

Rapid determination of 12 antibiotics and caffeine in sewage and bioreactor effluent by online column-switching liquid chromatography/tandem mass spectrometry

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Abstract This study presents a column-switching solid-phase extraction online-coupled to a liquid chromatography/tandem mass spectrometry (SPE-LC-MS/MS) method for simultaneous analysis of 12 antibiotics (7 sulfonamides and 5 fluoroquinolones) and caffeine detected in the sewage and effluent of a pilot anaerobic reactor used in sewage treatment. After acidification and filtration, the samples were directly injected into a simple and conventional LC system. Backflush and foreflush modes were compared based on the theoretical plates and peak asymmetry observed. The method was tested in terms of detection (MDL) and quantification limit (MQL), linearity, relative recovery, and precision intra- and inter-day in lab-made sewage samples. The method presented suitable figures of merit in terms of detection, varying from 8.00×10^{-5} to 6.00×10^{-2} ng (0.800 up to 600 ng L⁻¹; caffeine) with direct injection volume of only 100 μ L and 13 min of total analysis time (sample preparation and chromatographic run). When the method was applied in the

analysis of sewage and effluent of the anaerobic reactor ($n=15$), six antibiotics and caffeine were detected in concentrations ranging from 0.018 to 1097 μ g L⁻¹. To guarantee a reliable quantification, standard addition was used to overcome the matrix effect.

Keywords Antibiotics · Column switching · SPE online · Sewage and anaerobic reactor effluent

Introduction

Antibiotics have been reported in environmental matrices as a result of their wide use in human and veterinary medicine [1]. Sulfonamides are an important group of antibiotics mainly applied in veterinary medicine to treat urinary tract infections, ear infections, and bronchitis. The co-administration of trimethoprim-sulfamethoxazole enhances the treatment against bacterial infections inhibiting the synthesis of folic acid, essential for bacterial growth [2]. Fluoroquinolones are another important antibiotic group used for human and veterinary medicine. Both antibiotic groups have been found in concentrations of micrograms to nanograms per liter in sewage, wastewater, river water, wastewater treatment plant (WWTP) effluent, and other environmental waters [3–7].

Caffeine is a cardiovascular, cerebral, and respiratory stimulant found in analgesic and headache medication. Moreover, it is a component of a large variety of beverages and numerous food products. Thus, it has been reported in environmental matrices in concentration levels of nanograms to micrograms per liter [8, 9]. Based on these facts, caffeine is considered by researchers as an anthropogenic biomarker [10, 11].

These pharmaceutical compounds reach the sewage through animal or human excreta, disposal of expired or unused medicine, or its direct application into the environment,

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for example, fish farming [12]. Moreover, WWTPs are not designed for the removal of these compounds, and also around 82 % of Brazilian municipalities discharge sewage directly into the rivers [12–14].

Although sulfonamides and fluoroquinolones occur in low concentrations (ng L^{-1}), their ecotoxicological effects such as genotoxicity and mutagenicity have been described. Moreover, antibiotics in the environment may select resistant bacteria, or even enhance single, cross- and multi-resistance bacteria [15–17].

Both sulfonamides and fluoroquinolones are polar, water soluble, and chemically stable, which contributes to the antibiotic mobility in the environmental matrices and hinders the extraction and chromatographic separation [18, 19].

It is important to develop analytical methods that enable fast, sensitive, and reliable analysis mainly for monitoring antibiotic residues in environmental matrices. Liquid chromatography/tandem mass spectrometry (LC-MS/MS) is the instrumental technique of choice. A large variety of LC-MS/MS methods are available to analyze antibiotics in environmental matrices [6, 20–23]. Currently, priority falls on maximizing the number of antibiotics in a single method, and also on automating sample preparation and decreasing the sample volume used per analysis. Solid-phase extraction (SPE) is a sample preparation technique for environmental water analysis; however, in a conventional offline and manual setup, it is a tedious and time-consuming procedure [24]. On the other hand, an online setup overcomes those limitations, although it might require available dedicated devices or instrumental arrangement with valves and pumps. Recently, online SPE has been applied for the analysis of antibiotics in environmental matrices, although most of these methods only determine antibiotics from the same group, sulfonamides or fluoroquinolones [25–27]. Screening methods for multi-residue analysis of micropollutants using online SPE are also reported applying dedicated instruments for online sample preparation [28–30].

Regarding wastewater treatment techniques, new techniques are under development such as Fenton-like degradation using Fe^{3+} and H_2O_2 , photo-Fenton mediated by Fe^{3+} and oxalate, nanofiltration, electrochemical remediation, membrane bioreactor, and anaerobic reactors to evaluate the removal efficiency of pharmaceutical compounds [31–36]. The advancement of analytical instruments to determine compounds in sub-nanogram-per-liter level with minimum sample handling is essential to evaluate those new treatment technologies.

This study offers several advantages; firstly, the minimization of sample handling by using online column-switching sample preparation. Secondly, the injection of low sample volume of 100 μL was enough to perform the total analysis. Thirdly, a low nanogram-per-liter level was reached for simultaneous analysis of sulfonamides, fluoroquinolones, and

caffeine (major compounds found in environmental matrices), using only injection volume of 100 μL applied for sample pre-concentration. Fourthly, the method development, validation, and sample analysis was carried out in a conventional high-performance liquid chromatography (HPLC) system (with autosampler and six-port valves) capable of column switching, not requiring a dedicated device to perform online sample preparation. Only 13 min were necessary for sample preparation and chromatographic run to analyze 13 environmental-relevant compounds.

Finally, the method developed was successfully applied in the analyses of antibiotics found in the sewage and pilot-scale anaerobic reactor effluent. To the author's knowledge, this is the first published report of effluent analysis from vertical structured-bed reactor under intermittent aeration, a new reactor setup and the first publication of this online automated setup for this group of compounds in complex environmental liquid samples as sewage and wastewater effluent (see Electronic supplementary material (ESM) Table S1). There were seven compounds detected in all samples analyzed.

Materials and methods

Chemicals and reagents

Purified water was produced in the laboratory by a Milli-Q Plus Ultra (Billerica, MA) purification station.

All drug standards used were of high purity grade (>98 %). Sulfamethazine (SMZ), sulfamethoxazole (SMTX), sulfamerazine (SMER), sulfadiazine (SDIZ), sulfacetamide (SAC), sulfadimethoxine (SDMX), trimethoprim (TMP), norfloxacin (NOR), ofloxacin (OFLO), ciprofloxacin (CIP), pefloxacin (PEF), enrofloxacin (ENRO), and caffeine (CAF) were obtained from Sigma-Aldrich (St. Louis, MO). Isotopically labeled compounds used as internal standard (IS) ^{13}C -SMTX, ^{13}C -SDMX, ^{13}C -SMZ, TMP- D_9 , ENRO- D_5 , CIP- D_8 , and NOR- D_5 were also acquired from Sigma-Aldrich. Individual sulfonamide stock solutions at 500 mg L^{-1} were prepared in acetonitrile (Panreac, Barcelona), and 0.1 % of phosphoric acid or formic acid (Panreac) was added to improve dissolution. Stock solution of 500 mg L^{-1} of fluoroquinolones and CAF were prepared in methanol (Panreac).

Instrumentation

Chromatographic system

An Agilent 1200 LC series (Palo Alto, CA) was used in all analysis. This system consisted of one binary LC pumps; autosampler ALS 1200 with injection volume capacity from

0.1 to 100 μL ; thermostatted column compartment (TCC) 1200, and diode array detector (DAD) Infinity 1290. An additional LC pump, model LC-10 AVP from Shimadzu (Kyoto, Japan) was also used for sample loading.

ESI-MS/MS

A hybrid triple quadrupole-linear ion trap mass spectrometer QTRAP 5500 (AB SCIEX, Foster, CA) with turbo ion spray source was coupled to the LC system described above. Source-dependent parameters were optimized by flow injection analysis as follow: curtain gas (CUR) of 20 V, nitrogen collision gas (CAD) medium, source temperature (TEM) of 650 $^{\circ}\text{C}$, ion spray voltage of 5500 V, and ion source gases GS1 and GS2 at 50 psi.

Compound-dependent MS parameters (declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP)) were optimized by direct infusion of standard solution of each compound at concentration ranging from 10 to 50 $\mu\text{g L}^{-1}$. Entrance potential (EP) was set at 10 V.

QTRAP was operated in positive ionization mode with selected reaction monitoring (SRM) using a dwell time of 20 ms. For quantitative purposes, three different SRM transitions were monitored for each compound, except for OFLO and NOR which presented two intense and stable transitions. SRM transitions and other MS parameters are shown in Table 1. Resolution at the first and third quadrupole (Q1 and Q3) was set to unitary, and inter-scanning delay was 5 ms. All data was collected and processed in Analyst 1.6.1 software.

Column switching

A column-switching setup was used in the sample preparation procedure. The system consisted of two LC pumps, pump A for sample loading and pump B for gradient elution. Column switching was performed using a two-position six-port valve from Rheodyne (Rhonert Park, CA).

A SPE column with hydrophilic-lipophilic balanced polymer Oasis HLB (cartridge column, 2.1 \times 20 mm, 25 μm) was acquired from Waters (Milford, MA) and applied in the online sample preparation. The column-switching procedure was evaluated in forwardflush and backflush modes, as shown in Fig. 1.

In backflush mode, the sample was introduced in the SPE column by an aqueous mobile phase from pump A (position A). Simultaneously, the analytical column (Agilent Poroshel EC18 column 50 \times 2.1 mm \times 2.7 μm) was conditioned by the mobile phase flushed by that present in pump B. At 3.01 min, the valve switched to position B, allowing the pre-concentrated analytes to elute from the SPE column on the opposite direction of sample loading,

Table 1 MS/MS parameters for the target analytes by SRM and positive ionization mode

Compound	Q1 (m/z)	Q3 (m/z)	DP (volts)	CE (volts)	CXP (volts)
CAF	195	138	31	21	16
CAF	195	123	31	27	16
CAF	195	83	31	27	16
CIP	332	288	96	25	25
CIP	332	245	31	27	14
CIP	332	231	96	49	16
CIP-D ₈	340	296	81	25	14
CIP-D ₈	340	249	81	25	14
ENRO	360	316	81	37	12
ENRO	360	245	81	47	9
ENRO	360	286	81	47	12
ENRO-D ₅	365	347	91	55	9
ENRO-D ₅	365	245	91	55	9
ENRO-D ₅	365	321	31	27	16
NOR	320	302	66	29	14
NOR	320	276	66	23	14
NOR-D ₅	325	281	101	25	14
NOR-D ₅	325	238	101	25	14
OFLO	362	318	71	15	14
OFLO	362	261.1	71	21	12
PEF	334	233	61	35	12
PEF	334	205	61	47	8
PEF	334	290	61	25	14
SACET	215	156	46	15	8
SACET	215	92	46	29	14
SACET	215	108	46	27	12
SDIZ	251	156	46	21	10
SDIZ	251	92	46	35	14
SDIZ	251	108	46	31	8
SDMX	311	156	51	29	8
SDMX	311	92	51	45	14
SDMX	311	108	51	41	18
¹³ C-SDMX	317	162	76	41	14
¹³ C-SDMX	317	98	76	27	16
¹³ C-SDMX	317	114	76	41	16
SMER	265	156	61	23	8
SMER	265	92	61	37	14
SMER	265	108	61	35	16
SMTX	254	156	61	21	10
SMTX	254	92	61	37	14
SMTX	254	108	61	31	16
¹³ C-SMTX	260	98	96	39	16
¹³ C-SMTX	260	114	96	39	16
¹³ C-SMTX	260	162	96	33	6
SMZ	279	186	56	25	10
SMZ	279	92	56	41	16
SMZ	279	108	56	37	16
¹³ C-SMZ	285	98	96	39	14

Table 1 (continued)

Compound	Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	DP (volts)	CE (volts)	CXP (volts)
¹³ C-SMZ	285	114	96	39	9
¹³ C-SMZ	285	186	96	39	9
TMP	291	261	91	31	12
TMP	291	230	91	35	12
TMP	291	257	91	41	12
TMP-D ₉	300	234	106	35	12
TMP-D ₉	300	264	106	35	12
TMP-D ₉	300	123	106	35	12

towards to the analytical column and detector. Similarly, in forwardflush mode the sample was injected in the SPE column (position A), and after switching the valve to position B, the analytes were eluted towards the analytical column by pump B mobile phase but in the same flow direction as the sample loading.

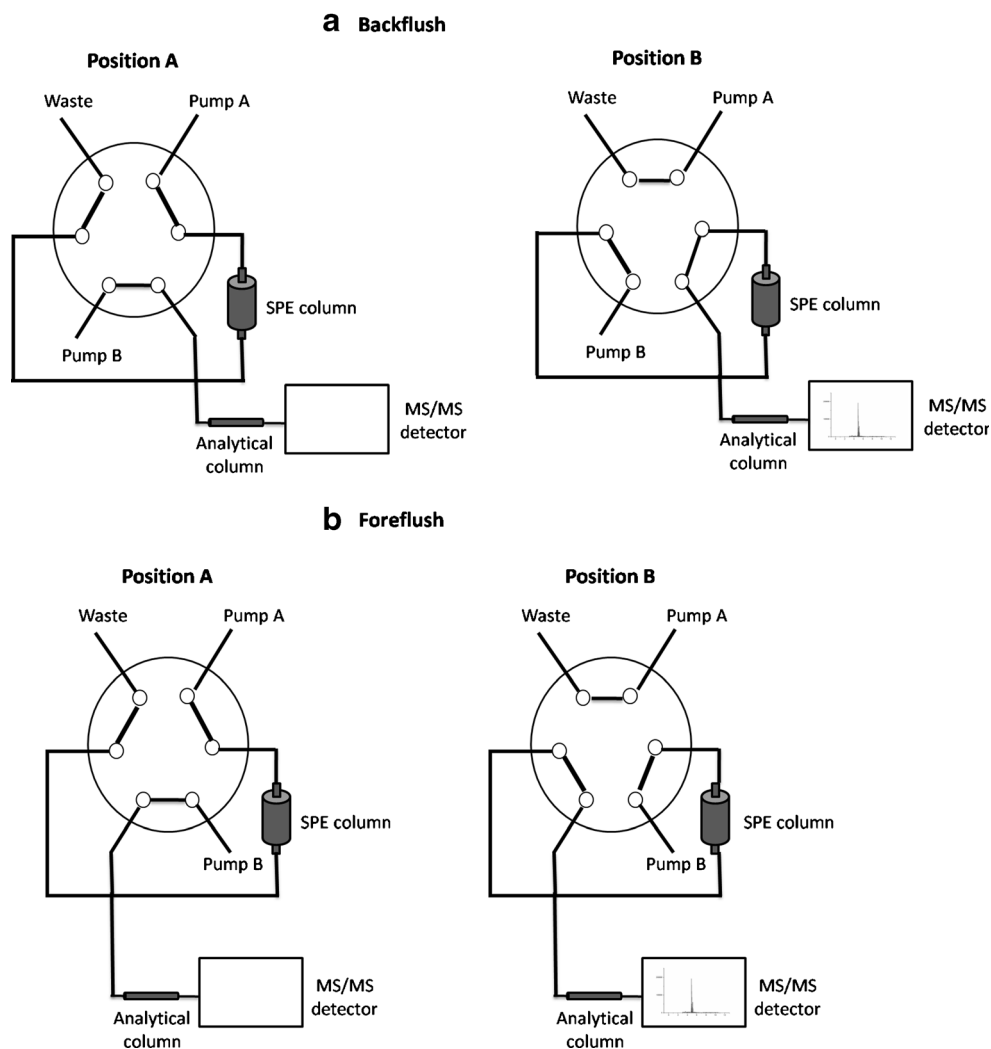
Chromatographic separation and method development

The SPE column retention factor (*k*) was evaluated for each compound using DAD detector monitoring at 254 and 270 nm wavelengths using different mixtures of mobile phase ranging from 2 to 50 % of B.

The mobile phase used in the binary pump B was a mixture of ultra-pure water (A) containing 0.1 % of formic acid and acetonitrile (B) also with 0.1 % of formic acid at a flow rate of 0.6 mL min⁻¹.

A gradient elution was programmed in pump B, starting with isocratic 95 % of A for 3.1 min. From 3.1 to 9 min, the mobile phase changed linearly down to 35 % of A and, then, changed linearly down to 5 % of A between 10 and 14 min. The injection valve was switched from positions A to B after 3.0 min, returning to its former position at 11.4 min, thereafter; it was submitted to 3 cycles of switching to avoid any carry-over effect. The analytical column was kept at 20 °C at TCC compartment.

Fig. 1 Column-switching setup. **a** Backflush and **b** foreflush



For sample loading, the mobile phase consisted of ultra-pure water at flow rate of 1.0 mL min^{-1} .

A rheodyne diverter valve was used to introduce mobile phase into MS detector ion source only after 3.0 min. It was programmed to deliver mobile phase for MS analysis for 10 min, as mentioned for the column-switching valve, this valve was also cycled to avoid carryover.

Backflush and foreflush were compared based on asymmetry (As) and number of theoretical plates (N).

Validation procedure

Linearity

For validation, injection volume of $100 \mu\text{L}$ and backflush mode was chosen. The method was validated considering international accepted criteria [37, 38]. From the stock solutions, two different dilutions of 1000 and $1 \mu\text{g L}^{-1}$ of each analyte were prepared in water. Thus, working solutions containing all compounds were prepared and used to spike lab-made sewage samples at seven different concentration levels to evaluate method linearity (matrix-matched calibration).

Table 2 shows the lab-made sewage composition. Before sample analysis, pH was adjusted to 3.0 with formic acid and filtered using $0.22 \mu\text{m}$ acetate cellulose membrane.

Calibration curves were obtained using external calibration and IS calibration. The sewage and anaerobic reactor effluent samples were quantified using both calibration methods.

The linear model was obtained using a weighted least-squares linear regression (WLSLR). Empirical weights of $1/x$, $1/x^2$, $1/x^{0.5}$, and $1/y^2$ were evaluated in order to determine the best weighting model. Linearity lack of fit was evaluated by analysis of variance (ANOVA).

Table 2 Lab-made sewage composition adapted from Mockaitis et al. [51, 52]

Component	Concentration (mg L^{-1})
Sucrose	47.8
Starch	148
Cellulose	47.2
Meat extract	215
Soybean oil	51.0
NaHCO_3	728
KH_2PO_4	120
NaCl	250
CaCl_2	7.00
MgCl_2	4.50
LAS (tensioactive)	15.0

Precision, limit of detection and quantification, relative recovery, and stability

Inter-day precision was evaluated at one level (medium level) while intra-day precision was measured at three concentration levels (low, medium, and high) also in a lab-made sewage matrix. Method detection limit (MDL) and method quantification limit (MQL) were determined as the concentration resulting in signal/noise (S/N) ratio of 3 and 10, respectively, after the same analytical procedure applied for real samples.

Relative recovery (RR) was measured by comparing the absolute area of each compound. Analytes were spiked into ultra-pure water and lab-made sewage samples at the concentration levels. Stability was evaluated in three different processes related to analysis, autosampler stability, frozen-thaw cycle storage at 4°C . The lab-made sewage sample was fortified and injected (t_0), the area values obtained were compared with sample aliquots analyzed after 2, 4, 6, 8, 24, 48, 120, and 504 h (3 weeks) for both studies.

Analysis of sewage and anaerobic reactor effluent

Samples of domestic sewage were collected in São Carlos, SP, Brazil. To remove nitrogen, carbon, and organic matter present in the sewage, a pilot-scale anaerobic reactor was applied. A vertical structured-bed reactor under intermittent aeration (volume of 650 L) described by Moura et al. [39] is promising technology for sewage treatment.

About 20 mL of sewage and bioreactor effluent were collected in 50 mL polypropylene sample bottles. The samples were submitted to a simple pre-treatment based on pH adjustment to 3.0 followed by filtration using $0.22 \mu\text{m}$ acetate cellulose membrane. Finally, the samples were analyzed using the SPE-LC-MS/MS system and quantified by standard addition method to better overcome the matrix effect. Standard addition was done at five different concentration levels spiking only the seven compounds detected in the samples. The first level refers to the sample without sample addition; the second level is the concentration which presented a signal 3.0 times higher than observed previously. Further levels were spiked taking into account a concentration ratio from the second level of 2.8 (third), 1.7 (fourth), and 1.2 (fifth).

Results and discussion

MS optimization

Antibiotics and caffeine were analyzed in positive-ion mode (ESI+) using formic acid as additive, while the selected reaction monitoring (SRM) mode was carried out for acquisition.

For fluoroquinolones, precursor $[\text{M}+\text{H}]^+$ and product ion were selected according to the highest signal obtained in the

SRM optimization. A neutral loss of CO₂ ([M+H-44]⁺) as well as related fragments of 3-carboxyl group ring [M+H-74]⁺ and piperazinic ring, [M+H-87]⁺, [M+H-101]⁺, [M+H-115]⁺, and [M+H-129]⁺ were selected. Also, loss of H₂O ([M+H-18]⁺) common for this antibiotics was avoided, only monitored for NOR. All those SRM transitions were used in a previous publication [40].

ESI⁺ also was used for sulfonamides, and product ions of *m/z* 186, 156, 108, and 92 were selected. Product ion at *m/z* 186 correspond to [C₆H₈N₃O₂S]⁺, *m/z* 156 to [C₆H₆NO₂S]⁺, *m/z* 108 to [C₆H₆NO]⁺, and *m/z* 92 to [C₆H₆N]⁺ as observed in previous studies [41–43].

For CAF, the product ions selected were at *m/z* 138 ([C₆H₈N₃O]⁺), 123 ([C₆H₇N₂O]⁺), and 83 ([C₄H₇N₂]⁺); *m/z* of 261 ([C₁₃H₁₇N₄O₂]⁺), 230 ([C₁₂H₁₄N₄O]⁺), and 123 ([C₈H₁₁O]⁺) were used for TMP as previously reported by Brenner et al. [44].

Column-switching online SPE

In order to avoid sample handling, cross contamination, tedious and time-consuming procedure, column-switching online SPE was selected as the sample preparation technique. In this technique, minimal procedures are necessary for sample analysis.

In current analytical market, there are different setups and column phases available. A column switching is a simple technique which requires a six-port valve found in many typical HPLC instruments. In previous studies, HLB column phase presented appropriate chemical properties to pre-concentrate and extract a wide variety of emerging contaminants [27, 45–50]. Here, particular preference was devoted to develop a method with a commercially available column, so that the method can be easily reproduced in any laboratory.

In order to warranty effective extraction, retention factor (*k*) of each compound was evaluated in the HLB SPE column using an isocratic mobile phase varying from 2 to 50 % ACN (B). Uracil was selected as void volume marker. According to Fig. 2, 5 % of B provided enough retention to all fluoroquinolones. For sulfonamides, a maximum 2 % of ACN was necessary to achieve enough retention of sulfacetamide; the same was observed to caffeine during loading procedure. Thus, sample loading used simple LC pump with an isocratic phase of ultra-pure water (pump A) to prevent the elution of analytes before the valve switching.

Analysis of lab-made sewage

Lab-made sewage was used to evaluate sample preparation performance. This solution is absent of antibiotics and caffeine, and also mimics sewage composition which allows to optimize and validate the proposed method in a complex sample.

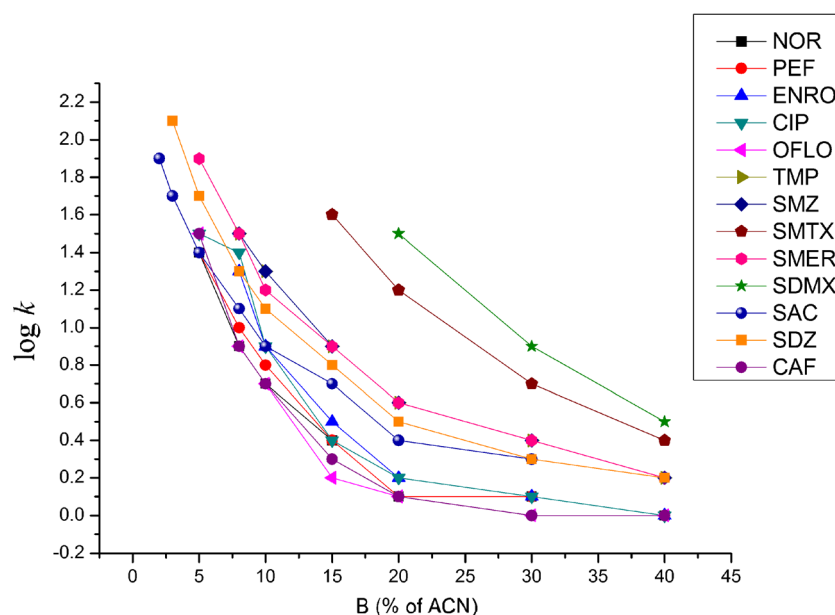
Furthermore, this lab-made sewage has been used in many studies in lab-scale anaerobic reactors and batch reactors as organic substrate for studies related to new technologies for wastewater treatment [51, 52].

Samples of lab-made sewage were injected into the SPE column, about 1.5 min were necessary to eliminate the main matrix components, as shown Fig. 3.

Elution mode

A gradient mode using pump B was used for the elution of analytes as described above. Backflush and foreflush were compared based on asymmetry and number of plates using a lab-made sewage spiked sample. SAC was used to calculate

Fig. 2 Log *K* of the target compounds using a mobile phase consisting of water containing 0.1 % of formic acid (a) and ACN (b) ranging from 98:2 to 50:50



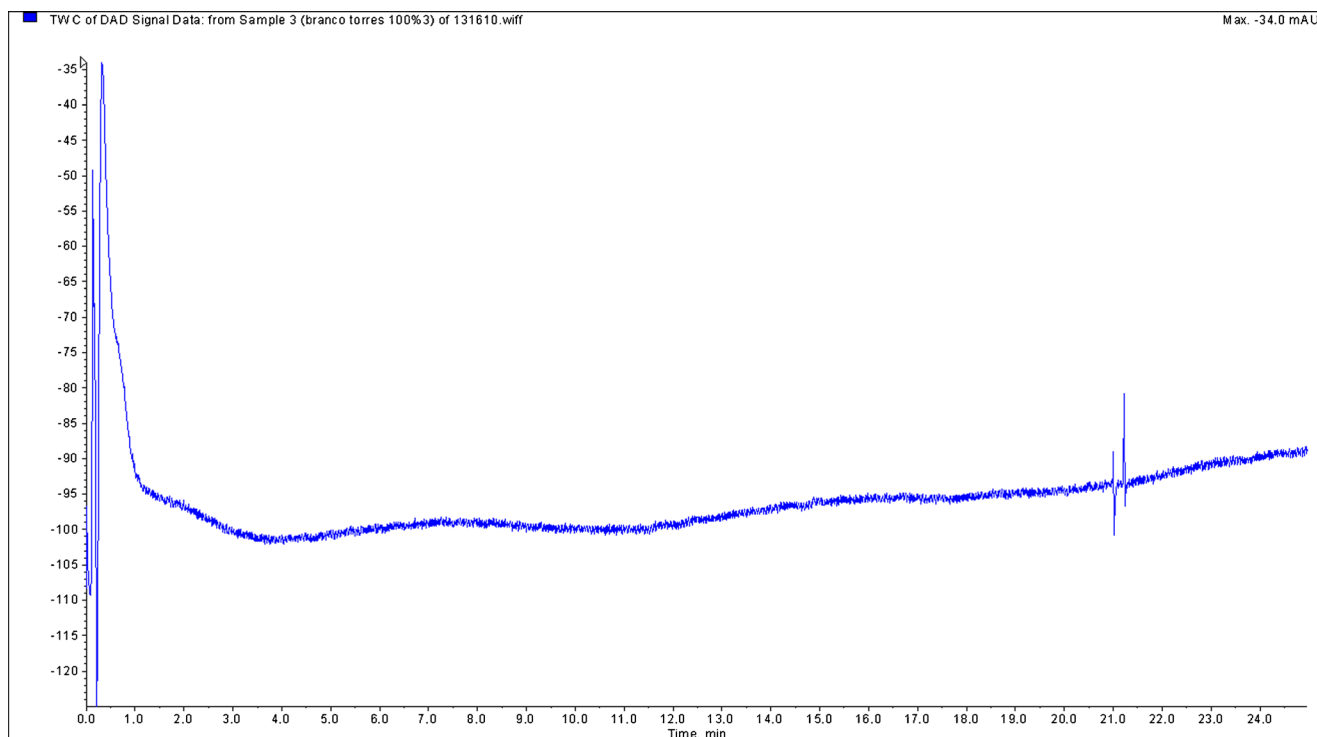


Fig. 3 Chromatogram showing removal of components in the lab-made sewage (peak between 0.15 and 1.5 min) after injection of 100 μL at 254 nm wavelength

these parameters since it requires 98 % of water to be retained in the SPE column.

In forwardflush mode, As and N were 2.5 and 256, respectively. As and N in backflush mode were 1.2 and 2304. Based on As and N data, a better peak focusing was obtained in the backflush mode, and this condition was adopted to elute all analytes during the method validation and sample analysis.

Chromatographic separation

The gradient and isocratic modes were tested using acetonitrile and water, both containing 0.1 % of formic acid in different mixtures. Most of the analytes co-eluted because of their structural similarity, however using the gradient mode provided a rapid determination. Besides the co-elution, the compounds were analyzed without any interference and cross-talk using the selected SRM transitions and MS/MS conditions.

The injection volume was evaluated from 5 to 100 μL (maximum autosampler capacity); 100 μL was used to obtain the lowest LOQs without compromising peak shape and efficiency.

A carryover study was necessary to avoid lack of precision and accuracy. The autosampler was programmed to clean internal and external needle parts with a mixture of acetonitrile, isopropanol, methanol, and water containing 0.1 % of acid formic (25 % (v/v) of each) pumped by a metering pump. In addition, the valve was switched to injection position to prevent adsorption of analytes. A minimal carryover of 0.5 % was

observed between the calibrator at the highest concentration level and subsequent blank injection.

Method validation

The sample pre-treatment was adjusting the pH to 3.0 and filtration through a 0.22- μm membrane. In pH=3.0, sulfonamides are mainly in neutral form and fluoroquinolones are in cationic form. HLB SPE phase showed enough selectivity to pre-concentrate and extract all of these polar compounds found in the lab-made sewage.

Figure 4 presents a typical chromatogram of spiked lab-made sewage at intermediate concentration levels.

Linearity

Linearity was evaluated spiking the analytes in the lab-made sewage in seven calibration points (3 orders of magnitude) and calculated by using least-squares linear regression analysis. Isotopically labeled IS were used in order to hinder the matrix effects. The calibration curves were tested using external standard method and IS. Both showed linearity without lack of fit. However, external standard was adopted since it contains the pure data obtained, also IS were not available for all analytes used in this proposed method, mainly for the less retained compounds, CAF and SAC.

Three SRM transitions were monitored, except for NOR and OFLO which presented two intense and stable transitions.

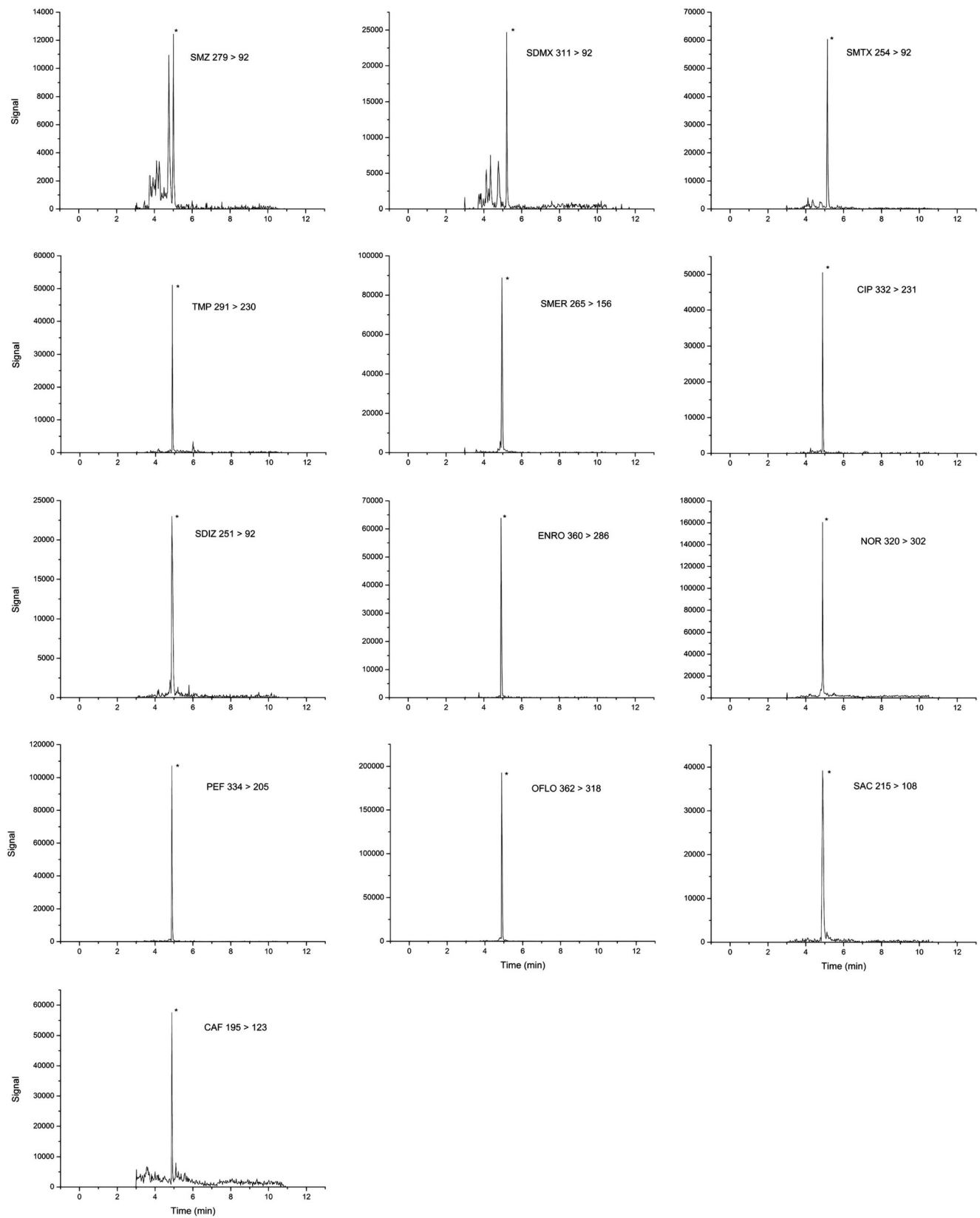


Fig. 4 Extracted ion chromatogram (XIC) from lab-made sewage sample spiked with $0.015 \mu\text{g L}^{-1}$ of SMZ, $0.149 \mu\text{g L}^{-1}$ of SDMX, $0.322 \mu\text{g L}^{-1}$ of SMTX, $0.645 \mu\text{g L}^{-1}$ of TMP, $0.660 \mu\text{g L}^{-1}$ of SMER, $0.594 \mu\text{g L}^{-1}$ of

CIP, $1.45 \mu\text{g L}^{-1}$ of SDIZ, $1.58 \mu\text{g L}^{-1}$ of ENRO, $3.92 \mu\text{g L}^{-1}$ of NOR, $3.92 \mu\text{g L}^{-1}$ of PEF, $5.40 \mu\text{g L}^{-1}$ of OFLO, $6.93 \mu\text{g L}^{-1}$ of SAC, and $14.9 \mu\text{g L}^{-1}$ of CAF

Table 3 Linearity, intra-day, and inter-day precision values achieved in lab-made sewage by the proposed method

Compound	MQL (ng L ⁻¹)	MDL (ng L ⁻¹)	Linear range (ng L ⁻¹)	Regression data (a, b, r ²)	Weighted calibration	Level ^a	Intra-day precision (RSD %)	Inter-day precision (RSD %)	RR (%)
SMZ	1.32	0.800	1.32–95.0	1.83×10 ⁴ 8.26×10 ² 0.984	NA	Low Medium High	2.96 16.8 8.95	– 8.95 –	42.7 34.1 32.8
TMP	16.5	5.00	16.5–1.19×10 ³	–5.38×10 ³ 1.05×10 ³ 0.984	1/x ²	Low Medium High	8.63 14.4 7.33	– 6.59 –	34.1 22.9 22.5
SAC	288	100	288–20.8×10 ³	1.30×10 ⁴ 2.99×10 ¹ 0.960	1/y ²	Low Medium High	2.58 4.00 2.36	– 11.7 –	9.08 7.90 8.54
SMTX	26.9	16.3	26.9–1.94×10 ³	–5.96×10 ³ 1.19×10 ³ 0.970	1/x ²	Low Medium High	8.91 10.3 11.8	– 10.8 –	38.6 38.4 34.8
SDZ	121	36.7	121–8.72×10 ³	4.72×10 ³ 7.25×10 ¹ 0.973	1/y ²	Low Medium High	12.8 4.53 3.05	– 13.0 –	12.2 12.6 12.3
SMER	55.0	33.3	55.0–3.96×10 ³	6.24×10 ² 5.11×10 ² 0.980	1/y ²	Low Medium High	6.06 7.56 4.15	– 8.96 –	33.7 26.6 27.4
SDMX	12.4	7.50	12.4–8.91×10 ²	–8.39×10 ² 7.55×10 ² 0.975	1/x ²	Low Medium High	10.6 11.3 14.5	– 14.6 –	72.5 47.9 50.2
NOR	450	198	450–32.4×10 ³	3.34×10 ⁴ 7.05×10 ¹ 0.997	1/y	Low Medium High	9.36 4.63 3.75	– 7.22 –	57.4 35.1 29.9
OFLO	326	198	326–23.5×10 ³	2.05×10 ⁴ 1.24×10 ² 0.985	1/y ²	Low Medium High	0.574 2.00 5.41	– 2.57 –	42.3 45.7 56.8
PEF	326	198	326–23.5×10 ³	4.15×10 ³ 5.76×10 ¹ 0.997	1/y	Low Medium High	6.49 10.8 4.20	– 5.12 –	42.3 45.3 43.9
ENRO	132	80.0	132–9.50×10 ³	9.48×10 ² 3.78×10 ¹ 0.942	1/x ²	Low Medium High	8.47 5.70 4.05	– 21.0 –	20.3 23.0 27.3
CIP	49.5	30.0	49.5–3.56×10 ³	2.25×10 ³ 2.42×10 ² 0.994	NA	Low Medium High	12.5 4.60 3.85	– 9.87 –	35.1 35.2 30.3
CAF	1.24×10 ³	500	1.24×10 ³ –8.91×10 ⁴	5.83×10 ³ 1.93 0.988	1/x	Low Medium High	8.23 9.85 8.11	– 7.06 –	12.2 7.20 10.4

“–” not evaluated, a intercept, b slope, r correlation coefficient, NA not applied, RR relative recovery

^aLow concentration (ng L⁻¹)=low concentration levels correspond to MQL levels studied. Medium concentration (ng L⁻¹)=high concentration levels correspond to upper limit of the linear range studied 37.1; NOR 1.35; OFLO 980; PEF 980; ENRO 396; CIP 148; and CAF 3.71. High concentration (ng L⁻¹)=high concentration levels correspond to upper limit of the linear range studied

Two transitions were used for confirmation and one for quantification. The quantification SRM transition was chosen according to the lack of fit, and the lowest values achieved to obtain the calibration curves were selected.

Weight calibration of $1/x^2$ were applied for SMTX, SDMX, TMP, and ENRO; $1/y^2$ for SDIZ, SAC, and OFLO; $1/y$ for PEF and NOR; and $1/x$ for CAF. The weighting factors were selected based on lower relative error and the sum of relative residual errors lower than 15 % in the LOQ level. Non-weighted calibrations were enough for CIP and SMZ. For the majority of analytes, regression data (R^2) was higher than 0.97, except for ENRO and SAC. These analytes presented a better R^2 value without weighted calibration, although with lower adjustment mainly for LOQ concentration level. Besides a lower R^2 value, better accuracy was observed using weighted calibration.

Figures of merit

The MDL and MQL were obtained based on a S/N ratio of 3 and 10, respectively. The MDL varied from 0.800 to 600 ng L⁻¹, and MQL from 1.200 to 1200 ng L⁻¹. These values are in the same range as previous studies applying offline and online SPE in the sample preparation step [4, 21, 29, 40, 46, 50, 53].

Table 3 shows in details the MDL, MQL, intra- and inter-day precision, and RR values. The intra-day and inter-day precision results are compatible with IUPAC and Anvisa guidelines [37, 38]. The intra- and inter-day precision values were lower than 16 and 21 %, respectively, which is acceptable for complex samples [4, 25, 29].

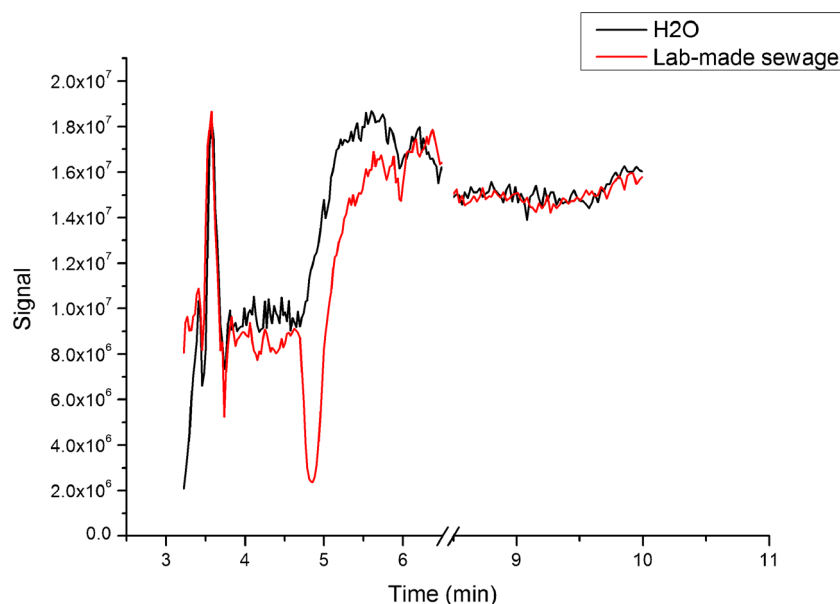
RR was evaluated by spiking three different concentration levels (MQL; medium and high) in ultra-pure water, and comparing those levels collected in the lab-made sewage. RR

values varied from 7 to 72 %, the lowest RR values were observed for CAF and SAC, the most polar compounds analyzed (log P -0.07 and -1.0). RR average varied from 23 to 48 % for fluoroquinolones and 8.5 to 56 % for sulfonamides. Similar RR using online SPE were obtained by Feitosa-Felizzola et al. [54] and Khan et al. [55]. In both studies, the respective authors related the low RR to matrix effect caused by ionization suppression. In fact, a RR study cannot discern between the effect of matrix on the extraction efficiency and on the ionization performance at detector. In order to determine extraction efficiency (by means of absolute recovery), the use of post-extraction spiking is recommended. However, for online SPE methods, it is a laborious, lengthy, and uncertain procedure. On the other hand, in order to infer about the matrix effect on electrospray ionization, the next section presents a qualitative matrix effect study by means of standard T-infusion test associated with matrix processing by the online extraction procedure. The stability of spiked samples in a lab-made sewage was evaluated in three different situations. Both samples were stable within 48 h as well as for long-term stability over 3 weeks (504 h). The highest stability was achieved when stored at 4 °C, analyte areas differ less than 20 % for t_0 injection; the samples were then kept at 4 °C.

Matrix effect

A T-infusion was used for matrix effect evaluation; ultra-pure water sample was used as reference and compared with the lab-made sewage. Figure 5 shows an intense signal suppression in the range of 4.7 and 5.0 min. This chromatographic region corresponds to the retention time of most compounds in this analysis. The matrix effect was expected since lab-made sewage is a complex sample containing inorganic and

Fig. 5 Evaluation of matrix effect on the ionization efficiency. Black line represents injection of ultra-pure water and red line the injection of lab-made sewage sample



organic compounds which may lead to an intense interference in the ionization of analytes.

Application to real samples

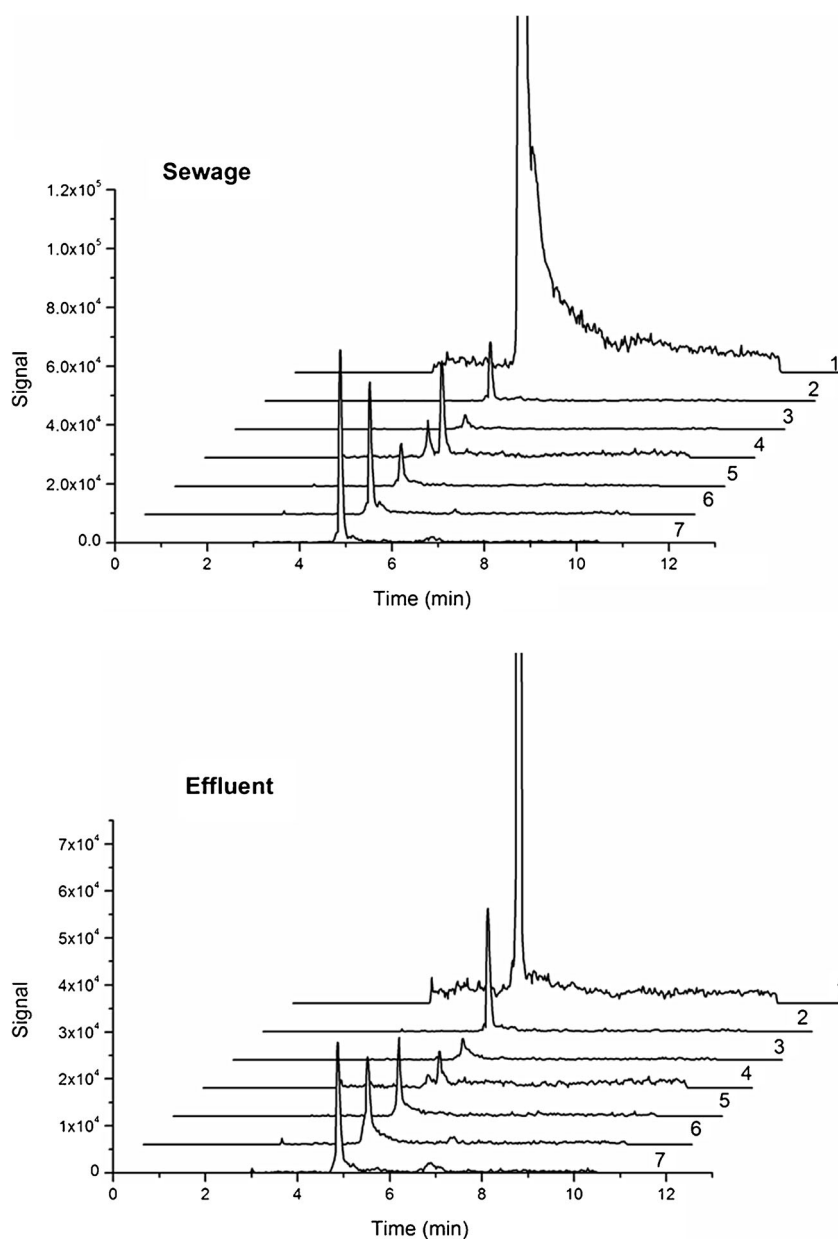
Real sewage samples and anaerobic pilot-scale reactor effluent samples ($n=15$) were analyzed. Six antibiotics and caffeine were detected and quantified in both matrices as shown in Fig. 6. A simple and rapid standard addition calibration was applied to overcome the observed matrix effect and improve the quality of the antibiotic concentration measured.

The concentration level in the anaerobic reactor effluent for SMZ, TMP, and OFLO in some samples was higher than in the sewage. This fact has been observed by hydrolysis of

conjugated metabolites of these antibiotics, typically found in the sewage [12, 25, 56, 57]. This phenomenon could be enhanced by the complex microbial community within the anaerobic reactor.

In all samples, the retention time deviation was less than ± 0.3 %. Moreover, the relative abundance of two different SRM monitored for each analyte varied less than ± 20 %. Thus, at least four identification points (IP) were accomplished in a minimum of three IPs as recommended by the EU criteria [58]. Of the seven compounds, five were confirmed as positive using these criteria. Only SMZ and CIP showed a SRM relative abundance transition with variation higher than ± 20 % in all samples. This could be due to the matrix effect. Moreover, both of these compounds are in low

Fig. 6 XIC of sewage and anaerobic reactor effluent sample. 1, CAF (195>138); 2, TMP (291>261); 3, SMZ (279>186); 4, SMTX (254>156); 5, OFLO (362>318); 6, NOR (320>276); 7, CIP (332>288)



nanogram-per-liter range, and the samples undergo much interference in their ion ratio.

Signal suppression was predominant in sewage and effluent samples for most of the analytes. However, signal enhancement was observed for SMZ and CIP which could be a key explanation for the SRM ion ratio deviation.

For the sewage, matrix suppression varied from 10 to 83 %, as observed in other studies [59, 60]. Previous studies using SPE offline related to analysis of hospital wastewater and urban influent wastewater demonstrated a ion suppression that varied from 30 to 90 % [57]. Even using online SPE, Ciofi et al. [61] observed ionization suppression for steroids analysis varying from 71.3 to 94.6 %. In the anaerobic reactor effluent, most of the samples showed suppression ranging from 12 to 91 %; however, some samples showed signal enhancement for SMZ, CIP, TMP, SMTX, NOR, and OFLO. Gros et al. [57] reported occurrence of signal enhancement for fluoroquinolones, and Zhou et al. [62] demonstrated the same behavior for SMZ and SMTX. This is attributed to the presence of dissolved organic matter which varied in the samples studied from 700 to 800 mg L⁻¹ of chemical oxygen demand (COD). Moreover, a slight fluctuation on the anaerobic effluent composition also explains this fact. This shift is caused by typical day-to-day oscillations in the sewage composition, which directly influenced the effluent analysis, mainly, for LC-MS/MS causing ion suppression or enhancement.

Table 4 shows the concentration levels found in the sewage and reactor effluent, a wide range varying from 0.018 µg L⁻¹

to SMZ until 1097 µg L⁻¹ of CAF was observed in accordance with previous publications [2, 63–65]. The sewage was collected from a densely populated neighborhood, close to small farm yards explaining the high concentrations of CAF and NOR.

Conclusions

An online SPE method was successfully developed and applied for routine analysis of several antibiotics and caffeine found in the sewage and anaerobic reactor effluent. This setup used a simple, conventional HPLC system (autosampler and six-port valves) with a column-switching technique. Sample pre-treatment was limited to filtration, pH adjustment and IS spiking. An online SPE HLB column showed suitable selectivity to pre-concentrate the antibiotics from two different classes and caffeine; both had different physico-chemical properties. More than 200 analyses were performed without signal loss or even without SPE and analytical column clogging. The method was completely validated in backflush mode, presenting linearity, selectivity, precision, and detectability in sub-nanogram-per-liter levels in complex samples similar to sewage material (lab-made sewage). Standard addition was applied to monitor six of the most common antibiotics and caffeine in the environmental samples without a time-consuming and labor-intensive sample preparation step. The method presented provides a good basis for further

Table 4 Antibiotic concentrations (µg L⁻¹) and signal-to-noise ratio of non-spiked sample (S/N) obtained through analysis of sewage and anaerobic reactor effluent

Sample number	Sample type	TMP	CAF	SMZ	SMTX	OFLO	NOR	CIP
1	Sewage	0.094 (13.1)	964 (504)	0.018 (24.0)	0.161 (378)	0.590 (49.6)	6.40 (50.6)	0.280 (100)
2	Effluent	0.083 (53.1)	40.0 (638)	0.023 (17.7)	0.049 (63.0)	0.600 (40.9)	4.50 (32.4)	0.190 (52.0)
3	Sewage	0.205 (249)	1097 (341)	0.043 (25.0)	0.317 (396)	2.05 (31.7)	14.0 (158)	0.790 (246)
4	Effluent	0.126 (188)	12.9 (128)	0.053 (23.8)	0.126 (189)	1.27 (43.0)	6.80 (42.0)	ND (NA)
5	Sewage	0.211 (106)	428 (712)	0.032 (34.7)	0.370 (52.7)	1.22 (40.3)	8.60 (66.4)	0.320 (110)
6	Effluent	0.116 (72.5)	ND (NA)	0.032 (74.2)	0.042 (57.9)	1.53 (54.1)	2.10 (24.4)	0.130 (70.8)
7	Sewage	0.118 (100)	830 (360)	0.031 (25.0)	0.216 (71.5)	1.63 (77.7)	10.4 (153)	0.500 (220)
8	Effluent	0.116 (37.7)	45.3 (174)	0.043 (46.8)	0.090 (109)	1.29 (31.1)	3.60 (59.7)	0.440 (163)
9	Sewage	0.138 (215)	341 (748)	0.021 (31.2)	0.342 (133)	0.660 (133)	6.30 (85.3)	0.350 (129)
10	Effluent	0.117 (87.8)	157 (445)	0.045 (48.9)	0.161 (13.5)	0.860 (48.3)	5.30 (51.9)	0.250 (67.0)
11	Sewage	0.204 (215)	456 (745)	0.048 (38.1)	0.616 (92.2)	0.750 (104)	10.1 (142)	0.370 (285)
12	Effluent	0.219 (117)	308 (226)	0.045 (31.0)	0.092 (14.1)	0.480 (26.5)	4.80 (69.1)	0.180 (57.1)
13	Sewage	0.327 (449)	539 (269)	0.029 (21.2)	0.656 (30.5)	1.69 (50.8)	8.00 (66.8)	0.440 (88.1)
14	Effluent	0.108 (56.5)	158 (159)	0.040 (24.1)	0.048 (12.1)	0.790 (36.0)	5.60 (53.3)	0.270 (40.7)
15	Effluent	0.113 (38.3)	122 (152)	0.040 (13.9)	0.088 (12.1)	0.690 (20.4)	5.60 (41.1)	0.260 (40.9)

ND not detected, NA not evaluated

studies on the removal and adsorption kinetics of antibiotics using lab-scale anaerobic reactors and batch studies.

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Conflict of interest The authors declare no conflict of interest relating to the material presented in this article.

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