Revista Brasileira de Ciências Ambientais
Brazilian Journal of Environmental Sciences

Low-temperature partitioning extraction optimized for antibiotics quantification in sewage sludge by liquid chromatography-tandem mass spectrometry

Extração de partição de baixa temperatura otimizada para quantificação de antibióticos em lodo de esgoto por cromatografia líquida-espectrometria de massas em tandem

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ABSTRACT

Antibiotics and their degradation byproducts are a relevant group of contaminants of emerging concern. Conventional wastewater treatment plants fail to remove them. Thus, uncontrolled disposal of antibiotics in the environment increases the spread of antibiotic-resistant bacteria and their genes. The present work describes the development and validation of an analytical method for the simultaneous determination of three antibiotics—sulfamethoxazole (SMX), sulfadiazine (SDZ), and trimethoprim (TMP)—in sewage sludge. The method is based on low-temperature partitioning extraction (LTPE) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LTPE is a low-cost extraction technique that does not require specific equipment, large amounts of solvents and samples, nor longer times to extract antibiotics from sludge. The LTPE procedure was optimized for dried anaerobic sludge from upflow anaerobic sludge blanket reactors fed with sewage. The influence of sample mass content, ultrasonic bath extraction time, and potential of hydrogen (pH) of the buffer solution on extraction efficiency was evaluated with experimental design methodology. The best conditions achieved to extract the target antibiotics were: 150 mg dried sludge, 20min of ultrasonic bath, and pH 7, in addition to 600 µL of McIlvaine buffer solution, and 600 µL organic solvent in

RESUMO

Antibióticos e seus subprodutos de degradação são um grupo relevante de contaminantes de preocupação emergente. As estações de tratamento de águas residuais convencionais não conseguem removê-los. Assim, o descarte descontrolado de antibióticos no ambiente aumenta a disseminação de bactérias resistentes a antibióticos e seus genes. O presente trabalho descreve o desenvolvimento e a validação de um método analítico para a determinação simultânea de três antibióticos — sulfametoxazol (SMX), sulfadiazina (SDZ) e trimetoprima (TMP) — em lodo de esgoto. O método é baseado na extração por partição de baixa temperatura (LTPE) seguida por cromatografia líquida-espectrometria de massas em tandem (LC-MS/MS). LTPE é uma técnica de extração de baixo custo que não requer equipamento específico, nem grandes quantidades de solventes e amostras, nem tempos mais longos para extrair antibióticos do lodo. O procedimento LTPE foi otimizado para lodo anaeróbico seco de reatores de manta de lodo anaeróbico de fluxo ascendente alimentados com esgoto. A influência do teor de massa da amostra, tempo de extração do banho ultrassônico e potencial de hidrogênio (pH) da solução tampão na eficiência da extração foi avaliada com metodologia de planejamento experimental. As melhores condições alcançadas para extrair os antibióticos alvo foram: 150 mg de lodo seco, 20min de banho ultrassônico e pH 7,

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**In memoriam*

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Conflicts of interest: the authors declare no conflicts of interest.

Funding: This study received financial support from the Coordination for Improvement of Higher Education Personnel (CAPES), Minas Gerais State Research Support Foundation (FAPEMIG), National Council for Scientific and Technological Development (CNPq), Agency for Financing Studies and Projects (FINEP), Brazilian National Health Foundation (FUNASA) – grant number 2510001557612017-21, and the Federal University of Ouro Preto (UFOP).

Received on: 07/11/2024. Accepted on: 02/04/2025.

https://doi.org/10.5327/Z2176-94782191

one cycle of extraction. The recovery values were 52.7% (SMX), 53.6% (SDZ), and 70.1% (TMP), and the method exhibited good repeatability (≤14.7%). The method detection and quantification limits ranged from 3.6 to 11.3 ng g^{-1} dw (dry weight) and 11.5 and 36.1 ng g^{-1} dw, respectively. Sewage sludge samples collected from two municipal wastewater treatment plants in Minas Gerais state/Brazil revealed the presence of TMP (174.2 and 134.8 ng g^{-1} dw) while sulfonamides were lower than the method detection limit. To the best of our knowledge, this is the first study based on LTPE followed by LC-MS/MS that was applied to simultaneously determine SMX, SDZ, and TMP antibiotics in sewage sludge.

Keywords: contaminants of emerging concern; organic contaminants; factorial design; ecofriendly extraction method; sludge sorption.

além de 600 µL de solução tampão McIlvaine e 600 µL de solvente orgânico em um ciclo de extração. Os valores de recuperação foram 52,7% (SMX), 53,6% (SDZ) e 70,1% (TMP), e o método apresentou boa repetibilidade (≤14,7%). Os limites de detecção e de quantificação do método variaram de 3,6 a 11,3 ng g^{-1} (peso seco) e 11,5 e 36,1 ng g^{-1}, respectivamente. Amostras de lodo de esgoto coletadas de duas estações de tratamento de águas residuais municipais no estado de Minas Gerais/Brasil revelaram a presença de TMP (174,2 e 134,8 ng g^{-1}) enquanto as sulfonamidas estavam abaixo do limite de detecção do método. Até onde sabemos, este é o primeiro estudo baseado em LTPE seguido por LC–MS/MS que foi aplicado para a determinação simultânea de antibióticos SMX, SDZ e TMP em lodo de esgoto.

Palavras-chave: contaminantes de preocupação emergente; contaminantes orgânicos; planejamento fatorial; método de extração ecologicamente correto; sorção de lodo.

Introduction

The presence of pharmaceuticals and personal care products (PPCPs) in sewage sludge has been widely investigated worldwide (Lindholm-Lehto et al., 2017; Ramos et al., 2019; Ajibola et al., 2020). The occurrence of PPCPs in wastewater effluents (ranging from ng L^{-1} to mg L^{-1}) and in biosolids (µg kg^{-1} to mg kg^{-1}) is a result of incomplete removal during conventional wastewater treatments (Göbel et al., 2005; Bajkacz et al., 2020; Mejías et al., 2021). Antibiotics and their degradation byproducts are responsible for the dissemination of antibiotic resistance bacteria and their related genes (Mejías et al., 2021). Thus, the presence of these contaminants of emerging concern (CECs) in the environment has become a global emerging health concern. Such antimicrobial agents can be released to the terrestrial environment through sewage sludge application on farmland intented to recycle nutrients and promote soil conditioning (Ajibola et al., 2020; Malvar et al., 2020).

A major concern arising from the application of sludge as a biofertilizer is the potential presence of CECs, which may cause bioaccumulation in the environment and even migrate to crops when sludge is applied in agricultural soils (Rodrigues et al., 2020). In Brazil, the National Environment Council (CONAMA) 498/2020 resolution establishes rules for biosolids production and its application in soils, even though it does not specify the maximum level allowed of antibiotics in biosolids intended for soil amendment or agricultural fertilizer. There is a literature gap on the occurrence of antibiotics in Brazilian sewage sludge which precludes risk assessment analysis for these CECs in the environment.

In order to assess the occurrence and fate of contaminants in wastewater treatment, analytical methods for antibiotic determination in sewage sludge matrices are needed. Methodologies for the extraction and quantification of CECs in aqueous matrices (e.g., influent and effluents of a wastewater treatment plant) are more common than in solid matrices (Dorival-García et al., 2013; Cavaillé et al., 2021). Thus, sewage sludge is challenging due to its complex nature and heterogeneous composition (Pérez-Lemus et al., 2019; Mejías et al., 2021). Analytical methods for antibiotics determination in environmental matrices are usually based on high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS).

Sludge preparation involves different extraction procedures, clean-up, and pre-concentration steps (Mejías et al., 2021). The most commonly used techniques for the extraction of antibiotics in sewage sludge include: ultrasonic solvent extraction (USE) (Ademoyegun et al., 2020; Malvar et al., 2020), pressurized liquid extraction (PLE) (Dorival-García et al., 2013; Malvar et al., 2020), matrix solid phase extraction (SPE) (Gama and Rocha, 2020), quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction (Ferhi et al., 2016; Benedetti et al., 2020; Malvar et al., 2020), and microwave-assisted extraction (MAE) (Dorival-García et al., 2013). The low-temperature partitioning extraction (LTPE) has been an alternative and versatile technique for the extraction of CECs in solid and semi-solid matrices (Paranhos et al., 2020). The LTPE procedure basically consists of extracting target compounds from dry sludge samples in aqueous and organic phases. A liquid-liquid partition equilibrium is established, and then analytes are extracted in the organic phase. When the temperature is lowered, the aqueous phase solidifies, whereas the organic supernatant containing the soluble analytes remains liquid (Gomes et al., 2017; Barros et al., 2019). LTPE combines several advantages, such as low-cost and simplicity; requires less solvents and toxic reagents; involves small sample size; does not require special equipment; and it is easier to perform the extraction compared to other techniques described in the literature (Gomes et al., 2017; Barros et al., 2019; Paranhos et al., 2020). It is particularly more accessible in developing and low-income countries (Paranhos et al., 2020) and may contribute to enhancing environmental monitoring of antibiotics and other CECs. As far as we know, this work employed, for the first time, the LTPE method for the simultaneous determination of three antibiotics in sewage sludge.

In this context, the aim of this study was to develop a simple, easy, rapid, and sensitive analytical method for the simultaneous determination of three antibiotics in sewage sludge based on the LTPE procedure followed by LC-MS/MS. The antibiotics investigated were sulfamethoxazole (SMX), sulfadiazine (SDZ), and trimethoprim (TMP). These antibiotics represent antibacterial agents widely used worldwide to treat many human and animal infectious diseases (An et al., 2015). Among them, SMX and TMP are mainly consumed in association (i.e., co-trimoxazole) in a 5:1 ratio (SMX:TMP) allowing synergistic effects (Thiebault, 2020). A design of experiments approach was used to determine the significance of the following extraction parameters: sample mass (mg), extraction time in ultrasonic bath (min), and pH of the buffer solution. This approach enabled the simultaneous determination of the effects of different variables on extraction efficiency. The developed method was validated and successfully applied to the analysis of antibiotic residues in sewage sludge from a wastewater treatment plant located in Minas Gerais state, Brazil. The proposed method can be applied in further studies on the antibiotic occurrence in sewage sludge to support normative deliberations that regulate the final disposal of sludge and biosolids.

Materials and Methods

Chemicals and reagents

Analytical standards of SMX, SDZ, and TMP were purchased from Inlab Confiança® (Diadema/SP, Brazil). All antibiotic standards were of high-purity grade (>95%). Ultra-pure water (18.2 MΩ.cm) was produced using an ion-exchange purification system. HPLC-grade methanol, acetonitrile, and formic acid (88%) were purchased from J.T. Backer® (Mexico), whereas acetone was purchased from Vetec® (Brazil). Disodium hydrogen phosphate (Na_2HPO_4) and anhydrous citric acid, for the preparation of McIlvaine buffer solution (McIlvaine, 1921) were obtained from Cromoline® (Brazil) and Synth® (Brazil), respectively. McIlvaine buffer solutions were prepared by mixing 0.1 M citric acid anhydrous and 0.2 M Na_2HPO_4, at different proportions to obtain three solutions of pH at 2.2, 4.5, and 7.0.

Individual standard solutions of each antibiotic were prepared at 1000 mg L^{-1} in methanol every six months. The working solutions containing all analytes were diluted using a methanol/water solution (75:25, v/v) with 0.1% formic acid. Stock and working solutions were stored in amber glass bottles at -20°C.

Sample collection and storage

Sewage sludge samples were collected in three upflow anaerobic sludge blanket (UASB) reactors fed with sanitary sewage and installed in the state of Minas Gerais, Brazil. The samples collected at the first reactor (UASB-1), was used to develop the analytical method. This reactor was operated on a pilot scale at the Sanitation Research and Training Center (CePTS), at the Federal University of Minas Gerais (UFMG)/Copasa (Arrudas Sewage Treatment Station, Belo Horizonte/MG, Brazil). The second (UASB-2) and third (UASB-3) reactors were operated on a full scale, for populations of around 500 and 50,000 inhabitants, respectively. Random samples with 5L of sludge of each reactor were collected in July 2020. Sewage sludge samples were collected after secondary treatment. Samples were stored in amber glass bottles and 1% (v.v^{-1}) methanol was added to reduce biological activity. Then, the samples were dried at 40°C until constant weight. The sludge was homogenized and stored in the dark at 4°C until extraction. Table 1 shows the anaerobic sludge characterizations.

Preparation of fortified samples

Sludge samples used for method development and validation were prepared by spiking the target antibiotics. The dried reference matrix samples (0.15 g dw) used for optimization were spiked at 500 ng g^{-1}. For validation studies, dry sludge samples were spiked at different concentrations (50, 200, and 500 ng g^{-1}) of each antibiotic (SMX, SDZ, and TMP) prepared in 10 mL of acetone. The samples were vigorously shaken for 10min and left overnight at room temperature to enable sufficient contact of analytes with the whole sludge and to remove the solvent added during spiking (Dorival-García et al., 2013; Ajibola et al., 2020).

Low-temperature partitioning extraction design of experiments

A complete 2^3 full factorial design was employed for evaluation of the simultaneous influence of three factors: sample mass (mg), extraction time in ultrasonic bath (min), and pH of the buffer solution. The experimental design was randomly performed (Table 2). The vari-

Table 1 – Main characteristics* of sludge produced by anaerobic reactors.

Parameter	Typical range
Dry solids content (%)	3–6
Density (kg/m^3)	1010–1020
Volatile solids/Total solids ratio	0.55–0.60
pH	6.6–8.0
Alkalinity (mg $L^{-1}CaCO_3$)	2500–3500
Nitrogen (N% of TS)	1.6–6.0
Phosphorous (P_2O_5% of TS)	1.5–4.0
Fats, grease (% of TS)	5–20
Protein (% of TS)	15–20

pH: potential of hydrogen; TS: total solids; *according to Kiely et al. (1997) and Chernicharo (2007).

ables and the levels to be examined were based on studies in the literature (Dorival-García et al., 2013; Ferhi et al., 2016; Ajibola et al., 2020) that used other techniques for the extraction of pharmaceuticals in sewage sludge. These three factors were studied on two levels and the analysis was performed in duplicate, totaling 16 experiments.

The dried fortified reference matrix (500 ng g^{-1}) was placed in an Eppendorf tube, at mass levels equal to what was predicted in the design (50 or 150 mg). A volume of 600 µL of McIlvaine buffer (pH 2.2 or 7.0) was added and the mixture was stirred in a vortex for 30s. Then, 600 µL of acetonitrile/methanol solution (95:5; v/v) was added and vortexed for 1min. The solution was maintained in an ultrasonic bath for 20 or 60min and centrifuged at 14800 rpm for 30min. Subsequently, the mixture was frozen at -20°C for at least 3h to ensure the complete freezing of the aqueous fraction and organic phase separation. A volume of 180 µL supernatant (organic phase) was carefully recovered and transferred to another Eppendorf tube. The extracts were centrifuged at 14800 rpm for 30min and 150 µL of supernatant was fully transferred to an insert and dried under nitrogen gas flow. The dried extract was kept at -20°C for a maximum of 24h and then resuspended in 150 µL of methanol/water (75:25, v/ v) with 0.1% formic acid before analysis by LC-MS/MS. Finally, the effect of the number of extraction cycles (1 and 2) was investigated. Thus, blank and fortified samples were extracted with one or two consecutive LTPE procedures. Cycle extractions were performed in five replicates with the addition of target antibiotics (500 ng g^{-1}).

Table 2 – Experimental design matrix for antibiotics extraction.

Tests	Mass sample (mg) – X_1	Extraction time in ultrasonic bath (min) – X_2	pH buffer solution – X_3
1 and 9	50 (-1)	20 (-1)	2.2 (-1)
5 and 8	150 (+1)	20 (-1)	2.2 (-1)
7 and 3	50 (-1)	60 (+1)	2.2 (-1)
2 and 11	150 (+1)	60 (+1)	2.2 (-1)
12 and 16	50 (-1)	20 (-1)	7.0 (+1)
14 and 10	150 (+1)	20 (-1)	7.0 (+1)
13 and 15	50 (-1)	60 (+1)	7.0 (+1)
4 and 6	150 (+1)	60 (+1)	7.0 (+1)

*Encoded values in parentheses.

Figure 1 presents the operating parameters of the extraction conditions and the final protocol.

Instrumentation

Chromatographic separation of target antibiotics was performed using HPLC Nexera X2 (Shimadzu, Japan), equipped with the analytical column Shim-pack XR-ODSIII C18 (150 × 2.0 mm; 2.1 µm). The column was maintained at 40°C and the total injection volume was 14 µL. Ultrapure water (A) and acetonitrile (B), both with 0.1% formic acid, were used as the mobile phases at a flow rate of 0.25 mL min^{-1}.

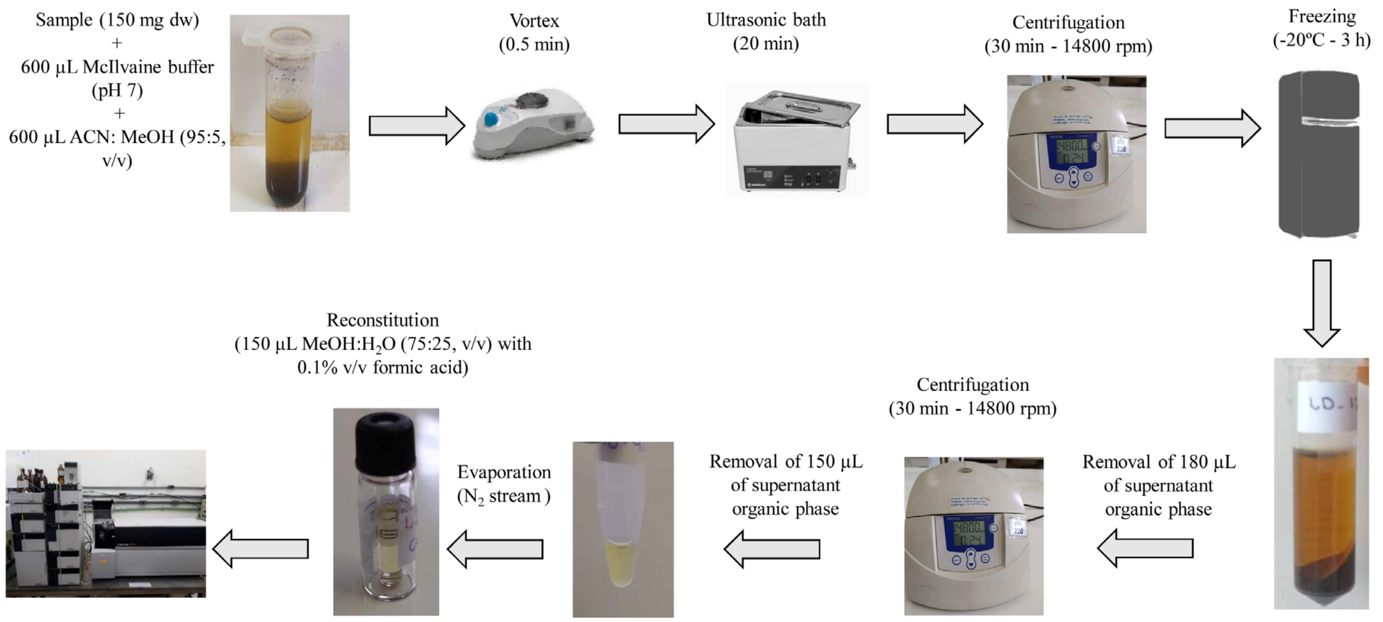

Figure 1 – Scheme of the low-temperature partitioning extraction procedure for extraction of antibiotics from sewage sludge samples.

The analytical separation was performed with the following gradient elution program: initial conditions 5% B; 0–1min, 5–10% B; 1–4min, 10–35% B; 4–5min, 35–95% B; 5–10min, 95% B; from 10 to 11min return to initial conditions; 11–13min, equalizing the column.

A Shimadzu LC-MS 8040 triple quadrupole, equipped with an electrospray ionization (ESI) source, operated at positive mode (ESI+), was employed to analyze the target antibiotics. The MS system was operated under the following conditions: interface voltage of +4.5 kV; desolvation line temperature of 230°C; heating block temperature of 400°C; drying gas (N_2) flow rate of 10 L min^{-1}; nebulizing gas (N_2) flow rate of 5 L min^{-1}; and collision-induced dissociation gas Argon 5.0 at 230 kPa. For each antibiotic, the optimization MS/MS conditions for multiple reaction monitoring (MRM) mode was performed using direct infusion of each standard antibiotic solution at a concentration of 1000 μg L^{-1}. Three transitions were monitored, and the most abundant one was used for antibiotic quantification. The retention times, mass-to-charge ratio for the precursor and ions of products, cone voltage, and collision energy for each compound are provided in Table 3. The LabSolution software (version 5.53, SP3, Shimadzu) was employed for both instrument control and data acquisition/analysis.

Method validation

The performance of the developed method was evaluated in terms of selectivity, linearity, initial precision and recovery (IPR), precision, accuracy, instrumental detection limit (IDL), method detection limit (MDL), and method quantification limit (MQL), as well as matrix effects. The method was validated using sewage sludge samples from UASB-1. In-house validation was performed based on the US Environmental Protection Agency (EPA) protocol with some changes (USEPA, 2016).

Due to the absence of certified reference materials, spiked sewage sludge samples were used as validation standards. Non-spiked samples were also analyzed since target antibiotics might be present. Thus, these amounts of antibiotics were used to offset the concentrations measured in the spiked sewage samples.

The method's selectivity was evaluated by three different MRM transitions, the ratio between confirmation and quantification fragment ions, and chromatographic peak retention time. Linearity was assessed by the coefficient of determination (R^2) using calibration analytical curves at the following levels: 2.0; 4.0; 10.0; 20.0; 40.0; 80.0; 120.0 ng L^{-1}. Matrix effect must be considered, mainly in complex matrices such as sewage sludge. Thus, the quantification of target antibiotics was based on the modified methodology proposed by Gomes et al. (2017). The basic procedure consists of two consecutive chromatographic runs. For the construction of analytical calibration curves, the first injection (X_1) was performed by adding 12 μL of standard mixture plus 2 μL of solvent. The second injection (X_2) was performed by adding 12 μL of the same standard mixture plus 2 μL of a known standard mixture solution (240 ng g^{-1}). The peak area of each injection was obtained. Thus, the ratios between the peak area from the first injection and the peak area difference (from the second minus the first injections) were calculated, as described in Equation 1 (Gomes et al., 2017). This approach was used as an equivalent to an internal standard to construct the analytical curves and quantify the selected antibiotics (SMX, SDZ, and TMP) in this study and other studies involving the antibiotics quantification in complex matrices (Rodrigues et al., 2020; Cunha et al., 2021; Rodrigues et al., 2021).

Linear regression analysis was used for the calibration of all analytes. Similarly, the area ratio obtained for each antibiotic in sample extract was interpolated to find the real concentration.

$$Area\ ratio = \frac{X_1}{(X_2 - X_1)} \tag{1}$$

Where:
X_1 = the first injection; and
X_2 = the second injection.

Table 3 – Target antibiotics, classified by their chromatographic retention time, and their optimized liquid chromatography coupled to triple quadrupole tandem mass spectrometry parameters by positive ionization mode.

Antibiotics	Rt (min)	Precursor ion (m/z)	Product ions (m/z)	Q1 pre-bias (V)	CE (eV)	Q3 pre-bias (V)
SMX	6.3	253.9	156.1	-24.0	-16.0	-29.0
			92.2	-25.0	-26.0	-17.0
			108.2	-24.0	-25.0	-21.0
SDZ	4.7	250.9	155.9	-24.0	-16.0	-30.0
			92.2	-24.0	-24.0	-17.0
			108.1	-24.0	-22.0	-19.0
TMP	3.9	290.8	230.1	-30.0	-23.0	-25.0
			123.1	-30.0	-24.0	-24.0
			261.1	-30.0	-26.0	-29.0

Rt: retention time; m/z: mass-to-charge ratio; CE: collision energy; SMX: sulfamethoxazole; SDZ: sulfadiazine; TMP: trimethoprim.

The IPR of the method were determined by spiking sludge samples at three concentration levels (50, 200, and 500 ng g^{-1}) of each target antibiotic. Each fortification level was carried out in quadruplicate. Recovery was calculated as the concentration of the spiked sample obtained after the whole LTPE (S_f) minus the concentration of the non-spiked sample (S_0) in comparison to the initial spiking level (S_i). The extraction recovery (R%) is reported as percentages, as described by Equation 2.

$$\mathrm{R}(\%) = \frac{S_f - S_0}{S_i} x100\% \qquad (2)$$

Where:
S_f = concentration of the spiked sample obtained after the whole low-temperature partitioning extraction (LTPE);
S_0 = concentration of non-spiked sample; and
S_i = initial spiking level.

Method accuracy and precision, expressed as intra-day repeatability in terms of relative standard deviation, were estimated by spiking experiments at concentration levels specified for the IPR essay (50, 200, and 500 ng g^{-1}).

The IDLs and IQLs were determined as the minimum detectable amount of each antibiotic MRM mode with 3- and 10-times signal-to-noise ratio (S/N=3), respectively. The MDLs and MQLs for SMX, SDZ, and TMP were determined by spiking seven replicates of sludge samples prior to extractions at a low concentration level. These seven aliquots were prepared by LTPE and injected in LC-MS/MS, and the standard deviation of replicates was calculated. The MDLs were calculated by multiplying the standard deviation by 3.14 (the Student's *t*-test value at 6 degrees of freedom) while the MQLs were found by multiplying the MDLs by 3.18 (USEPA, 2016).

Statistical analysis

The statistical software R Core Team (2013) was used to analyze data from the experimental design experiments. The quality of the model fit was evaluated using adjusted R^2 and relative standard error values. The Tukey, Shapiro-Wilk, Breusch-Godfrey, and Bartllet (α=0.05 significance) were applied to verify the additivity assumptions, normality of errors, independence of errors, and homogeneity of variances, respectively.

The designs were repeated twice for this experiment matrix. The uniform experimental conditions allowed this experiment to run in a completely randomized design (CRD), which is the most efficient of the experimental designs, since the number of degrees of freedom for the residue is the largest possible. An experiment in a complete factorial 2^3 and CRD was used, according to the linear model of Equation 3.

$$Yijkr = \mu + \alpha i + \beta j + \gamma k + \alpha\beta ij + \alpha\gamma ik + \beta\gamma jk + \varepsilon ijkr \qquad (3)$$

Where:
Y = response variable (extraction efficiency expressed as recovered mass of each antibiotic);
μ = overall average;
α_i = level *i* effect of the mass;
β_j = level *j* effect of the time;
γ = level *k* effect of the pH;
$\alpha\beta_{ij}$ = interaction effect of level *i* of mass with level *j* of time;
$\alpha\gamma_{ik}$ = interaction effect of level *i* of mass with level *k* of pH;
$\beta\gamma_{jk}$ = interaction effect of level *j* of time with level *k* of pH;
ε = residual error; and
ε_{ijkr} = independent random variables with normal distribution of zero mean and common variance (σ2).

All assumptions were met, thus ensuring reliable results for inference. The effect of each factor was tested using the Fischer test with a corresponding p-value (p). The factors whose p-values were less than 0.05 were considered statistically significant.

Results and Discussion

Extraction procedure for sludge samples

The LTPE methodology was selected to develop a fast and reproducible method for SMX, SDZ, and TMP antibiotics determination in sewage sludges. The effect of different extraction parameters on extraction efficiency was evaluated to obtain optimal relative extraction conditions for target antibiotics from anaerobic sludge. Some extraction parameters were fixed based on preliminary tests involving the same extraction technique and previous literature studies (Dorival-García et al., 2013; Ferhi et al., 2016; Ajibola et al., 2020) which include: Eppendorf tube as the extraction vessel, organic extracting solution (acetonitrile/methanol; 95:5, v/v), sample volume (600 µL), organic extracting solution volume (600 µL), and the use of McIlvaine buffer solution to keep pH at the desired value. McIlvaine buffer solution was selected due to the pH range (2.2 to 8.0) and its wide use in drug extraction from sludge (Dorival-García et al., 2013; An et al., 2015; Ferhi et al., 2016).

Experimental designs were used to assess the influence of *k* factors and their simultaneous interactions. Three variables were selected: sample mass, extraction time in an ultrasonic bath, and pH of the buffer solution. The mass recovered from the extraction of each target antibiotic was used as a response. The estimated coefficients from the models were standardized in order to verify and compare the influence of variables on the best fit model. The main effects were determined by the F test, calculated for each factor and these results are shown in Table 4.

The statistical testing of the model was carried out using analysis of variance (ANOVA). The quality of fit for each model was verified by adjusted R^2 values and the residual standard error (RSE) (Table 5). The R^2 of an experiment measures the proportion of the total variations that

Table 4 – Standardized coefficients, p-value, and interactions between factors to assess operating parameters of low-temperature partitioning extraction for antibiotics in full factorial design 2^3. Values in bold indicate a significant effect at a 95% probability level ($p<0.05$).

Antibiotic	X_1	p	X_2	p	X_3	p	X_{12}	p	X_{13}	p	X_{23}	p
SMX	0.7329	**0.0000**	0.1935	0.0566	-0.3000	**0.0080**	0.2736	**0.0129**	-0.4092	**0.0012**	-0.1494	0.1256
SDZ	0.8779	**0.0000**	0.0698	0.5543	-0.1699	0.1481	0.0248	0.8220	-0.2599	**0.0386**	-0.1543	0.1845
TMP	0.5890	**0.0000**	-0.1021	0.2003	0.5986	**0.0000**	-0.0897	0.2553	0.4688	**0.0001**	-0.0847	0.2812

X_1: sample mass (mg); X_2: time in the ultrasonic bath (min); X_3: pH of the buffer solution; p: p-value; SMX: sulfamethoxazole; SDZ: sulfadiazine; TMP: trimethoprim.

Table 5 – Adjusted coefficient of determination and residual standard error of the models proposed for each antibiotic, generated by analysis of variance.

Antibiotic	Adjusted R^2	RSE
SMX	0.8824	0.1318
SDZ	0.8342	0.0960
TMP	0.9181	0.1984

R^2: coefficient of determination; RSE: residual standard error; SMX: sulfamethoxazole; SDZ: sulfadiazine; TMP: trimethoprim.

could be illustrated by the model. The adjusted R^2 were 0.8824 (SMX), 0.8342 (SDZ), and 0.9181 (TMP). The obtained RSE was <19.84%, indicating that the models were satisfactory at 95% confidence level. The plots of predicted values against the observed values of the analytical response (Figure 2) corroborate the goodness-of-fit information.

ANOVA results showed that the factors X_1 (sample mass; mg) and X_3 (pH of the buffer solution) significantly influenced the extraction efficiency, while factor X_2 (time in the ultrasonic bath; min) did not influence the %RSE obtained for any tested antibiotic (Table 3). Even though, some second-order interactions were shown to be statistically significant to explain the best fit model. Therefore, the cross-effect graphs were interpreted to observe the influence of the combination between the levels of significant variables. Figure 3 shows the cross-effect graphs of significant interactions.

Mass was the factor that had the greatest influence on extraction efficiency ($p=1.87.10^{-5}$) of SMX. The results showed that increasing the amount of mass (50 to 150 mg) had a positive effect on the system. It was observed that the effect of pH variable was negative, which means an inverse relationship between pH and response. Ultrasonic bath time was not statistically significant for the system (p=0.0566). The interactions between mass and ultrasonic bath time and between mass and pH were statistically significant. In general, it was found that mass was more significant for increasing the extraction efficiency and there is evidence that the ultrasonic bath time variable has a smaller influence on the extraction than the pH variable.

According to the ANOVA data for SDZ (Table 4), the results suggest that mass had a strong influence on the system. The effects of variables time and pH of the buffer solution were not statistically significant—a fact confirmed by the non-significance of the variables through ANOVA. The second order interaction between mass and pH of the buffer was statistically significant. It was observed that there is a trend towards higher extraction efficiency of SDZ at pH 2.

Concerning TMP, the ANOVA suggests that the variables mass and pH of the buffer solution strongly affected the responses, whereas the ultrasonic bath time was not a statistically significant variable in extraction efficiency (p=0.2003). The interaction between mass and pH was statistically significant, and it was noted that pH dominated TMP extraction, with better results at pH 7 than pH 2. According to the cross-effect graphs of statistically significant interactions for the three antibiotics at the highest mass level, it is observed that the pH of the buffer solution exerts a greater influence on TMP extraction than on SMZ and SDZ. It is important to highlight that there was a considerable decrease in extraction efficiency of TMP at pH 2, and this is related to its speciation form.

When developing multi-residue analytical methodologies, one limitation is the fact that a single condition to better extract all target analytes may not be achieved (Cavaillé et al., 2021). It is considered that the analysis of the extraction parameters performed for each compound can be essential to support future works that employ this methodology for a single residue. However, the analyzed parameters were chosen to ensure a unique analysis method that had lower analysis costs and time.

The amount of sludge mass is important to establish the sensitivity of the method since it is directly related to the matrix effect (Topuz et al., 2014). The sample amount was evaluated in studies that used other extraction techniques (Göbel et al., 2005; Dorival-García et al., 2013; Topuz et al., 2014; Benedetti et al., 2020). Göbel et al. (2005) evaluated the sample amount parameter in the optimization of PLE for sulfonamides and macrolides. However, no significant influence on extraction efficiency of the antibiotics from varying sample amounts was observed. Dorival-García et al. (2013), on PLE and MAE optimization for quinolones, analyzed the sample weight (0.5 and 2.0 g) and found that the sludge mass had a negative effect on recoveries for target quinolones, indicating the extraction of more interferents. Topuz et al. (2014) also evaluated different amounts of sludge (0.1, 0.2, 0.5, and 1.0 g) for quantifying diclofenac by USE. These authors pointed out that the presence of excessive content of interferences resulted in sign suppression of diclofenac when 1 g of sludge sample was extracted. In the present study, the sludge mass was the most influential variable with a positive effect on the antibiotic extraction efficiency. Thus, 150 mg of dry sewage sludge was used to provide better extraction efficiencies.

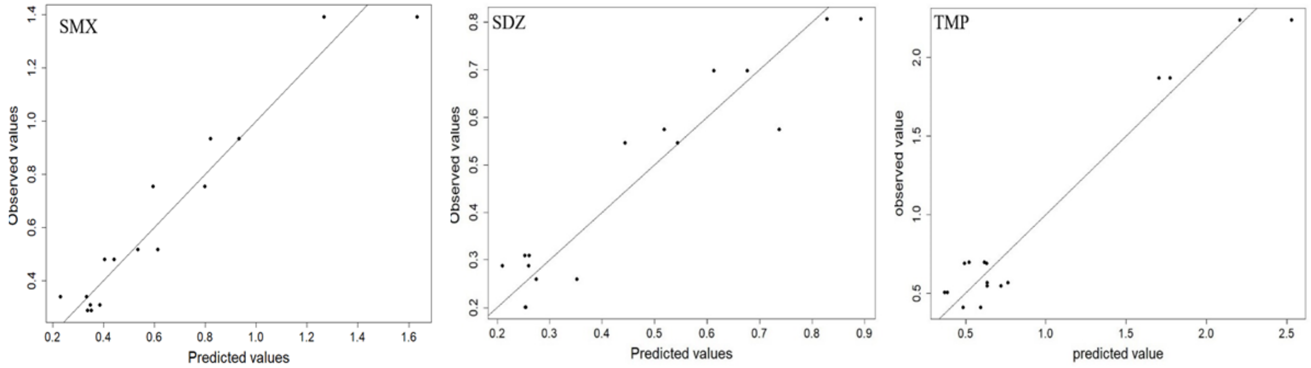

Figure 2 – Plots of predicted and observed values of target antibiotics analyzed by liquid chromatography coupled to triple quadrupole tandem mass spectrometry after low-temperature partitioning extraction extraction.
SMX: sulfamethoxazole; SDZ: sulfadiazine; TMP: trimethoprim;

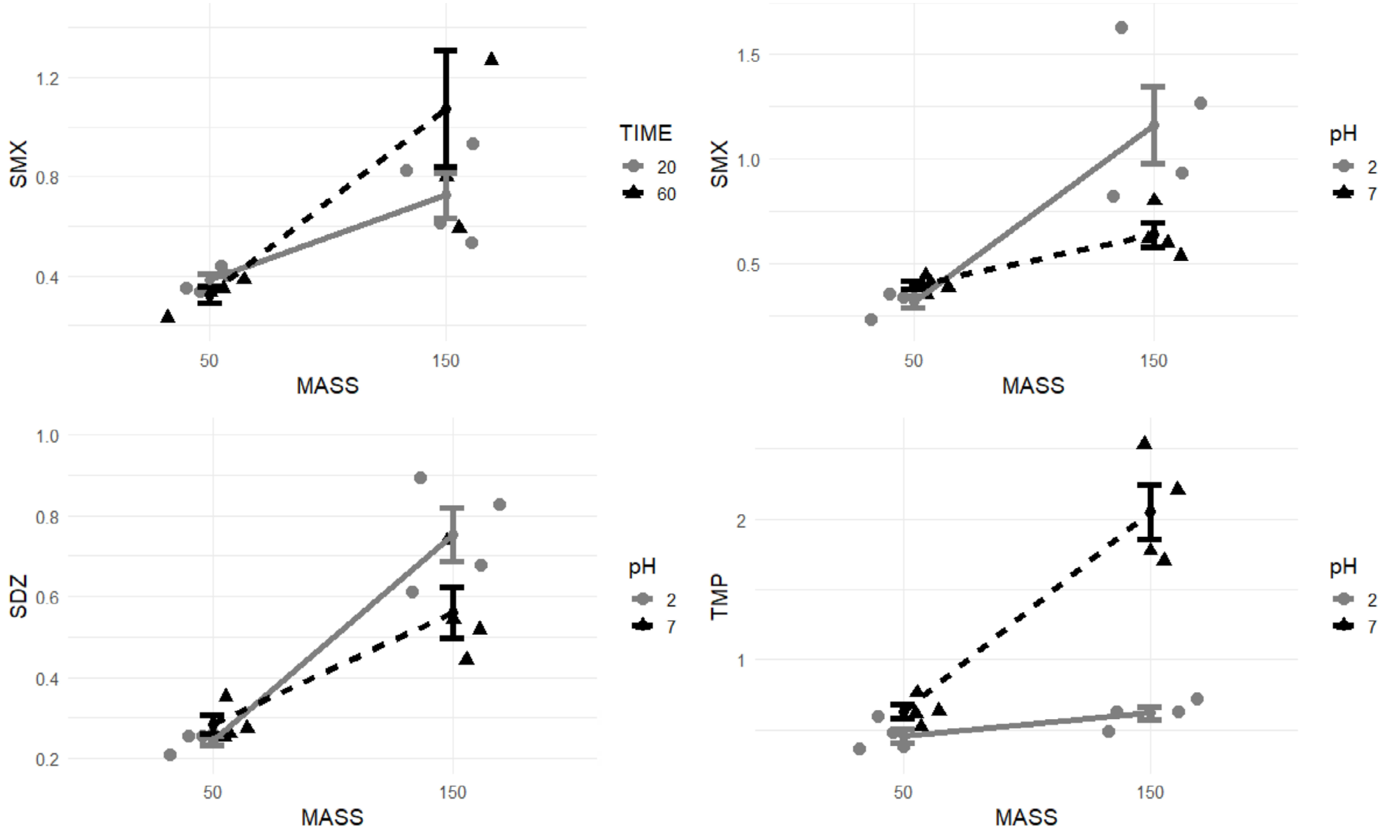

Figure 3 – Cross-effect plots of statistically significant interactions for the extraction of sulfamethoxazole, sulfadiazine, and trimethoprim from sewage sludge samples.
SMX: sulfamethoxazole; SDZ: sulfadiazine; TMP: trimethoprim.

The effect of extraction time is a commonly investigated variable in sludge extractions using other methods such as PLE, USE, accelerated solvent extraction (ASE), and QuEChERS (Göbel et al., 2005; Dorival-García et al., 2013; Topuz et al., 2014; Malvar et al., 2020). An influence of the cycle time on PLE efficiencies may be expected due to the higher extraction temperature used, resulting in a reduction of the viscosity of the solvent (Göbel et al., 2005). In ASE, it is expected that a long exposure to solvent allows the matrix to expand, improving the solvent penetration into the sample interstices and the contact with the analytes (Gama and Rocha, 2020). The results found in the present work showed that time in the ultrasonic bath did not significantly affect the extraction efficiency of SMX, SDZ, and TMP; therefore, the shortest time (20min) was selected for method validation.

Regarding the pH of the buffer solution, two hypotheses based on the antibiotic partition equilibrium were drawn to evaluate the LTPE efficiency in relation to the pH of the medium, as follows: 1. Antibiotic were transferred from solid particles of sludge sewage to the aqueous phase; and 2. Antibiotics were transferred from the aqueous phase to the organic phase. Firstly, the tendency of the antibiotics sorbed onto sludge particles to partition into the aqueous phase was evaluated. Then, the partitioning equilibrium between the aqueous and the organic phases was analyzed. It is expected that the compound in its molecular form has its solubility in water reduced when compared with its ionic form, which increases its partition to the organic phase (Barros et al., 2019).

The sorption behavior of CECs may be estimated by their hydrophobic and electrostatic interactions with organic matter, the main component of sewage sludge, and also by their acid-base properties (Dorival-García et al., 2013). Sorption of contaminants onto the sludge matrix can occur by absorption and adsorption (Suárez et al., 2008). Absorption is the process in which molecules present in a fluid are encapsulated or embedded by a suspended solid particle or immiscible liquid droplet which have more affinity to them than the fluid itself (Suárez et al., 2008; Aquino et al., 2013). For instance, it may refer to the interactions of the aliphatic and aromatic groups of a compound with the lipophilic cell membrane of the microorganisms and the lipid fractions of the sludge, being well characterized by K_{ow} (Suárez et al., 2008). On the other hand, adsorption involves the phenomenon of mass transfer at the interface between a solid (in this case sludge) and a liquid (in this case water) phase (Aquino et al., 2013), and two main types of interactions (physical and chemical) may occur. Physical sorption occurs due to weak Van der Waals attraction forces, whereas in chemisorption, chemical bonding between sorbate and sorbent molecules takes place (Patel et al., 2020). In this process, stronger forces, such as covalent bonds or electrostatic interactions are involved (Patel et al., 2020). In the case of electrostatic interactions, it may involve the attraction of positively charged groups of compounds with negatively charged surfaces of microorganisms, which are the main solids of anaerobic sludge. Thus, it is related to the tendency of a substance to be ionized or dissociated in the aqueous phase, which is indirectly characterized by the dissociation constant (K_a) (Suárez et al., 2008).

All antibiotics studied are hydrophilic (log $K_{ow}<2.5$). Therefore, interactions with sludge were mainly increased and attributed to adsorption of electrostatic nature. Indeed, many pharmaceuticals detected in sludge are lipophilic compounds, which have polar functional groups ($-NH_2$ or COOH groups) and can adsorb onto particulate organic matter or minerals via electrostatic interactions (Mejías et al., 2021). High concentrations of hydrophilic antibiotics such as norfloxacin (log $K_{ow}=0.46$, up to 3919 ng g^{-1} dw) and TMP (log $K_{ow}=0.91$, up to 196 ng g^{-1} dw) have been quantified in sewage sludge possibly by such mechanism (Göbel et al., 2005; Ferhi et al., 2016).

The rationale for the hypothesis that the sorption mechanism of antibiotics in the sludge is predominantly by electrostatic adsorption was supported by analyzing the pH values of the samples, antibiotics' pK_a values, and the isoelectric point (PI) or point of zero charge (PCZ) of the sludge. The pH of the solution has a significant impact on the sorption of analytes since it determines the surface charge of the adsorbent (sludge) and the degree of ionization and speciation of the adsorbate (antibiotics) (Elouear et al., 2009). Since no buffer was added into the system, the equilibrium pH was equal to the system pH (Elouear et al., 2009). Regarding the analysis of sludge surface charge, the discussion was based on the pH of PI. According to Elouear et al. (2009), the PI can be interpreted as the pH value where the net charge is equal to zero. Literature data reveal that the pH value of PI in sewage sludge is approximately 2.9 (Mejías et al., 2021). In aqueous solution, the first step for antibiotic adsorption onto sludge is the development of the surface charge of sludge following its hydration. Since sewage sludge has a low PI, a net positive surface charge is only expected under strong acidic conditions (pH<2.9), and a negative net surface charge should be expected under neutral environmental conditions. The charges of the compounds under the respective conditions were assumed given the analysis of the surface charge of sewage sludge in contact with the buffer solution (pH 2.2 and 7.0).

Under acidic conditions at a pH below pK_{a1}, SMX (pK_{a1} 1.97 and pK_{a2} 6.18) and SDZ (pK_{a1} 1.60 and pK_{a2} 6.36) exist as a cation; between pK_{a1} and pK_{a2}, the molecule carries a neutral charge, and above pK_{a2}, it should be negatively charged (Shafrir and Avisar, 2012). Electrostatic repulsions are expected to occur between the partially cationic sulfonamides and the positively charged sewage sludge at pH 2.2. Similarly, repulsions are also expected at pH 7.0 since partially anionic sulfonamides are repelled by the negatively charged groups (hydroxyl and carboxyl) ubiquitously present in the humic and fulvic acids that make up the sludge (Shafrir and Avisar, 2012). Such behavior strengthens the hypothesis that sulfonamides in ionic forms preferentially have a greater affinity for the aqueous phase. Therefore, it is suggested that the proportion of neutral molecules in the respective pH values considered had a significant influence on the extraction efficiency. Theoretical calculations of the percentage of molecules in ionic and neutral form were made to prove this hypothesis. At pH 2.2, it is expected to be about 62.9 and 79.9% of neutral molecules of SMX and SDZ, respectively. At pH 7, around 13.1 and 18.6% of molecules in neutral form are expected. When comparing the proportions of the compounds in the analyzed pH values, it is observed that at pH 2.2, there is a greater proportion of molecules in the neutral form, which are less soluble in the aqueous phase, thereby explaining the greater efficiency extraction of SMX and SDZ at pH 2.2. These results show that the hypotheses raised are plausible to explain the pH influence on extraction efficiency.

TMP has an ionizable functional group with pK_a 7.0 (Thiebault, 2020). Thus, TMP at pH 2.2 is fully protonated, therefore electrostatic repulsions between TMP (positively charged) and sludge (also positively charged due to PI=2.9) are expected. In this case, the positively charged TMP will tend to preferentially remain in the aqueous phase rather than go to the organic phase. This correlates well with the form the species assume in solution (ionic or neutral) and that will certainly influence their partitioning. On the other hand, at pH close to neutral, equal concentrations of cationic and neutral species are expected (Suárez et al., 2008). Therefore, it is suggested that electrostatic attraction forces between TMP$^+$ and the negatively charged sludge will occur with at least half of the TMP molecules present in the sludge. Thus, the other half of the TMP molecules, which will be in neutral form, will not tend to adsorb onto sludge; this will make them prone to interact with hydrophobic liquids and would facilitate its partitioning from the aqueous phase to the organic phase. Indeed, the obtained results reveal a correlation of TMP extraction efficiency with pH, with the best results observed at pH 7.

TMP exhibited greater dependence on the variable pH of the buffer solution than SMX and SDZ, so pH 7 was chosen as a parameter for the validation of the developed method.

The number of extraction cycles was assessed in this study. One and two consecutive extractions of the same sample were performed to evaluate the ability of the method to quantitatively extract SMX, SDZ, and TMP. The recovery (R%) are shown in Figure 4.

Extraction cycles are a critical factor in the LTPE procedure regarding the efficiency and frequency of the applied method. With each new extraction cycle, the introduction of fresh organic fraction can maintain a higher gradient concentration and a favorable solvent/sample balance, therefore, improving the partition to the organic phase by increasing recoveries. The results show that the recoveries were statistically different, with the highest rates being obtained with two consecutive extractions for all antibiotics. However, when considering the cost-benefit of the analysis, a single extraction was selected since the increment in recovery was from 30 to 50% for SMX and SDZ, and more than 100% for TMP. Moreover, performing two consecutive extraction steps reduces the analytical frequency.

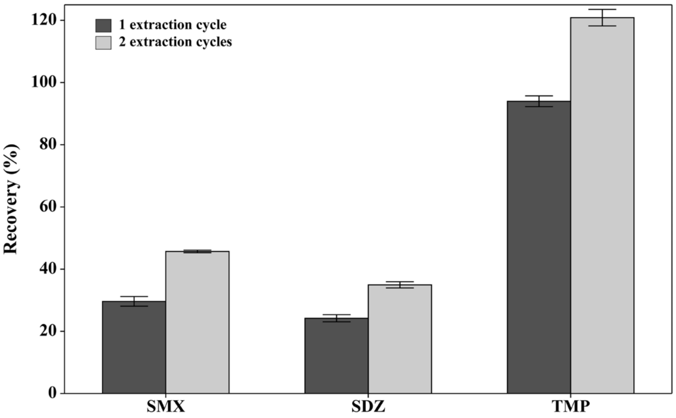

Figure 4 – Mass recovery (%) of antibiotics in one and two consecutive extractions of the same sample. Error bars represent standard deviation (n=5)

SMX: sulfamethoxazole; SDZ: sulfadiazine; TMP: trimethoprim.

Analytical performance parameters

The analytical performance parameters of the developed method are summarized in Table 6. All target antibiotics showed linear behavior within the studied range. The R^2 of calibration curves ranged from 0.9937 to 0.9958. IDLs ranged from 0.6 to 12.0 pg. These values indicate the high sensitivity of the mass spectrometer to detect target antibiotics at low concentrations, which makes it suitable for analyzing environmental samples.

IPR was determined by spiking samples at three levels (50, 200, and 500 ng g^{-1} dw). It was measured in terms of %RSD and ranged from 3.2 to 14.7%, which was acceptable considering the specifications of the AOAC 2016 (AOAC, 2016). In addition, SMX, SDZ, and TMP exhibited recoveries varying between 36.6–52.7, 29.1–53.6, and 63.8–70.6%, respectively. The relatively low recoveries obtained may be related to the complexity of the sludge matrix, once the extraction of interferents influences the partition equilibrium during the LTPE procedure. Another possible reason may be attributed to the hydrophilic character of the antibiotics. Similar recoveries from sludge samples of 64, 63, and 51% for SMX, SDZ, and TMP, respectively, were achieved by Göbel et al. (2005), employing pressurized liquid extraction with further SPE clean-up. Average recovery values of 35% for SMX and 56% for TMP were also achieved by Ajibola et al. (2020) using QuEChERS based on extraction with dispersive-SPE clean-up. In the present study, the recoveries for SMX and SDZ at 50 ng g^{-1} dw were higher than at 200 and 500 ng g^{-1} dw. This fact might be attributed to the competition between analytes and interferents for the organic fraction. Ajibola et al. (2020) also observed recovery rates at different levels of the antibiotics in sewage sludge. Thus, the recovery rates obtained in the present study for the selected antibiotics were acceptable, considering simultaneous extraction of three antibiotics in a real complex environmental matrix.Although SMX and SDZ exhibited low recovery values at some fortification levels, the method showed sensitivity, precision, and reproducibility. Nevertheless, it is recommended that future work test the salting-out effect and assess sample purification by dialysis for reducing matrix effects caused by components co-extracted from the sludge matrix.

The precision (%RSD) ranged from 7.3 to 18.9%, hence this method can be considered precise <20%, which is quite acceptable considering the matrix complexity. The accuracy values ranged from -13.8 to 19.9%, which is also acceptable (Göbel et al., 2005; Lindholm-Lehto et al., 2017; Ajibola et al., 2020).

The MDL and MQL values were in the ng g^{-1} order of magnitude. For SMX, the MDL was 11.3 ng g^{-1} whereas for SDZ and TMP, it was 3.6 and 9.3 ng g^{-1}, respectively. Low MDLs and MQLs were achieved, despite using a small sample mass for extraction. In fact, the MDLs and MQLs calculated in this study are comparable to those obtained by other analytical methods (Göbel et al., 2005; Shafrir and Avisar, 2012; Gago-Ferrero et al., 2015; Ferhi et al., 2016).

Table 6 – Analytical method validation parameters: linearity, instrumental detection limits, method detection and quantification limits, initial precision and recovery, accuracy, and precision at three concentrations (50, 200, and 500 ng g^{-1} dw).

Antibiotics	Linearity (R^2)	IDL (pg injected)	MDL (ng g^{-1})	MQL (ng g^{-1})	IPR (%R)			Accuracy (%)			Precision (%RSD)		
					50 ng g^{-1}	200 ng g^{-1}	500 ng g^{-1}	50 ng g^{-1}	200 ng g^{-1}	500 ng g^{-1}	50 ng g^{-1}	200 ng g^{-1}	500 ng g^{-1}
SMX	0.9958	0.6	11.3	36.1	52.7 (5.2)	36.6 (9.9)	45.3 (7.1)	-6.9	-5.6	19.9	11.6	7.3	10.3
SDZ	0.9937	12	3.6	11.5	53.6 (4.3)	29.1 (14.2)	37.7 (3.2)	10.2	5.4	16.1	13.3	9.0	10.3
TMP	0.9948	6	9.3	29.7	63.8 (14.7)	69.1 (6.0)	70.6 (9.1)	1.7	-13.8	-12.6	18.9	18.0	9.5

IDL: instrumental detection limit; MDL: method detection limits; MQL: method quantification limits; IPR: initial precision and recovery; SMX: sulfamethoxazole; SDZ: sulfadiazine; TMP: trimethoprim; RSD: residual standard deviation.

Blank analysis for method development and validation is needed. It should be noted that spiking samples that already contain the target compounds in larger amounts can affect the determination of recovery, precision, MDL, and MQL. Thus, spiking concentration in the sludge before extraction is negligible in relation to the high concentration of the compounds in the blank (non-spiked sample). Consequently, errors associated with the difference between spike samples and the concentration of the non-spike samples can be large, which leads to high %RSD values.

Table 7 – Concentrations (ng g^{-1}) of three selected antibiotics in sewage sludge from two UASB reactors treating sanitary sewage in Minas Gerais, Brazil.

Antibiotics	UASB-2	UASB-3
SMX	Nd	Nd
SDZ	Nd	Nd
TMP	174.2±5.3	134.8±8.7

UASB: upflow anaerobic sludge blanket; nd: not detected; ±: standard deviation.

Application to real sewage sludge samples

The method developed was applied in two sewage sludge samples (UASB-2 and UASB-3) in triplicates. The results are summarized in Table 7.

SMX and SDZ were not detected in any of the two samples analyzed. At environmental pH (6–8), SMX is either in its neutral or anionic form and is not expected to accumulate in sludge due to electrostatic repulsing by negatively charged particles (Lindholm-Lehto et al., 2017). Furthermore, sulfonamides generally have strong hydrophilicity with low solid-liquid partition coefficient (K_d), being mainly associated to the liquid phase. Therefore, it was not expected to find significant amounts of these antibiotics in the sludge. Indeed, the obtained results are in agreement with other studies (Göbel et al., 2005; Shafrir and Avisar, 2012; Gago-Ferrero et al., 2015; Ferhi et al., 2016).

In contrast, a slight amount of TMP in the sludge samples analyzed was quantified (Table 7). At environmental pH ranges (Bajkacz et al., 2020; Malvar et al., 2020), TMP can be found partially protonated (Shafrir and Avisar, 2012). The presence of a positive charge on the TMP molecule results in an increased probability of sorption into sludge. Consequently, its detection in the samples analyzed was expected. These values are in the same range as those found in the literature for TMP for similar matrices. Overall, results in the same order of magnitude were previously reported by Göbel et al. (2005), Ademoyegun et al. (2020), and Ajibola et al. (2020).

Conclusions

An alternative analytical method for the determination of antibiotics in sewage sludge has been developed and validated. The method involves extraction of SMX, SDZ, and TMP by LTPE followed by LC-MS/MS analysis. A complete 2^3 full factorial design was employed to understand which parameters most affected the quantification of the selected antibiotics. Sample mass and pH of buffer solution significantly influenced the extraction efficiency, being the first and the most influential variables. The best extraction conditions were: 150 mg of dry sludge, 600 µL of McIlvaine buffer solution, 600 µL of organic solvent, 20min of ultrasonic extraction, and pH 7. In addition, one cycle of extraction was enough for SMX, SDZ, and TMP recovery. Therefore, the LTPE procedure proved to be an applicable extraction method to the selected antibiotics, and besides being cost-effective, it is an environmentally friendly method for employing low volumes of organic solvents. The method was validated with satisfactory results and applied to the analysis of sludge samples from UASB reactors. SMX and SDZ were not detected in anaerobic sludge, whereas TMP was detected in concentrations from 134.8 to 174.2 µg g^{-1}.

Acknowledgments

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for their financial support, Minas Gerais State Research Support Foundation (FAPEMIG), National Council for Scientific and Technological Development (CNPq), Agency for Financing Studies and Projects (FINEP), Brazilian National Health Foundation (FUNASA), Federal University of Ouro Preto (UFOP), and Multicenter Post-Graduation Program in Chemistry of Minas Gerais (*Programa de Pós-Graduação Multicêntrico em Química de Minas Gerais*). We would like to thank for all the good days we had with André Barros, who left us unexpectedly and whose contribution to our laboratory and our lives will remain for posterity.

Authors' contributions

Cunha, C.C.R.F.: conceptualization, methodology, investigation, writing — original draft. **Freitas**, M.G.: methodology, investigation. **Rodrigues**, D.A.S.: methodology, investigation. **Barros**, A.L.C.: methodology, investigation, formal analysis. **Ribeiro**, M.C.: methodology, software, formal analysis. **Paranhos**, A.G.O.: writing — review & editing. **Aquino**, S.F.: conceptualization, visualization, writing — review. **Sanson**, A.L.: conceptualization, visualization, writing — review & editing. **Afonso**, R.J.C.F.: resources, conceptualization, writing — review & editing, project administration, funding acquisition, supervision.

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