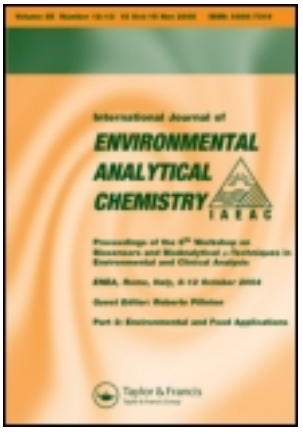

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Determination of cephalosporin antibiotics in water samples by optimised solid phase extraction and high performance liquid chromatography with ultraviolet detector

Penghua Wang [a], Tao Yuan [a], Jiangyong Hu [b] & Youming Tan [c]
[a] School of Environmental Science and Engineering, Shanghai Jiao Tong University, Shanghai 200240, China
[b] Division of Environmental Science and Engineering, National University of Singapore, 10 Kent Ridge Crescent 119260, Singapore
[c] School of Public Health, Shanghai Jiao Tong University, Shanghai 200025, China

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Determination of cephalosporin antibiotics in water samples by optimised solid phase extraction and high performance liquid chromatography with ultraviolet detector

Penghua Wang[a], Tao Yuan[a]*, Jiangyong Hu[b] and Youming Tan[c]

[a]*School of Environmental Science and Engineering, Shanghai Jiao Tong University, Shanghai 200240, China;* [b]*Division of Environmental Science and Engineering, National University of Singapore, 10 Kent Ridge Crescent 119260, Singapore;* [c]*School of Public Health, Shanghai Jiao Tong University, Shanghai 200025, China*

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A reliable and robust analytical method based on solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detector was developed for the simultaneous determination of five cephalosporin antibiotics (Ceftazidime, Cefradine, Cefaclor, Cefotaxime and Cefoperazone) in various water samples. Under optimised conditions, it was applicable to preconcentrate up to 500 ml of water samples in the OASIS HLB cartridges with reasonable recoveries for all the cephalosporin antibiotics tested. Recoveries were as follows: deionised water, tap water and groundwater, between 84.2 and 98.9%; surface water, between 71.2 and 81.0%; influent and effluent of wastewater treatment plant (WWTP), between 56.9 and 72.1%. The method detection limits (MDLs) for different water samples were in the range of 26 to 59 $ng\,l^{-1}$. Real water samples were analysed using the proposed approach to demonstrate the applicability and validation. Negative results were obtained for the tap water and groundwater. However, all the selected cephalosporin antibiotics were identified in the influent and effluent of a local WWTP at $ng\,l^{-1}$–$\mu g\,l^{-1}$ level. In addition, Ceftazidime was found in surface water with a concentration of 0.75–2.60 $\mu g\,l^{-1}$. The results indicate that the 'pseudo-persistent' contamination of cephalosporin antibiotics in the water environment could not be neglected.

Keywords: cephalosporin antibiotics; water sample; solid phase extraction (SPE); high performance liquid chromatography (HPLC)

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are a diverse group of environmental emerging chemicals with a low level of analytical measurements, comprising human and veterinary drugs (including antibiotics, antiphlogistics/anti-inflammatory drugs, β-blockers, lipid regulators, antiepileptics, etc.), diagnostic agents (such as X-ray contrast media) and other consumer chemicals (such as cosmetics, fragrances and sun-screen agents) as well as inert ingredients or excipients used in PPCPs' formulations and manufacture [1–4]. Unlike 'persistent, bioaccumulative toxic' (PBT), 'persistent organic pollutants' (POPs) and other 'bioaccumulative chemicals of concern' (BCCs), which have

*Corresponding author. Email: taoyuan@sjtu.edu.cn

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been investigated for decades, PPCPs have been studied during recent years. Additionally, antibiotics pollution in the aquatic environment has become one of issues of most concern raised by PPCPs.

As a matter of fact, antibiotics have become the kind of 'pseudo-persistent' pollutants in the aquatic environment since they have been widely and continually used in daily life, although their half-lives are relatively shorter than those of POPs. To some degree, antibiotics' residue in the environment is responsible for the appearance of drug-resistant bacteria. Furthermore, the health and ecological risks of antibiotics' residue cannot be ignored, as adverse effects on human beings and the ecological environment by them have been constantly reported worldwide [5–9]. Antibiotics could find their way into municipal sewage or hospital wastewater in their original structure or metabolites after being taken by human beings and animals. Although all these wastewaters are to be treated through wastewater treatment plants (WWTPs), ineffective operations of WWTPs make their widespread appearance in the aquatic environment possible [10–11]. Different levels of antibiotics have been detected in the wastewater from antibiotics factories and hospitals [12]. In addition, antibiotics could enter the environment from some other sources, including run-off from animal feeding operations, infiltration from aquaculture activities, leaching from landfills and from compost made from animal manure containing antibiotics [10,12–13]. Recently, an increasing number of antibiotics were found in the aquatic environment (including surface, ground, drinking water and sludge), generating a growing concern from public authorities and the population. Most of the identified antibiotics in the environment belong to fluoroquinolones, sulfonamides, trimethoprim and macrolides, etc. [14–17].

In recent years, there has been a rapid development of β-lactam antibiotics, in which most attention has been focused on cephalosporin antibiotics. Nowadays these antibiotics hold a large share in the global market and can be considered to be one of the most important and most frequently used groups of antibiotics [18]. It was noted that, to date, the occurrence reports of cephalosporin antibiotics in the aquatic environment have been relatively scarce, compared with those of the antibiotics mentioned above. One of the major reasons is that this group of antibiotics contains a common chemically unstable β-lactam nucleus in the molecular, which is highly sensitive to pH, heat and β-lactamase enzymes, etc. However, as mentioned above, it could also occur as 'pseudo-persistent' pollutants in the environment owing to the large amount of daily application.

It has been reported that China, in 2003, was one of world's largest producers of β-lactam antibiotics, and it is ranked first among nations for cephalosporin production [19]. Additionally, China is known anecdotally for the inappropriate use of antibiotics products. As such, the residue of cephalosporin antibiotics in the Chinese aquatic environment might be a critical issue, albeit with the contamination by β-lactam antibiotics being of minor importance to the mixed-watershed in other places such as northern Colorado [20]. In 2008, several cephalosporin antibiotics (Cefalexin, Cefotaxime and Cefazolin) were detected in influent and effluent samples from four sewage treatment plants (STPs) in Hong Kong as well as in influent samples from one STP in Shenzhen, China [21]. The concentrations of these ranged from ng l^{-1} to μg l^{-1}. It was also indicated that the occurrences and concentrations of cephalosporin antibiotics would be affected by the regional variations in the prescription and use patterns of antibiotics [21].

Unfortunately, the methods for the determination of cephalosporin antibiotics in environmental water samples have not been well documented to date, although the corresponding methods for biological fluids were critically reviewed by

El-Shaboury *et al*. [18]. It was noted that the method development for these antibiotics is much more difficult to some extent due to the chemical instability of the common β-lactam nucleus and the minor differences in chemical structures between the analogues. Niu *et al*. [22] attempted to extract several cephalosporin antibiotics, sulfonamides and phenolic compounds, from aqueous solution using carbon nanotubes as a solid-phase extraction adsorbent. However, the method was only validated for sulfonamides in real water samples. Recently, Gulkowska *et al*. [23] and Cha *et al*. [20] covered three cephalosporin antibiotics (Cefalexin, Cefotaxime and Cefazolin) and one (Cephapirin) cephalosporin antibiotic, respectively, in their methods developed for the determination of antibiotics in water samples. In addition, Rao *et al*. [24] developed a method to determine antibiotics, including several cephalosporins (Cefaclor, Cefadroxil, Cefdinir, Cefprozil, Ceftiofur and Cefuroxime axetil), in surface waters. However, these methods relied on high performance liquid chromatography coupled with tandem mass spectrum (HPLC-MS/MS), which may not be available and/or applicable in most laboratories. Moreover, none of them focuses on the method for the determination of cephalosporin antibiotics. Thus, less information would be provided for detection method development about other cephalosporin antibiotics when regional variations in the prescription and application patterns are concerned. More recently, Puig *et al*. [25] developed and optimised the capillary electrophoresis based method for determinations of two cephalosporin antibiotics, Ceftiofur (a cephalosporin antibiotic approved for veterinary use) and Cefoperazone, in water samples. However, to achieve a satisfactory detection limit, what was required was a complicated preconcentration process such as large-volume sample stacking to improve sensitivity. On the other hand, as mentioned above, since there has been a lack of detailed study on the occurrence, fate and behaviour of cephalosporin antibiotics in the water environment, it is also imperative to develop a direct and applicable analytical method to measure the concentrations of these antibiotics in water samples.

As mentioned above, a number of researchers have successfully determined cephalosporin antibiotics in biological samples using HPLC with UV detector [18]. Moreover, solid phase extraction (SPE), which allows a large sample volume to be concentrated and purified in one step, has been widely applied in the analysis of trace level of analytes in water samples.

In view of the above, the main objective of this study was to develop a method for the simultaneous determination of the selected cephalosporin antibiotics (Ceftazidime, Cefradine, Cefaclor, Cefotaxime and Cefoperazone), which are widely used antibiotics, in water samples using SPE coupled with HPLC method. The accuracy and precision of HPLC analysis and method detection limit (MDL) were described. The SPE optimisation and analytes' stability were also discussed in this study. Finally, the method was validated by investigating the occurrence of these cephalosporin antibiotics in the aquatic matrices, such as tap water, groundwater, surface water, influent and effluent of a local WWTP. To our knowledge, this is the first time that the selected cephalosporin antibiotics have been simultaneously determined in the aquatic matrices using SPE with HPLC.

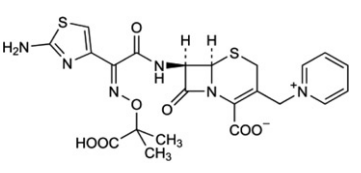
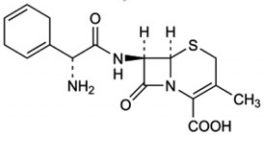
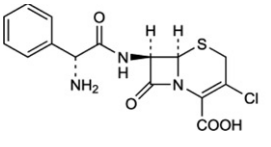
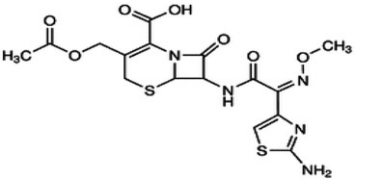
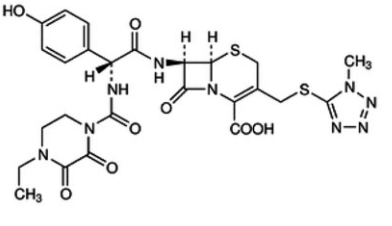
2. Experimental

2.1 *Chemicals and reagents*

Chemical standards of Ceftazidime (purity, >84.2%), Cefradine (>91.8%), Cefaclor (>93.2%), Cefotaxime (>89.3%), Cefoperazone (>96.5%) were purchased from National

Institute for the Control of Pharmaceuticals and Biological Products of China. The CAS numbers, molecular formulas, molecular weights, molecular structures and other information of the selected cephalosporin antibiotics are summarised in Table 1. HPLC-grade methanol and formic acid (96%) were purchased from Tedia, USA (by Shanghai Dahu Scientific Instrument Ltd.). Disodium ethylenediamine tetra-acetate (Na_2EDTA, ≥99%) was purchased from Sigma-Aldrich, USA (by Shanghai Dahu Scientific Instrument Ltd.). Ultrapure water was used throughout the study unless otherwise stated.

Table 1. Basic information of the selected cephalosporin antibiotics in this study.

Antibiotics	CAS number[a]	Molecular formula and weight[a]	pKa[a]	logKow[b]	Chemical structure[a]
Ceftazidime	72558-82-8	$C_{22}H_{22}N_6O_7S_2$ 546.60	1.9, 2.7, 4.1	−1.36	
Cefradine	38821-53-3	$C_{16}H_{19}N_3O_4S$ 349.41	2.5, 7.3	0.41	
Cefaclor	53994-73-3	$C_{15}H_{14}ClN_3O_4S$ 367.81	1.5, 7.2	0.35	
Cefotaxime	63527-52-6	$C_{16}H_{17}N_5O_7S_2$ 455.46	3.8	0.64	
Cefoperazone	62893-19-0	$C_{25}H_{29}N_9O_8S_2$ 645.67	2.6	−0.74	

Notes: [a]Data collected from Clarke's Analysis of Drugs and Poisons (http://medicinescomplete.com/mc/clarke/current/).
[b]Data collected from PhysProp Database (http://www.syrres.com/esc/physdemo.htm).

2.2 *Sample collection and preparation*

It was found that some other β-lactam antibiotics can be readily adsorbed on the glassware walls [26]. This indicates that cephalosporin antibiotics adsorption on the glassware should not be ignored, especially in the case of trace analysis in this study. In addition, various metal ions can catalyse the rate of inactivation or hydrolytic opening of cephalosporin antibiotics [27]. To eliminate these factors, all of the glassware used in experiment were washed under ultrasonic, then rinsed with deionised water, 5% Na_2EDTA and ultrapure water 3 times, respectively. At last, all glassware (wrapped with silver paper) was heated at 130°C for over 4 h before use.

Water samples included tap water (directly collected from a local family), groundwater (collected from a well in Nanhui District in Shanghai), surface water (collected at a depth of 0.25 to 1.0 m from a creek flowing through Minhang campus of Shanghai Jiao Tong University), influent and effluent of WWTP (collected from a local WWTP with A^2/O as the treatment processes, average daily inflow of 50,000 $m^3\,day^{-1}$, which includes urban (65%) and industrial (35%) wastewater and serves 150,000 people from areas in the vicinity of 28 km^2). All the samples were collected as grab sample and at least in triplicate. To avoid contamination and analytes' loss, polypropylene bottles for water sampling were rinsed with methanol, deionised water and subsequently corresponding water sample before sample collection. The water samples were transported to the laboratory as soon as possible in order to keep analytes in their original modality. In the lab the water samples were first centrifuged at 8000 r min^{-1} for 10 min at 4°C, and then filtered through qualitative filter paper (Whatman, Grade 1: 11 μm) and 0.22-μm millipore film to remove particulate matter prior to extraction. Considering the tendency towards biodegradation and/or hydrolysis of the labile cephalosporin antibiotics, all samples were stored at 4°C prior to SPE. Extraction and measurement were performed within 48 h after collection.

2.3 *Solid-phase extraction (SPE)*

In this study, SPE was performed using a 12-fold vacuum extraction manifold device (Supelco, USA). To achieve satisfactory performance, a series of SPE cartridges, i.e. ENVI-18 (500 mg/3 ml, silica-based reverse phase type, Supelco), Discovery DPA-6S (250 mg/3 ml, polymeric reverse phase type, Supelco), Bond Elut Plexa (200 mg/6 ml, polymeric reverse phase type, Varian) and OASIS HLB (200 mg/6 ml, polymeric reverse phase type, Waters), were tested and compared. In addition, some factors influencing recovery, such as pH and loading volume of samples, extraction flow rate and elution process, were investigated.

Under the optimised conditions, 500 ml water samples were prepared for extraction by adding 5 ml of 5% Na_2EDTA and being acidified to pH 2.5 with 0.5 M HCl to inhibit further biological degradation and enhance trapping of the acidic compounds on the SPE sorbent. Before sample concentration, the cartridges were first preconditioned with 4 ml methanol and then with 4 ml ultrapure water. It was followed by pretreated-samples passing through the cartridges at flow rate of 5–10 ml min^{-1}. Then the cartridges were washed with 6 ml ultrapure water to remove excess Na_2EDTA and other impurity. The cartridges were air dried under vacuum for about 2 h to remove excess water. The analytes retained were eluted with 3×2 ml of methanol at normal pressure. The eluates were collected in tubes and evaporated subsequently to dryness under a gentle stream of nitrogen. The extracts were redissolved with 1 ml of ultrapure water. After thoroughly

mixing, 1 ml of extract was filtered by filter holder (Puradisc25NYL) and transferred into an amber vial (to prevent photodegradation) for further analysis.

2.4 *High performance liquid chromatography (HPLC)*

The LC system was LC-10A HPLC (Shimadzu, Japan) including an SPD-10A UV detector and a couple of pumps. Cephalosporin antibiotics were separated using a Shim-pack VP-ODS (150 mm × 4.6 mm, 5 μm) chromatogram column in combination with a guard column GVP-ODS (10 mm × 4.6 mm, 5 μm). Methanol was used as mobile phase A and 0.1% formic acid in water was used as mobile phase B (A/B = 40/60 (v/v)). Analyses were carried out at a flow rate of 0.5 ml min^{-1}. The UV detector wavelength was dually set at 254 and 270 nm to improve method accuracy. Column temperature was kept at 35°C. The injection volume was 10 μl. Quantification was performed using external calibration and peak area measurements.

2.5 *Stability of cephalosporin antibiotics in water*

The experiment of stability of the selected cephalosporin antibiotics in water was carried out before sample collection and calibration curve establishment. Mixed working solutions of different concentrations (1, 3 and 5 μg ml^{-1} in water) were made from stock solution (1 mg ml^{-1} in ultrapure water, stored at 4°C in refrigerator) by dilution with ultrapure water. The mixed working solutions were analysed at different time intervals by HPLC. The stability was evaluated according to their changes of concentration.

2.6 *Method validation*

To assess the accuracy and precision of HPLC analysis for cephalosporin antibiotics, repeatability experiments were carried out with 1, 3 and 5 μg ml^{-1} of mixed standards solution, respectively. Five replicates for each concentration were run within three different days. Limit of detection (LOD) and limit of quantification (LOQ) were determined using the software of CLASS-VP Ver.6.X on the signal-to-noise ratios of 3 and 10, respectively. A series of mixed working solutions were freshly prepared by diluting the stock solution with ultrapure water. For each cephalosporin antibiotics, at least seven concentration points were employed to plot the calibration curves.

For recovery studies, 500 ml of deionised water, tap water, groundwater, surface water and effluent of WWTP were spiked with 5 μg l^{-1} of Ceftazidime, Cefradine, Cefaclor, Cefotaxime and Cefoperazone, and 500 ml of influent of WWTP spiked with 10 μg l^{-1} before extraction. The recovery was determined by Equation (1).

$$\text{Recovery}(\%) = [(C - C_0)/C_1] \times 100 \quad (1)$$

where C was concentration of spiked samples, C_0 was concentration of original samples and C_1 was spiked concentration.

Several other tap water, groundwater, surface water, influent and effluent wastewater samples were collected to analyse whether they contained detectable quantities of the analytes of interest or not. And samples with no analytes were selected as the reference water samples. Method detection limit (MDL) was determined according to the method recommended by US EPA [28]. Briefly, MDL was calculated by Equation (2) in which SD

stands for the standard deviation of samples spiked some amount of analyte, n for repeated number and t (n, 0.99) for t-Statistic on the basis of 99% confidence level with $n-1$ degrees of freedom.

$$\mathrm{MDL} = \mathrm{SD} \times t(n, 0.99) \tag{2}$$

In this study, reference samples for MDL calculation were spiked at $50\,\mathrm{ng\,l^{-1}}$ ($n=7$) for deionised water, tap water, groundwater, surface water and effluent of WWTP, and at $100\,\mathrm{ng\,l^{-1}}$ ($n=7$) for influent of WWTP, respectively. Additionally, to avoid system error and contamination of analytes, 500 ml deionised water was applied as blank sample every 10 measurements in the processes of method development and real sample analysis.

3. Results and discussion

3.1 *Stability of cephalosporin antibiotics in water*

The stability of β-lactam antibiotics standard in various solutions during their storage was reported elsewhere. Lindberg *et al.* [12] found that no significant decline of $80\,\mathrm{mg\,l^{-1}}$ amoxicillin and ampicillin in deionised water at 4°C after one month of storage occurs. Fagerquist *et al.* [29] did not observe any significant degradation for amoxicillin, ampicillin, oxacillin and Penicillin G at 10, 1 and $0.1\,\mu\mathrm{g\,ml^{-1}}$ in methanol–water (v/v = 50/50) for 12 days at −20°C while 20% degradation for cloxacillin and 10% degradation for dicloxacilline occurs. In this study, the result showed that the degradation of the selected cephalosporin antibiotics in mixed working solutions were not significant for 4 days stored at 4°C. However, the degradation percentage began to increase greatly from the fifth day. Thus, the mixed working solution was replaced every 4 days using ultrapure water in order to avoid analytical error.

3.2 *SPE optimisation*

A series of parameters were evaluated to establish the optimum conditions for the SPE procedure. The factors include the selection of SPE cartridges, pH of the sample, washing solutions, composition and volume of the eluting solutions and water samples' volume.

In this study, four types of SPE cartridges were evaluated to simultaneously preconcentrate the five cephalosporin antibiotics in ultrapure water which were spiked at $5\,\mu\mathrm{g\,l^{-1}}$ standards in triplicate. Their recoveries were compared at two pH conditions (pH 2.5 and 7.5). The results were summarised in Figure 1. It was noted that when pH was 7.5, most recoveries of cephalosporin antibiotics were very poor, except for that of Cefotaxime and Cefoperazone (88.6 and 113.8, respectively) obtained by OASIS HLB cartridge. When pH was adjusted to 2.5, both ENVI-18 and OASIS HLB cartridges could achieve satisfactory recoveries for the selected cephalosporin antibiotics, which were 60.7–90.8% and 87.6–105.9%, respectively. In addition, the performance of OASIS HLB was generally better than that of ENVI-18, especially for Cefaclor. Thus, OASIS HLB cartridge was applied in the following experiments.

On the other hand, as the aquatic solutions of cephalosporin antibiotics can be present as neutral, anionic or cationic forms, their extraction behaviour is pH dependent. In this study, to further evaluate the effect of sample pH on the extraction recoveries, a set of 500 ml ultrapure water samples spiked at $5\,\mu\mathrm{g\,l^{-1}}$ of standards, with pH values between 1.5 and 8.5 (acidified with 0.5 M HCl), were loaded into OASIS HLB cartridges and analysed

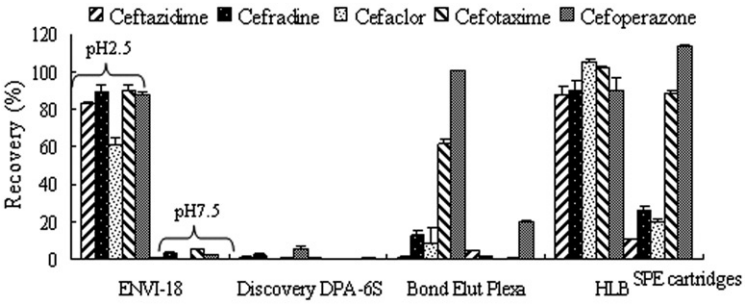


Figure 1. Recoveries of cephalosporin antibiotics using different SPE cartridges at pH2.5 and 7.5. Note: As indicated by ENVI-18 data, the left five columns and the right five columns for each of the analytes show data obtained under pH2.5 and 7.5, respectively.

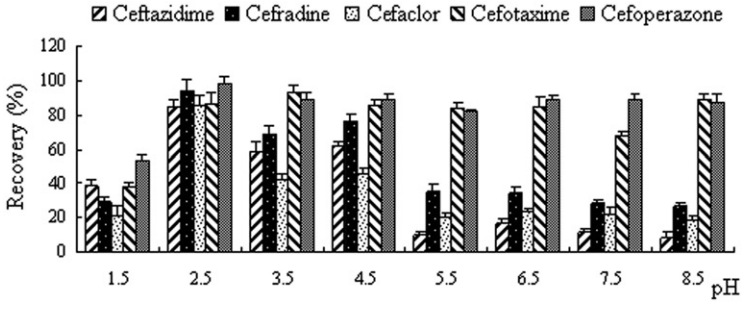


Figure 2. Effects of sample pH on the HLB cartridges' extraction recoveries.

using the procedure described in Section 2.4. It was found that the best recovery results were obtained at pH 2.5 with recoveries ranging from 84.7 to 98.1% (Figure 2).

OASIS HLB cartridges contain the copolymer made from a balanced ratio of hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. At neutral pH, the cephalosporin antibiotics were negatively charged and their interactions with the sorbent were lower than when the analytes were uncharged as happens at pH 2. Moreover, as to OASIS HLB cartridges, the polar interactions between the sorbent and the analytes were less influenced by the analyte charge. Therefore, sample was adjusted to pH 2.5 before SPE loading in the later experiments.

Before samples' loading, the SPE cartridges were preconditioned with organic reagents (methanol was used in this study) and water. Loading velocity is another important factor affecting recovery. Extraction will not be efficient when loading velocity is too fast. However, when loading velocity is slow, it makes extra degradation of cephalosporin antibiotics possible. Yang *et al*. [30] achieved higher recovery and repeatability when the loading velocity was below $5\,\mathrm{ml\,min^{-1}}$ for tetracycline and sulfonamide antibiotics. However, in this study, satisfactory recovery and repeatability were obtained when the loading velocity was around 5–10 $\mathrm{ml\,min^{-1}}$ with ultrapure water samples spiked at $5\,\mu\mathrm{g\,l^{-1}}$ standards.

There are many kinds of organic compounds in real water samples. They can be adsorbed on the cartridges as well as the analytes do. Without cartridges clean-up, they will be eluted with the analytes, resulting in baseline drift and even spillover with the

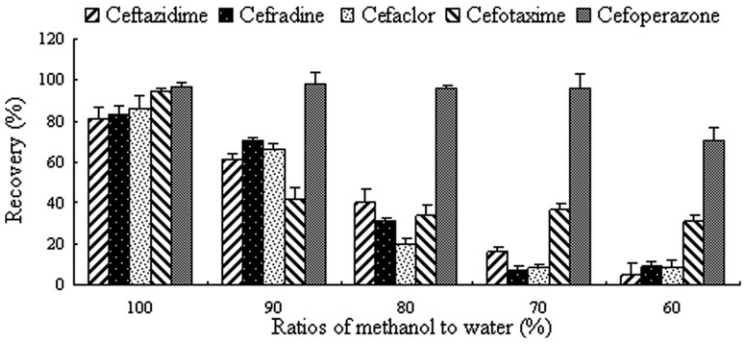


Figure 3. Effects of the ratios of methanol and water on the HLB cartridges' extraction recoveries.

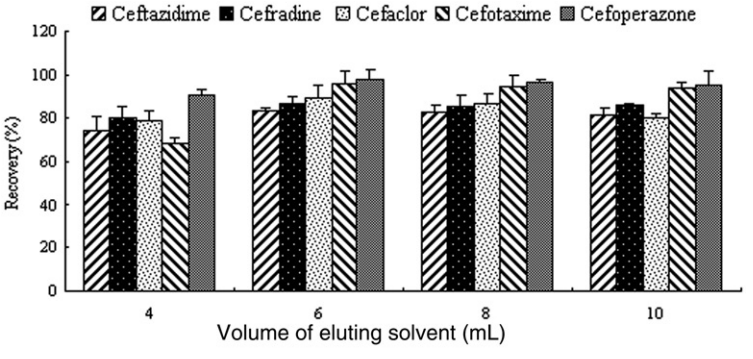


Figure 4. Effects of the volume of eluting solvent on the HLB cartridges extraction recoveries.

analytes while carrying out quantitative analysis with the HPLC. Therefore, cartridges were washed to remove some non-targets before elution process. It was found in the preliminary experiment that 6 ml of ultrapure water was enough to achieve cleaning.

As the polarity and acidity-alkalinity of eluting solution will affect the performance of elution, the kind of eluting solvent is needed not only to elute most of the targets but also to reduce the non-targets. In order to select the proper eluent to elute the retained cephalosporin antibiotics, different ratios of organic solvent/water mixtures (ratios of methanol and water are 100, 90, 80, 70 and 60%, respectively) were tested. The result showed that 100% methanol had better recovery (above 80%) than other eluting solvents (Figure 3). In addition, 3×2 ml of methanol was enough to elute the analytes (Figure 4).

In this study, breakthrough of the SPE sorbent was investigated using ultrapure water samples (from 500 ml to 1000 ml) spiked at $5\,\mu g\,l^{-1}$ of standards. The result showed no breakthrough occurred when the volume of ultrapure water samples is 1000 ml. However, the SPE cartridges were observed to be gradually blocked when loading 1000 ml of wastewater samples. The presence of matrix components in real water samples can reduce the preconcentration efficiency of antibiotics in SPE step. Higher sample volumes with higher organic matter content will augment the clogging of the SPE cartridge and negatively affect the retention of the antibiotics. Thus, sample volume of 500 ml was applicable for SPE loading in this study.

3.3 *HPLC method development*

In this study, additional reference wavelength was applied to improve the accuracy of analytes qualification. Our preliminary experiments revealed that 254 nm was the optimum analytical wavelength for the selected cephalosporin antibiotics, since both satisfactory chromatographic response and segregative sensitivity were achieved. Considering that some unknown interfering compounds in real samples would possibly have same retention time with the analytes, a reference wavelength of 270 nm was introduced to check if there is unknown interfering compound co-eluted with analytes. In this study, the peak areas ratios of 254 to 270 nm for Ceftazidime, Cefradine, Cefaclor, Cefotaxime and Cefoperazone are 2.2, 1.9, 1.5, 2.0 and 1.5, respectively. So far, no unknown interfering compounds were co-eluted from the real samples, which may be attributed to the specific structure of cephalosporin antibiotics and the optimised SPE procedure. Lastly, regarding the real samples, the standard addition strategy was also applied to further guarantee the method reliability (data was shown below in Section 3.5).

As to the mobile phase of HPLC analysis, a number of solvents were employed for the determination of cephalosporin antibiotics [18,20]. In this study, methanol and 0.1% formic acid were chosen as the mobile phases, as better results were achieved by them, compared with other solvents applied.

A series of preliminary experiments were carried out for HPLC analysis under different flow rates and ratios of mobile phase. The results showed that the satisfactory chromatogram was achieved when the rate was $0.5\,\mathrm{ml\,min^{-1}}$ and the ratio of methanol/0.1% formic acid was 40/60 (Figure 5). Thus, these HPLC conditions were applied in the later experiments.

3.4 *Method validation*

3.4.1 *Accuracy, precision, linearity and LOD/ LOQ of the HPLC analysis*

The results showed that the relative standard deviations (RSD) of retention times obtained in different days were below 0.12, 0.32, 0.22, 0.30 and 0.42 for Ceftazidime, Cefradine, Cefaclor, Cefotaxime and Cefoperazone with different concentrations (1, 3, $5\,\mu\mathrm{g\,ml^{-1}}$), respectively (refer to supplementary online material – see notes to Table 1).

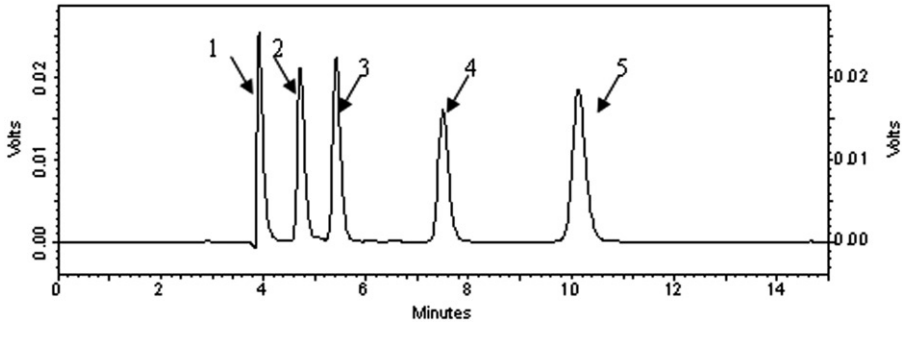


Figure 5. Typical chromatogram under the optimised separation conditions of HPLC.
Note: 1. Ceftazidime 2. Cefradine 3. Cefaclor 4. Cefotaxime 5. Cefoperazone; Mobile A: methanol, Mobile B: 0.1% formic acid in water (A/B = 40/60 (v/v)), 254 nm, $0.5\,\mathrm{ml\,min^{-1}}$, 35°C.

This demonstrated the satisfactory stability and reliability of the instrument analysis. Detected concentrations of Ceftazidime, Cefradine, Cefaclor, Cefotaxime and Cefoperazone with different concentrations (1, 3, 5 $\mu g\,ml^{-1}$) (Table 2) confirmed the satisfactory accuracy of analysis.

Instrumental LOD and LOQ were between 11.2 to 19.9 $\mu g\,l^{-1}$ and 32.2 to 60.2 $\mu g\,l^{-1}$, respectively (Table 2). In addition, according to the LOD, all blank samples were negative of analytes of interest in this study. Corresponding linear ranges and determination coefficient ($R^2 > 0.99$) are also summarised in Table 2.

3.4.2 *Recoveries and method detection limit (MDL)*

Recoveries and MDLs of the selected cephalosporin antibiotics in different water samples are given in Table 3 and Table 4, respectively. It was noted that the recoveries of influent and effluent of WWTP could achieve above 60% (except for Ceftazidime in influent). Other water samples obtained were above 70% of recoveries for all selected cephalosporin antibiotics. The MDLs of the selected cephalosporin antibiotics in deionised water, tap water, groundwater and surface water were from 26 to 45 $ng\,l^{-1}$. However, slightly higher MDLs, from 32 to 59 $ng\,l^{-1}$, were found in influent and effluent of WWTP.

Table 2. Accuracy and parameters of quantitative analysis of five cephalosporin antibiotics by HPLC.

Antibiotics	LOD ($\mu g\,l^{-1}$)	LOQ ($\mu g\,l^{-1}$)	Linear range ($\mu g\,ml^{-1}$)	R^2	Detected concentrations ($n=8$)		
					1 $\mu g\,ml^{-1}$[a]	3 $\mu g\,ml^{-1}$	5 $\mu g\,ml^{-1}$
Ceftazidime	11.2	32.2	0.02–5.00	0.9967	0.98[b] (0.68)[c]	2.97 (0.52)	4.95 (0.39)
Cefradine	14.1	51.2	0.05–10.00	0.9936	0.96 (0.47)	2.97 (0.52)	4.96 (0.30)
Cefaclor	14.2	52.3	0.05–10.00	0.9992	0.98 (0.92)	2.98 (0.30)	4.98 (0.45)
Cefotaxime	15.1	45.7	0.02–5.00	0.9999	0.98 (0.54)	2.97 (0.29)	4.94 (0.17)
Cefoperazone	19.9	60.2	0.04–20.00	0.9995	0.98 (0.66)	3.01 (0.37)	4.98 (0.29)

Notes: [a]Theoretical concentration.
[b]Average value.
[c]Relative standard deviation (%RSD).

Table 3. Spiked recoveries of five cephalosporin antibiotics in water samples.

Antibiotics	Recovery (%, $n=3$)					
	Deionised water[a]	Tap water[a]	Groundwater[a]	Surface water[a]	Influent of WWTP[b]	Effluent of WWTP[a]
Ceftazidime	84.2[c] (4.8)[d]	90.7 (4.6)	88.1 (6.5)	75.5 (6.1)	56.9 (11.5)	66.1 (12.5)
Cefradine	93.8 (5.1)	90.3 (2.9)	89.4 (3.7)	78.8 (8.5)	65.9 (11.1)	63.8 (12.6)
Cefaclor	98.9 (9.9)	87.2 (6.3)	85.1(4.9)	76.7 (9.0)	61.3 (6.9)	70.2 (5.9)
Cefotaxime	95.9 (8.7)	92.4 (2.4)	92.5 (3.3)	81.0 (9.6)	63.9 (8.4)	60.3 (9.7)
Cefoperazone	94.2 (5.5)	91.9 (3.4)	84.2 (7.1)	71.2 (10.1)	68.5 (13.8)	72.1 (10.7)

Notes: [a]Spiked at a concentration of 5 $\mu g\,l^{-1}$.
[b]Spiked at a concentration of 10 $\mu g\,l^{-1}$.
[c]Average value.
[d]%RSD.

Table 4. MDLs of five cephalosporin antibiotics in water samples.

Antibiotics	MDL ($ng\,l^{-1}$, $n=7$)					
	Deionised water	Tap water	Groundwater	Surface water	Influent of WWTP	Effluent of WWTP
Ceftazidime	26	30	30	29	52	33
Cefradine	33	33	31	34	50	44
Cefaclor	30	29	29	34	42	35
Cefotaxime	34	36	35	34	55	37
Cefoperazone	40	40	41	45	59	46

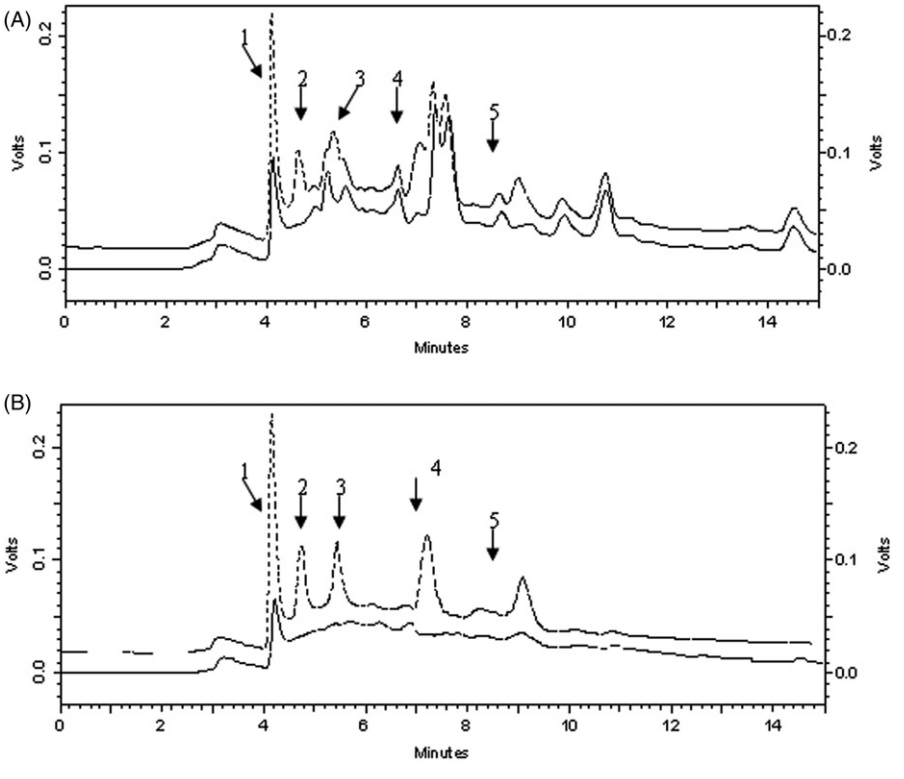


Figure 6. Chromatograms of original (real line) and spiked (dashed line) wastewater samples. Note: A and B indicate influent and effluent wastewater samples, respectively; numbers 1 to 5 indicate Ceftazidime, Cefradine, Cefaclor, Cefotaxime, Cefoperazone, respectively; baselines of the spiked samples were intentionally moved up for comparison purposes.

3.5 *Analysis of cephalosporin antibiotics in various water samples*

The developed method was successfully applied to analyse the selected cephalosporin antibiotics in various water samples. All the samples were collected as grabbed and replicate samples in the Winter of 2007 and Spring of 2008. Chromatograms of original and spiked wastewater samples are shown in Figure 6. The results are listed in Table 5.

Table 5. Concentrations of five cephalosporin antibiotics in real water samples during different periods ($\mu g\,l^{-1}$).

Antibiotics	Tap water ($n=5$)[a]	Groundwater ($n=5$)	Surface water ($n=5$)	Influent ($n=4$)	Effluent ($n=3$)
Ceftazidime	n.d.[b]	n.d.	0.75–2.60	1.85–3.68	0.12–0.60
Cefradine	n.d.	n.d.	n.d.	0.40–2.14	n.d.–0.43
Cefaclor	n.d.	n.d.	n.d.	n.d.–0.97	n.d.–0.43
Cefotaxime	n.d.	n.d.	n.d.	n.d.–0.82	n.d.–0.13
Cefoperazone	n.d.	n.d.	n.d.	n.d.–0.94	n.d.–0.53

Notes: [a]Times of determination.
[b]Not detected.

The values reported in this study were not corrected by corresponding recoveries. It was found that none cephalosporin antibiotics were detected in the samples of tap water and groundwater. However, cephalosporin antibiotics were detected in surface water, influent and effluent from WWTP (Table 5). Rao *et al.* [24] attempted to identify some antibiotics including several other cephalosporin antibiotics (such as Cefadroxil, Cefdinir, Cefprozil, Ceftiofur and Cefuroxime axetil) in surface waters using a HPLC-MS/MS based method. According to the results, the cephalosporin antibiotics they included were not found in surface water [24]. It was reported that Cefalexin were identified at $ng\,l^{-1}$ level ($182\,ng\,l^{-1}$) in surface waters collected from Victoria Harbour, Hong Kong [23]. Cha et al. [20] investigated the occurrence and fate of Cephapirin in both wastewater from the Drake Water Reclamation Facility (a WWTP in Greely) and surface water from the Cache la Poudre River through pristine, urban and agricultural landscapes in northern Colorado. They found that most of samples (60 surface water samples, 72 influent and 72 effluent wastewater samples) were negative for Cephapirin, except one sampling site (Site 5) with the greatest influence of agriculture, was detected once at $9\,ng\,l^{-1}$ of Cephapirin [20]. However, in our study, Ceftazidime was found in surface water and all selected cephalosporin antibiotics were identified in the influent and effluent wastewater samples, some of which were even at $\mu g\,l^{-1}$ level (Table 5). To our knowledge, this is the first report for simultaneous determination of the selected five cephalosporin antibiotics in water matrices. It is also the first analytical method and occurrence study of Ceftazidime and Cefradine in environmental water samples, to our knowledge.

4. Conclusions

A reliable method was developed for the simultaneous detection of five cephalosporin antibiotics in various water samples. SPE procedure was optimised in terms of SPE cartridge selection, sample pH, loading velocity, sample volume and elution process. In this study, 500 ml of water sample was acidified to pH 2.5 to enhance trapping of the analytes on the OASIS HLB cartridges (200 mg/6 ml, Waters). After extraction, the cartridges were then washed and eluted with 6 ml ultrapure water and 3×2 ml methanol, respectively. In addition, HPLC analysis had been reinforced to achieve satisfactory chromatographic response and segregative sensitivity. A reference wavelength of 270 nm was introduced to avoid possible interfering compounds in real water samples. The recoveries for influent and effluent of WWTP could achieve 56.9–68.5% and

60.3–72.1%, respectively. As to other water matrices, the recoveries ranged from 71.2% to 98.9%. The satisfactory MDLs of the selected cephalosporin antibiotics could be achieved in all tested samples, which were from 26 to 59 $ng\,l^{-1}$.

Finally, the proposed method was validated by analysis of real samples. Negative results were obtained for the selected cephalosporin antibiotics in tap water and groundwater. However, all the selected cephalosporin antibiotics were identified in the influent and effluent of a local WWTP. In addition, Ceftazidime was found in surface water with a concentration of 0.75–2.60 $\mu g\,l^{-1}$. To our knowledge, this is the first study for the simultaneous detection of cephalosporin antibiotics in water matrices using SPE and HPLC. It is also the first occurrence report of Ceftazidime and Cefradine in water environmental samples, to our knowledge. The relatively high occurrences of cephalosporin antibiotics in the local water environment are consistent with the fact that there is mass production and inappropriate use of this group of antibiotic products in China. The results indicate that the 'pseudo-persistent' situation of cephalosporin antibiotics could not be neglected, as the ecological risk of these antibiotics or their degradation products in the water environment is unclear.

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