and all techniques. In some cases, the same compounds experienced signal enhancement on one instrument and suppression on another, while using the same ion source. This can be explained by the phenomenon of post-interface signal suppression in the Orbitrap, as demonstrated by Kaufmann *et al.*[25] One of differences between the QqQ and the Q-Exactive is presence of an S-lens in the Q-Exactive. The ion optics and the first quadrupole will always affect the signal the same way for both techniques used (methylphenidate, methadone). Another source of the signal changes can be found in filling of the C-trap which is limited both with ion population and time. In the presence of a rich matrix, minor ions can be influenced in full scan mode but not in HRPS, where ion abundance is much lower, e.g. norbuprenorphine glucuronide. Unfortunately, all above-mentioned processes can affect the resulting signal as can be seen from Table 5.

The trueness of the detection was evaluated using the ratio of the target analytes determined in spiked wastewater to the nominal concentration after subtraction of 'blank' wastewater at three concentration levels. Recoveries for the target compounds were calculated using the response factor of the matrix-matched standard. For most of compounds good recoveries were observed at three fortification levels (MS/MS and HRPS detections), with some exceptions for cathinone, MDA and norbuprenorphine glucuronide when too low recoveries were obtained (Table 4). Regarding HRFS detection, a larger number of compounds showed recoveries out of the acceptable range at concentrations 20 and 200 ng L^{-1}.

LOQs

LOQs for the triple quadrupole instrument were in the range 1.3–15 ng L^{-1} for most of the analyzed compounds (25 out of 27). Exceptions were THC-COOH, amphetamine and MDA with LOQs 20, 24 and 181 ng L^{-1}, respectively.

For the Q-Exactive operated in full scan mode, LOQs ranged from 0.46 to 13 ng L^{-1} for 26 of the analytes. Higher LOQ was observed for amphetamine (54 ng L^{-1}). The LOQs for all of the tested compounds, established using HRPS mode on the Q-Exactive, were 1.7–11 ng L^{-1}.

LOQ values for all the target compounds are presented in Table 6. LOQs for both quantification and confirmation transitions are shown.

While LOQ values were approximately at the same level for the most of the tested drugs, signal-to-noise (S/N) ratio was significantly better for HRMS, for some compounds in HRPS it is difficult to measure S/N ratio due to zero noise in measured window. Examples of chromatograms for methamphetamine are shown in Fig. 1.

Selectivity and mass extraction window

Selectivity is a very important parameter of method performance, especially when a difficult matrix such as untreated wastewater is analyzed. Co-extracted matrix

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Table 6. Quantitative performance of three MS methods for the tested drugs (LOQs and interferences)

Analyte	LOQ quan[a] (ng L^{-1})			LOQ qual[b] (ng L^{-1})			Interferences quan[a]			Interferences qual[b]		
	MS/MS	HRFS	HRPS	MS/MS	HRFS	HRPS	MS/MS	HRFS	HRPS	MS/MS	HRFS	HRPS
2-Oxy-3-hydroxy-LSD	2.5	0.88	3.7	6.6	1.1	3.1	No	No	No	No	No	No
Amphetamine	24	54	3.5	75	63	43	No	Yes	No	Yes	Yes	Yes
Benzoylecgonine	11	6.3	3.2	11	23	3.5	No	No	No	No	No	No
Cathinone	15	17	5.3	79	77	3.7	Yes	Yes	No	Yes	Yes	No
Citalopram	8.1	7.3	8.6	8.3	5.1	15	No	No	No	No	No	No
Cocaine	4.5	12	5.4	7.1	29	7.2	No	Yes	No	No	Yes	No
Codeine	13	5.6	2.7	13	6.8	4.5	No	No	No	No	Yes	No
EDDP	5.3	0.46	7.7	5.4	1.9	7.2	No	No	No	No	Yes	No
Ketamine	3.7	2.1	3.8	14.0	9.9	5.2	No	No	No	No	No	No
LSD	7.1	3.1	2.9	8.7	18	5.2	No	No	No	No	Yes	No
MBDB	5.5	4.2	4.8	8.0	4.1	5.3	No	Yes	No	No	Yes	No
MDA	181	10	4.3	158	58	5.4	Yes	Yes	No	No	Yes	No
MDEA	4.6	4.1	6.4	5.9	4.9	6.7	No	No	No	No	Yes	No
MDMA	4.1	2.7	7.5	4.6	4.4	7.9	No	No	No	No	No	No
Mephedrone	2.2	2.3	7.6	4.5	9.6	7.7	No	No	No	No	No	No
Metamphetamine	4.0	1.7	6.9	4.2	2.1	7.8	No	No	No	No	No	No
Methadone	5.8	0.90	7.1	6	0.71	7.3	No	No	No	No	No	No
Methylphenidate	2.3	3.9	6.3	24	2.8	8.9	No	No	No	Yes	No	Yes
Midazolam	4.4	3.0	1.5	3.6	2.6	3.4	No	No	No	No	No	No
Norbuprenorphine glucuronide	4.6	37	23	188	26	125	No	No	No	Yes	Yes	Yes
Norketamine	1.3	1.9	4.5	136	7.9	5.1	No	No	No	No	No	No
Oxazepam	6.1	3.5	4.5	7	2.4	5.6	No	No	No	No	No	No
Oxycodone	4.8	13	5.1	6.4	53	22	No	Yes	No	No	Yes	No
Risperidone	4.1	7.2	6.8	12	3.9	121	No	No	No	No	Yes	No
THC-COOH	20	2.5	1.7	22	11	13	No	Yes	No	Yes	Yes	Yes
Tramadol	15	11	11	17	5.9	[c]	No	No	No	No	No	[c]
Venlafaxine	11	6.4	7.9	11	6.2	9.1	No	No	No	No	No	No

[a]Quantification transition.
[b]Qualification transition.
[c]No confirmation ion.

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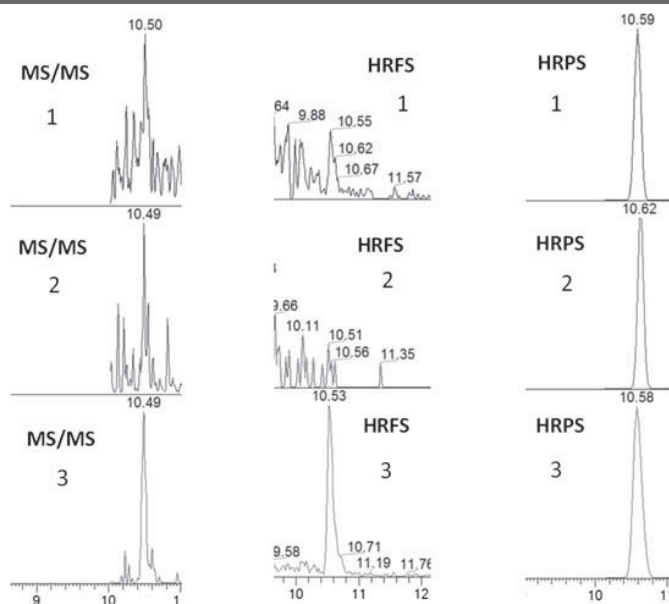

Figure 1. Examples of chromatograms for THC-COOH at the concentration level 10/100 (native/labeled) ng L^{-1}, MEW = 5 ppm (1 quantification ion, 2 confirmation ion, 3 internal standard).

components can cause interferences and affect the reliability of measured data. It is important to evaluate mass resolving power to achieve good balance for selectivity and sensitivity. For QqQ detection a resolution of 0.7 Da at FWHM is routinely used and allows good selectivity/sensitivity ratio. Thus, triple quadrupole instruments with MS/MS detection have demonstrated advantage for the analysis of licit and illicit drugs in complex matrices. In HRMS detection not only resolving power, but also the mass extraction window should be considered in order to improve selectivity. Kaufmann *et al*.[26] have reported that in HRFS a resolution of ≥50 000 (*m/z* 200) and MEW 5 ppm is sufficient to produce equal or better selectivity compared to SRM acquisition at unit mass resolution. Maximum MEWs for our study were calculated according Xia *et al*.[27] The maximum MEW for HRFS acquisition at resolving power 70 000 is 14 ppm, for HRPS acquisition – 57 ppm. Narrower MEWs were tested in order to reduce interferences. As a result of MEW optimization 5 ppm was chosen to be an adequate mass window for the construction of XICs. Narrowing of the MEW down to 2 ppm has resulted in peak distortion for some compounds (Fig. 2). This was addressed more to mass accuracy than mass stability. The masses were stable within the range 0.16 to 0.60 ppm but the differences from theoretical mass were found in the range 0 to 2.2 ppm. The MEW set to 5 ppm covers both uncertainties satisfactorily and there is no need to change the quantification method setting after every recalibration of the mass spectrometer.

In our study, good selectivity for 25 of the 27 target compounds was demonstrated for the triple quadrupole instrument (Table 6). The QqQ was not selective enough for cathinone and MDA because of interferences.

The improved selectivity of Orbitrap-based detection is the result of its high resolution and accurate mass detection, which minimize matrix interferences. Nevertheless, the selectivity of the Q-Exactive in full scan operating mode was not as good as that of the QqQ, because interferences were observed for 7 of the 27 target analytes, complicating their analysis and leading to false positive results. Much better selectivity was obtained when targeted HRPS was chosen. The exact masses of all of the target analytes were added to the inclusion list and time windows were set on the basis of expected retention time for each analyte. Collision energy values were optimized. The HRPS approach showed good results in terms of selectivity for all of the tested compounds. In addition, compounds such as cathinone were detected in MS/MS mode (poor confirmation on qualification ion), but were not found in HRPS mode. We can state that MS/MS can provide false positive data compare to HRPS. There were no interferences observed at the quantification transition in HRPS, and only four interferences on the qualifying ion were found.

Comparison of the three studied MS methods

The advantages and disadvantages of each MS detection method, based on the above-mentioned performance parameters, are summarized in Table 7. For eight (ketamine,

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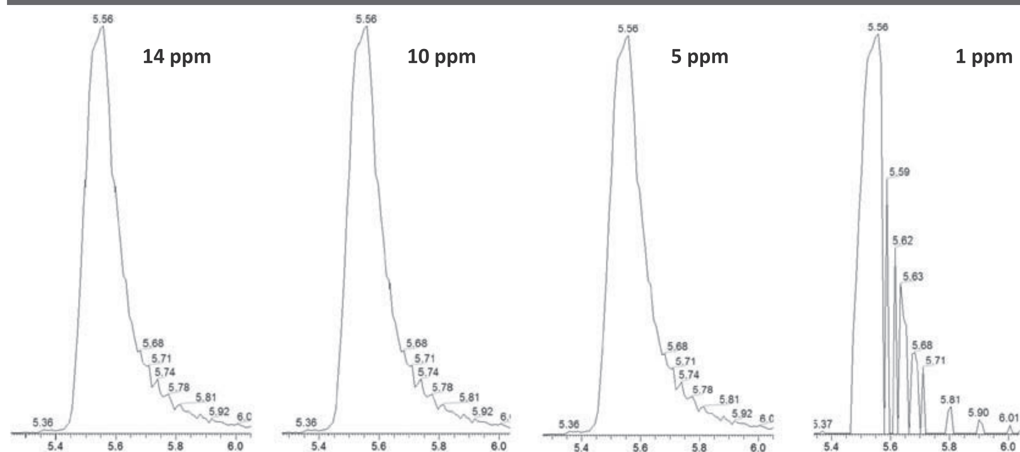

Figure 2. Optimization of MEW for methamphetamine (HRFS detection).

Table 7. Summary of the method performance advantages for each detection

	Repeatability			Matrix effect			LOQ (Selectivity)			Total score		
Analyte	**MS/MS**	**HRFS**	**HRPS**	**MS/MS**	**HRFS**	**HRPS**	**MS/MS**	**HRFS**	**HRPS**	**MS/MS**	**HRFS**	**HRPS**
2-Oxy-3-hydroxy-LSD	1	0	1	1	0	0	1 (1)	1 (1)	1 (1)	4	2	3
Amphetamine	1	1	1	1	0	1	0 (1)	0 (0)	1 (0)	3	1	3
Benzoylecgonine	1	1	1	1	0	1	0 (1)	1 (1)	1 (1)	3	3	4
Cathinone	0	0	1	0	0	0	0 (0)	0 (0)	1 (1)	0	0	3
Citalopram	1	1	1	0	0	1	1 (1)	1 (1)	1 (1)	3	3	4
Cocaine	1	1	1	1	1	1	1 (1)	0 (0)	1 (1)	4	2	4
Codeine	1	1	1	0	0	0	0 (1)	1 (1)	1 (1)	2	3	3
EDDP	1	1	1	0	1	1	1 (1)	1 (0)	1 (1)	3	3	4
Ketamine	1	1	1	1	1	1	1 (1)	1 (1)	1 (1)	4	4	4
LSD	1	1	1	0	0	1	1 (1)	1 (0)	1 (1)	3	2	4
MBDB	1	1	1	1	1	1	1 (1)	1 (0)	1 (1)	4	3	4
MDA	1	1	1	1	0	1	0 (0)	1 (0)	1 (1)	2	2	4
MDEA	1	1	1	1	1	1	1 (1)	1 (0)	1 (1)	4	3	4
MDMA	1	1	1	1	1	1	1 (1)	1 (1)	1 (1)	4	4	4
Mephedrone	1	1	1	1	1	1	1 (1)	1 (1)	1 (1)	4	4	4
Metamphetamine	1	1	1	1	1	0	1 (1)	1 (1)	1 (1)	4	4	3
Methadone	1	1	1	0	1	1	1 (1)	1 (1)	1 (1)	3	4	4
Methylphenidate	1	1	1	0	0	0	1 (1)	1 (1)	1 (1)	3	3	3
Midazolam	1	1	1	0	0	0	1 (1)	1 (1)	1 (1)	3	3	3
Norbuprenorphine glucuronide	0	1	1	0	0	1	1 (1)	1 (1)	1 (1)	2	3	4
Norketamine	1	1	1	0	0	0	1 (1)	1 (1)	1 (1)	3	3	3
Oxazepam	1	1	1	0	0	0	1 (1)	1 (1)	1 (1)	3	3	3
Oxycodone	1	1	1	0	1	1	1 (1)	0 (0)	1 (1)	3	2	4
Risperidone	1	1	1	0	0	0	1 (1)	1 (1)	1 (1)	3	3	3
THC-COOH	1	1	1	1	1	0	0 (1)	1 (0)	1 (1)	3	3	3
Tramadol	1	1	1	1	1	1	1 (1)	1 (1)	1 (1)	4	4	4
Venlafaxine	1	1	1	1	0	1	1 (1)	1 (1)	1 (1)	4	3	4
1 means good performance, 0 means bad performance.										85	77	97

MDMA, mephedrone, midazolam, norketamine, oxazepam, risperidone and tramadol) out of 27 target compounds, all three tested methods had good performance.

The Q-Exactive operated in full scan mode did not show any outstanding results, while targeted HRPS mode showed significant improvements, and had the best performance for

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seven of the analyzed drugs. Improved LOQs and selectivity were observed for amphetamine, benzoylecgonine, cathinone, codeine, MDA, and THC-COOH. For LSD, HRPS was much better in terms of matrix effect, and was the only configuration where the matrix effect was not significant.

For compounds including amphetamine, cathinone, cocaine, MBDB, MDA, oxycodone, and THC-COOH HRFS was inadequate, as interferences were observed and the analysis was not selective enough and led to false positive detection of MDA. In this case, the method of choice was HRPS with the improved selectivity and LOQ parameters.

For two compounds, detection using a triple quadrupole instrument provided the best results. Methylphenidate and norbuprenorphine glucuronide showed better sensitivity and selectivity when analyzed in MS/MS mode, using the QqQ.

Using the quantification of performance parameters given in Table 7, we can order the detection methods as follows: HRFS < MS/MS < HRPS, where the best ranked is HRPS.

CONCLUSIONS

All three tested methods showed good linearity and repeatability. High-resolution techniques (HRFS and HRPS) had advantages in terms of signal-to-noise ratio due to noise absence. For some compounds, a significant improvement in LOQs was achieved with HRFS and HRPS, while for others MS/MS still remains the detection technique of choice.

HRFS did not show good results regarding selectivity. For seven compounds, this approach was unsuitable as too much interference was observed. Carrying out targeted HRPS significantly reduced interference and improved selectivity.

The results obtained allow us to conclude that for most of the tested drugs, the Q-Exactive is equally suitable or better than state of the art MS/MS based on the triple quadrupole. The advantage of this instrument lies in the combination of high-performance quadrupole precursor selection with high-resolution, accurate-mass Orbitrap detection. This detection method significantly improved selectivity and, for some of the analytes, also LOQs. Consequently, HRPS allowed for a more accurate determination of drug residues in a complex matrix such as wastewater. In conclusion, it should be stated that use of HRFS for screening shows some limitations for low molecular weight compounds lacking specific heteroatoms such as chlorine in their structure.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic (CENAKVA No. CZ.1.05/2.1.00/01.0024), the Ministry of Education, Youth and Sports of the Czech Republic (Kontakt II LH12179), the Grant Agency of the University of South Bohemia (No. 047/2010/Z), and the Swedish Foundation for Strategic Environmental Research. We thank the Edanz Agency for English language corrections.

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CHAPTER 3

APPLICATION OF PASSIVE SAMPLING APPROACH FOR WASTEWATER SAMPLING

Fedorova, G., Golovko, O., Randak, T., Grabic, R., 2013. Passive sampling of perfluorinated acids and sulfonates using polar organic chemical integrative samplers. Environmental Science and Pollution Research 20 (3), 1344–1351.

It was allowed by publisher on 6th April, 2013 to include the paper in this Ph.D. thesis.

Environ Sci Pollut Res
DOI 10.1007/s11356-012-1003-0

RESEARCH ARTICLE

Passive sampling of perfluorinated acids and sulfonates using polar organic chemical integrative samplers

Ganna Fedorova · Oksana Golovko · Tomas Randak · Roman Grabic

Received: 29 March 2012 / Accepted: 21 May 2012
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Abstract The applicability of a polar organic chemical integrative sampler (POCIS) for detection and determination of perfluorinated acids and sulfonates in water was studied under field conditions. Standard POCIS configurations (i.e., pharmaceutical and pesticide) were deployed in effluent from a wastewater treatment plant for 1, 2, and 3 weeks. Ten of 15 target compounds were found in POCIS, five of which were quantified in wastewater. Pest-POCIS appeared more effective for the sampling, while Pharm-POCIS had a more rapid uptake kinetic, which leads to faster saturation or equilibrium. The results showed that the pesticide configuration is probably more suitable for the sampling of this class of compounds. Based on average concentration in water over the sampling period and amount of compound adsorbed in the POCIS, we calculated sampling rates for five studied compounds and obtained values of 0.034 to 0.222 L day^{-1}.

Keywords POCIS · Perfluorinated chemicals · Uptake kinetics · Sampling rate · Passive sampling · Field conditions

Introduction

Perfluorinated chemicals (PFCs) belong to the class of chemicals in which the molecular structure includes a polyfluorinated alkyl chain. These chemicals possess unique physical–chemical properties of water and oil repellency and thermal and oxidative resistance, and are widely used in consumer and industrial applications such as food packaging, non-stick polymers, paints, textile coatings, fire-fighting materials, and inert surfactants for semi-conductor etching (Plumlee et al. 2008; Zushi et al. 2011). The beneficial properties also lead to their persistence, toxicity, and bioaccumulation. Recently, studies have reported extensive distribution of PFCs in various environmental compartments, biota, and humans (Giesy and Kannan 2001; Kannan et al. 2004; Karrman et al. 2010; Wang et al. 2011). Perfluorinated acids and sulfonates (PFSAs) are the PFCs of primary concern in water pollution. Due to their solubility and low volatility and vapor pressure, these compounds tend to persist in water (Yamashita et al. 2008). Perfluorinated acids and PFSAs are not only released from point sources. The most abundant representatives of this group, perfluorooctylsulfonate (PFOS) and perfluorooctanoic acid (PFOA), can also be formed as breakdown products of precursor chemicals (Dinglasan et al. 2004; Pan et al. 2011). Perfluorooctylsulfonate has been classified as a persistent organic pollutant under the Stockholm Convention (Tittlemier and Braekevelt 2011).

Domestic and industrial wastewaters are assumed to be the main source of perfluorinated chemicals in aquatic environments (Murakami et al. 2008). Most PFCs are resistant to hydrolysis, photolysis, microbial degradation, and metabolism (Jahnke and Berger 2009). Hence their removal presents significant challenges. Perfluorinated chemicals have been found in wastewater treatment plant (WWTP) effluents and also in river water in several countries (Ericson et al. 2008; Lin et al. 2010; Pan et al. 2011; Eschauzier et al. 2010). Thus, monitoring of PFCs presence in WWTP effluent is necessary to conduct risk assessment for aquatic organisms.

Conventional sampling for perfluorinated chemicals in water is done by grab sampling or the use of automatic samplers

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-012-1003-0) contains supplementary material, which is available to authorized users.

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Published online: 06 June 2012

that produce pooled water samples. There is the lack of information about the passive sampling of PFCs in water, although the approach could be effective, as concentrations of contaminants can fluctuate as a result of sporadic release. In such cases passive sampling can overcome limitations of conventional sampling and provide data on time-integrated concentrations (Bueno et al. 2009). A further advantage of passive sampling is the ability to mimic biological uptake, avoiding use of aquatic organisms for biomonitoring (Alvarez et al. 2005; Kot et al. 2000).

Polar organic chemical integrative sampler (POCIS) is an effective sampling tool for many water soluble compounds. POCIS comprises a sequestration phase (sorbent) sandwiched between two polyethersulfone (PES) membranes. The sampler enables estimation of the cumulative exposure to bioavailable hydrophilic organic chemicals (Arditsoglou and Voutsa 2008). POCIS has been used for sampling polar organic chemicals such as pharmaceuticals, pesticides, and illicit drugs, as well as hormones and UV-blockers (Harman et al. 2011; Lissalde et al. 2011; Zenker et al. 2008; Zhang et al. 2008). Unfortunately, available calibration data for polar organic compounds is limited to a small number of pesticides and pharmaceuticals and was obtained under laboratory conditions (Mazzella et al. 2008; Soderstrom et al. 2009; Bartelt-Hunt et al. 2011). Very recent paper from Kaserzon et al. deals with the laboratory calibration of modified POCIS configuration for 11 selected PFCs (Kaserzon et al. 2012). For now, this is the only published study which reports calibration data for this class of compounds.

The aim of this research was to evaluate the potential of standard POCIS configuration as a sampling tool for PFCs in water and to estimate sampling rates under field conditions.

Materials and methods

Chemicals and solvents

Two standardized configurations of POCIS (Pharm-POCIS and Pest-POCIS) were purchased from Exposmeter AB (Tavelsjö, Sweden). Two hundred milligrams Oasis HLB was used as a sorbent in the Pharm configuration. The OASIS HLB consists of hydrophilic–lipophilic balanced copolymer of [poly(divinylbenzene)-co-N-vinylpyrrolidone]. Sequestration medium of Pest-POCIS consists of a triphasic admixture of a hydroxylated polystyrene-divinylbenzene resin (Isolute ENV+) and a carbonaceous adsorbent (Ambersorb 1500) dispersed on a styrene divinylbenzene copolymer (S-X3 Bio Beads). Detailed information on sorbents is presented in the publication of Alvarez and coauthors (Alvarez et al. 2004).

The following compounds were analyzed: perfluorobutanesulfonate (PFBS), perfluoro-hexanesulfonate (PFHxS), perfluoro-heptanesulfonate (PFHpS), perfluorooctanesulfonate (PFOS), perfluoro-decanesulfonate (PFDS), perfluoro-n-pentanoic acid (PFPeA), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA), perfluoro-n-decanoic acid (PFDA), perfluoro-n-undecanoic acid (PFUdA), perfluoro-n-dodecanoic acid (PFDoA), perfluoro-n-tridecanoic acid (PFTrDA), and perfluoro-n-tetradecanoic acid (PFTeDA). Mixtures of perfluoroalkylcarboxylic acids and perfluoroalkylsulfonates in methanol were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). The surrogate standard solutions of seven mass-labeled (^{13}C) perfluorinated acids (C_4, C_6, C_8, C_9, C_{10}, C_{11}, and C_{12}) and two mass-labeled (^{18}O and ^{13}C) perfluoroalkylsulfonates (C_6 and C_8) in methanol were obtained from the same company.

Methanol (LiChrosolv Hypergrade), acetonitrile (LiChrosolv Hypergrade), toluene (Suprasolv), and dichloromethane (Suprasolv) were purchased from Merck (Darmstadt, Germany). Formic acid was used to acidify mobile phases (Labicom, Olomouc, Czech Republic). Ultra pure water was obtained from the aqua-MAX-Ultra system (Younglin, Kyounggi-do, Korea).

Mixtures of standard solutions of all analytes and surrogate standards were prepared in methanol at a concentration 1 $\mu g\ mL^{-1}$ and stored at 4 °C.

Field deployment of POCIS

Sampling was carried out at the WWTP in České Budějovice, Czech Republic from the January 27 to the February 16, 2011. The site is a mechanical–biological treatment plant with activation, partial nitrification, and thermophile anaerobic sludge stabilization. It has a capacity of 90,000 $m^3\ day^{-1}$ and serves 112,000 inhabitants. The major source of input is wastewater from domestic use, with wastewater from two breweries and a dairy making up less than 5 %.

Both pharmaceutical and pesticide configurations of POCIS were used in the study. Triplicates of each POCIS placed in protective cages were deployed in the effluent water (parcial channel) for 1, 2, and 3 weeks. Mean water velocity at the outlet was 530 Ls^{-1}, and average integrated flow was 45,620 $m^3\ day^{-1}$. At the end of the exposure period, samplers were cleaned with ultrapure water and transported on ice to the laboratory, where they were stored at −18 °C until the extraction procedure.

Simultaneously, pooled water samples were taken by automated sampler (time proportional sampling, ASP-STATION 2000 sampler, Endress+Hauser). Samples were taken at 15-min intervals, and after 24 h were mixed to obtain the day's mean. Each pooled sample was divided into three subsamples, immediately frozen, and stored until analysis.

Water temperature in the effluent channel was continuously monitored during the experiment (Fig. 2).

Extraction of PFCs: POCIS extracts

Chemical residues of interest were extracted from the passive samplers according to standardized procedures (Alvarez et al. 2005). POCIS were carefully disassembled and the sorbent was transferred into glass gravity-flow chromatography columns (1 cm i.d.) plugged with glass wool. Sequestered analytes were recovered from the sorbent by organic solvent elution. Methanol (40 mL) was used for pharmaceutical POCIS configuration. For Pest-POCIS, PFCs were eluted with 50 mL of mixture of dichloromethane/methanol/toluene (8:1:1, *v/v/v*). The internal standard was added at the absolute amount of 20 ng per sample. Extracts were reduced to approximately 1 mL by rotary evaporation, transferred to autosampler vials, and further evaporated to 0.5 mL under a gentle stream of nitrogen.

Quality control was confirmed by analysis of blank samples to assure that target analytes were not introduced from sampling or laboratory procedures and sample handling. Nine isotope-labeled compounds were used as internal standard.

Extraction of PFCs: water samples

Pooled water samples were subjected to minimal treatment procedures. Before analysis they were thawed at room temperature, filtered through 0.45-μm regenerated cellulose filters (Labicom, Olomouc, Czech Republic), and spiked with the same internal standards as POCIS at 1 ng 10 mL^{-1} of sample. Samples were further analyzed using the in-line-SPE-liquid chromatography (LC)-MS/MS method.

LC-MS/MS analysis

POCIS extracts were diluted twice with ultra pure water and then analyzed by conventional LC injection (5 μL of sample injection). Triple-stage quadrupole MS/MS TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA, USA) coupled with Accela 1250 and Accela 650 LC pumps (Thermo Fisher Scientific, San Jose, CA, USA), and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used for analysis.

A Hypersil GOLD Phenyl column (50×2.1 mm ID×3-μm particles, Thermo Fisher Scientific, San Jose, CA, USA), preceded by a guard column (10×2.1 mm i.d, 3-μm particles) of the same packing material and from the same manufacturer, was used for the separation of target compounds. A gradient of MeOH in water, with all solvents acidified by 0.1 % formic acid, was used for elution of analytes. The elution conditions were optimized for the best separation of the matrix from the target compounds.

Heated electrospray in negative ion mode was used for ionization of target compounds. The key parameters were set as follows: ionization voltage, 3.5 kV; sheath gas, 35 arbitrary units and auxiliary gas, 15 arbitrary units; vaporizer temperature, 200 °C; capillary temperature, 325 °C; collision gas, argon at 1.5 mL min^{-1}. Both first and third quadrupole were operated at a resolution of 0.7 FMWH. Two SRM transitions were monitored for all analytes except PFPeA and PFHxA. For PFOS only one transition was used for quantification (m/z 499→499). It was done to prevent underestimation of the concentration due to the structural isomerization of PFOS. Response factors for structural isomers differ in MS/MS detection. For example, when applying transition m/z 499→99 difference in calculation of total PFOS could be 30–40 % depending on the composition of branch isomers (Berger et al. 2011).

Filtered and spiked water samples were analyzed with in-line-SPE-LC-MS/MS configuration, with C18 column (Hypersil Gold, 20×2.1 mm i.d, 12-μm particles, Thermo Fisher Scientific, San Jose, CA, USA) used as an extraction column.

In-line-SPE allows pre-concentration and analysis of the sample in a single run, making large volume samples and time-consuming SPE extraction procedures unnecessary. The extraction and analytical process required 16 min and used only 1 mL of sample.

A high-flow Accela 650LC pump was used for sample loading, and a low-flow Accela 1250LC pump was used for liquid chromatography at the analytical column, which was the same as that used in the POCIS analysis. Analytes retained in the extraction column were flushed with reversed flow of the gradient of MeOH in water and introduced into the analytical column where they were separated and consequently detected by MS/MS. All details of analytical method (mass transitions, collision energies, LC gradients etc.) are given in "Electronic supplementary material" (ESM) Tables S1–S3.

Results and discussion

Method validation and matrix effects

Five point (POCIS analysis from 1 to 100 ng mL^{-1}) and six-point (water samples from 10 to 1,000 ng L^{-1}) calibration curves were prepared by spiking 1 mL of water/methanol mixture (1:1, *v/v*) and 10 mL of water, respectively. A consistent amount of surrogate standard was added, as mentioned. The developed LC-MS/MS (in-line-SPE-LC-MS/MS) chromatographic procedures exhibited linearity, with average R-squared values of R^2=0.9914 and R^2=0.9865 for water and POCIS analysis, respectively.

Limits of quantification (LOQ) were defined as the concentration of the lowest point of the calibration curve (relative standard deviation, RSD, of average response factor <30 %) divided by 4. LOQs ranged from 2 to 11 ng L^{-1} for wastewater samples and 0.3 to 2 ng $POCIS^{-1}$ for POCIS extracts. Both POCIS extracts and water samples were analyzed in triplicate. Complete data with average concentrations and RSDs are given in ESM Table S4.

Co-eluting compounds, originating from the matrix, can cause ion suppression or enhancement (Salvador et al. 2007), consequently, affecting repeatability and trueness of the data. An option for eliminating this effect is the use of isotope-labeled internal standards. In addition to using nine internal standards in the analysis, the matrix effect was evaluated by adding native compounds to the matrix at levels of 1,000 ng L^{-1} to wastewater samples and 100 ng per POCIS to POCIS extract samples. It was assumed that the spiked concentration was approximately two orders of magnitude of the initial concentration in the sample. Matrix effects were assessed by comparing differences between responses obtained for spiked matrices (matrix-matched standard) and calibration standards prepared in solvent or water. POCIS extracts from 1, 2, and 3 weeks of exposure were spiked with target compounds to evaluate the difference dependent on the duration of exposure.

A significant matrix effect (more than 20 % difference from the solvent standard - emphasized with bold italics) was found for four compounds in wastewater samples (Table 1). In POCIS extracts, five compounds were affected by matrix: PFBA, PFBS, PFOA, PFDS, and PFTeDA. For PFBS, there were significant differences among 1, 2, and 3 weeks of exposure. Evidently, duration of the exposure can have an impact on quantification of some of the compounds. Thus, the matrix effect was corrected separately for each week.

Uptake kinetics of target compounds

The calibration experiment was carried out under the conditions of the WWTP's effluent: high water velocity, relatively high content of target analytes, heavy matrix, and possible biofouling. Water concentration of target compounds in effluent was stable (Fig. 1). No trends in concentration changes were observed during the exposure period.

Another important factor that can affect sampling rate is temperature. The kinetics of diffusion processes at the membrane–water phase is temperature dependent (Bueno et al. 2009). During the exposure period, temperature and total 24-h integrated flow were stable (Fig. 2). This provided good conditions for calibration.

We aimed to evaluate the suitability of POCIS as a sampling tool for selected PFCs and to determine kinetic regimes and the corresponding sampling rates. Ten of 15 target compounds were found above the limits of quantification in POCIS. The five PFCs with the longest alkyl chain (one perfluoroalkylsulfonate and four perfluoroalkylcarboxilic acids) were not detected in either Pharm or Pest-POCIS configuration.

Differences among uptake kinetics are shown in Fig. 1. Perfluorooctanoic acid, PFOS, and PFHxS showed the typical accumulation pattern. For PFHxS, both POCIS configurations showed linear uptake during 21 days of exposure. This was also found for PFDA, whereas accumulation of PFHpS was linear only for Pharm-POCIS; and PFNA only for Pest-POCIS (coefficients of linear regression for all compounds are given in Table 2).

Table 1 Matrix effect in wastewater (WW) samples and POCIS extracts compound

	WW, %	Pharm-POCIS, %			Pest-POCIS, %		
		7 days	14 days	21 days	7 days	14 days	21 days
PFPeA	***38***	−5	−2	−2	−2	−7	−8
PFBS	***55***	***−33***	8	***22***	11	16	19
PFHxA	−9	6	8	9	8	10	6
PFHxS	2	−1	5	9	4	7	6
PFHpA	5	5	4	3	7	5	−2
PFHpS	−2	−14	−15	−8	−11	−7	−10
PFOA	−11	***23***	20	15	***23***	18	10
PFOS	−2	−17	−1	9	2	1	13
PFNA	−12	−18	−2	−9	9	0	−4
PFDA	−7	10	11	11	12	7	8
PFUdA	−12	10	11	11	13	12	11
PFDS	***43***	***−83***	***−25***	***21***	−18	−13	19
PFDoA	8	8	12	16	14	11	10
PFTrDA	16	−12	−1	9	−3	−4	−3
PFTeDA	***−43***	***−47***	***−35***	***−25***	***−35***	***−35***	***nn***

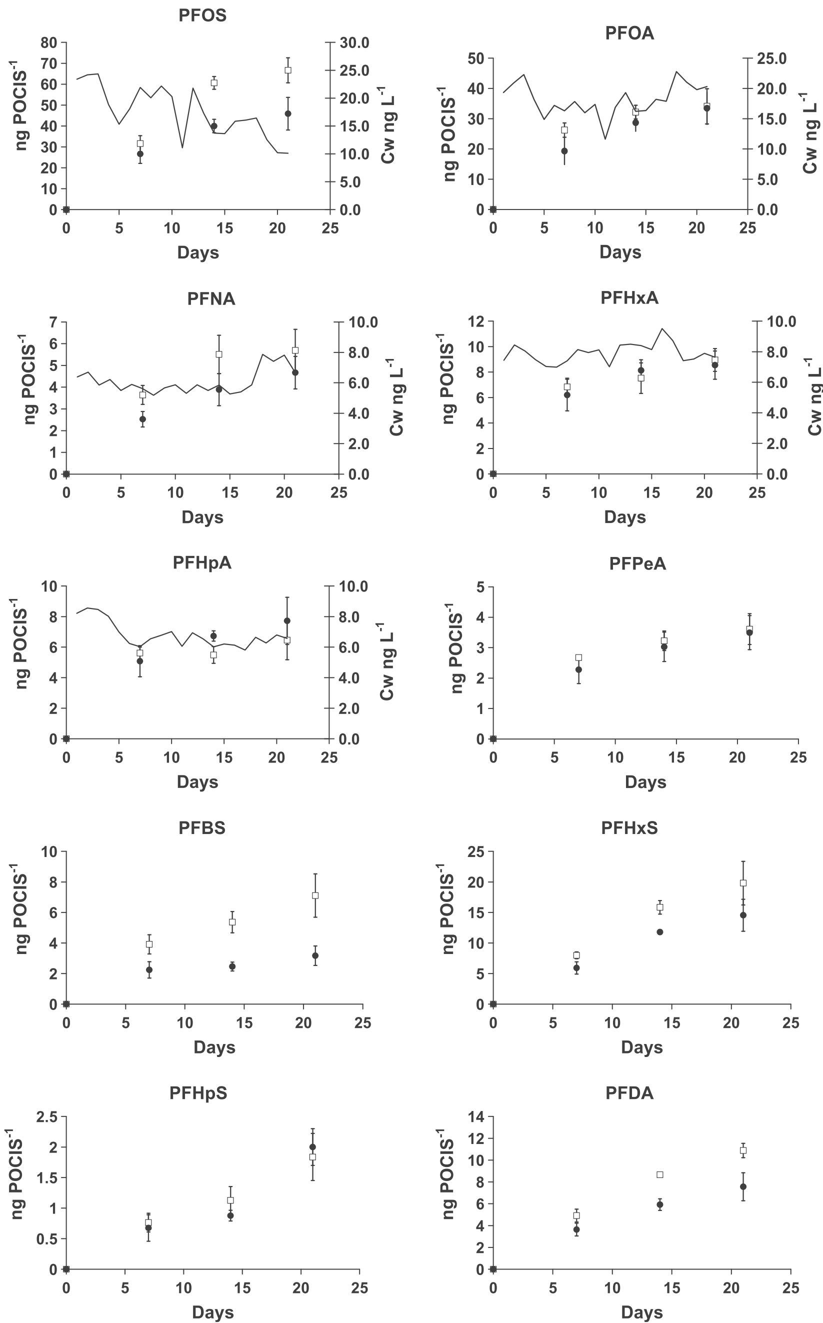

Fig. 1 Uptake in POCIS (*left axis*, *n*=3) and water concentrations (*right axis*) of target compounds (*white*—Pharm-POCIS, *black*—Pest-POCIS)

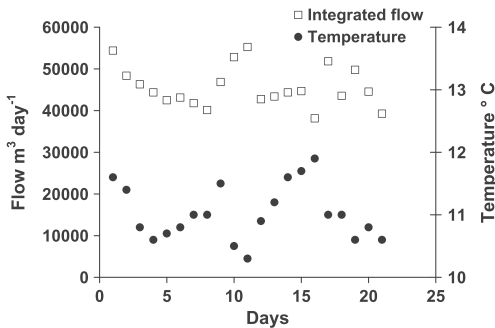

Fig. 2 Wastewater temperature and flow rates (total integrated 24-h flow) during the exposure period

Perfluorooctylsulfonate and PFOA are typical representatives of curvilinear uptake, which was observed for both configurations. However, for PFOS it was possible to calculate sampling rates for a 14-day period, as uptake was linear during that time (coefficients of linear regression were 0.964 and 0.999 for Pest and Pharm POCIS, respectively). The accumulation curve of PFOA for Pharm-POCIS demonstrated that dynamic equilibrium was reached rapidly or the sampler became saturated, i.e., uptake was not linear even during the initial 2-week period. The correlation coefficients in Table 2 confirm that the average slopes over 14 days also do not show linearity for PFHxA, PFHpA, PFPeA, and PFOA.

For the pesticide configuration of POCIS, only PFBS did not fit linear uptake. Hence, Pest-POCIS possesses a higher capacity for sampling PFCs, while Pharm-POCIS reached equilibrium or saturation much faster. Biofouling is not involved in this process, because it should equally affect both configurations. The uptake differences between POCIS configurations can be probably explained by the different sorbents used.

Sampling rates

Sampling rates of selected PFCs were calculated from amount of the compounds adsorbed in POCIS and water concentration according to the following equation:

$$Rs = M_{\mathrm{POCIS}} \cdot C_{\mathrm{W}}^{-1} \cdot t^{-1} \quad (1)$$

Where Rs is sampling rate (liters per day), M_{POCIS} is the amount of target compound in the POCIS (nanograms), C_{W}=average water concentration (nanograms per liter), and t=exposure period (days). This equation is valid for the linear stage of uptake. Thus, for each compound we used the period of time during which linear uptake was observed.

Sampling rates of the studied PFCs for both POCIS configurations are listed in Table 2. As only five compounds were found in water samples above LOQ, sampling rates for five PFCs were calculated only. All sampling rates for both types of samplers and different exposure periods are plotted on the ESM Fig. S1. For PFHxA, PFHpA, and PFOA no

Table 2 Average sampling rates (Rs) and uptake linearity of PFCs in pesticide and pharmaceutical POCIS

Compound	Pest-POCIS				Pharm-POCIS			
	Rs, L day^{-1}	RSD[a], %	Linearity over 14 days[b]	Linearity over 21 days[b]	Rs, L day^{-1}	RSD[a], %	Linearity over 14 days[b]	Linearity over 21 days[b]
Perfluorosulfonates								
PFOS	0.147[c]	7	0.965	0.913	0.222[c]	16	0.999	0.943
PFBS	ND	–	0.817	0.840	ND	–	0.935	0.943
PFHxS	ND	–	1	0.976	ND	–	1	0.979
PFHpS	ND	–	0.911	0.929	ND	–	0.960	0.984
Perfluorocarboxylates								
PFHxA	0.073[c]	10	0.916	0.813	–	–	0.817	0.797
PFHpA	0.063[c]	5	0.920	0.871	–	–	0.733	0.707
PFOA	0.117[c]	10	0.962	0.917	–	–	0.882	0.783
PFNA	0.034[d]	21	0.971	0.938	0.065[c]	7	0.967	0.857
PFPeA	ND	–	0.922	0.875	ND	–	0.873	0.811
PFDA	ND	–	0.983	0.968	ND	–	0.994	0.973

ND compounds were not detected in water samples

[a] Relative standard deviation of the triplicates

[b] Correlation coefficients of linear regression

[c] Calculated for 14-days exposure period

[d] Calculated for 21-days exposure period

sampling rates are reported for Pharm-POCIS, as the uptake was not linear even for a 2 week sampling period. Rs values ranged from 0.034 to 0.222 $\mathrm{L\,day^{-1}}$. Consequently, use of POCIS for water sampling equates to sampling and concentration of about 3 L water over 14 days. It would give an advantage over the common extraction techniques with respect to limits of detection.

Conclusions

These data represent the applicability of POCIS for time-integrative water sampling for perfluorinated carboxylates and perfluorinated sulfonates. Ten of 15 analyzed PFCs were found in both standard POCIS configurations, with only five of them detected in wastewater. The duration of linear uptake was determined for both POCIS. Pest-POCIS seems to have a higher capacity for sampling of PFCs while Pharm-POCIS has a faster uptake kinetic, which leads to saturation or equilibrium. Linearity of uptake is superior for the Pest configuration. Up to date, these are the first calibration data available for the standard POCIS.

Acknowledgments This study was supported by Ministry of Education, Youth and Sports of Czech Republic CENAKVA no. CZ.1.05/2.1.00/01.0024, the Grant Agency of the University of South Bohemia no. 047/2010/Z, and Ministry of Education, Youth and Sports of Czech Republic Kontakt II LH12179. We thank Jiri Stara and other colleagues at CEVAK a.s. (Budweiss, Czech Republic) for help with sample collection. We thank Lucidus Consultancy for language correction of the manuscript.

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CHAPTER 4

WASTEWATER ANALYSIS: PERSPECTIVES AND COMPLICATIONS

4.1. Singer A.C., Järhult J.D., Grabic R., Khan G.A., Fedorova G., Fick J., Lindberg R.H., Bowes M.J., Olsen B. Söderström H., 2013. Compliance to oseltamivir among two populations in Oxfordshire, United Kingdom affected by influenza A(H1N1)pdm09, November 2009 – a wastewater epidemiology study. PloS One 8 (4), 1–8.

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Compliance to Oseltamivir among Two Populations in Oxfordshire, United Kingdom Affected by Influenza A(H1N1)pdm09, November 2009 – A Waste Water Epidemiology Study

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Abstract

Antiviral provision remains the focus of many pandemic preparedness plans, however, there is considerable uncertainty regarding antiviral compliance rates. Here we employ a waste water epidemiology approach to estimate oseltamivir (Tamiflu®) compliance. Oseltamivir carboxylate (oseltamivir's active metabolite) was recovered from two waste water treatment plant (WWTP) catchments within the United Kingdom at the peak of the autumnal wave of the 2009 Influenza A (H1N1)pdm09 pandemic. Predictions of oseltamivir consumption from detected levels were compared with two sources of national government statistics to derive compliance rates. Scenario and sensitivity analysis indicated between 3–4 and 120–154 people were using oseltamivir during the study period in the two WWTP catchments and a compliance rate between 45–60%. With approximately half the collected antivirals going unused, there is a clear need to alter public health messages to improve compliance. We argue that a near real-time understanding of drug compliance at the scale of the waste water treatment plant (hundreds to millions of people) can potentially help public health messages become more timely, targeted, and demographically sensitive, while potentially leading to less mis- and un-used antiviral, less wastage and ultimately a more robust and efficacious pandemic preparedness plan.

Citation: Singer AC, Järhult JD, Grabic R, Khan GA, Fedorova G, et al. (2013) Compliance to Oseltamivir among Two Populations in Oxfordshire, United Kingdom Affected by Influenza A(H1N1)pdm09, November 2009 – A Waste Water Epidemiology Study. PLoS ONE 8(4): e60221. doi:10.1371/journal.pone.0060221

Editor: Richard Joseph Sugrue, Nanyang Technical University, United States of America

Received November 15, 2012; **Accepted** February 18, 2013; **Published** April 15, 2013

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Funding: The authors received funding for the collection and analysis from the Swedish Research Council Formas, the Natural Environment Research Council–Knowledge Transfer (PREPARE) Initiative contract NE/F009216/1, the Ministry of Education, Youth and Sports of the Czech Republic CENAKVA No. CZ.1.05/2.1.00/01.0024 and the Grant Agency of the University of South Bohemia No. 047/2010/Z. The authors received in-kind support from G.F.F. Hoffman – La Roche Ltd. through donated deuterated OC. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors received funding from a commercial source (La Roche Ltd.). There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

An influenza pandemic is regarded as one of the most significant civil emergency risks with major global human health consequences and the potential to cause significant social and economic damage and disruption [1,2]. One of the few options for alleviating the human health burden from an influenza pandemic is the use of pharmaceuticals such as antivirals. Vaccine provision and non-pharmaceutical measures (e.g., closing schools and/or borders, hand-washing) represent two other widely used infection mitigation approaches employed in national preparedness plans. Numerous countries world-wide distributed courses of antivirals for prophylaxis and treatment of influenza-like illness (ILI) during the 2009 Influenza A(H1N1)pdm09 pandemic, with the majority of the antiviral stockpiles consisting of oseltamivir (Tamiflu®) [3].

The United Kingdom Health Protection Agency (HPA) QSurveillance National Syndromic Surveillance System monitored a range of clinical and syndromic indicators that were indicative of influenza activity. This system was established when antiviral drugs were deployed during the pandemic in the U.K. The National Pandemic Flu Service (NPFS) was responsible for the provision of information, syndromic diagnosis, and prescription and dispensing of antiviral courses in the UK.

Compliance rates to the prescribed antiviral course (i.e., one dose of Tamiflu per day for prophylaxis and two doses per day for treatment) was first reported in the U.K. three months after the outset of the pandemic [4–8]. Compliance was highly varied, ranging between 48 to 97%, with a narrow age range of study participants, <14 yrs of age. To our knowledge, compliance rates for the period of the second, autumnal wave of the 2009 influenza pandemic in the U.K. was not collected, nor was there any significant body of research to draw upon for predicting compliance in >14 yr olds at any point during the 2009 pandemic. This knowledge gap greatly hinders the ability of

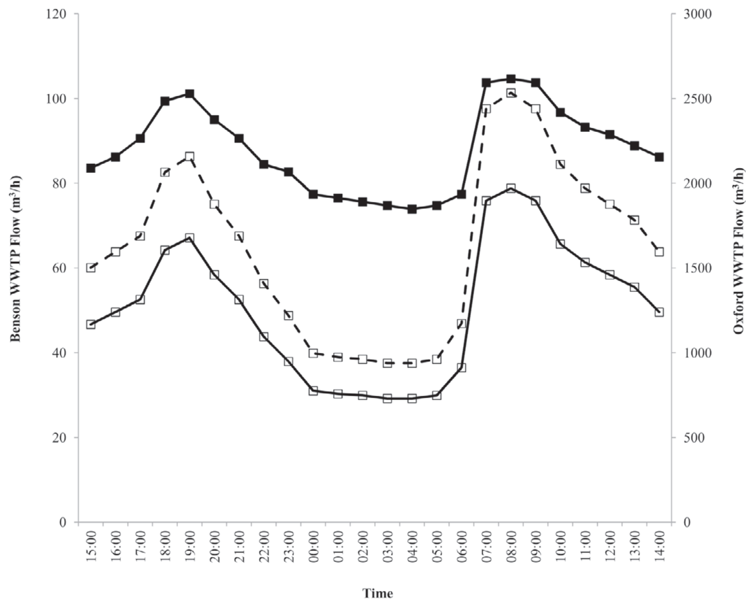

Figure 1. Waste Water Treatment Plant Flow. Hourly flow (m^3/h) from Benson (open) and Oxford (shaded) WWTP on sampling days 11 November 2009 (solid line) and 10 May 2011 (dashed line).
doi:10.1371/journal.pone.0060221.g001

government and public health planners to proactively address the problem of compliance and the issues it generates.

Poor compliance drains resources by diverting limited antiviral stocks from those who may need it most. Mis- and un-used antivirals can lead to the hastening of antiviral resistance in cases where influenza-infected people do not comply with the prescribed course and dosing regimen. The provision of antivirals that remain unused also represents a significant financial cost to governments [9]. Antiviral non-compliance can also influence the success of inter-related public health plans, such as combating secondary bacterial infections in influenza cases. The provision of antivirals is expected to decrease the need for antibiotics by an estimated ~50% owing to a reported decline in secondary infections in antiviral users [3,10,11]. For these reasons, the pandemic influenza medical response and the national pandemic preparedness plan will remain unnecessarily vulnerable without greater certainty with respect to human behaviour – more specifically, antiviral compliance.

Oseltamivir carboxylate (OC; oseltamivirs active metabolite) is frequently demonstrated to be a conservative chemical in waste- and fresh-water systems [12–20], and as such, represents an ideal tracer for the waste water forensic epidemiology approach [21–23]. In this epidemiological study we evaluated the load of OC in waste water as an unbiased measure of Tamiflu consumption during an influenza pandemic. The OC levels in influent of two waste water treatment plants (WWTPs) located at Benson (51.61562, −1.10945) and Oxford (51.71384, −1.21545), in Oxfordshire, England, were measured during the peak of the 2009 Influenza A(H1N1)pdm09 pandemic [24]. Measured OC was compared with two complementary sources of national government statistics to assess compliance rates. It is proposed that an empirically-derived estimate of compliance, recorded in near-real time, can help to inform and prioritise public health messages at the spatial resolution of the WWTP catchment, which can range from a population of a few thousand in rural areas to over a 1 million in highly urban areas. We argue that this granular understanding of non-compliance can help public health messages become more targeted and efficacious, leading to less mis- and un-used antivirals, cost savings and a more robust preparedness plan.

Methods

Waste Water Sampling

An urban and a rural WWTP were chosen for this study to reflect potentially different pharmaceutical use patterns in the two catchment populations. The rural WWTP at Benson England, serves a population of 6,230 people with a consented dry weather flow of 2,517 m^3/d and an annual average dry weather flow (DWF) of 1,368 m^3/d. The Benson WWTP has a hydraulic retention time of 7–8 h at dry weather flow and consists of trickling filters as the main biological treatment step. The urban WWTP at Oxford serves a population of 208,000 with a consented dry weather flow of 50,965 m^3/d and an annual mean DWF of 38,000 m^3/d. The Oxford WWTP has a hydraulic retention time of 15–18 h, and utilizes activated sludge as the main biological treatment step. Both WWTPs have primary and secondary sedimentation steps. The Oxford and Benson sewer systems receive flow from a number of pumping stations either running in series to the site along the sewer network or in parallel from sub-catchments.

Thames Water Utilities Limited provided access to both WWTP; all necessary permits were obtained for described field

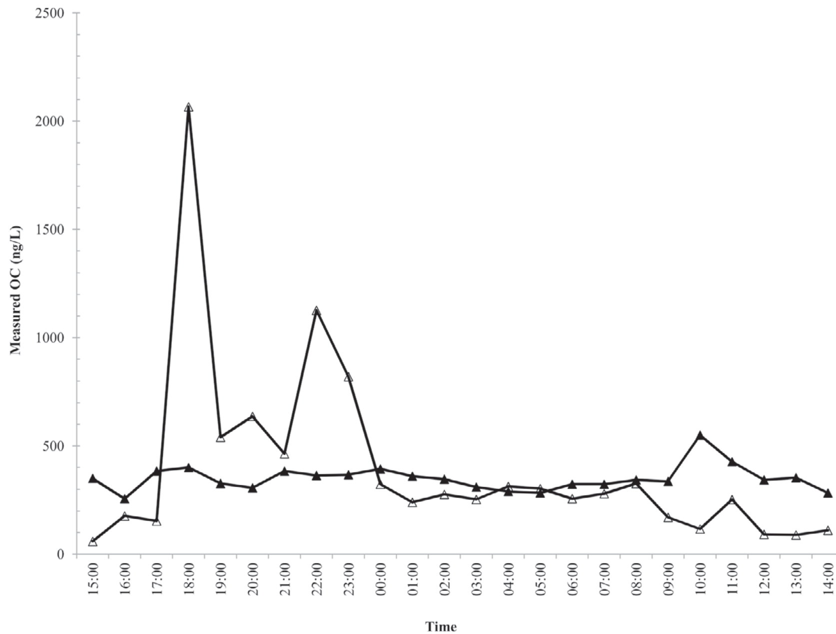

Figure 2. Oseltamivir Carboxylate Concentration in Waste Water. Hourly time-proportional influent concentration of OC (ng/L) in Oxford WWTP (shaded) and Benson WWTP (open).
doi:10.1371/journal.pone.0060221.g002

studies, including the Thames Water Operational Safety Authorization (TWOSA). Each WWTP was sampled using an automated sampler scheduled to recover a time-proportional sample (approximately 750 mL) of influent every hour for 24 hours. Sampling commenced at 15:00 on Nov 10th, with the last sample taken at 14:00 on Nov 11th 2009. At its completion, samples were stored, in triplicate, in 50-mL borosilicate glass vials with PTFE-lined caps at −80°C. Samples were shipped frozen to Umeå University, Sweden, where they were stored at −20°C until analysis.

OC was converted to mass loading using hourly WWTP flows for the sampling period (Figure 1). Flows were determined in consultation with Thames Water, the WWTP operator. Flows at both WWTPs peaked between 07:00 to 9:00 and again from 18:00 to 19:00. An additional 24-h sampling was initiated at 10:00 on 15 May, 2011 from only the Benson WWTP effluent for the purpose of confirming the background concentration of antiviral during the inter-pandemic period, which officially began on 10 August, 2010 [25].

Environmental Conditions

Precipitation on 11 November, 2009 (3.3 mm) was approximately 1.0 mm below the monthly average for November (4.25 mm) [26]. The 24-hour mean flow for Benson WWTP was 1,210 m^3/d, 13% below the average annual dry weather flow. Oxford WWTP's daily flow was 52,828 m^3/d, 28% higher than the annual average dry weather flow for the same time period. No precipitation occurred within the previous 48 h of the May 11, 2011 sampling point of the Benson WWTP influent where the mean 24-h flow was 1556 m^3/d. The temperature during the sampling period ranged from 0.7 to 10.5°C and 6 to 19.6°C over the November 11, 2009 and May 11, 2011 sampling periods, respectively [26].

Measurement of OC in Waste Water

An on-line solid phase liquid extraction/liquid chromatography-tandem mass-spectrometry (SPE/LC-MS/MS) system was used to measure the OC levels in the samples collected at Benson and Oxford WWTPs in southern Oxfordshire, England. The SPE/LC-MS/MS system used has been evaluated and described in details previously [27]. Briefly, 1 mL of 5 mL pre-filtered (0.45 μm pore size) sample was analyzed by the SPE/LC-MS/MS system. The samples were quantified using a deuterated OC internal standard, with six calibration points. The limit of quantification (LOQ) was 2 ng/L.

Predicting Tamiflu Consumption

Two methods were used for predicting Tamiflu consumption within the two WWTP catchments, based on data from the National Pandemic Flu Service (NPFS) [24] and the HPA/QSurveillance National Syndromic Surveillance System (HPA) [28]. The NPFS recorded the collection of 1,079,179 courses of antiviral treatment in England between the launch of NPFS in July 2009 to February 2010, when it ceased operation, equating to ~2% of the population. Using these values, an estimated 132 courses of antivirals were dispensed in the Benson WWTP catchment and 4,401 in the Oxford WWTP catchment over the same period of time. Approximately 66,218 courses of antiviral

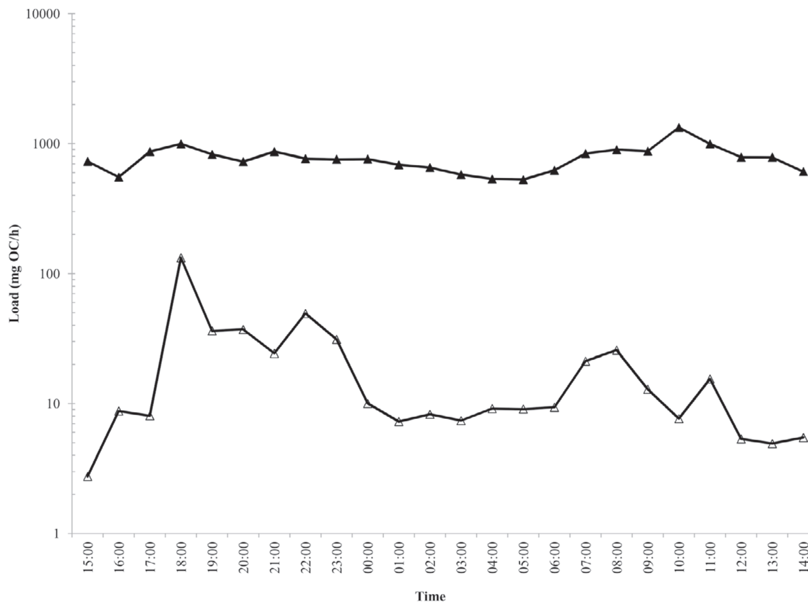

Figure 3. Oseltamivir Carboxylate Load in Waste Water. Calculated hourly influent load of OC (mg/h) for Oxford WWTP (closed) and Benson WWTP (open).
doi:10.1371/journal.pone.0060221.g003

(6% of all antivirals dispensed) were dispensed in Week 43 (3 weeks prior to the WWTP sampling), the national peak for the autumnal wave of the 2009 influenza pandemic [24], equating to approximately 0.13% of the population of England receiving antiviral. An exact amount of antivirals dispensed for Week 46 (the week of the study) was not available, however, it is estimated that there was less than a 10% decline in antiviral dispensing by Week 46, thereby making any antiviral allocation differences between Week 43 and 46 negligible (see Figure 15 in ref [24] for antiviral collections during the pandemic). These predictions translate into: 8 and 270 courses of antiviral collected within the Benson and Oxford WWTPs catchment during the week of sampling (10 November, 2009). The following compliance scenarios were examined: 40, 45, 50, 55, 60, 70 or 100% compliance (i.e. 40–100% of those collecting Tamiflu would use it, as directed). The standard dosing regime was assumed: 0.075 g per dose, consumed twice per day (0.150 g/d).

The HPA dataset reports 54.2 people per 100,000 with ILI in the Oxfordshire PCT during Week 46 (inclusive of both the Oxford and Benson WWTP catchments). This prediction translates to 3·4 and 112·7 cases of ILI in the Benson and Oxford catchments, respectively. In addition to the seven compliance scenarios previously mentioned for the NPFS dataset, an additional scenario was needed for the HPA dataset: 50 or 100% of the cases of ILI were prescribed antiviral. This additional scenario was examined because only ~50% of ILI cases are clinically-diagnosed with influenza. The antiviral prescription rate for ILI might be expected to more closely approximate 100% than the more clinically-accurate 50% prescription rate, as syndromic diagnosis prevails during a pandemic, with clinical diagnosis more the exception than the rule.

Predicted OC Concentrations in Waste wsater

The projected concentration of OC in the waste water (ng/L) was calculated using Equation 1,

$$\frac{D_P \times D_M}{P \times L} \times 10^9 \tag{1}$$

where the product of the population of each catchment (P; Benson = 6230 and Oxford = 208000) and the volume of waste water per person (L) (230 L) was divided into the product of population predicted to consume Tamiflu (D_p) and the mass equivalent of OC consumed per day in grams (D_M; 0·15 g/d is the defined daily dose (DDD) for Tamiflu for treatment purposes).

An additional scenario that assumes either 80 or 100% of the Tamiflu dose was recovered as OC in WWTP influent was employed when predicting concentrations of OC in waste water or back-calculating from waste water to Tamiflu users. This scenario was employed as 100% of the Tamiflu dose is excreted into the waste water, with up to 20% in the form of the prodrug oseltamivir. However, the prodrug can potentially transform in the waste water to the active antiviral, OC, leading to a theoretical maximum of 100% of consumed Tamfilu recovered as OC [29]. This scenario was necessary because the parent prodrug was not monitored in the waste water. If the ratio of parent compound to active metabolite was found to be approximating 1:4, a default scenario of 80% would be sufficient.

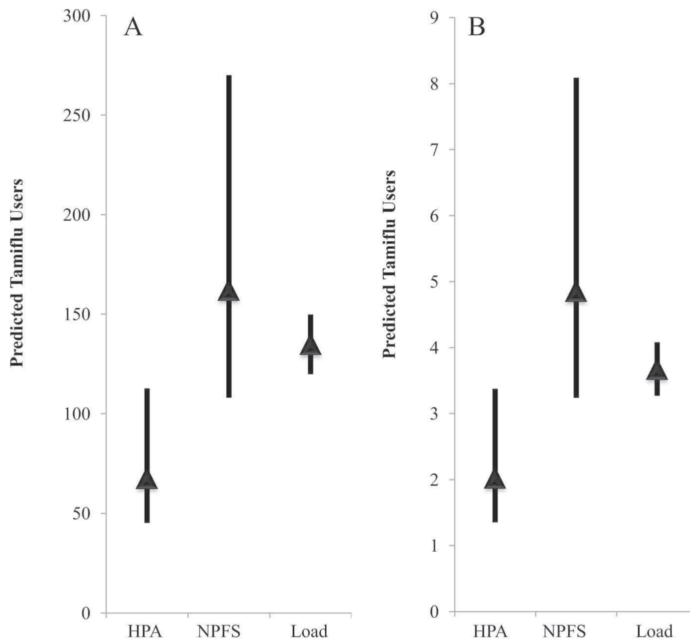

Figure 4. Scenario projections for Tamiflu-consumption. Tamiflu consumption during the 24 hour influent sampling at Oxford (A) and Benson (B) WWTPs based on HPA or NPFS statistics and varying compliance, Tamiflu metabolism, and ILI prescription rates were as detailed in the Materials and Methods. Load represents back-calculated predicted number of Tamiflu users for the 24-h sampling period assuming 80 or 100% OC in the waste stream. The mean of the scenarios is represented by a shaded triangle.
doi:10.1371/journal.pone.0060221.g004

Results

Measured Antiviral Concentrations or Load in Waste water

The concentration of OC in the influent of Benson WWTP ranged from 59 to 2,070 ng/L, with a mean of 394±435 ng/L (Figure 2). The average load for the 24 h period was 490±26.9 mg/h (Figure 3). The concentration of OC in the influent of Oxford WWTP ranged from 257 to 550 ng/L, with a mean of 350±60 ng/L (Figure 2). The average load for the 24 h period was 17,979±179 mg/h (Figure 3). OC was not recovered (<2.0 ng/L) in the effluent of Benson WWTP during the 'inter-pandemic' sampling period, as anticipated.

Predicted Use of Antiviral from Scenarios

When the full range of compliance (40, 45, 50, 55, 60, 70, 100%) and antiviral allocation rates (50 or 100% of ILI cases) were considered using the HPA population statistics, the Tamiflu-using population was predicted to be between 1.35 and 3.37 people in Benson and 45.1 and 112.7 people in Oxford WWTP catchments (0.02–0.05% of the respective catchment populations; Figure 4).

When the full range of compliance scenarios were considered using the NFPS Tamiflu allocation statistics, the Tamiflu-using population was predicted to be between 3.24 and 8.09 people in Benson and 108 and 270 in Oxford WWTP catchments, respectively (0.05–0.13% of the respective catchment populations; Figure 4).

Predicted Antiviral released into Waste Water from Scenarios

Predicted concentrations of OC in the Benson and Oxford WWTP (calculated using Equation 1 including all scenarios discussed in the Material & Methods Section), range from 57 to 847 ng/L (Figure 5). HPA-based scenarios ranged from 57 to 282 ng/L, while NPFS-based scenarios ranged from 270 to 846 ng/L. All the HPA-based scenarios yielded OC concentrations below the measured concentration for Benson (mean 394 ng/L), while only one scenario exceeded the measured concentration for Oxford (mean 350 ng/L). This one scenario was the most conservative, assuming: 100% compliance, 100% of ILI cases were allocated antiviral, and 100% of Tamiflu was recoverable as OC (353 ng/L). Given the unlikely nature of these conservative assumptions, it is argued that the NFPS dataset is a better reflection of antiviral use in the community.

Predicted Consumption of Antiviral from Measured Load

Measured concentrations of OC were converted to units of Tamiflu users per day. All calculations considered the possibility that the measured concentration of OC reflects either 80 or 100% of the daily dose (0.15 g/d), and in the case of the HPA dataset, it also considered the option that either 50 or 100% of people recorded with ILI were prescribed antiviral. Predicted Tamiflu use from the total 24-h load of OC ranged from 3 to 4 people (0.5–0.6% of the catchment population) in the Benson WWTP catchment and from 120 to 154 people (0.6 to 0.7%) in the

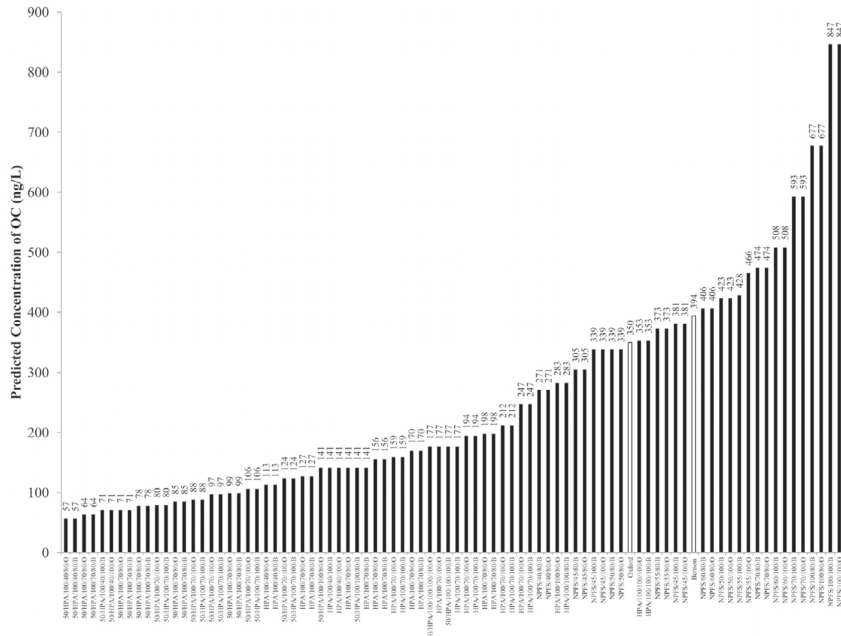

Figure 5. Scenario predictions of OC influent concentration (ng/L). Scenario codes can be interpreted as follows: percent of ILI cases prescribed OC, used only when HPA data was employed (assumed =100, unless specified as 50)/Source of data (NPFS or HPA)/percent compliance (40, 45, 50, 55, 60, 70, 100%)/percent of parent compound converted to OC before WWTP inlet (80, 100%)/O = Oxford WWTP and B = Benson WWTP. Mean 24-h OC concentrations at Benson and Oxford are noted by the WWTP name, only. Daily water usage was assumed to be the UK national average of 230 L/capita [3].

doi:10.1371/journal.pone.0060221.g005

Oxford WWTP catchment (Figure 4). Projections remained consistent even when the 24-h mean concentration was used in lieu of load (3 to 4 and 123 to 154 people for Benson and Oxford, respectively).

Discussion

In this study, Tamiflu use and compliance were predicted from measured OC in waste water influent and through the use of national government statistics on cases of ILI (HPA) and antiviral dispensing (NFPS). Measured concentrations of OC in waste water were consistent with NFPS-derived scenarios. This is not surprising given that NFPS statistics reflect actual antiviral dispensing, albeit at a spatial scale of the UK (62 million), quite dissimilar from the spatial scale of the WWTP (i.e. 6,230 and 240,000 people). HPA-derived scenarios were shown to routinely underestimate Tamiflu use, reflecting the fact that this statistic is derived from regional case rates of ILI determined from patient visits to the general practitioner (GP). Owing to the syndromic diagnosis of ILI during the pandemic via the NPFS website and phone hotline, the HPA-ILI statistic reflects only a fraction of the national ILI-population, as visits to the GP were actively discouraged once the NPFS was fully operational in late-July 2009 [24].

The recovered oseltamivir carboxylate in the Benson and Oxford WWTP catchments during the peak of the autumnal wave in southern England can be best explained by a compliance rate of 45–60% based on NPFS estimates of oseltamivir collection (Figure 5). In a recent study, Singer et al. (2011) modelled Tamiflu use and environmental concentrations of OC during an influenza pandemic of differing severities: $R_0 = 1.65$, 1.9 and 2.3, with $R_0 = 1.65$ being a good reflection of the 2009 influenza pandemic (R_0 is the basic reproductive number reflecting the number of cases one case generates on average over the course of their infectious period). Extrapolating from Singer et al. (2011), a pandemic of $R_0 = 1.65$ was projected to generate one Tamiflu user within the Benson WWTP catchment and 168 people within the Oxford WWTP catchment on the day of the peak of the pandemic. The model scenario assumes negligible antiviral prophylaxis and the provision of antivirals for treatment of 30% of those with access to antivirals [3]. Projections by Singer et al (2011) were entirely consistent with national antiviral allocation statistics and estimates generated in this study using the waste water epidemiology approach.

The variability in load and concentration of OC in the Benson WWTP influent (relative standard deviation (RSD) = 132 and 110%, respectively) was much greater than that of Oxford (RSD = 23 and 17%, respectively). This variability is, to a large extent, a function of the difference in population between Benson and Oxford (6230 and 208,000 respectively). Assuming, on average, a person flushes the toilet five times a day [30], the number of flushes per user of Tamiflu (i.e., doses ×5) in Benson ranges from 6.5 to 40 and 225 to 1350 in Oxford (based on NPFS estimates). The low number of flushes per day in Benson and for some scenarios in Oxford would make it difficult to get a representative sampling of Tamiflu users in these catchments using hourly time proportional sampling. A theoretical threshold of 1000 flush events per chemical within a WWTP catchment was proposed as a guide for the minimum flush events needed to justify the use of a time-proportional hourly sampling frequency [30]. Catchments with fewer flush events per day might require more frequent sampling to ensure measured analytes were representative of the pharmaceutical use habits of the catchment population. However, the pumped nature of both of these waste water systems contributes to the mixing of discrete flushing events, particularly important in the smaller Benson catchment during off-peak flow periods. We argue this mixing in the waste water system has alleviated some of the variability associated with sampling small populations at an hourly time interval. However, future studies should give suitable consideration towards minimising the confounding effects of the population size on the waste water sampling.

Antiviral compliance during the 2009 Influenza A(H1N1)pdm09 pandemic has been estimated in a few countries worldwide, most of which were assessed during the early phase of the pandemic on a very small demographic. A study in England examined the degree to which 11–12 yr old pupils of a secondary school complied with a 10-day prophylaxis (once-daily) dosing regimen of Tamiflu at the outset of the pandemic in the UK (April 29, 2009) [4]. The authors found compliance was very high, with 77% taking the full course of Tamiflu [4]. A considerably lower compliance rate of 48% was estimated in a subsequent study that also investigated pupils of a similar age (14 yr) at a boarding school [5]. An online survey of pupils from one primary and two secondary schools in London at the outset of the pandemic in the UK reported only 48% of primary schoolchildren completed a full prophylaxis course, compared to 76% of secondary schoolchildren [6]. A study of compliance in 1–11 yr olds within a nursery, primary school and afterschool club in Scotland reported 97% of the children completed the full prophylaxis regime [7]. The authors proposed that the high compliance might have been related to the socioeconomic status of the population under investigation. Fifty-three (adult) staff and 273 pupils (7–12 yrs old) at a primary school in Sheffield, England were provided Tamiflu for prophylaxis during the latter part of the first wave of the pandemic in the UK (June, 2009) [8]. Of this group, 84% of the pupils and 80% of the staff completed the course of antivirals. It is clear that the survey approach will always be biased towards a demographic and limited in it's scope. In an effort to address these limitations, (web-based) surveys have been implemented during or shortly after the pandemic (e.g., Flusurvey [31,32]) to fill the knowledge gap. However, such approaches are still biased and thus must be balanced with other sources of information. Here we present the sampling of waste water treatment plant influent as an unbiased method for the determination of drug use and compliance. This study represents the only published report of non-survey based oseltamivir compliance globally and the first report on oseltamivir compliance for the second wave of the pandemic in the UK. Estimated compliance from this study is consistent with the lower range of published compliance rates. The integrated sampling from waste water is proposed as a better measure of compliance as compared to surveys, as this study incorporates all age groups in an unbiased manner, while survey-based studies typically focused on a limited sample size and narrow demographic (<14 year olds).

The accuracy of a waste water epidemiology model for determining drug use and compliance is potentially confounded by inappropriate drug disposal into the waste water itself. In most cases, the active drug that is consumed is excreted into waste water over a period of several hours. In the case of inappropriate drug disposal, the entire dose enters the waste water at once. Presumably when one disposes of drugs in this manner, it entails the disposal of multiple doses at once. Such a bolus of drug might appear realistic if only one waste water sample was taken owing to potential uncertainties with respect to compliance, biodegradation and prescription rates. However, the bolus would appear completely unfeasible with sufficiently high sampling frequency as the quantity of drug passing per unit time would, in comparison to other time points, be unachievable on a per capita basis [30,33]. Additional supporting evidence for the origin of OC in waste water can come from measuring the ratio of OC:OP, as described in a number of previous studies [17,34,35]. However, as OC (the active antiviral) is generated from the prodrug oseltamivir phosphate (OP), it can be assumed that all OC found in waste water in this study was the result of the consumption and (*in vivo*) metabolism of the prodrug.

In summary, we propose waste water forensics can be a valuable tool in monitoring population behaviour and a valuable resource for public health planning. Insight into the proportion of the population that does not utilise allocated antiviral or is not compliant with the dosing regimen could help to inform the development and prioritization of public health provisioning during an influenza pandemic. Owing to the fact that the unit of measurement is a WWTP catchment, the public health message can be targeted and focused towards a particular demographic, with potentially greater efficacy and cost savings. More resolved statistics on the provision of antivirals at the level of the WWTP catchment (e.g., Primary Care Trust) would further improve the power of the model. The forensic epidemiological approach employed in this study might also be applicable to other pharmaceuticals that are highly conserved in the waste stream for which compliance rates are in question.

Author Contributions

Conceived and designed the experiments: ACS JDJ JF HS. Performed the experiments: ACS JDJ RG GAK GF JF RHL MJB BO HS. Analyzed the data: ACS HS. Contributed reagents/materials/analysis tools: ACS JF MJB HS. Wrote the paper: ACS HS.

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STORAGE EFFECT ON THE ANALYSIS OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN WASTEWATER

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Abstract

Pharmaceuticals and personal care products (PPCPs) are receiving considerable attention and have been recognized as emerging pollutants in aquatic environments. Wastewater monitoring is of great importance as wastewater treatment plants are the main source of those pollutants. In this study, the stability of 124 target analytes in influent and effluent wastewater samples during short-term and long-term storage was assessed. The most common storage scenario was considered, in which samples were frozen immediately after sampling without any pre-treatment. More analytes remained stable during short-term storage at 4 °C than under freezing conditions. During long-term storage, three types of behavior were observed: constant concentrations throughout the experimental period, decreasing concentrations with time, and disappearance of the compound from the sample after freezing. Differences between effluent and influent samples were observed for 50 out of 124 tested PPCPs. The amount of stable analytes decreased with time during long-term storage. Specifically, 72% and 56% of the target compounds in the effluent and influent wastewater, respectively, remained stable during 2 months of storage, while the amount of stable compounds decreased to 40% and 32%, respectively, over 4 months. The results stress the importance of storage factors during analysis of pharmaceuticals in wastewater. Thus, the stability of target compounds in the samples under the planned storage conditions should be checked before starting the experiment to obtain reliable data.

Keywords: pharmaceuticals; personal care products; wastewater; stability; storage conditions.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are primarily introduced to the water environment via anthropogenic sources. Since PPCPs are compounds of medium to high polarity, they end up in the water compartment after being used. Furthermore, many pharmaceuticals have been designed to stay active for long periods of time to fulfill their therapeutic function; thus, they can persist in the environment, remaining active and affecting aquatic life. Some studies have already shown negative effects of PPCPs on aquatic ecosystems in general, as well as in certain aquatic species. Indeed, endocrine disruption, effects on reproductive

function, and toxicity to aquatic organisms have been documented (Crofton et al., 2007; Kvarnryd et al., 2011; Ricart et al., 2010). Antibiotics are also of great concern owing to their contribution to the development of antibiotic-resistant bacteria (Heuer et al., 2009; Rhodes et al., 2000).

Wastewater treatment plants (WWTPs) are considered to be the main source of PPCPs in the environment (Daughton and Ternes, 1999). Specifically, these compounds are detected in WWTP effluent, after which they enter surface water (Reemtsma et al., 2006; Watkinson et al., 2009). Thus, the removal of these emerging pollutants during the treatment process should be thoroughly investigated. There is also increasing interest in wastewater monitoring to enable further risk assessment for aquatic organisms.

Such PPCPs monitoring is already challenging owing to the low concentrations, complex matrices and wide range of compounds with broad physical-chemical properties. Accordingly, simple, reliable and rapid methods are needed to enable fast, sensitive, and selective determination of these emerging pollutants. Significant efforts have been made in this field, resulting in different analytical procedures (Khan et al., 2012; Salvador et al., 2007; Segura et al., 2007). One of the proposed methods represents a new trend in environmental water analysis: in-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC-MS/MS) analysis, which allows pre-concentration and analysis of the sample in a single run, making large volume samples and time-consuming SPE extraction procedures unnecessary. However, since most of these methods are focused on one or two pharmaceutical classes, some improvements should be introduced.

Additionally, very little attention has been paid to the sampling methods and storage of the samples, despite the great importance of these activities (Madrid and Zayas, 2007). Nevertheless, only a few studies have evaluated the effects of storage and preservation conditions on the data obtained (Baker and Kasprzyk-Hordern, 2011; Hillebrand et al., 2013). Currently, there is no uniform procedure for the storage and pre-treatment of samples before analysis. Most studies dealing with wastewater analysis report the preservation of samples by freezing without any pre-treatment (Bijlsma et al., 2012; Bijlsma et al., 2009; Hernandez et al., 2011; Loganathan et al., 2009; Xu et al., 2007), while others subject the samples to pH adjustment (Chen et al., 2012; Gheorghe et al., 2008; Nurmi and Pellinen, 2011). Some studies have also reported storing of wastewater in a refrigerator before analysis (Berset et al., 2010; Salem et al., 2012).

Freezing of samples without any treatment is the most common method of storage because it avoids complications during field sampling. Freezing also enables storage for a long time, which is beneficial to long-term monitoring programs, as it is impossible to keep wastewater in the refrigerator for more than a few days because PPCPs are bioactive and hence susceptible to breakdown by bacteria or other transformation reactions (Castiglioni et al., 2011).

Therefore, we conducted this study to investigate how freezing affects the original concentrations of such pollutants in wastewater. To accomplish this, samples of untreated and treated wastewater were collected from the influent and effluent of the WWTP in Ceske Budejovice, Czech Republic and analyzed for 124 PPCPs.

2. Materials and methods

Chemicals and reagents

LC-MS grade methanol and acetonitrile (LiChrosolv Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Labicom (Olomouc, Czech Republic). Ultrapure water was obtained from an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). A detailed description of pharmaceutical standards has been provided by Grabic et al. (2012). Trimethoprim (13C3) and sulfamethoxazole (13C6) were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Carbamazepine (D10) and amitriptyline (D6) were acquired from CDN Isotopes (Pointe-Claire, Quebec, Canada). Stock solutions of all pharmaceuticals were prepared in methanol at a concentration of 1 mg mL^{-1} and stored at -20 °C. A spiking mixture was prepared by diluting stocks in methanol to a final concentration of 1 µg mL^{-1} for each compound, after which it was stored at -20 °C.

Wastewater samples

Samples of treated and untreated wastewater were collected by grab sampling the effluent and influent, respectively, of the WWTP in Ceske Budejovice. High density polyethylene bottles were used for sampling and storage. Wastewater was spiked with target compounds at a concentration of 1 µg L^{-1} to obtain a proper response for all analytes. Three sets of samples were frozen and kept at -18 °C for 1 week, 2 months and 4 months before analysis. The samples were filtered through 0.45 µm regenerated cellulose filters (Labicom, Olomouc, Czech Republic) immediately prior to analysis, but not before storage. This was done to simulate the most common situation, in which samples are taken by the staff of the WWTP, frozen, and then transported to the laboratory for analysis. Three time points were studied, 7 days, 60 days and 120 days. Time 0 corresponds to the initial concentrations of the target compounds measured directly after sampling. A second set of the samples was held at 4 °C for 7 days, after which they were analyzed. All samples were analyzed in triplicate.

LC/LC-MS/MS conditions

A triple stage quadrupole MS/MS TSQ Quantum Ultra Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Accela 1250 LC pump (Thermo Fisher Scientific), Accela 600 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used for analysis. A Hypersil GOLD phenyl column (50 mm × 2.1 mm ID × 3 µm particles; Thermo Fisher Scientific, San Jose, CA, USA) and a Cogent Bidentate column (50 mm × 2.1 mm ID × 4 µm particles; Microsolv Technology Corporation, Eatontown, NJ, USA) were used for the separation of target analytes.

Heated electrospray (HESI-II) and atmospheric pressure chemical ionization coupled with atmospheric pressure photoionization (APCI/APPI) in positive and negative ion modes were used for ionization of the target compounds. Both the first and third quadrupoles were operated at a resolution of 0.7 full width at half

maximum (FWHM). The mass transitions of most of the studied compounds were the same as those described by (Grabic et al., 2012). All details pertaining to the analytical method (mass transitions – *m/z*, collision energies, and retention times) are presented in Table S1 of the electronic supplementary material (ESM). Optimized parameters for both ion sources are summarized in Table S2 (ESM).

The in-line SPE-LC-MS/MS configuration was the same as that used by Khan et al. (Khan et al., 2012). Target compounds were analyzed in three runs (two for HESI with different columns and one for APCI/APPI) to achieve better separation. The LC gradients are given in Tables S3-S5 (ESM).

Method performance

The method was validated by determining its linearity, repeatability, trueness and limits of quantification (LOQ) at the concentration ranges of interest. The linearity of the calibration curves was tested at 10 ng L^{-1} to 2500 ng L^{-1}. The calibration curve was measured on each day of analysis at the beginning and end of the sequence to check the instrumental stability. The repeatability was assessed by analysis of 10 replicate wastewater samples (influent and effluent) that had been spiked with the target PPCPs.

Trueness was evaluated based on the concentration ratio of wastewater samples spiked with 2 µg L^{-1} of the target analytes to the nominal concentration (recovery) after subtraction of the blank wastewater. Matrix effects were assessed for each compound, and corrections for ion suppression or enhancement were conducted using matrix-matched standards. Matrix matched standards were prepared from sampled wastewater by spiking with both internal standard (IS) and native compounds at 500 ng L^{-1} and 5000 ng L^{-1} respectively. The peak area/IS ratio determined for the non-spiked samples was subtracted from peak area/IS ratio in a matrix matched standard to achieve the matrix affected response factor.

LOQ was defined as the concentration of the lowest point of the calibration curve (relative standard deviation, RSD, of the average response factor < 30%) divided by 4.

Two transitions were monitored for each analyte according to the European Commission Decision 2002/657/EC of 17 August 2002 concerning the performance of analytical methods and interpretation of results.

Quality control was confirmed by analysis of blank samples to assure that target analytes were not introduced from sampling or laboratory procedures and sample handling. Four isotope labeled compounds were used as the internal standard.

3. Results and discussion

Method performance

Method validation was based on evaluation of the linearity, trueness, repeatability, and LOQ. All method performance parameters are presented in the Table S6 (ESM).

A six-point calibration curve was prepared by spiking tap water with the target compounds at 10 to 2500 ng L^{-1}. A consistent amount of surrogate standard was added (500 ng L^{-1}). The R-squared values were satisfactory, ranging from 0.975 to 0.999 (for most of the compounds r^2 > 0.991).

The LOQs were defined as the concentration of the lowest point of the calibration

curve (RSD of the average response factor < 30%) divided by 4. The LOQs for target PPCPs ranged from 0.59 to 49 ng L^{-1} for wastewater samples, which enables determination of their presence in trace amounts.

Repeatability was assessed by analyzing treated and untreated wastewater samples replicated 10 times. The RSD for measured replicated samples was lower than 20% for the majority of analytes (Table S6).

The trueness of the detection was evaluated from the ratio of target analytes determined in wastewater samples spiked with 2 μg L^{-1} to the nominal concentration after subtraction of the blank wastewater. With this type of instrument configuration, it is difficult to define recovery of the target analyte separately from the matrix effect when the extraction and pre-concentration of the sample is carried out on-line. The observed matrix effects are summarized in Table S7 (ESM). Matrix effects were assessed by comparing differences between responses obtained for spiked matrices (matrix matched standards) and calibration standards prepared in tap water. Significant matrix effects (above 20%, bold in the Table) were observed for most analytes. Comparatively, ion enhancement was prevalent in the majority of the compounds and of greater magnitude. Only 20 of all analyzed PPCPs did not seem to be affected by the matrix.

Stability of PPCPs during storage

The stability of 124 target PPCPs during wastewater freezing/thawing was evaluated. In this study, we considered an analyte to be stable if its concentration during storage was in the range of 60-120% of the initial concentration. Three sets of influent and effluent wastewater samples were stored at -18 °C for 1 week, 2 months and 4 months. Another set of wastewater samples was kept at 4 °C in the refrigerator; however, the samples became turbid and turned pink after 1 week of storage and could not be analyzed after longer storage times. The results for different storage conditions are summarized in Table 1. Storage at 4 °C for 1 week appeared to be more effective than freezing because a higher percentage of analytes remained stable.

Table 1. *Amount of compounds (%) which remained stable during different storage conditions.*

Total number of compounds	Storage conditions							
	1 week				2 months		4 months	
	4 °C		-18 °C		-18 °C		-18 °C	
	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent
124	86	83	78	61	72	56	40	32

Based on these findings, it is better to analyze samples as soon as possible while avoiding long-term storage and freezing/thawing if possible. This approach is unfortunately unrealistic for most of the cases. Freezing of samples immediately after their collection is the easiest way of sample preservation.

The stability of all PPCPs tested during long-term storage at -18 °C is presented in Table 2. During our experiment, three types of behavior were observed: constant concentrations throughout the experimental period, decreasing concentrations with time during the experiment and disappearance of the compound from the sample after freezing (Fig. 1). Moreover, the percentage of stable analytes decreased with time during long term storage.

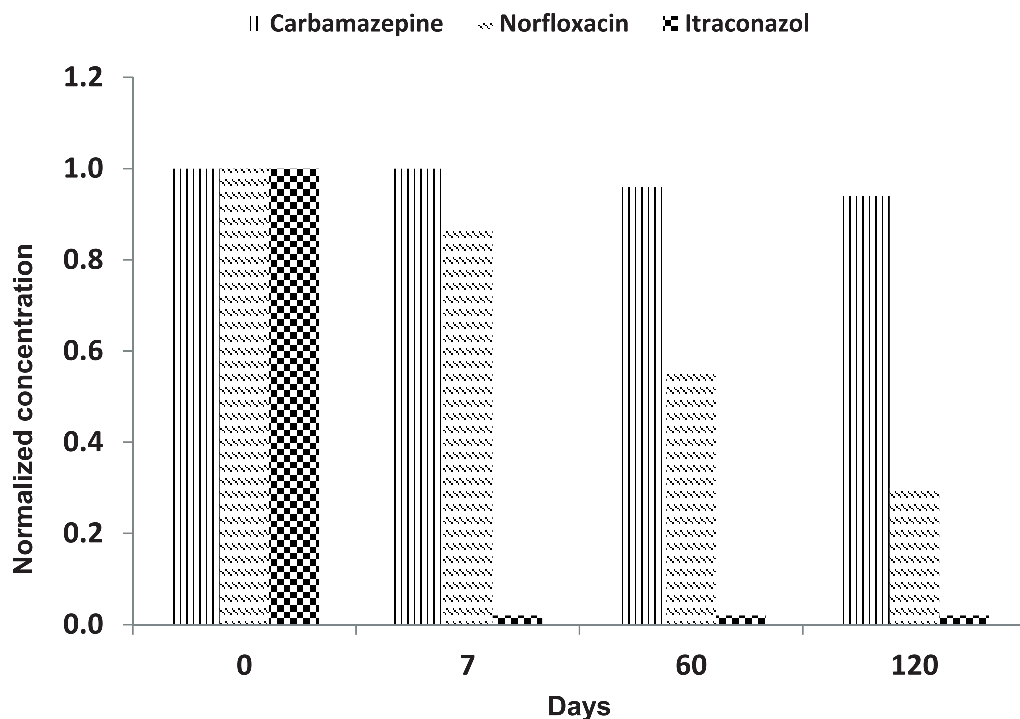

Figure 1. Compound behavior during long-term storage.

In general, better results were obtained for the effluent than the influent wastewater. For example, 27 target compounds were unstable in treated wastewater, while 49 were unstable in untreated wastewater. It is important to know which compounds are unstable during studies of the effectiveness of wastewater treatment. For example, false negative WWTP effectiveness is likely for some fluoroquinolones (difloxacin, enoxacin, enrofloxacin, lomefloxacin, oxolinic acid) because they will be present after the F/T cycle in the effluent sample and absent in the influent sample (Table 2). Overall, 25 of the 124 target PPCPs, including fenofibrate, amiodarone and all compounds of imidazole class (econazole, miconazole) disappeared from samples of treated and non-treated wastewater after freezing. These findings indicate that wastewater samples should not be analyzed for those compounds after a freezing/thawing cycle.

Table 2. *Stability of PPCPs in wastewater samples during long-term storage at -18 °C.*

Stable for 4 months[a]		Stable for 2 months[a]		Stable for 1 week[a]		Not stable[a]	
Effluent	**Influent**	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**	**Influent**
Atenolol	Atenolol	Alfuzosin	Atorvastatin	BP4	Alfuzosin	Amiodarone	Amiodarone
Atorvastatin	Azithromycin	Atracurium	Atracurium	Cyproheptadine	BP4	Chlorpromazine	Chlorpromazine
Azithromycin	Bezafibrate	Budenoside	BP3	Dipyridamole	Flecainide	Chlorprothixene	Chlorprothixene
Bezafibrate	Biperiden	Ciprofloxacin	Bupropion	Doxycycline	Flumequine	Clemastine	Clemastine
Biperiden	Bisoprolol	Chloramphenicol	Chloramphenicol	Furosemide	Mirtazapine	Clomipramine	Clomipramine
Bisoprolol	BP1	Desloratadine	Ciprofloxacin	Ketoprofen	Roxithromycin	Dicycloverine	Clonazepam
BP1	BP2	Dihydroergotamine	Clindamycine	Risperidone		Duloxetine	Cyproheptadine
BP2	Budenoside	Donepezil	Erythromycin	Sulfamoxol		Econazole	Desloratadine
BP3	Buprenorphine	Enrofloxacin	Finasteride			Felodipine	Dicycloverine
Buprenorphine	Carbamazepine	Erythromycin	Florfenicol			Fenofibrate	Difloxacin
Bupropion	Cilazapril	Florfenicol	Fluconazole			Fulvestrant	Dihydroergotamine
Carbamazepine	Citalopram	Flumequine	Ibuprofen			Haloperidol	Donepezil
Cilazapril	Clarithromycin	Haloperidol	Levofloxacin			Indometacin	Doxycycline
Citalopram	Diclofenac	Hydroxyzine	Naproxen			Itraconazole	Duloxetine
Clarithromycin	Diltiazem	Ibuprofen	Norfloxacin			Levomepromazine	Econazole
Clindamycine	Diphenhydramine	Levofloxacin	Oseltamivir			Loperamide	Enoxacin
Clonazepam	Dipyridamole	Maprotiline	Oseltamivir_carboxylate			Meclozine	Enrofloxacin
Diclofenac	Disopyramide	Naproxen	PBS			Miconazole	Felodipine
Difloxacin	Eprosartan	Nefazodone	Rosuvastatin			Paroxetine	Fenofibrate
Diltiazem	Fexofenadine	Norfloxacin	Sulfadiazine			Perphenazine	Flutamide
Diphenhydramine	Furosemide	Oseltamivir	Sulfadimethoxine			Sertaline	Fulvestrant
Disopyramide	Glibenclamide	Oxolinic_acid	Sulfamethazine			Sulconazole	Glimepiride
Enoxacin	Haloperidol	Oxytetracycline	Sulfamethizole			Tamoxifen	Hydroxyzine
Eprosartan	Irbesartan	PBS	Sulfamethoxazole			Terbinafine	Indometacin
Fexofenadine	Isradipine	Promethazine	Sulfamoxol			Tetracycline	Itraconazole
Finasteride	Memantine	Ropinirole	Sulfaphenazole			Tonalid	Ketoprofen

Table 2.

Stable for 4 months[a]		Stable for 2 months[a]		Stable for 1 week[a]		Not stable[a]	
Effluent	**Influent**	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**	**Influent**
Flecainide	Metoprolol	Roxithromycin	Tramadol			Triclosan	Levomepromazine
Fluconazole	Naloxone	Sulfadimethoxine	Trimethoprim				Lomefloxacin
Flutamide	Orphenadrine	Sulfamerazine	Venlafaxine				Loperamide
Glibenclamide	Oxazepam	Sulfamethazine					Maprotiline
Glimepiride	Ropinirole	Sulfamethizole					Meclozine
Irbesartan	Sotalol	Sulfamethoxazole					Mianserin
Isradipine	Sulfamerazine	Sulfaphenazole					Miconazole
Lomefloxacin	Sulfamethoxypyridazine	Sulfaquinoxaline					Nefazodone
Memantine	Sulfaquinoxaline	Sulfathiazole					Oxolinic_acid
Metoprolol	Sulfathiazole	Tramadol					Oxytetracycline
Mianserin	Terbutaline	Trimethoprim					Paroxetine
Mirtazapine	Trihexyphenidyl	Valsartan					Perphenazine
Naloxone	Valsartan	Verapamil					Promethazine
Orphenadrine	Verapamil						Risperidone
Oseltamivir_carboxylate							Sertaline
Oxazepam							Sulconazole
Rosuvastatin							Sulfasalazine
Sotalol							Sulfapyridine
Sulfadiazine							Tamoxifen
Sulfamethoxypyridazine							Terbinafine
Sulfapyridine							Tetracycline
Sulfasalazine							Tonalid
Terbutaline							Triclosan
Trihexyphenidyl							
Venlafaxine							

a Concentration of those compounds were in the range 60–120% of the initial concentration

Thirty target compounds, including atenolol, carbamazepine, diclofenac, and oxazepam, remained stable throughout the experimental period in both effluent and influent wastewater (Table 2). For such compounds, storage of samples at -18 °C is acceptable. Some compounds, including BP4, doxycycline and risperidone, showed declining concentrations with the storage time. Accordingly, this should be considered when selecting the duration of the storage period. Overall, the decision regarding storage conditions should be made after careful consideration of the analytes and the purpose for wastewater analysis.

4. Conclusions

This paper describes the effects of long-term storage of wastewater samples on the analysis of PPCPs. Short-term stability (1 week) of 124 PPCPs in wastewater was tested by comparing samples stored at 4 °C and -18 °C, while long-term stability was evaluated during storage at -18 °C. No sample pre-treatment before storage was carried out to simulate the most common method of sample handling. For the short-term storage, keeping the samples in fridge showed better results than freezing/thawing: 85% (83%) of stable analytes in wastewater effluent (influent) and 78% (61%) respectively. The amount of stable analytes decreased with time during long-term storage, and only 40% and 32% of the target compounds in the effluent and influent wastewater, respectively, remained stable throughout the experiment. Additionally, differences in the stability of 50 of the tested PPCPs in effluent and influent samples were observed. Accordingly, care should be taken when the efficiency of wastewater treatment is evaluated as some false results could be obtained.

Overall, the results of this study indicate that multi-residue analysis of wastewater samples is always a compromise concerning not only the analytical method development and extraction procedure, but also the choice of storage conditions. Because little attention is given to the storage factor, the stability of target compounds in samples should be checked under the planned storage conditions before starting any experiment.

Acknowledgments

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic CENAKVA No. CZ.1.05/2.1.00/01.0024, the Grant Agency of the University of South Bohemia No. 087/2013/Z and the Czech Science Foundation GACR P503/11/113. We thank Edanz agency for English language corrections.

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CHAPTER 5

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Emerging organic pollutants have gained considerable interest over last several decades. Special attention is paid to such compounds as pharmaceuticals, illicit drugs, some polar pesticides, perfluorinated compounds. Major issue of concern is that these compounds are constantly used and released and thus are distributed in aquatic environment due to their hydrophilic properties. Their potential adverse effects on aquatic organisms are largely unknown. Therefore, studying fate of emerging contaminants in aquatic ecosystem is of great importance, as these data form the basis for carrying out risk assessment for aquatic organisms.

In the frame of presented thesis occurrence of emerging pollutants in different compartments of aquatic environment was studied. Different environmental matrices were covered: wastewater, surface water and fish with the focus on pharmaceuticals, illicit drugs and perfluorinated compounds. Water sampling was carried out using both grab and passive sampling. Application of POCIS for wastewater sampling was studied.

The critical step in the assessment of pollutants' fate is the development of suitable analytical methodology to monitor them in the environment. Although numerous pharmaceuticals have been already detected in aquatic environment all over the world (Kolpin et al., 2002; Nikolaou et al., 2007), still there is need for new reliable methods for their detection and quantification. The output of the study is three multi-residue methods for the analysis of pharmaceuticals and illicit drugs in water and fish tissues. Their main advantage is wide range of compounds covered, which enables obtaining maximum information with minimum analytical effort.

Multi-residue method developed by Grabic and co-authors allows determination of hundred pharmaceuticals in water samples in one analytical run (Grabic et al., 2012). The method is based on liquid chromatography coupled to triple quadrupole mass spectrometric detection. While numerous methods are already available in the literature (Barrek et al., 2009; Gros et al., 2006; Huerta-Fontela et al., 2010), any of them does not cover so broad range of compounds. Currently, multi-residue analytical methods are becoming essential tools that provide reliable information about the occurrence and fate of pharmaceuticals in the environment. The selection of the pharmaceuticals included in this method was based on relevant ecotoxicological criteria using fish steady state plasma concentration model (Fick et al., 2010). Thus, pharmaceuticals which have a potential to bioconcentrate in fish are analyzed. Forty-nine studied pharmaceuticals in surface water and 47 in wastewater were lacking an analytical protocol for their determination in environmental samples. Pharmaceuticals included in the method represent 27 classes, and therefore broad range of physical-chemical properties. It constitutes a challenge not only for their analysis, but also for the extraction procedure. Single SPE for the extraction of target compounds from water samples is proposed, while many published methods use different sorbent materials for each pharmaceutical class which lead to the efficient extraction of a wide range of analytes, but also requires time-consuming sample preparation (Sacher et al., 2001). The method was applied for the screening of pharmaceuticals in the effluents of six WWTP in Sweden (population equivalents ranged from 6400 to 330000 inhabitants). Forty-five to 64 target compounds were found above limit of quantification (LOQ). High concentrations were observed for representatives of anti-hypertension drugs (up to several μg L^{-1}), some antibiotics (hundred ng L^{-1}), and

anti-depressants (up to half µg L^{-1}). Widespread occurrence of pharmacologically active compounds in WWTP effluents is a matter of concern, as these compounds will eventually reach surface waters and can affect aquatic life. Therefore, WWTP monitoring is crucial for the understanding of pollutants' fate in the environment.

In addition, wastewater analysis can be an effective tool for the estimation of drug consumption and compliance rates. A wastewater epidemiology approach was employed to estimate oseltamivir (Tamiflu®) compliance in the United Kingdom (Singer et al., 2013). Predictions of oseltamivir consumption from the levels detected in wastewater influent were compared with two sources of national government statistics. The derived compliance rates ranged from 45 to 60%, which indicated that approximately half of the medication is unused. Mis- and un-used antivirals can lead to the hastening of antiviral resistance in cases where influenza-infected people do not comply with the prescribed course and dosing regimen. Up to date, this is the only non-survey based approach for the estimation of oseltamivir compliance.

This approach is also promising for the obtaining realistic data on the consumption of illicit drugs. Zuccato and co-authors have proposed a methodology for the back-calculation of drug consumption in the local community from the drug residues in the influent wastewater (Zuccato et al., 2008). Thus, not only forensic, but also environmental analysis of illicit drugs is important. Most of the methods dealing with the analysis of illicit drugs in wastewater are based on LC/MS/MS (Karolak et al., 2010; Terzic et al., 2010). QqQ mass spectrometer is the instrument of choice for the analysis of complex matrices. Nevertheless, high resolution mass spectrometry is gaining interest for the analysis of illicit drugs. A new generation Q Exactive mass spectrometer combines high-performance quadrupole precursor selection with high-resolution, accurate-mass Orbitrap detection. It allows the combination of non-targeted screening in full scan with targeted MS/MS analysis, thus representing an excellent tool for fast and easy environmental screening. The comparison of three methods based on different MS detection (QqQ MS/MS, full-scan HRMS and MS/HRMS) for the analysis of 27 drugs of abuse in wastewater was carried out (Fedorova et al., 2013d). Up to date, this is the first report of the use of in-line-SPE-LC/MS/HRMS for the environmental analysis of drug residues. The performance of the methods was assessed regarding linearity, LOQs, trueness, precision and selectivity. For nine (codeine, ketamine, MDEA, MDMA, mephedrone, midazolam, norketamine, oxazepam and tramadol) out of 27 target compounds, all three tested methods had good performance. Q Exactive operated in full scan mode did not show any outstanding results, while targeted MS/HRMS mode showed significant improvements, and had the best performance for seven of the analyzed drugs. Improved sensitivity and selectivity were observed for amphetamine, benzoylecgonine, cathinone, MDA, oxycodone, and THC-COOH. For compounds including amphetamine, cathinone, cocaine, MBDB, MDA, oxycodone, and THC-COOH HRMS, full scan was inadequate, as interferences were observed and the analysis was not selective enough. In this case, the method of choice was MS/HRMS with the improved selectivity and sensitivity parameters. For three compounds, detection using a triple quadrupole instrument provided the best results. 2-Oxy-3-hydroxy-LSD, methylphenidate and norbuprenorphine glucoronid showed lower LOQs and better selectivity when analyzed in MS/MS mode, using QqQ. Obtained results allow us to conclude that Q Exactive is equally suitable or better than state of the art MS/MS based on triple quadrupole for most of the tested drugs. Consequently, MS/HRMS allowed more accurate determination of drug residues in a complex matrix such as wastewater.

Taking into account that WWTPs are the main source of pharmaceuticals in aquatic environment, a lot of efforts are put into the developing of new and modifying of existing analytical methods for the wastewater analysis, however less attention is paid to sample collection and preservation. Up to date, there is no uniform procedure for sample collection and handling. Only few studies have been published on the stability of PPCPs in environmental samples. The studies are limited in number of tested matrices and compounds, as well as in tested conditions of storage (Baker and Kasprzyk-Hordern, 2011; Glassmeyer and Shoemaker, 2005; Vanderford et al., 2011). Stability of 124 PPCPs in influent and effluent wastewater samples was tested in the presented study (Fedorova et al., 2013b). The most common ways of short-term (Berset et al., 2010; Salem et al., 2012) and long-term storage were assessed (Bijlsma et al., 2012; Hernandez et al., 2011; Loganathan et al., 2009). It is crucial to know, whether type of storage effects the concentrations of target analytes in the sample or they remain stable during whole period of storage. Obtained results showed that storage in cold at 4°C for one week is more effective than freezing for the same time period, as higher percentage of analytes remained stable. Unfortunately, this way of sample preservation is not suitable when long-term monitoring is carried out and storage of the samples for longer time is needed. In this case it is difficult to avoid preservation of wastewater using freezing. This type of sample preservation was reported to be the most effective for the analysis of illicit drugs in wastewater (Castiglioni et al., 2011). During long-term storage three types of behavior were observed: constant concentrations during whole period of experiment, decreasing of compound amount with time during the experiment and disappearing of compound from the sample once being frozen. In general, better results were obtained for the effluent than for the influent wastewater. For example, 27 target compounds were unstable in treated wastewater, while 49 – in untreated. For 50 out of 124 tested PPCPs differences for effluent and influent samples were observed. We should be aware of this fact when carrying out the analysis for the study of the efficiency of wastewater treatment. For instance, in case of some fluoroquinolone antibiotics (difloxacin, enoxacin, enrofloxacin, lomefloxacin, oxolinic acid) it is likely to obtain false negative WWTP efficiency, as those compounds will be present after freezing/thawing cycle in the effluent sample and absent in the influent one. Thirty target compounds remained stable for 4 months in experimental condition both in effluent and influent wastewater. For such compounds, as for example atenolol, carbamazepine, diclofenac, oxazepam, it is possible to obtain relevant data on their concentrations in wastewater, which was stored at -18 °C. Concentrations measured after 4 month of the sample storage will reflect the initial concentrations of those compounds in the sample. The amount of stable analytes decreased during the long-term storage. 72% and 56% of the target compounds in the effluent and influent wastewater respectively remained stable during 2 months of storage, while for the period of 4 months the amount of stable compounds decreased to 40% and 32% respectively. Obtained results stress the importance of storage factor for the analysis of pharmaceuticals in wastewater. Thus, the stability of target compounds in the samples under the planned storage conditions should be checked before starting the experiment to get reliable data.

One possible way to overcome above mentioned limitations of grab sampling is use of passive sampling approach. It is beneficial as it allows obtaining time weigh average concentrations of pollutants over certain period of time, and thus gives more representative information on the pollution situation. Another advantage is

pre-concentration of target analytes, which is crucial in case of environmental analysis of POCs, which are present at trace concentrations. In the frame of the presented study, calibration of POCIS in wastewater effluent was carried out. Sampling rate values for 5 perfluorinated compounds (Fedorova et al., 2013a), 32 PPCPs and 20 pesticides (Fedorova unpublished data) were calculated. Lack of the reported calibration data for POCIS samplers is a limitation of application of passive sampling approach for environmental monitoring of hydrophilic pollutants. Without knowing sampling rates it is impossible to back calculate the amount of certain compound in the sampler to water concentration. In this case, POCIS can only be used for comparative purposes. For instance, set of passive samplers can be deployed along the river to assess its contamination profile. POCIS has been used for sampling polar organic chemicals such as pharmaceuticals, pesticides, and illicit drugs, as well as hormones and UV-blockers (Harman et al., 2011; Lissalde et al., 2011; Zenker et al., 2008; Zhang et al., 2008). Available calibration data for polar organic compounds is limited to a small number of pesticides and pharmaceuticals and was obtained under laboratory conditions (Bartelt-Hunt et al., 2011; Mazzella et al., 2008). Only few studies have conducted calibration experiments under field conditions (Harman et al., 2011; Ly et al., 2013). Regarding perfluorinated compounds only one study reports application of POCIS for their sampling (Kaserzon et al., 2012). Calibration data was obtained for 7 compounds for the modified configuration of POCIS under laboratory conditions. Unlike the experiment of Kaserzon and co-authors, calibration of POCIS in this study was carried out under the conditions of the WWTP's effluent. It was shown that optimum exposure period for both standard POCIS configurations is 14 days. Ten of 15 target compounds were found in POCIS, five of which were quantified in wastewater. This fact stresses the advantage of passive sampling over the grab sampling, as more compounds could be identified.

Analysis of biota samples is an essential part in the assessment of pollutants' fate in aquatic environment. In the presented study multi-residue method for the analysis of 32 antibiotics in aquaculture products was developed and validated (Fedorova et al., 2013c). This kind of analysis is important for several reasons. Antibiotics are widely applied in aquaculture both for prevention and treatment of bacterial diseases. Excessive use of antibiotics in industrial aquaculture results in the presence of residual antibiotics in commercialized fish and shellfish products. Uncontrolled consumption of antibiotics by fish consumers is leading to the development of antibiotic resistance in bacteria that are pathogenic to humans. Therefore, analysis of edible fish tissues for the presence of these compounds is needed to assess human health risk. In addition, obtained data present information on the potential of antibiotic bioconcentration in fish. Antibiotics of different classes were considered in the study: those ones approved for use in aquaculture, forbidden ones, and also those, which are normally used for human treatment only. The developed method was applied for the screening of antibiotics in different fish samples available at the Czech market. The fish and shrimp samples were bought from the three biggest supermarkets in České Budějovice, which belong to a network of shops covering the whole Czech Republic (CR). Wild and farmed fish (carp, salmon, trout, tilapia, pangasius, etc.), and shrimp of foreign (European, Asian, African) and Czech origin were obtained. In total 97 samples were obtained. Up to date, this is the first study of this kind in the CR, which covered broad range of compounds, not only those with set maximum residue limits (MRL), and which was focused both on farmed and wild fish. The most frequently occurred antibiotic is flumequine – it was detected in

9 of 15 positive samples. It is a member of fluoroquinolone family. Other antibiotics belonging to this family were also prevailing – they were present in 12 of 15 samples. This group of antibiotics was of high interest. Some of fluoroquinolone antibiotics are authorized for use in aquaculture in Europe, but they are forbidden in USA, Australia and Canada (except of flumequine and oxolinic acid) due to their high persistence. The fact that not only farmed fish samples were positive was intriguing. Two samples of squid, one mackerel, two herrings and one shark sample contained at least one target analyte. All those fishes were wild and it was supposed that they should be free of any antibiotic residues. The possible explanation of this phenomenon is that wild fish inhabiting areas near to the fish farms can consume antibiotics together with the leftovers of pellets which were enriched with antibiotics for the fish treatment. This is another drawback of excessive use of antibiotics in aquaculture and proof that POCs can bioconcentrate in aquatic organisms. All found concentrations were lower than MRLs established in European Union (Commission Decision (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, 2010). Still, presence of antibiotic residues in edible fish tissues could be a matter of concern and the sign of improper use of antibiotics in aquaculture industry, what consequently can cause adverse effects for the environment and human health. No antibiotics were detected in the fish of Czech origin.

During analytical method development for different environmental matrices the importance of the use of matrix-matched standards was shown. Obviously, for such complex matrices using only isotope dilution approach is not enough to correct ion suppression or enhancement that occur in the ion source. Especially, it concerns multi-residue methods as isotopic labeled compounds are not available for all of the target analytes. It was shown, that combination of isotope dilution approach with the use of matrix-matched calibration curve is the best solution for environmental analysis (Grabic et al., 2012).

Conclusions and future perspectives

This study presents multi-residue analytical methods, which were applied for the monitoring of POCs in various environmental matrices. These methods have significant advantages over the existing ones: they allow measuring broad range of pollutants in one analytical run and their consequent quantification at trace levels. Important factors as sample storage and matrix effect were studied to avoid obtaining irrelevant data. Application of novel sampling approach was investigated, which appeared to be more effective than the commonly used one. Passive sampling of polar compounds is still under development and it has some shortcomings. Further research is needed to overcome the limitations and allow PS to be extensively used in monitoring programs.

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ENGLISH SUMMARY

Fate of polar organic pollutants in aquatic environment

Ganna Fedorova

Polar organic pollutants present an important aspect of current environmental research. Such compounds as pharmaceuticals and personal care products, illicit drugs, perfluorinated compounds, synthetic musk are recognized to be emerging pollutants. They are introduced to the aquatic ecosystem by anthropogenic inputs, and are not effectively removed by wastewater treatment plants (WWTP), which are considered to be the main source of POCs in aquatic environment. Their continuous release to the environment is a matter of concern, even though trace concentrations are observed. Their potential adverse effects on aquatic organisms are largely unknown. Therefore, studying fate of emerging contaminants in aquatic ecosystem is of great importance.

In this thesis occurrence of emerging pollutants in different compartments of aquatic environment was studied. Different environmental matrices were covered: wastewater, surface water and fish tissues with the focus on pharmaceuticals, illicit drugs and perfluorinated compounds. Although numerous pharmaceuticals have been already detected in aquatic environment all over the world, still there is need for new reliable methods for their detection and quantification. The output of the study is three multi-residue methods for the analysis of pharmaceuticals and illicit drugs in water and fish samples. Their main advantage is that wide range of compounds from different classes is covered, which enables obtaining maximum information with minimum analytical effort. All the methods were validated including such key parameters as limits of quantification, selectivity, recovery and repeatability, and showed excellent performance allowing determination of target compounds at trace levels. Consequently, the methods were applied for the monitoring of POCs in different environmental samples. Wide occurrence of pharmaceuticals in Swedish WWTP effluents was shown. Rather high concentrations for the representatives of anti-hypertension drugs, some antibiotics and anti-depressants were detected. This fact stresses the importance of WWTP′ effluent monitoring as it is the main source of PPCPs in the aquatic environment.

In addition, wastewater analysis can be an effective tool for the estimation of drug consumption and compliance rates. A wastewater epidemiology approach was employed to estimate oseltamivir (Tamiflu®) compliance in the United Kingdom. The derived compliance rates ranged from 45 to 60%, which indicated that approximately half of the medication is unused. Up to date, this is the only non-survey based approach for the estimation of oseltamivir compliance. This approach is the only way to obtain representative data on illicit drugs consumption in the local community.

Passive sampling approach was tested for the monitoring of POCs in wastewater. Both standard configurations of POCIS were calibrated under the field conditions for a broad range of contaminants. Sampling rate values were calculated for the case of wastewater effluent. This is a significant contribution to the potential widespread application of POCIS for environmental monitoring programs.

Important issues concerning wastewater analysis were assessed, including matrix effects and storage. Matrix effects were reported for the prevalent number of target

analytes. Thus, use of matrix-matched calibration curve together with isotope labeled compounds is suggested. Stability of 124 PPCPs in wastewater samples during storage was tested. Results of the study indicate that a lot of target compounds are affected by the storage conditions. Thus, sampling should be very carefully planned, depending on the purposes of the monitoring.

Analysis of biota samples is an essential part in the assessment of pollutants' fate in aquatic environment. Therefore, the screening of antibiotics in fish available at the Czech market was carried out. Thirty-two antibiotics from different classes were analyzed. The fish and shrimp samples were bought from the three biggest supermarkets in České Budějovice, which belong to a network of shops covering the whole Czech Republic. In total 97 samples were obtained. Up to date, this is the first study of this kind in the CR, which covered broad range of compounds, not only those with set maximum residue limits (MRL), and which was focused both on farmed and wild fish. Fifteen samples were positive for at least one antibiotic from the list. Found concentrations did not exceed MRL values established in the European Union. No antibiotics were detected in the fish of Czech origin. Still, presence of antibiotic residues in edible fish tissues could be a matter of concern and the sign of improper use of antibiotics in aquaculture industry, what consequently can cause adverse effects for the environment and human health.

CZECH SUMMARY

Osud polárních organických polutantů ve vodním prostředí

Ganna Fedorova

Polární organické polutanty patří mezi důležité aspekty současného výzkumu životního prostředí. Sloučeniny, jako například farmaka a produkty osobní spotřeby (PPCP), nelegální drogy, perfluorované sloučeniny, syntetické pižmo, jsou považovány za nově vznikající polutanty z důvodu jejich širokého rozšíření ve vodním prostředí. Do vodního prostředí se dostávají prostřednictvím antropogeniích zdrojů a čistírny odpadních vod (ČOV) nejsou schopny je efektivně odstranit, což je považováno za hlavní zdroj polárních organických látek (POC) ve vodním prostředí. Jejich permanentní uvolňování do životního prostředí je velmi znepokojující, přestože se jedná pouze o stopové koncentrace. Jejich potenciální negativní účinky na vodní organismy jsou do značné míry neznámé. Z tohoto důvodu je studium nově vznikajících znečišťujících látek ve vodním ekosystému velmi důležité.

Tato práce zkoumala výskyt nově vznikajících polutantů v různých částech vodního prostředí. Byly použity různé matrice životního prostředí: odpadní vody, povrchové vody a ryby se zaměřením na farmaka, nelegální drogy a perfluorované sloučeniny. Přestože již bylo po celém světě ve vodním prostředí objeveno značné množství farmaceutických látek, nové spolehlivé metody vedoucí k jejich zjištění a kvantifikaci jsou stále potřebné. Výstupem studie jsou tři multireziduální metody analyzující farmaka a nelegální drogy ve vodě a ve vzorcích ryb. Jejich hlavní výhodou je, že široká škála sloučenin z různých tříd již byla pokryta, což umožňuje získat maximum informací při minimálním analytickém úsilí. Všechny metody byly validovány včetně klíčových parametrů, jako jsou například mezní hodnoty stanovitelnosti, selektivity, výtěžnosti a opakovatelnosti, a prokázaly vynikající účinnost, díky čemuž bylo možné určit cílové sloučeniny ve stopovém množství. Metody byly následně aplikovány na monitorování POC v různých vzorcích životního prostředí. Ve Švédsku byl prokázán široký výskyt farmak v oblasti odtoků z ČOV. Jednalo se o poněkud vysoké koncentrace zástupců léků na zvýšený krevní tlak, některá antibiotika a antidepresiva. Tento fakt zdůrazňuje význam monitorování ČOV, jelikož čistírny patří mezi hlavní zdroje PPCP ve vodním prostředí.

Navíc, analýza odpadních vod může představovat účinný nástroj pro odhad spotřeby drog a míry dodržování. Byly použity metody epidemiologie odpadních vod pro odhad dodržování oseltamiviru (Tamiflu®) ve Velké Británii. Odvozené míry dodržování se pohybovaly v rozsahu mezi 45 až 60 %, což naznačovalo, že zhruba polovina medikamentů zůstala nevyužita. V současnosti je toto jediná nepřímá metoda vedoucí k odhadu kompliance oseltamiviru. Tato metoda je jediným způsobem, jak získat reprezentativní údaje o spotřebě nelegálních drog v místní komunitě.

Pro monitorování POC v odpadních vodách byla testována metoda pasivního vzorkování. Obě standardní konfigurace polárního chemického integrativního pasivního vzorkovače (POCIS) byly v polních podmínkách kalibrovány na širokou škálu kontaminantů. Hodnoty vzorkovacích rychlostí byly vypočítány na odtok odpadních vod. Jedná se o významný přínos pro potenciální rozsáhlé použití vzorkovače POCIS pro monitorovací programy životního prostředí.

Byly zhodnoceny důležité otázky týkající se analýzy odpadních vod, včetně

matricových jevů a skladování. Matricové jevy byly zaznamenány u převládajícího počtu cílových analytů. Z toho důvodu je navrhováno použití kalibrační křivky zohledňující vliv matrice spolu s izotopově značenými sloučeninami. Byla testována stabilita 124 polutantů PPCP ve vzorcích odpadních vod během skladování. Výsledky studie naznačují, že velké množství cílových sloučenin je ovlivněno podmínkami skladování. Vzorkování by tak mělo být velmi pečlivě naplánováno v závislosti na účelech monitorování.

Analýza vzorků bioty je nezbytnou součástí hodnocení stavu polutantů ve vodním prostředí. Z toho důvodu byla provedena kontrola antibiotik obsažených v rybách, které jsou dostupné na českém trhu. Bylo analyzováno 32 druhů antibiotik z různých tříd. Vzorky ryb a krevet byly zakoupeny ve třech největších supermarketech v Českých Budějovicích, které patří do sítě obchodů pokrývajících celou Českou republiku. Celkem bylo získáno 97 vzorků. V současné době se jedná o první studii tohoto typu v ČR, která pokryla širokou škálu nejen sloučenin, u kterých je nastaven maximální reziduální limit (MRL), a která se zaměřila jak na volně žijící ryby, tak i ryby z farmových odchovů. 15 vzorků mělo pozitivní výsledek na alespoň jedno z antibiotik uvedených na seznamu. Nalezené koncentrace nepřevýšily hodnoty MRL stanovené Evropskou Unií. V rybách českého původu nebyla nalezena žádná antibiotika. Avšak přítomnost reziduí antibiotik v jedlých tkáních ryb by mohla být velmi znepokojující a mohla by znamenat nesprávné použití antibiotik v průmyslu akvakultury, což může mít následně nežádoucí vliv na životní prostředí a lidské zdraví.

ACKNOWLEDGEMENTS

It is a pleasant task to express my thanks to all people who contributed in many ways to the success of this study. It was an unforgettable experience for me and I am thankful to all my colleagues and friends who shared best and worst moments of my doctoral journey.

I am very grateful to my supervisor Roman Grabic, Ph.D., for his encouragement, guidance and support from the beginning to the end. It has been a great opportunity to work under the guidance of such an experienced and caring teacher. Thank you Roman for your constant supports both in scientific and day-to-day life. Your comments along the way were of great help and your optimistic view on things always made me carry on with confidence. You are simply the best supervisor in the world. I am also very thankful to your family; their support and kind attitude mean a lot to me.

I would like to sincerely acknowledge Prof. Dipl.-Ing. Otomar Linhart, DSc. for giving me an opportunity to study in the Faculty of Fisheries and Protection of Waters.

It is difficult to overstate my gratitude to all my dear colleagues from the Laboratory of Environmental Chemistry and Biochemistry headed by Assoc. Prof. Dipl.-Ing. Tomáš Randák, Ph.D.: Assoc. Prof. Dipl.-Ing. Vladimír Žlábek, Ph.D., Dipl.-Ing. Jan Turek, Ph.D., Jitka Kolářová, DVM, M.Sc. Zhihua Li, Ph.D., M.Sc.Oksana Golovko, M.Sc.Viktoriia Burkina, Dipl.-Ing. Kateřina Grabicová, Dipl.-Ing. Daniel Červený and Pavla Simandlová. Special thanks to Tomáš for leading us the way he did and for taking care of everyone. Members of the Laboratory of Aquatic Toxicology and Ichtyopathology also deserve my sincerest thanks. Thank all of you for your help, good discussions and valuable suggestions, for sharing many good times with me in the laboratory and in the field. Thank you for providing a stimulating and fun filled environment, for all amazing parties we have had. I am blessed to have such colleagues like you.

One page is definitely not enough to acknowledge everyone namely. I express my thanks to all staff of the Faculty of Fisheries and Protection of Waters. I am proud to be the part of this team. Also my thanks go to my friends outside of the faculty: to Ukrainian ones for supporting me even being thousands kilometers away and to Czech ones for making me feel like home.

Special thanks are extended to colleagues from the Laboratory of Environmental Chemistry and Biogeochemistry, Umea University, especially to Prof. Mats Tysklind and Assos. Prof. Jerker Fick. I do appreciate your help. I am grateful for the cooperation throughout the entire period of the present work. Time spent in Umea University was a great experience for me. I would like to thank all people from Chemistry Department for keeping warm atmosphere in the cold North.

Finally, I would like to express my heartfelt thanks to the most important people in my life. My dear Mum and Dad, Valentina and Vladimir, my brother Pavel and my Sergej – your love provided my inspiration and was my driving force. Your understanding and indescribable support throughout my whole life are invaluable. I am very grateful for your care, your patience, your love and trust in me.

The study was supported by the following financial sources:

- Ministry of Education, Youth and Sports of the Czech Republic CENAKVA No. CZ.1.05/2.1.00/01.0024.

- The Czech Science Foundation GACR P503/11/113.
- The Grant Agency of the University of South Bohemia No. 047/2010/Z.
- The Grant Agency of the University of South Bohemia No. 038/2011/Z.
- The Grant Agency of the University of South Bohemia No. 087/2013/Z.
- Ministry of Education, Youth and Sports of Czech Republic Kontakt II LH12179.
- The Swedish Foundation for Strategic Environmental Research (MISTRA) (within the research project MISTRAPHARMA).

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Fedorova, G., Grabic, R., Randak, T., 2011. Residues of antibiotics in fish meat available at Czech market. AQUA 2012, Prague, Czech Republic, 1–5 September 2012. (oral presentation)	2012
Fedorova, G., Golovko, O., Grabic, R., 2011. Different Approaches for the Monitoring of Pharmaceuticals and Personal Care Products in Wastewater. SETAC, Glasgow, Scotland, 11–16 May 2013. (poster presentation)	2013

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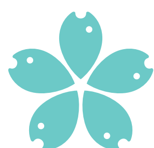

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a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

ISBN 978-80-87437-72-8