Talanta 85 (2011) 1238–1245

Contents lists available at ScienceDirect

Talanta

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

Measurement of trace levels of antibiotics in river water using on-line enrichment and triple-quadrupole LC–MS/MS

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ARTICLE INFO

Article history:
Received 16 February 2011
Received in revised form 29 April 2011
Accepted 5 May 2011
Available online 31 May 2011

Keywords:
Antibiotics
On-line solid phase extraction
Liquid chromatography–tandem mass spectrometry
River water

ABSTRACT

This study presents the development of an automated on-line solid phase extraction (SPE)–liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of 23 antibiotics in environmental water samples. After optimisation of LC–MS/MS conditions, SPE parameters such as sorbent type, sample pH or sample volume were optimised. Antibiotic recoveries ranged from 64% to 98% and compared favourably with those achieved using off-line SPE. Limits of detection were in the range 0.5–13.7 ng L^{-1}.

This on-line SPE–LC–MS/MS procedure was applied to the analysis of water samples taken in three rivers within the Seine River basin, near Paris (France). The obtained results revealed the occurrence of 12 antibiotics, including tylosin, erythromycin, tetracycline, amoxicillin, trimethoprim, sulfamethoxazole, oxolinic acid, flumequine, norfloxacin, ciprofloxacin, ofloxacin, and vancomycin (2–1435 ng L^{-1}).

1. Introduction

Antibiotics are an important group of pharmaceuticals which has been widely used in both human and veterinary medicine [1–3] The worldwide total antibiotic consumption is estimated between 100 000 and 200 000 tons per year [4]. In 1999, more than 13 000 tons of antibiotics were used in the European Union plus Switzerland [2]; France had the highest consumption with 729 tons used for human medicine purposes and 1295 tons used for veterinary medicine in 2002 [5].

After administration, and depending on compound chemical properties, 5–90% of the antibiotic dose may be excreted as metabolites or parent compounds [2,6–8]. Therefore, large amounts of antibiotics are susceptible to enter in aquatic systems directly via wastewater treatment plants (WwTPs) effluent discharge or via aquaculture activities. However, the application of sludge and animal manure to agricultural fields as fertilizers may contaminate agricultural soils, and lead to indirect input of antibiotics into hydrosystems, by leaching to surface waters and/or infiltration into groundwater [1].

Antibiotics have been detected in wastewater, surface water, ground water and drinking water [9–13], as well as in soil [14,15] and in river bed sediment [16,17]. Moreover, it has been showed that some antibiotics may persist for up to several months in surface waters [18,19] and in sediments [20–23]. Several studies have demonstrated that antibiotic contamination may have deleterious impacts on ecosystems [7,24–26] and that it could also favour the development of resistant bacterial strains [27–29].

The analysis of antibiotics in environment samples represents a difficult task due to the high complexity of the matrices analysed and to the low concentrations of these compounds in environmental samples [30]. Most published analytical methods for the determination of antibiotics in environmental liquid matrices relied on off-line solid-phase extraction (SPE) and liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS) [30]. A few studies, however, reported on the development of on-line SPE–LC–MS/MS methods that allow for a reduction of sample preparation time and for an increase of productivity. Such studies were usually restricted to a few classes of antibiotics only [31–34].

Thus, the aim of the present work was to develop an automated multi-residue analytical method for the simultaneous determination in river water of 23 antibiotics belonging to 9 classes. Once this on-line SPE–LC–MS/MS procedure was optimised and validated, it was applied to the analysis of water samples collected from three rivers in the Seine River basin (nearby Paris, France).

2. Experimental

2.1. Reagents and chemicals

The following antibiotics were purchased from Sigma–Aldrich (St Quentin Fallavier, France): *Sulfonamides* (SFs): sulfamethazine

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doi:10.1016/j.talanta.2011.05.013

Table 1
Acquisition parameters for antibiotic analysis in SRM mode. Frag: fragmentor; CE: collision energy.

	Precursor ion (*m*/*z*)	Product ion (*m*/*z*) (quantification)	Frag (V)	CE (eV)	Product ion (*m*/*z*) (confirmation)	Frag (V)	CE (eV)
TLS	916.5	174.1	185	40	772.5	185	28
ERY	734.5	158.1	175	28	116.1	175	48
CTE	479.1	444.1	140	28	462.1	140	12
TET	445.2	410.2	165	16	154.1	165	24
AMO	366.1	114.0	100	17	208.1	100	9
CEF	456.1	396.0	120	4	125.0	120	60
TRI	291.2	123.1	145	24	261.1	145	24
ORM	275.2	123.1	145	21	259.2	145	25
SMZ	279.1	186.1	120	12	124.0	120	24
SMX	254.1	92.0	110	24	156.0	110	10
OXO	262.1	243.7	110	12	216.0	110	28
NAL	233.1	215.1	87	8	187.1	87	24
FLU	262.1	244.1	115	16	202.1	115	32
PIP	304.1	286.2	105	12	215.1	105	36
ENR	360.2	316.2	140	16	342.2	140	16
ENO	321.1	303.1	125	16	232.1	125	36
LOM	352.2	265.1	140	20	308.1	140	12
SAR	386.1	342.2	145	16	368.1	145	20
NOR	320.1	276.2	135	12	233.1	135	24
CIP	332.1	314.2	135	16	231.1	135	40
OFL	362.2	318.2	140	16	261.1	140	24
ORN	220.0	128.1	97	12	82.1	97	28
VAN[a]	725.0	144.1	135	8	100.1	135	40
NOR-d_4	325.2	281.1	135	12	237.1	135	16
SMX-d_4	258.1	96.1	100	24	160.0	100	12
AMO-d_4	370.1	114.0	85	16	212.0	85	4

[a] Precursor ion: $[M+2H]^{2+}$.

(SMZ) and sulfamethoxazole (SMX), *Quinolones* (Qs): oxolinic acid (OXO) and nalidixic acid (NAL); *Fluoroquinolones* (FQs): flumequin (FLU), enrofloxacin (ENR), ciprofloxacin (CIP), ofloxacin (OFL), norfloxacin (NOR), pipemidic acid (PIP), enoxacin (ENO), lomefloxacin (LOM) and sarafloxacin (SAR); *Tetracyclines* (TCs): tetracycline (TET) and chlorotetracycline (CTE); *Macrolides* (MLs): tylosin (TLS) and erythromycin (ERY); *β-lactams* (β-Ls): amoxicilin (AMO) and cefotaxim (CEF); *Diaminopyrimidines* (DMs): trimethoprim (TRI) and ormethoprim (ORM); *Nitro-imidazoles* (N-IDs): ornidazole (ORN); and *Glycopeptides* (GLs): vancomycin (VAN). All standards had a purity higher than 90%, except TLS (>79%).

Deuterium-labelled compounds were used as internal standards (ISs): *amoxicillin-d_4* (AMO-d_4) and sulfamethoxazole-d_4 (SMX-d_4) were supplied by TRC (Toronto-Canada) while norfloxacin-d_4 (NOR-d_4) was purchased from Sigma–Aldrich. All ISs had purity higher than 95% (isotopic purity >99%).

LC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from VWR (Fontenay Sous Bois, France). Ultrapure water (UP-water) was dispensed from an Elga Purelab Maxima water purification system (Elga LabWater, Le Plessis Robinson, France). Analytical grade formic acid (99%), ortho-phosphoric acid (85%), EDTA disodium salt (Na_2-EDTA) (99%) and NaOH 50% were purchased from Sigma–Aldrich.

For most analytes, stock standard solution of each compound was prepared in MeOH (1 mg mL^{-1}). FQ standards, however, were prepared in MeOH with 0.4% NaOH 50% to increase their solubility. It should be noted that, for the same reason, AMO, AMO-d_4, VAN and CEF standards were prepared in UP-water. These stock solutions were stored in amber glass vials at −18 °C. The stock solutions of AMO, CEF and AMO-d_4 were renewed monthly because of their limited time stability. Working solutions containing a mixture of all compounds (1 μg mL^{-1} each) were freshly made in UP-water/MeOH (90:10, v/v) and stored at −18 °C.

2.2. Sample collection and preparation

In December 2009, river water samples were collected in 1 L aluminium bottles from three rivers in the Seine River basin. This watershed is characterised by a high population density within the Paris conurbation and the Seine River itself receives large amounts of domestic effluents.

Seine River water samples were collected upstream of the Paris conurbation (Marnay s/Seine) and in Paris downtown (Quai d'Austerlitz). Note that the water samples taken at Marnay s/Seine, which is considered as a reference station, were also used for on-line SPE method development.

Samples were also taken from two small rivers flowing south of the Paris conurbation: the Charmoise and the Prédecelle rivers. The Charmoise River receives effluents from the Fontenay-Les-Briis WwTP, which treats both domestic and hospital effluents. On the contrary, the Prédecelle River receives effluents of the Briis-sous-Forges WwTP which receives domestic wastewater only. For these two rivers, water samples were collected upstream and downstream of the WwTP effluent outfall.

After collection, samples were transported to the laboratory and 500-mL subsamples were filtered through 47 mm glass fiber GF/F filters, nominal cut-off size 0.7 μm (Whatman, Fontenay Sous Bois, France). Then 20 mL of the so-obtained filtrate was passed through 0.2 μm nylon membrane filters (Millipore, France) and 0.1% Na_2-EDTA 1 M and ISs (100 ng L^{-1} each) was added. EDTA was used as a chelating agent to reduce antibiotic binding to major cations, thereby promoting analyte retention on the SPE cartridge [30].

Filtered samples were analysed within 3 days if stored at 4 °C and within 3 weeks if stored at −18 °C. The sample pH was adjusted at 4 or 7 by addition of 5% ortho-phosphoric acid or 5% NaOH immediately prior to extraction.

2.3. On-line SPE–LC–MS/MS analysis of antibiotics

The analytical system consisted of a pre-concentration module coupled to a LC–MS/MS system (both from Agilent Technologies, Massy, France).

The on-line SPE system consisted of an automated liquid sampler (ALS) fitted with a 900 μL injection loop with multi-draw capability. A programmable 6 ports/2 positions valve was used to switch between the load or elution modes.

C_{18} HD cartridges (HySphere, Spark Holland, 2 mm × 10 mm) were conditioned with 3 mL of MeOH (A) and 3 mL of UP-water (B), which pH was adjusted at 4 or 7, depending on the pH value of the water sample to be subsequently extracted. A quaternary pump delivered the loading buffer at a flow rate of 1 mL min^{-1} (100% solvent B) and 1800 μL (i.e. twice 900 μL) samples were loaded onto a C_{18} HD cartridge. At 0.1 min after injection, the valve switched position and the analytes retained on the C_{18} HD cartridge were progressively transferred to the LC analytical column in back-flush mode, using the LC solvent gradient described below. Separation was achieved using an Agilent Zorbax Eclipse Plus C_{18} column (2.1 mm I.D. × 150 mm, 3.5 μm particle size). Mobile phase solvents were UP-water + 0.1% formic acid (C) and ACN + 0.1% formic acid (D) in an initial ratio (C:D) of 90:10. Separation was achieved at 35 °C using a flow rate of 0.5 mL min^{-1} with the following (C:D) gradient: 90:10 to 75:25 in 2 min; 65:35 at 4 min; 25:75 at 7 min; 0:100 at 7.1 min for 3 min. Then, the system was equilibrated for 2.4 min prior to the next injection (total run time: 12.5 min).

During MS/MS analysis, the sample injection loop was flushed as follows to prevent cross-contamination: 100% B for 2 min, 100% A for 4 min (0.5 mL min^{-1}).

Note that two runs were performed for each sample: in the first series of analyses, sample pH was adjusted at pH 4 while, in the second series, it was adjusted at pH 7.

Procedural blanks consisting of UP-water spiked with ISs were analysed as a control of contamination during sample handling in the laboratory and to assess potential memory effect in the on-line SPE apparatus.

The Agilent 6410 triple quadrupole mass spectrometer was equipped with an electrospray ionisation (ESI) source and it was operated in positive mode. Argon (99.9%, Air Liquide, Paris, France) was used as collision gas while nitrogen was used as the nebulising gas (11.0 L h^{-1}, nebuliser pressure 35 psi) and was produced via a nitrogen generator (Claind, France). Source temperature was set at 350 °C and MS/MS signal acquisition was performed in Selected Reaction Monitoring (SRM) mode (Table 1). For each analyte, two signals were monitored, corresponding to the transition between the precursor ion and the two most abundant product ions. The most abundant one was used for quantification while the other one was used for confirmation. The ratio of quantification transition area to confirmation transition area was compared to that obtained with an authentic standard (margin applied: 20%). Data were collected using Mass Hunter software from Agilent Technologies, which was also used for analyte quantification. In order to improve sensitivity, each run was divided into five acquisition windows and dwell time was set at 40–200 ms, depending on the time segment.

For quantification, each IS was used to quantify one or several classes of antibiotics: AMO-d_4 was used for β-Ls, NOR-d_4 was used for Qs, FQs and TCs, and SMX-d_4 was used for SFs, MLs, DMs, N-IDs and GLs. Quantification was carried out by calculating the response factor of each analyte relative to its corresponding IS and concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio.

2.4. Off-line SPE procedure

Off-line pre-concentration of antibiotics was performed on Oasis HLB cartridges (60 mg/3 mL) from Waters (Guyancourt, France). Oasis HLB cartridges were conditioned with 3 mL of MeOH, followed by 3 mL of UP-water. Samples (pH 4) were passed through the cartridges at a flow rate of 2–3 mL min^{-1}. Then, cartridges were rinsed with 3 mL of UP-water/MeOH (90:5, v/v), and dried under vacuum during 10 min. Finally, analytes were eluted with 5 mL of MeOH and extracts were evaporated under a nitrogen stream at 40 °C and reconstituted to 0.5 mL in UP-water/MeOH (90/10, v/v) with 0.1% formic acid. Extracts were then passed through 0.2 μm syringe filters.

Analyses were carried out using the system described above (without the pre-concentration module). The injection volume was set at 10 μL.

2.5. Method validation

For the determination of analyte recovery rates using the on-line SPE procedure, UP-water or river water samples were spiked with 50–200 ng mL^{-1} of each analyte prior to extraction. For each analyte, the absolute recovery was calculated as the ratio of the peak area in spiked samples extracted by on-line SPE and in standards directly injected onto the analytical column (i.e. without on-line pre-concentration). Recovery experiments were also performed for the off-line procedure, as follows: 500-mL filtered water samples were spiked with each antibiotic (200 ng L^{-1} each), 0.1% of Na_2EDTA 1 M was added and pH was adjusted at 4 using 5% ortho-phosphoric acid. Samples were then passed through Oasis HLB cartridges as described above.

2.5.1. Sample storage

A filtered river water sample (0.2 μm) was spiked with a mixture of 23 antibiotics (200 ng L^{-1} each). This spiked sample was then divided in 10-mL aliquots, which were transferred in to amber glass vials. A set of subsamples was then stored at 4 °C and was analysed daily during one week. Meanwhile, another set of sub samples was stored at −18 °C and was analysed weekly over a one month period.

2.5.2. Matrix effects

Matrix effects were evaluated using the following equation:

$$\text{matrix effect}\,\% = \left[\frac{A_m + s - A_m}{A_0 - 1}\right] \times 100$$

where $A_m + s$ is the peak area of the analyte in a spiked river water sample, A_m is the peak area of the analyte in an unspiked river water sample and A_0 is the peak area of the analyte in UP-water [31]. Positive values indicate signal enhancement and negative values represent signal suppression due to the sample matrix. Matrix effects were investigated at analyte concentrations of 50 and 200 ng L^{-1}.

2.5.3. Method validation

Validation method was further evaluated in terms of linearity, repeatability, accuracy and sensitivity. As regards linearity, five point calibration curves were constructed using spiked river water samples at concentrations ranging from 5 to 5000 ng L^{-1}. Precision was defined as the relative standard deviation (RSD) of a triplicate analysis of river water samples spiked at two different concentrations (50 and 200 ng L^{-1}). Accuracy was evaluated by analysing river water samples spiked with both native antibiotics and labelled ISs. Furthermore, for each analyte, the limits of detection (LODs) and the limits of quantification (LOQs) were determined as the concentrations with a signal to noise ratio of 3 and 10, respectively, in samples spiked at 50 ng L^{-1}.

3. Results and discussion

3.1. LC–MS/MS optimisation

3.1.1. ESI-MS/MS parameters

Antibiotics were firstly identified in full-scan mode (m/z 50–1500) by direct infusion of individual standard solutions (1 μg mL^{-1}) at a flow rate of 0.5 mL min^{-1}, using UP-water/ACN (95:5, v/v) with 0.1% formic acid. Typically, the protonated molecule

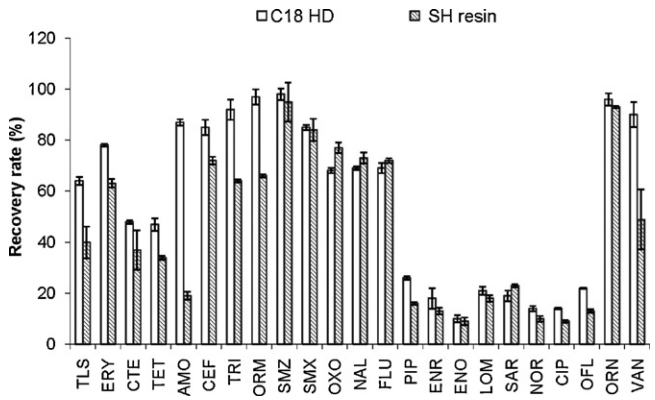

Fig. 1. Analyte recovery determined from the extraction of UP-water samples spiked at 200 ng L^{-1} on SH and C_{18} cartridges (n = 3).

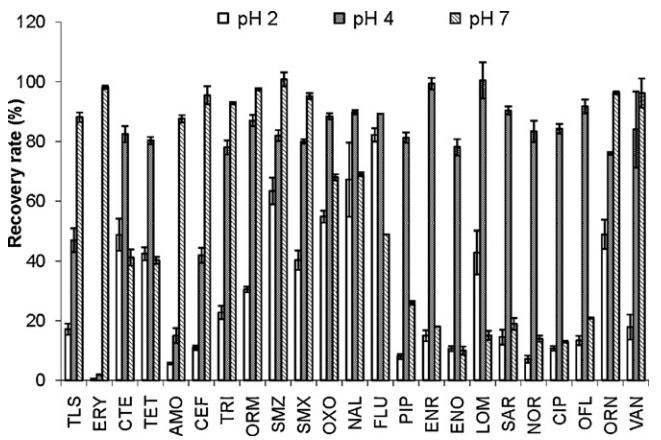

Fig. 2. Effect of sample pH on antibiotic recovery rates (C_{18} cartridge, UP-water spiked at 200 ng L^{-1}, triplicate analysis).

$[M+H]^+$ produced the most intense ion and was selected as the precursor ion, except for VAN which showed a doubly charged ion $[M+2H]^{2+}$. For each compound, product ion scanning was performed and once product ions were selected, fragmentor voltage and collision energy were optimised using Agilent Optimizer software (Table 1).

Then, the following ESI-MS/MS parameters were also optimised: nebuliser gas flow rate (9.0–11.0 L min^{-1}), nebuliser pressure (30–40 psi) and source temperature (300–400 °C). The optimised conditions were as follows: nebulising gas flow rate was set at 11 L h^{-1}, while nebuliser pressure was set at 35 psi and source temperature was set at 350 °C.

The linearity of the MS analyser response was investigated by performing duplicate injections standard solutions. The range tested was 5–5000 ng L^{-1}, except for AMO/CEF (50–5000 ng L^{-1}), and for ORN/CTE (5–3000 ng L^{-1}). A linear response was observed and correlation coefficients (R^2) ranged from 0.997 to 0.999 for all analytes.

3.1.2. LC separation optimisation

The initial composition of the mobile phase was set at UP-water/MeOH 90:10 to promote the retention of the most polar analytes. Then, we investigated the influence of the following parameters: organic modifier (MeOH vs. ACN), concentration of formic acid as mobile phase additive (0.01–0.1%, v/v), flow rate (0.4–0.6 mL min^{-1}) and column temperature (30–50 °C).

For most compounds, sharper chromatographic peaks were observed when using ACN instead of MeOH as organic modifier. Using ACN, 13 resolved peaks were observed while using MeOH only 9 peaks were observed. As regards column temperature, 35 °C was found to be the optimum setting, yielding the highest resolution, the greatest number of separated peaks and the strongest analyte response. Finally, we also observed that the optimal flow rate was 0.5 mL min^{-1}, providing maximum sensitivity and the best analyte separation. Using these optimised conditions, all of the compounds were eluted within 7 min.

3.2. On-line SPE optimisation

3.2.1. SPE adsorbent

The first step of the on-line SPE optimisation was to determine the most appropriate adsorbing. Two types of cartridges were used: SH resin (functionalized PS-DVB, 35 μm particle size) and C_{18} HD cartridges (high C density, end-capped, 7 μm particle size), both form Spark Holland.

For each type of sorbent, 0.9 mL UP-water samples (pH ~ 7) spiked with all antibiotics (200 ng L^{-1}) were loaded onto the cartridge at a flow rate of 1 mL min^{-1}. Although recoveries for SMX, SMZ, NAL, OXO, FLU and ORN were similar with both cartridges, for most analytes the highest recovery rates were obtained by using with C_{18} cartridge (Fig. 1). For FQs, however, the recoveries were low with both C_{18} and SH cartridges (<40%). Moreover, the chromatograms obtained with SH cartridges showed unacceptable peak broadening (data not shown), possibly because of large particle size (35 μm instead of 7 μm). Thus, C_{18} HD cartridges were selected for further method development.

3.2.2. Sample pH

Antibiotics may be found as neutral, cationic, anionic or even zwitterionic species, depending on sample pH [35]. It has been shown by others that analytes are best retained when present as neutral species in water samples; this point has been discussed in details elsewhere [30]. Sample and eluant pH have thus previously been shown to be crucial parameters for antibiotics preconcentration using SPE [11]. It should also be noted that, at pH below 7, ERY is immediately converted into its main degradation product ERY-H_2O [30] and that β-Ls are readily degraded under strong acidic and basic conditions [36].

Therefore, using C_{18} HD cartridges, the effect of sample pH on analyte recovery was investigated. Cartridges were preconditioned with 3 mL of methanol and 3 mL of UP-water, which pH was adjusted at 2, 4 or 7, depending on the pH value of the water sample to be subsequently extracted. Then, 0.9 mL of UP-water (pH 2, 4 or 7) spiked with all antibiotics (200 ng L^{-1} each) was loaded onto the cartridge. We observed that analyte recovery was greatly affected by pH, since all selected antibiotics exhibited pH-dependent recoveries (Fig. 2). At pH 2, recoveries were less than 40% for most analytes. At pH 4 recoveries of FQs and TCs were higher than 78% but less than 50% at pH 7. Conversely, MLs and β-Ls recoveries were lower than 50% at pH 4 and higher than 80% at pH 7. Meanwhile, the recovery rates of Qs, SFs, DMs, N-IDs and GMs showed no strong pH dependence; their recoveries were in the range 60–100% (pH 4 or 7). But recovery rates of SFs, DMs, N-IDs and GMs were somewhat higher at pH 7 and recovery rates of Qs were somewhat higher at pH 4.

Thus, for subsequent extractions on river water samples, two runs were performed for each sample, one at pH 4 and another one at pH 7. pH 7 was selected as the optimal extraction condition for SFs, DMs, N-IDs, GMs, MLs and β-Ls but pH 4 was selected for Qs, FQs and TCs.

3.2.3. Sample volume

Sample volume is another critical parameter in on-line SPE–LC–MS/MS, since it may affect both signal suppression and

Table 2
Recovery rates, limits of detection (LODs) and limits of quantification (LOQs) of antibiotics in spiked river water samples analysed by on-line SPE–LC/MS–MS (C_{18} sorbent). Results are expressed as mean value ± standard deviation ($n = 5$).

	Spike level (ng L^{-1})					
	50		LOD (ng L^{-1})	LOQ (ng L^{-1})	200	
	Recovery (%)	RSD (%)			Recovery (%)	RSD (%)
TLS	87 ± 4	4.1	0.6	1.9	87 ± 2	2.7
ERY	91 ± 1	0.7	0.8	2.5	86 ± 3	2.9
CTE	78 ± 3	3.4	2.3	7.7	75 ± 4	4.6
TET	75 ± 3	4.2	1.8	6.0	76 ± 2	2.9
AMO	70 ± 1	1.9	12.0	39.2	64 ± 3	4.8
CEF	68 ± 3	4.4	13.7	45.6	66 ± 2	3.4
TRI	80 ± 0	0.6	1.5	4.8	90 ± 1	1.6
ORM	87 ± 0	0.5	1.9	6.2	88 ± 2	2.6
SMZ	98 ± 2	2.4	1.4	4.7	96 ± 4	3.6
SMX	98 ± 1	1.0	0.6	2.0	94 ± 2	1.7
OXO	80 ± 1	1.2	1.7	5.7	75 ± 1	1.3
NAL	81 ± 1	0.9	1.3	4.3	76 ± 2	2.9
FLU	83 ± 0	0.1	1.1	3.5	79 ± 3	3.1
PIP	78 ± 2	2.1	5.0	16.5	76 ± 2	3.0
ENR	84 ± 2	2.2	3.3	11.0	83 ± 2	2.5
ENO	75 ± 3	3.6	2.6	8.7	78 ± 2	2.8
LOM	88 ± 4	4.7	3.3	11.0	81 ± 5	6.4
SAR	80 ± 1	1.6	1.1	3.6	79 ± 4	5.4
NOR	78 ± 4	4.6	2.1	7.0	76 ± 4	4.6
CIP	74 ± 2	2.2	1.0	3.3	76 ± 3	3.7
OFL	81 ± 2	2.8	0.5	1.7	81 ± 3	3.9
ORN	84 ± 0	0.5	4.3	14.1	87 ± 2	2.1
VAN	85 ± 5	5.6	2.0	6.7	89 ± 6	7.1

antibiotic recovery rate [32,33]. Using UP-water samples spiked with antibiotics (200 ng L^{-1}), we investigated the influence of sample volume in the range 0.9–2.7 mL. Breakthrough was observed when sample volume was set at 2.7 mL (recoveries <60% for all analytes), while breakthrough was slightly higher for 1.8 mL-samples than for 0.9-mL samples (average: 3%). As a matter of fact, and considering the low levels antibiotics in surface waters, sample volume was set at 1.8 mL so as to lower method detection limits while keeping acceptable recovery rates.

3.2.4. Sample loading flow rate

The influence of sample loading flow rate was investigated over the range 1.0–2.0 mL min^{-1} (1.8 mL UP-water samples, pH adjusted at 7 and spiked at 200 ng L^{-1}). For 10 out of 23 analytes, increased breakthrough was observed at 2 mL min^{-1} (average: 10%). Lower flow rates were not investigated, since we aimed at keeping the loading step duration under 4 min. Therefore, sample loading flow rate was set at 1 mL min^{-1}.

3.2.5. Recoveries and detection limits

The extraction of antibiotics from river water samples was evaluated by performing recovery experiments on samples taken from a reference site (Marnay s/Seine), spiked at two levels. Good antibiotic recoveries were achieved regardless of spike levels (range 64–98%) (Table 2). Depending on the analyte and the sample matrix, such recoveries are similar to those achieved by others, using on-line SPE with sorbents such as Oasis HLB or C_{18} cartridge [31–33,37]. Furthermore, precision was also satisfactory since RSDs were in the range 0.5–7.1% ($n = 5$). LODs were in the range 0.5–13.7 ng L^{-1} while LOQs were in the range 1.7–46 ng L^{-1}, which is lower than values reported by others [31,32,37].

3.2.6. Matrix effects

Several studies showed that matrix effects were relatively low for compounds such as FQs, SFs and TLs [31,34]. In the present study, we observed that, regardless of analyte concentration (i.e. 50 or

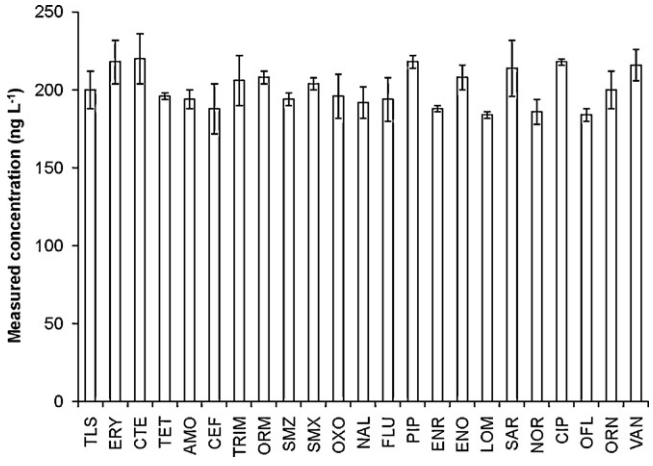

Fig. 3. Evaluation of method quantification performance on river water samples spiked at 200 ng L^{-1}.

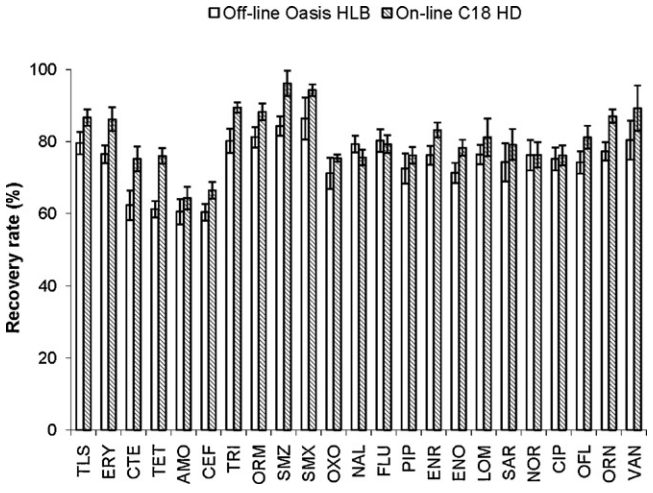

Fig. 4. Antibiotic recovery rates from UP-water samples spiked at 200 ng L^{-1}: off-line SPE (Oasis HLB) vs. on-line SPE (C_{18}).

Table 3
Signal suppression (%, mean ± S.D., $n = 3$) for selected analytes: comparison of on-line SPE using C_{18} sorbent with off-line using Oasis HLB sorbent.

Classes	Antibiotics	Signal suppression (%)		
		On-line (C_{18})		Off-line (HLB)
		200 ng L^{-1}	50 ng L^{-1}	50 ng L^{-1}
Macrolides	Tylosin	14 ± 1	12 ± 2	18 ± 4
	Erythromycin	12 ± 2	13 ± 3	19 ± 4
Tetracyclines	Chlorotetracycline	12 ± 3	11 ± 2	6 ± 3
	Tetracycline	14 ± 3	10 ± 2	7 ± 3
β-Lactams	Amoxcilin	26 ± 3	24 ± 3	30 ± 3
	Cefotaxim	26 ± 4	23 ± 3	30 ± 3
Diaminopyrimidines	Trimethoprime	8 ± 1	7 ± 1	16 ± 3
	Ormethoprime	5 ± 1	7 ± 2	12 ± 1
Sulfonamides	Sulfamethazine	13 ± 2	13 ± 2	14 ± 2
	Sulfamethoxazole	13 ± 1	13 ± 1	15 ± 5
Quinolones	Oxolinic acid	18 ± 2	18 ± 2	23 ± 6
	Nalidixic acid	21 ± 1	19 ± 1	24 ± 5
Fluoroquinolones	Pipemidic acid	17 ± 1	17 ± 1	20 ± 3
	Flumequin	19 ± 2	16 ± 2	21 ± 3
	Enrofloxacin	19 ± 3	18 ± 3	19 ± 4
	Enoxacin	18 ± 2	19 ± 2	22 ± 4
	Lomefloxacin	18 ± 2	16 ± 2	19 ± 2
	Sarafloxacin	16 ± 3	17 ± 2	21 ± 2
	Norfloxacin	18 ± 2	17 ± 2	20 ± 3
	Ciprofloxacin	19 ± 3	19 ± 3	23 ± 2
	Ofloxacin	19 ± 2	17 ± 1	20 ± 2
Nitro-imidazoles	Ornidazole	11 ± 1	10 ± 1	19 ± 2
Glycopeptides	Vancomycin	12 ± 2	9 ± 2	19 ± 3

200 ng L^{-1}), signal suppression was usually lower than 20%, except for AMO and CEF (up to 25%) (Table 3); this is in good agreement with previous reports [36,38]. Furthermore, ion suppression was generally higher using the off-line SPE procedure, likely as a consequence of higher amounts of co-extracted matrix constituents (Table 3).

These results also showed that antibiotics within the same class generally exhibited a similar degree of matrix effects. Since ion suppression could lead to inaccurate quantification, we opted for the use of appropriate isotope-labelled ISs to overcome this artefact. Results shown in Fig. 3 provide evidence that accurate quantification was achieved for all analytes in spiked river water samples.

3.2.7. On-line SPE vs. off-line SPE

Fig. 4 shows the recovery rates of target compounds using two SPE procedures: on-line SPE (C_{18} HD) vs. off-line SPE (Oasis HLB). The latter procedure is widely used and may be considered as a reference procedure [30]. Good recoveries were achieved with both techniques (>60% for all compounds). For 12 out of 23 compounds, however, recoveries achieved using on-line SPE were higher than those achieved using off-line SPE, which agrees with previous findings [33].

3.2.8. Sample storage and stability

Sample storage is another critical step that is too often overlooked. In this study, the stability of target compounds in river water samples filtered at 0.2 μm was investigated under two different storage conditions: up to 6 days at 4 °C and up to 4 weeks at −18 °C. As shown in Fig. 5, analyte stability strongly depended on storage temperature and different degradation kinetics were observed at −18 °C and at 4 °C. At 4 °C, most antibiotics were stable over the 6-day period (degradation <10%), except AMO and CEF that degraded up to 20% after 3 days. Stability was greatly increased when samples were stored at −18 °C. However, even at that low temperature, significant degradation of AMO and CEF was still observed if storage time exceeded 21 days. Thus, for subse-

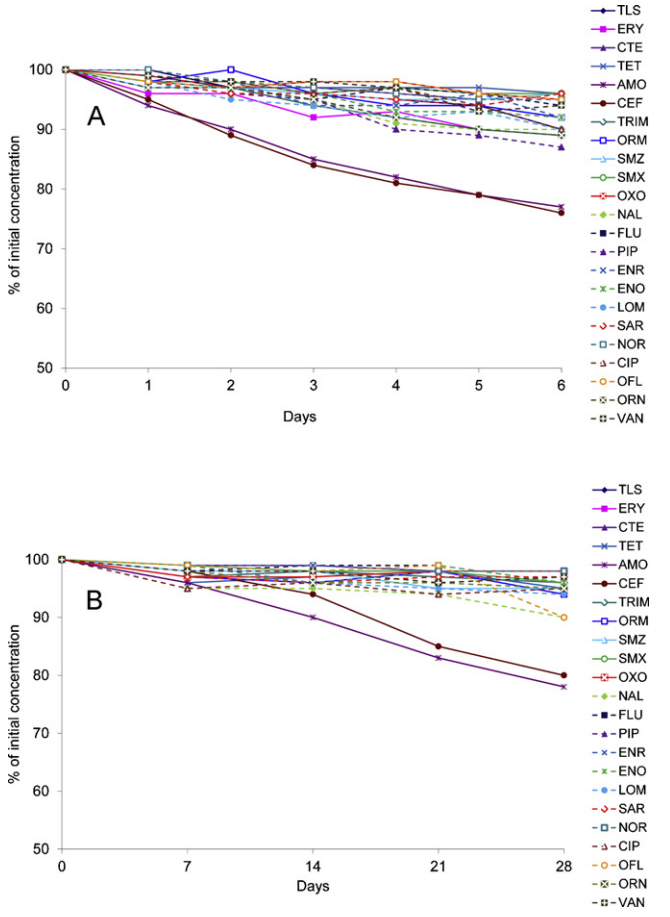

Fig. 5. Influence of sample storage conditions on antibiotic stability in river water samples filtered at 0.2 μm and spiked at 200 ng L^{-1}.

Table 4
Antibiotic concentrations determined in river water samples (ng L^{-1}), (–) <LOQ: limit of quantification (see Table 2).

	Seine River		Prédecelle River		Charmoise River	
	Marnay	Jussieu	Upstream WwTP	Dowstream WwTP	Upstream WwTP	Downstream WwTP
TLS	2.8 ± 0.1	–	–	–	–	–
ERY	–	4.0 ± 0.3	–	4.2 ± 0.8	–	131 ± 3
CTE	–	–	–	–	–	–
TET	–	–	–	7.4 ± 0.3	–	–
AMO	–	68 ± 6	–	–	–	–
CEF	–	–	–	–	–	–
TRI	–	–	–	8.0 ± 0.3	–	254 ± 6
ORM	–	–	–	–	–	–
SMZ	–	–	–	–	–	–
SMX	3.6 ± 0.6	18 ± 1	–	25 ± 1	5.6 ± 0.4	1435 ± 41
OXO	–	23 ± 1	–	–	–	–
NAL	–	–	–	–	–	–
FLU	–	4.6 ± 0.5	–	–	–	–
PIP	–	–	–	–	–	–
ENR	–	–	–	–	–	–
ENO	–	–	–	–	–	–
LOM	–	–	–	–	–	–
SAR	–	–	–	–	–	–
NOR	–	37 ± 2	–	75 ± 6	–	17 ± 2
CIP	–	17 ± 1	–	–	3.5 ± 0.2	135 ± 2
OFL	2.3 ± 0.1	18 ± 1	3.5 ± 0.2	65 ± 3	4.3 ± 0.3	231 ± 4
ORN	–	–	–	–	–	–
VAN	–	–	–	–	–	90 ± 12

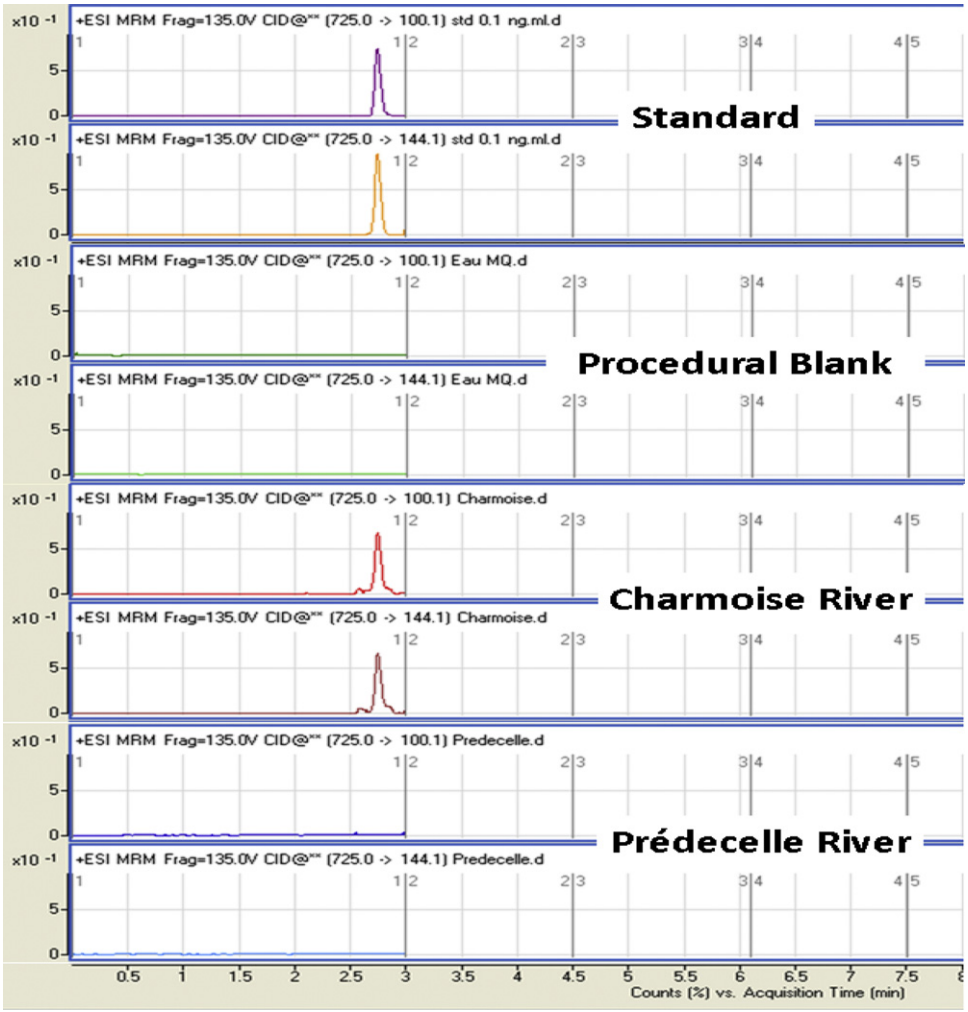

Fig. 6. Vancomycin chromatograms: standard solution (100 ng L^{-1}), procedural blank, Charmoise River water extract (89.5 ng L^{-1}), Prédecelle River water extract (<LOD).

quent series, samples were analysed within 3 days after collection if stored at 4 °C, whereas storage time could be extended up to 3 weeks at −18 °C.

3.3. Antibiotic levels in river water samples

Table 4 provides a summary of the antibiotic concentrations measured by on-line SPE–LC–MS/MS in river water samples taken from three rivers within the River Seine basin. Overall, 12 antibiotics belonging to 8 classes were detected in these samples and concentrations ranged from 2.3 to 1434.7 ng L^{-1}.

Only 4 antibiotics, namely CIP, OFL (human use), SMX (human and veterinary use) and TLS (veterinary use only) could be detected upstream of known point sources (e.g. WwTPs) at low concentrations (∼3 ng L^{-1}). In the case of the Charmoise River, the occurrence of SMX, CIP and OFL may possibly be explained by runoff from agricultural fields located on the river banks and amended with sewage sludge. As regards the Seine River, TLS presence may be due to agricultural activities (i.e. cattle farming).

WwTPs processes often achieve incomplete removal of antibiotics, resulting in discharge of these chemicals into surface waters [39,40]. In this study, antibiotic levels dramatically increased downstream of WwTP effluent outfall in both the Charmoise River and in the Prédecelle River, which supports the assumption that WwTPs are a major source of antibiotics in such riverine sytems. Antibiotic levels in the Charmoise River were higher than in the Prédecelle River, likely as a result of hospital effluent input. Meanwhile, antibiotic concentrations in the Seine River were less than in Charmoise River and Prédecelle River as a result of higher dilution rates.

The antibiotic concentrations reported in this study were similar to those reported previously for the Seine River [12]. They are, however, higher than those observed in Spain and Italy [32,34,41]. The antibiotic concentrations detected in the Charmoise River were higher than those observed in small rivers in Germany [42].

Finally, it should be noted that 5 antibiotics (namely ERY, SMX, OFL, CIP and NOR) in river water were most frequently detected. Moreover, VAN a compound that is used only in hospitals, was detected in the Charmoise River only, which is the only one to receive treated hospital effluent (Fig. 6). Its potential as a tracer of hospital effluent discharge is currently under investigation.

4. Conclusion

An on-line SPE–LC–MS/MS multi-residue method was developed for the determination of 23 antibiotics at trace level in river water samples. The advantages of this method is automation, minimum sample handling, low sample volume required, low solvent consumption, high-throughput (25 min/sample), high recoveries (>70% for most compounds), good reproducibility (RSDs < 11%), improved accuracy, high sensitivity (LOD ∼ ng L^{-1} compatible with environmental levels) and high selectivity. It was also demonstrated that the use of selected ISs allowed for reliable analyte quantification.

This method has been successfully applied to the determination of 8 antibiotic classes in river water samples. A total of 12 antibiotics were detected in samples collected from the Seine river basin.

Acknowledgments

PhD funding for Quoc Tuc Dinh was provided by the French Ministry for Higher Education. The Piren Seine project (http://www.sisyphe.upmc.fr/piren/) funded this work while Agilent Technologies kindly organised the loan of an on-line SPE system and provided technical support.

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