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Automated method for the solid phase extraction of tetracyclines in wastewater followed by fluorimetric determination

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ABSTRACT

This work presents a multisyringe flow injection analysis (MSFIA) system for the automatic extraction and determination of tetracyclines in wastewater samples. The sample was adjusted with Na₂EDTA buffer before solid-phase extraction with an Oasis HLB column used for the analyte preconcentration. The europium (Eu³⁺)-based and citrate-mediated method (using Tris-HCl buffer) was selected for the fluorimetric analysis ($\lambda_{exc/em} = 400/612$ nm). For fluorescence detection, a low-cost system consisting of an USB 2000 CCD detector and a 3D-printed support that holds a LED light source was used. Under optimized conditions, the proposed method provided low limits of detection ($9.4 \mu\text{g L}^{-1}$) and quantification ($31 \mu\text{g L}^{-1}$), and good values for intra-day (<4 %) and inter-day precisions (<6 %). Recoveries of spiked TCs in wastewater samples ranged from 87 to 106 %. The results of this work were in good agreement with the measurements obtained by liquid chromatography coupled to a fluorescence detector.

1. Introduction

Tetracyclines (TCs) are widely used antibiotics in human and veterinary medicine due to their low cost, high efficiency and good water solubility. In agriculture and livestock, they are used to treat bacterial diseases or to promote the growth of plants or animals [1,2]. However, the widespread application of these pharmaceuticals leads to their accumulation in food, plant soil, human and animal excreta. TCs can also be released into wastewater from municipalities, pharmaceutical industries, hospitals, or farms [3]. The concentration levels of these antibiotics in wastewater range from $\mu\text{g L}^{-1}$ to mg L^{-1} . Consequently, TCs of the untreated or inadequately treated effluents from wastewater treatment plants (WWTP) enter water bodies and contaminate aquatic ecosystems. TCs residues in the environment promote bacterial resistance which can harm human and ecological health by increasing the risk of infections. TCs also cause endocrine disruption and growth problems in certain species [1,4]. In WWTP, these antibiotics can inhibit the activity of microorganisms from aerobic and anaerobic wastewater treatments, affecting the sludge properties and the removal of nutrients and COD [5–7]. Therefore, the determination of TCs is very important for routine monitoring in WWTP.

Techniques to determine TCs in water include high or ultra-performance liquid chromatography (HPLC/UPLC) with different types of detection such as ultraviolet (UV) [8,9], fluorescence (FLD) [10,11], mass spectrometry (MS) [10,12] and chemiluminescence [13]. However, methods using these techniques require expensive instruments and have slow and cumbersome detection. Therefore, the development of spectrofluorimetric methods has currently brought great interest as an alternative due to its easy operation, high specificity, low cost and rapid response [14,15].

TCs fluoresce in presence of UV light. Furthermore, TCs can enhance their fluorescence intensity when complexed with metal ions, especially europium (Eu³⁺ or Eu(III)). TCs absorb light strongly at 400 nm and transfer the excitation energy to europium, whose complex emits at 612 nm. The formation of the Eu-TC complex allows an intense, stable and long-lasting emission [16]. The europium method has been used satisfactorily in the analysis of different samples (food samples, natural waters, residual water, etc.) [17–19]. The emission intensity of the Eu-TC complex, and the selectivity between these two, is improved by adding other auxiliary sensitizing agents such as citrate [20] or cetyltrimethylammonium chloride/bromide (CTAC/CTAB) [21]. Citrate is a more suitable co-ligand than the other two mentioned because the

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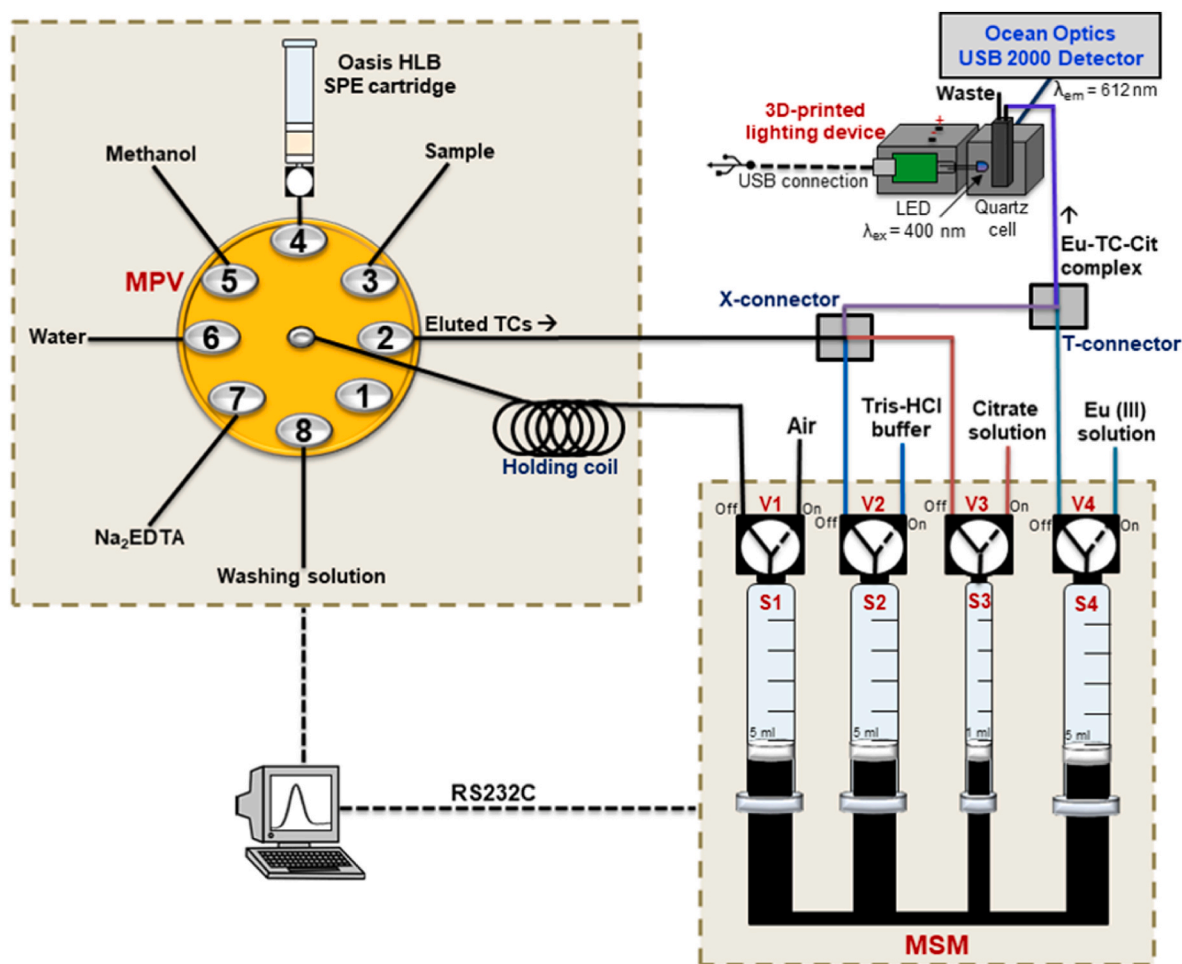


Fig. 1. Schematic representation of the MSFIA system with fluorescence detector for the determination of tetracyclines in wastewater.

Eu-TC-Cit complex does not react with phenols unlike the complexes formed with CTAC or CTAB. In addition, it is a less toxic, cheaper and more available reagent in laboratories. Citrate enhances the fluorescence intensity by chelating Eu^{3+} and displacing water molecules coordinated with it [20].

Since antibiotics are generally found at trace levels in media with complex matrices (such as wastewater), a solid-phase extraction (SPE) procedure is required as a cleanup step to extract and preconcentrate analytes and remove potential interferents [12]. SPE is the preferred extraction technique due to its high selectivity and low solvent consumption. Many solid phases have been used for SPE, such as C18, cation-exchange, mixed-mode and hydrophilic-lipophilic balance (HLB). Oasis HLB cartridges are preferred for the extraction of TCs because of their high recovery rates and good repeatability [3].

Nevertheless, SPE involves tedious and time-consuming procedures [22]. Therefore, automation techniques are useful to reduce the workload of sample pretreatment along with compound detection. The referenced automated methods for the TCs analysis in different samples imply an extraction in liquid or solid phase, with subsequent analysis by liquid chromatography [8,23,24]. In this work, a different approach is proposed, which involves the use of a low-cost fluorescent detection system. This involves using a miniature detector together with a UV light source, powered through computer's USB outputs, thus replacing the large and expensive UV lamps used in previous works. This light source device has already been used successfully in other automated [25] and portable [26] systems developed by our research group.

In this paper, a multi-syringe flow analysis (MSFIA) system for the automatic extraction and fluorimetric determination of tetracyclines in

wastewater samples is presented. SPE was performed with an Oasis HLB column. The europium (Eu^{3+})-based and citrate-mediated method was selected for the fluorimetric analysis. For fluorescence detection, a low-cost system consisting of a USB 2000 CCD (charge-coupled device) detector and a 3D-printed support that holds a LED light source was used. Extraction and complex formation parameters were optimized to obtain a high sensitivity. Finally, the results of the proposed methodology were compared with those obtained by the reference method that uses liquid chromatography coupled to a fluorescence detector (UPLC-FLD).

2. Experimental

2.1. Reagents, standard solutions and samples collection

All reagents were of analytical grade. Ultrapure water obtained from a Milli-Q system (Millipore, USA, resistivity $>18 \text{ M}\Omega \text{ cm}$) was used to prepare all solutions. TCs (98–102 %, Sigma Aldrich, USA) stock solutions were prepared in methanol at a concentration of 1 g L^{-1} and stored in fridge at $4 \text{ }^\circ\text{C}$. Dilutions were performed daily with ultrapure water. Ethylenediamine tetra-acetic acid disodium salt (Na_2EDTA) (99 %, Sigma-Aldrich, USA), tris (hydroxymethyl)-aminomethane ($\text{C}_4\text{H}_{11}\text{NO}_3$) (Merck, Germany), sodium tetraborate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) (Acros Organics, Belgium), europium (III) chloride hexahydrate ($\text{EuCl}_3 \cdot 6\text{H}_2\text{O}$) (99 %, Sigma Aldrich, USA), sodium citrate tribasic dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) (Carlo Erba Reagents, Italy), hydrochloric acid (HCl) (37 %, Sigma Aldrich, USA) and sodium hydroxide (NaOH) (Merck, Germany) were used. Europium and citrate stock standard solutions were prepared at 0.1 M in ultrapure water. The buffer solutions

were prepared as follows: 0.1 M Na₂EDTA, 1 M Tris-HCl and 0.05 M Na₂B₄O₇·10H₂O. The pH was adjusted to the desired values with either 1 M HCl or NaOH. HPLC grade methanol, ethanol, acetone, acetonitrile and dimethylsulfoxide were purchased from Sigma Aldrich (USA). Oasis HLB extraction cartridges (6 cm³, 200 mg) were purchased from Waters (Ireland).

Ammonium acetate (C₂H₇NO₂) (98 %) and calcium chloride (CaCl₂) (97 %) used as additives in the aqueous mobile phase of chromatographic validation method were obtained from Alfa Aesar (Germany).

A multi-element standard solution with anions and cations (Carl Roth GmbH & Co. KG, Germany), glucose hydrated (C₆H₁₂O₆·H₂O) (Merck, Germany) and erythromicine (Sigma Aldrich, USA) were tested as interferences.

Samples were collected from the inlet, outlet and from a secondary settler of a wastewater treatment plant (Bouc-bel-air, France). Samples were filtered through 0.45 and 0.22 μm PES filters prior to use.

2.2. System description

Fig. 1 shows a schematic representation of the Multi-Syringe Flow Injection Analysis (MSFIA) system with spectrofluorimetric detection used for the determination of tetracyclines in wastewater. The set up included a multisyringe module (MSM) used to aspirate and dispense the liquids, an 8-port rotary multiposition-valve (MPV) (Crison, Spain) used to select the flow channel, and the detection system, described later.

MSM was equipped with three 5 mL-syringes (Hamilton, France) (S1, S2 & S4) and with one 1 mL-syringe (S3); each of them has a three-way solenoid valve (V1–V4) on its head (N-Research, USA), allowing the multicommutation operations. S2–S4 (Tris-HCl buffer, citrate and Eu (III) solution) were connected with the manifold in the OFF position (desactivated) and with the reagent container in the ON position (activated). S1 was attached to the center port of the MPV in the OFF position and was open to air in the ON position. The MPV peripheral port configuration was as follows: eluted TCs (2), sample or standard solution (3), Oasis HLB SPE cartridge (4), methanol (5), water (6), Na₂EDTA buffer (7), washing solution (8). Manifold was constructed with polytetrafluoroethylene (PTFE) tubes with an internal diameter of 0.8 mm. The connection between S1 and the MPV included a 5 mL holding coil (HC). X-connector (polymethylmethacrylate - PMMA) was attached to port 2 of the MPV, to the OFF position of S2–S3, and to one of the inputs of T-connector. In turn, the latter was attached to the OFF position of S4 and to the tube connecting to the flow cell. The instruments were connected to a computer via RS-232 serial interface. Instrumental control and data acquisition were performed with the AutoAnalysis 5.0 software (Sciware Systems, Spain).

The detection system consisted of a 3D-printed holder device used to host a monochromatic UV light LED ($\lambda_{exc} = 400$ nm) as a radiation source, a flow-through quartz cell (10 mm optical light path and 450 μL of volume) and an USB2000 CCD detector (Ocean Optics Inc., USA). The cuvette holder was connected to (a) the illumination device by means of a nut and to (b) the spectrofluorimeter by means of a fiber optic, at 90° with respect to the illumination source. An electronic circuit supplied the LED with a voltage of 5 V and at the same time adjusted the lighting intensity by means of two buttons (\pm). A more detailed description of this model is presented in previous authors' papers [25,26].

2.3. Analytical procedure

The detailed analytical procedure is shown in Table S1 of the supporting material and is summarized in six main steps, as follows: in the first step (conditioning of the SPE column), 2 mL of methanol were aspirated into the HC connecting S1 (OFF) to the MPV. Next, this volume was dispensed into the Oasis HLB cartridge (port 4). This same step was repeated with 2 mL of Na₂EDTA buffer. In the second step (sample loading), the HC was completely filled with 5 mL of sample and then discharged into the cartridge. This step was repeated until the sample

had passed completely through the column. In the third step (washing), 2 mL of the washing solution was loaded into the HC and then propelled through the extraction phase. In the fourth step (elution), 1 mL of the elution solution was aspirated into the HC and then dispensed into the cartridge. Subsequently, the eluate was introduced into the HC and, with the valves in the ON position, air was aspirated into S1 and the reagent solutions into S2–S4. The eluate was dispensed through port 2 to the X-connector. In the fifth step (complex formation and detection), the buffer (1 mL) and citrate (0.2 mL) solutions were dispensed through the OFF position of S2 and S3, respectively, and mixed with the eluate at the X-connector. After that, the europium solution (1 mL) was dispensed through the OFF position of the S4 and mixed into the T-connector with the outlet mix from the X-connector. The formed complex was led to the flow cell of the fluorimetric detector ($\lambda_{exc/em} = 400/612$ nm), and then, to the waste container. The emitted intensity was evaluated as a function of the height of the peak and three replicates were carried out for each standard solution or sample. In the sixth and final step (cleaning), cleaning of the HC and the path between port 2 and the detection cell was performed with 50 % (v/v) methanol and then with water (4 mL each).

A flow rate of 10 mL min⁻¹ was set for the conditioning, washing, and cleaning of the SPE column, as well as for the transport of the eluate, solutions, and mixtures products. Note that the cartridge is inverted (upside down) in the column conditioning, sample loading, and washing steps, so that, the liquid falls by gravity into a waste container. This avoids aspiration of the loaded volumes on the column and reduces the total procedure time. In the elution and cleanup steps, the cartridge is positioned as seen in Fig. 1 and is taped to the MPV module. In this way, the liquid is kept inside the tube of the cartridge and can be aspirated either to mix with the MSM solutions or to clean the system in the last steps.

2.4. Validation method

The chromatographic analysis was performed on a C18 column (2.1 × 50 mm, 1.8 μm; Agilent Zorbax RRHD Eclipse) using an UPLC-FLD system from Agilent Technologies (USA) (Agilent 1290 Infinity: quaternary pump, autosampler and Agilent 1260 Infinity FLD detector). The flow rate was set at 0.3 mL min⁻¹ and the injection volume was 10 μL. The column temperature was kept at 20 °C. The mobile phase consisted of an aqueous solution (A) with additives (C₂H₇NO₂ buffer (10 mM) pH 7.0 with CaCl₂ (35 mM) and Na₂EDTA (25 mM) in ultrapure water) and methanol (B) [10]. The aqueous mobile phase was filtered through 0.22 μm PES filters and was prepared immediately prior to use. The gradient conditions were as follows: A from 90 % to 57 % for 1.5 min, hold at 57 % for 1 min, next 57 % to 34 % for 2.5 min, then 34 % to 90 % for 0.5 min and hold at 90 % up to 9.0 min. The wavelengths for excitation and emission were 365 nm and 500 nm, respectively.

3. Results and discussion

Tetracycline (TC) was selected from the tetracyclines group (oxytetracycline, chlortetracycline, etc.) to perform the analytical method due to its prevalence in wastewaters and its high fluorescence intensity. HLB cartridges were chosen for TC extraction because they do not contain silanol groups, thus avoiding the binding of metal ions and the irreversible binding of TC to them.

3.1. Selection of solvent elution

Selection of the eluent was performed on-line with an aqueous solution containing 500 μg L⁻¹ of TC. Methanol (MeOH), ethanol (EtOH), acetone (ACE), acetonitrile (ACTN) and dimethylsulfoxide (DMSO) were studied as eluent solvents. Initially, the column was conditioned as explained in the analytical procedure (section 2.3). Afterwards, 5 mL of the aqueous solution (500 μg L⁻¹, adjusted with Na₂EDTA buffer at pH

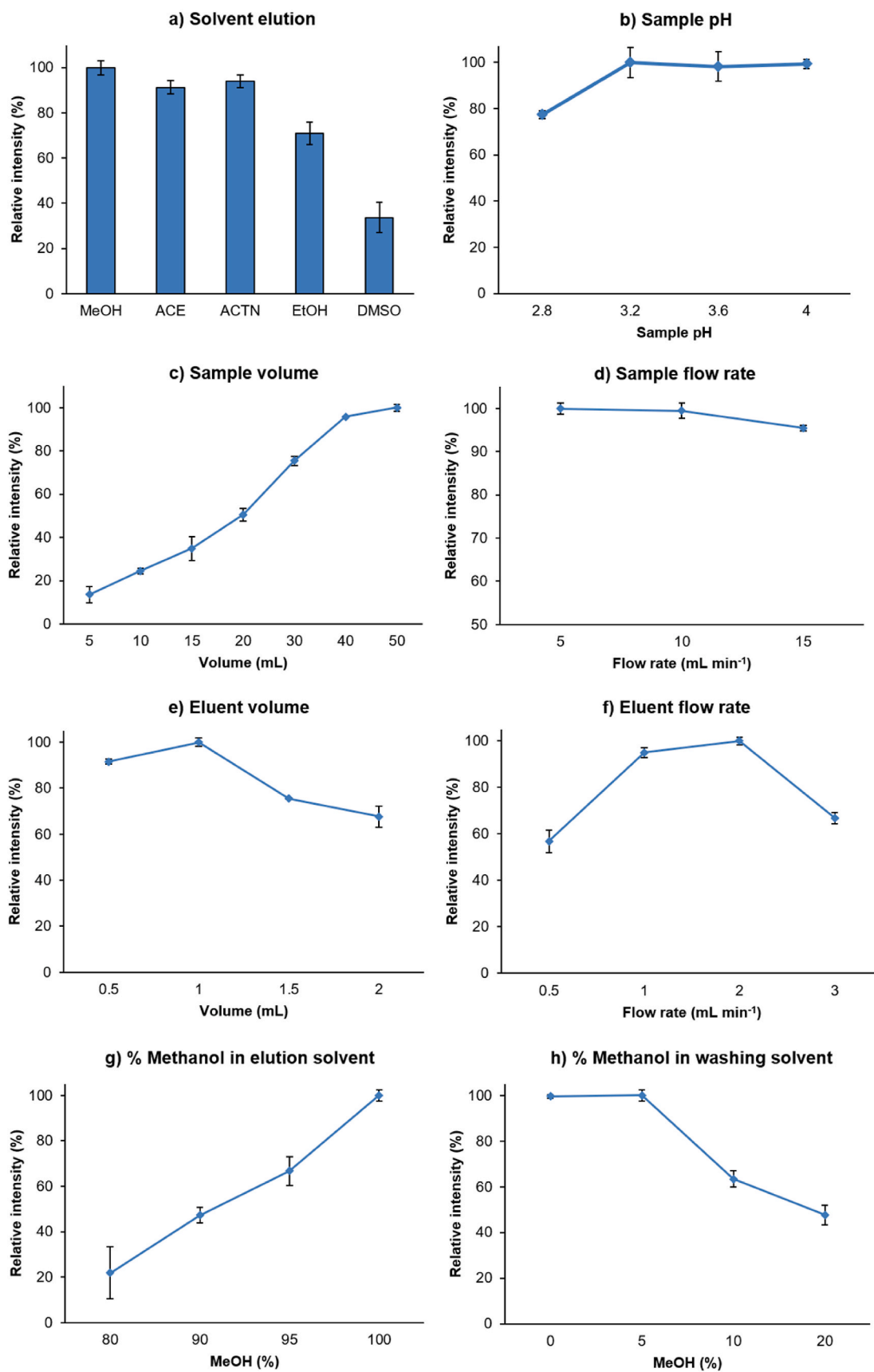


Fig. 2. Results of the optimization of extraction parameters (triplicate results with error bars).

4.0) was passed through the Oasis HLB cartridge at a flow rate of 5 mL min⁻¹. The column was rinsed with 2 mL of water. Next, the elution was performed using 1 mL of each tested solvent at a flow rate of 2 mL min⁻¹. Finally, the Eu-TC complex was formed by mixing the TC eluate with 5 mM europium and Tris-HCl (pH 8.5).

As can be seen in Fig. 2a, MeOH showed the highest extraction efficiency among the solvents tested. Therefore, MeOH was selected as elution solvent. The results are presented as relative intensity (%) assigning 100 % to the solvent with the best performance and the others compared to it. DMSO was found to be the least effective in TC elution (relative intensity ≤ 34 %), which can be attributed to its low polarity. Tetracyclines are relatively polar compounds and therefore their extraction is favored with highly polar solvents.

The compatibility of MeOH with the reagents involved in the complex formation made the evaporation and drying of the eluent unnecessary. Skipping these time-consuming steps enhanced throughput and eliminated the possibility of TC loss during evaporation.

3.2. Optimization of extraction parameters

The main parameters affecting the SPE procedure were tested in order to study their influence on the pre-concentration efficiency. The selected variables and their initial value were: pH sample (4.0), sample volume (5 mL), sample loading flow rate (5 mL min⁻¹), washing solution composition (100 % water), elution solution concentration (100 % MeOH), eluent volume (1 mL) and eluent flow rate (2 mL min⁻¹). The

variables were optimized following the present sequence. Once each variable was optimized, it was fixed for future assays. Sample solution was initially prepared spiking 500 $\mu\text{g L}^{-1}$ TC. The fluorescence intensity expressed as a percentage of the relative intensity was used as the signal response. The value of the variable with the highest signal intensity was assigned 100 % and the other values were compared to it.

3.2.1. Optimization of the pH of the conditioning solvent and the sample

To improve extraction efficiency, Na₂EDTA buffer was used for column conditioning and sample pH adjustment. Na₂EDTA buffer is used to chelate metals or multivalent cations that are highly soluble in water. This also avoids the formation of TC chelating complexes with metal ions and prevents their competitive binding to the HLB cartridge adsorption sites [27].

The effect of sample pH and column conditioning buffer was investigated. The pH of the sample was studied in a pH range of 2.8–4.0 by adding 1 mL of the Na₂EDTA buffer solution (0.1 M) adjusted with HCl (1 M). The column was conditioned with 2 mL of the buffer solution at the same pH studied for the sample pH adjustment. Since no differences were found in the pH range of 3.2–4.0 (Fig. 2b), it was decided to set the pH to 4.0. Under this pH, the zwitterionic state of tetracyclines (pH > pK_{a1} 3.3) [28] is favored, which is preferred for the maximum adsorption of polar amphoteric compounds in the HLB phase due to charge neutrality.

In other works, Na₂EDTA is usually used together with phosphate and citric acid (Na₂EDTA McIlvaine buffer) to enhance the chelating

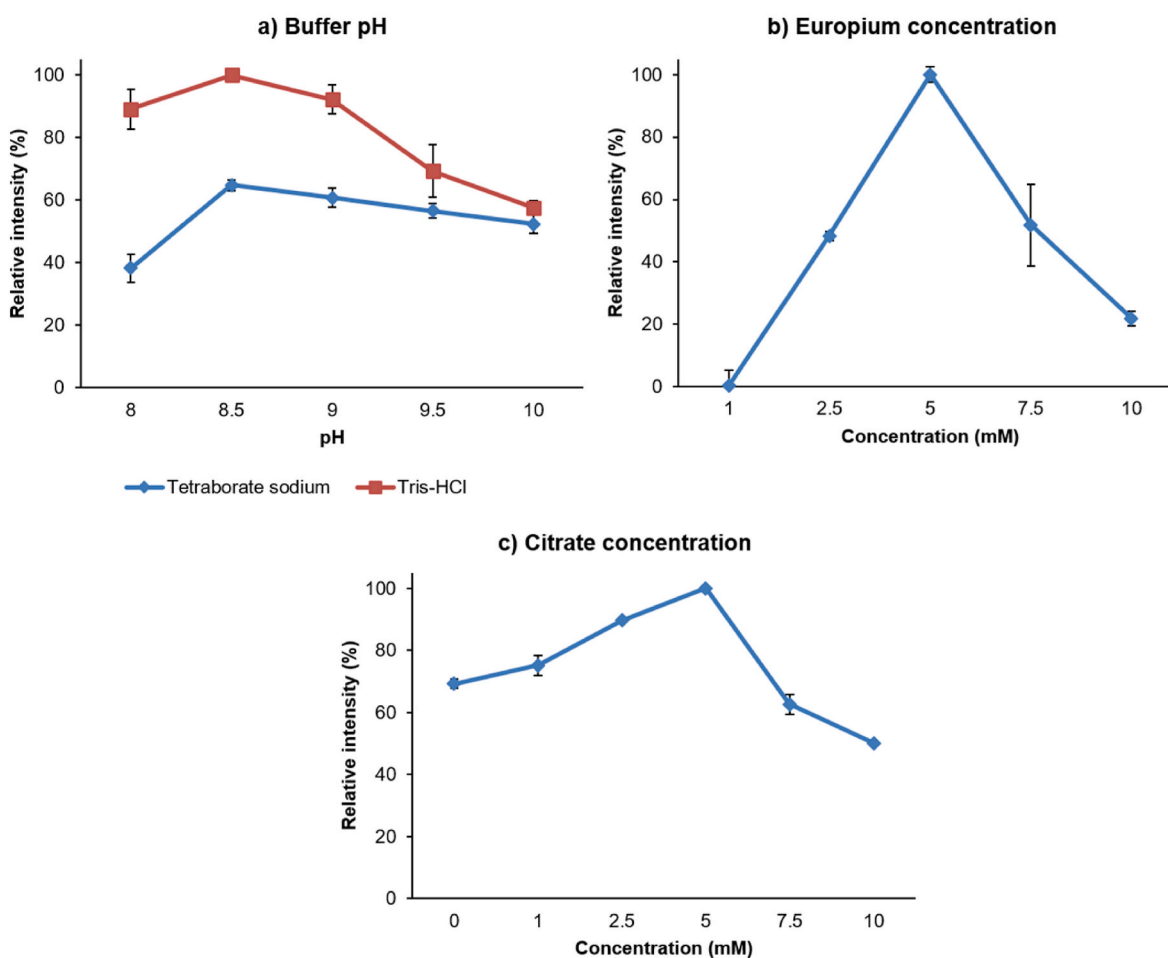


Fig. 3. Results of the optimization of complex formation parameters (triplicate results with error bars).

effect [14,18,24]. However, in this work, they were not used for two reasons: phosphate can form a precipitate with Eu(III), affecting the formation of the complex [18] and citric acid prevents studying the influence of citrate (the sensitizing agent) on the fluorescence intensity of the Eu-TC-Cit complex (section 3.4.3).

3.2.2. Sample volume and flow rate

Different sample volumes (5, 10, 15, 20, 30, 40 and 50 mL) were examined (Fig. 2c). As expected, the signal intensity increased when increasing sample volume. We can observe that the signal grew slowly between 40 and 50 mL, obtaining the highest intensity with this last volume. Consequently, a volume of 50 mL was selected for future studies in order to obtain a higher sensitivity in the detection. Sample volumes larger than 50 mL were not evaluated in order to have a compromise between the preconcentration of the analyte and the extraction time.

Next, sample flow rates of 5, 10 and 15 mL min⁻¹ were tested (Fig. 2d). The results show that low flow rates (≤ 10 mL min⁻¹) favored the analyte recovery. When the flow rate was increased to 15 mL min⁻¹, the signal dropped by 4 %. There were no significant differences between 5 and 10 mL min⁻¹; hence, 10 mL min⁻¹ was used in the following assays to decrease analysis time.

3.2.3. Eluent volume, flow rate and concentration

Different MeOH volumes (0.5, 1.0, 1.5 and 2.0 mL) and flow rates (0.5, 1.0, 2.0 and 3.0 mL min⁻¹) were studied. According to Fig. 2e, the highest signal was achieved using 1.0 mL of MeOH. As expected, the signal intensity decreases as the eluent volume increases. Volumes greater than 1 mL resulted in low analyte enrichment. On the contrary, volumes below 1 mL did not allow a complete elution of the analyte, leading to a lower response intensity. For this reason, 1 mL was chosen as the optimal MeOH volume for elution.

As it is shown in Fig. 2f, flow rates outside the range between 1.0 and 2.0 mL min⁻¹ led to poor response. Among the tested flow rates, 2.0 mL min⁻¹ gave the higher signal intensity and had minimal non-significant difference with 1.0 mL min⁻¹. Therefore, 2.0 mL min⁻¹ was selected as optimum elution solution flow rate.

Subsequently, methanol solutions with different concentrations in water (80, 90, 95, 100 % v/v) were assessed to investigate the potential effect of methanol percentage on the formation of the Eu-TC complex and fluorescence intensity. The results (Fig. 2g) showed that the TC intensity was higher when it was eluted with 100 % methanol. Thus, it was decided to elute with undiluted methanol.

3.2.4. Washing solution composition

The composition of the washing solution was studied using a real sample (outlet wastewater) filtered and spiked with 500 $\mu\text{g L}^{-1}$. Pure water and water (2 mL) with 5, 10 and 20 % of MeOH were tested to partially remove organic interferents retained by the HLB cartridge prior to the elution step. The results (Fig. 2h) showed that TC starts to elute from the HLB cartridge when the methanol percentage exceeds 5 %. The addition of 5 % of MeOH did not provide a significant improvement with respect to the water wash. Nevertheless, it was decided to wash with 5 % of MeOH to ensure the elimination of possible interferents in samples with more complex matrices [12,22].

3.3. Reusability

The cartridge is reusable up to 20 times with standard solutions and 10 times with wastewater samples from the outlet and from the settler. For inlet wastewater samples, the resin can be reused only 5 times without affecting repeatability (RSD ≤ 10 %).

3.4. Optimization of complex formation parameters

3.4.1. Effect of pH and buffer solution

Tetracyclines show high fluorescence in basic media due to

deprotonation of the complex-forming groups: tricarbonyl ring (pK_{a1} 3.3) and β -diketone (pK_{a2} 7.7) are chelated by multivalent cations such as europium (Eu^{3+}). In this way, the deprotonated tetracycline acts as a nucleophile or donor [17,29]. Taking this into account, the borate buffer and the Tris-HCl buffer were tested at different pH values in an alkaline pH range (8.0, 8.5, 9.0, 9.5 and 10). The effect of pH and buffer solution on the fluorescence intensity of the Eu-TC complex was studied by mixing 1 mL of the eluate (200 $\mu\text{g L}^{-1}$ TC) with 1 mL of the europium solution (5 mM) and 1 mL of the buffer solution in the X-connector of the automated system. The volumetric proportion of the mixture was previously studied in manual tests (data not shown).

As can be seen in Fig. 3a, both buffers at pH 8.5 presented the highest emission intensity and declined at lower or higher pH values. Compared with borate, Tris-HCl exhibits a 35 % increase in fluorescence intensity at optimum pH. Therefore, Tris-HCl buffer at pH 8.5 was used for further measurements. These results are similar to those obtained by other works [20,29,30]. A pH greater than 9 forms a slightly yellow cloudy solution that decreases the fluorescence intensity. This is possibly attributed to the formation of a europium precipitate under alkaline conditions. Regarding the buffers, Tris-HCl provides a greater stability of the complex formed, while the ionic species of the borate probably cause the quenching of the fluorescence [30].

3.4.2. Effect of europium concentration

The influence of Eu(III) concentration is a common studied factor in the formation of the Eu-TC complex, as the fluorescence intensity of TC is dependent on the concentration of Eu(III) added to the reaction mixture. Although the concentration of Eu(III) has already been investigated in various previous works [20,29,30], in this current research it was decided to study this parameter due to the variability of the results obtained between manual mixtures and mixtures in flow systems. In this latter case, the analyte concentration was detected while the fluid moved through the cell.

The effect of the Eu(III) concentration was determined by measuring the fluorescence intensity of the Eu-TC complex at different Eu(III) concentrations (1.0, 2.5, 5.0, 7.5, and 10 mM), while the antibiotic concentration was kept constant. As seen in Fig. 3b, the fluorescence intensity increased by increasing the europium concentration from 1.0 to 5.0 mM and declined considerably thereafter. Europium in excessive amounts has been found to cause a quenching effect [30]. Thus, 5 mM was selected as the optimal europium concentration for the proposed flow method.

The reaction of TC with Eu(III) is rapid and can be measured after 10 s when the fluorescence intensity is maximum and constant.

3.4.3. Effect of citrate concentration

The coupling of Eu(III) with the oxygen atoms of the three carboxyl groups and the hydroxyl group of the citrate removes the water molecules surrounding the Eu(III) coordination sites, leading to the formation of a new complex (Eu-TC-Cit) with a higher fluorescence intensity [20, 29]. Thus, in order to find the optimal citrate concentration that had a positive effect on the fluorescence intensity of the complex, the following citrate solutions were prepared: 1.0, 2.5, 5.0, 7.5 and 10 mM. The complex in the absence of citrate (Eu-TC) is indicated with 0 mM. The experiment was performed by mixing in the X-connector: 1 mL of the eluate (200 $\mu\text{g L}^{-1}$ TC) with 1 mL of the Tris-HCl buffer (pH 8.5) and 0.2 mL of citrate solution. Next, 1 mL of the europium solution (5 mM) was added to this mixture in the T-connector.

As it is shown in Fig. 3c, an increase in fluorescence intensity was observed when the citrate concentration increased up to 5 mM. After this point, the signal decreased. Adding 5 mM citrate to the mixture (Eu-TC-Cit) succeeded in increasing the fluorescence intensity of the complex without citrate (Eu-TC) by 30 %. Thus, 5 mM citrate was selected for this work.

Table 1
Analytical parameters of the proposed method for the determination of TC.

Analytical parameters	TC
Linear range ($\mu\text{g L}^{-1}$)	30–1000
Linear equation	$I = 775.1 (C) + 216.6$
Determination coefficient (R^2)	0.998
LOD ($\mu\text{g L}^{-1}$)	9.4
LOQ ($\mu\text{g L}^{-1}$)	31
Enrichment factor (EF)	40
Intra-day precision (% RSD, $n = 5$, $C = 50/500 \mu\text{g L}^{-1}$)	1.9–3.9/0.5–2.0
Inter-day precision (% RSD, $n = 5$, $C = 200 \mu\text{g L}^{-1}$)	4.1–5.5

LOD: Limit of detection, LOQ: Limit of quantification.

3.5. Interferences evaluation

Wastewater contains other organic and inorganic compounds that can potentially interfere with fluorimetric measurements. Substances such as sugars, metal ions and other antibiotics could coordinate with Eu (III) or react with TC, affecting the fluorescence of the Eu-TC-Cit complex [31].

Therefore, under previously established optimized conditions, the influence on the fluorescent response of a $200 \mu\text{g L}^{-1}$ TC by the following interferents was evaluated: glucose, erythromycin and a multielement standard solution containing anions ($100 \mu\text{g L}^{-1} \text{F}^-$, $2000 \mu\text{g L}^{-1} \text{Cl}^-$, $500 \mu\text{g L}^{-1} \text{Br}^-$, $300 \mu\text{g L}^{-1} \text{NO}_2^-$, $500 \mu\text{g L}^{-1} \text{NO}_3^-$, $800 \mu\text{g L}^{-1} \text{PO}_4^{3-}$, $600 \mu\text{g L}^{-1} \text{SO}_4^{2-}$) and cations ($800 \mu\text{g L}^{-1} \text{NH}_4^+$, $800 \mu\text{g L}^{-1} \text{Ca}^{2+}$, $400 \mu\text{g L}^{-1} \text{K}^+$, $200 \mu\text{g L}^{-1} \text{Li}^+$, $400 \mu\text{g L}^{-1} \text{Mg}^{2+}$, $400 \mu\text{g L}^{-1} \text{Na}^+$). Glucose and erythromycin were evaluated at three concentrations (100, 200 and $400 \mu\text{g L}^{-1}$). A compound was considered as interferent when its concentration caused a significant change ($\geq 5\%$) in the value of the fluorimetric signal. As depicted in Fig. S1, high concentrations of ions, antibiotic and glucose exhibited a slightly greater effect on fluorescence responses, although they did not achieve a significant change ($\leq 3\%$). These results indicate that the proposed method can be used for the selective detection of TCs in wastewater.

3.6. Analytical parameters

Table 1 presents the analytical features for the TC determination by the automated method. The calibration curve for TC under optimal preconcentration conditions is given as $F = 775.1C + 216.6$ in the range of $30\text{--}1000 \mu\text{g L}^{-1}$ ($R^2 = 0.998$) (F , is the fluorescence intensity, C , is the analyte concentration in $\mu\text{g L}^{-1}$). Without SPE extraction, TC responds linearly up to 40mg L^{-1} ($R^2 = 0.991$). The limit of detection (LOD) and the limit of quantification (LOQ) were determined as 9.4 and $31 \mu\text{g L}^{-1}$, respectively. These values were calculated according to IUPAC criteria, by dividing three times and ten times the standard deviation of the blank ($n = 10$) by the slope of the curve. The limits of detection and quantification are enough to detect and quantify the typical concentrations present in wastewater samples. Tetracycline concentration ranges between 50 and $270 \mu\text{g L}^{-1}$ have been found in raw wastewater and maximum concentrations of $5116 \mu\text{g L}^{-1}$ have been found in WWTPs [3]. The enrichment factor (EF) was calculated as the ratio of the slope of the calibration lines obtained with and without SPE procedure. The EF of 40 obtained for TC shows the high preconcentration capacity of the method.

Eu(III) also forms a complex with other TCs as oxytetracycline (OTC) and chlortetracycline (CTC). The calibration curves, linear range, LOD, LOQ and EF obtained for OTC and CTC are displayed in Table S2. The results indicated that the response decreased in the order $\text{TC} > \text{OTC} \geq \text{CTC}$.

3.7. Application and validation

The proposed automated method was applied to wastewater samples collected from the inlet, outlet and from a secondary settler of a

Table 2
Application of the developed method to wastewater samples and add-recovery test at three spiking levels.

Sample	Spiking level ($\mu\text{g L}^{-1}$)	Found ($\mu\text{g L}^{-1}$) *	Recovery (%)	RSD (%)
Inlet Wastewater (S1)	0	ND		
	50	48.2 ± 0.9	96.4	1.9
	200	183.5 ± 8.3	91.8	4.5
Inlet Wastewater (S2)	0	ND		
	50	46.7 ± 1.8	93.4	3.9
	200	187.1 ± 7.6	93.6	4.1
Secondary settler	0	ND		
	50	49 ± 0.9	97.9	1.9
	200	206.6 ± 8.4	103.3	4.1
Outlet wastewater (S1)	0	ND		
	50	49.4 ± 1.8	98.8	3.7
	200	192 ± 9.5	96	4.9
Outlet wastewater (S2)	0	ND		
	50	501 ± 4.6	100.2	0.9
	200	183.2 ± 10.2	91.6	5.5
	500	478.7 ± 2.5	95.7	0.5

S1: Sample 1, S2: Sample 2, ND: not detected. *Average \pm standard deviation ($n = 5$).

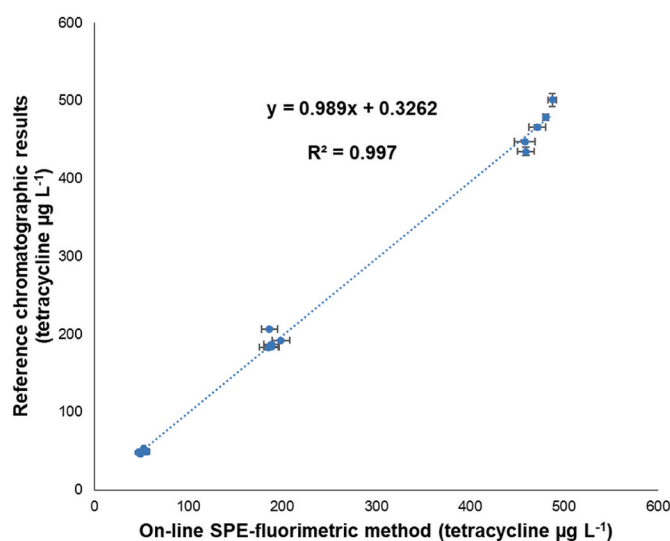


Fig. 4. Regression line for comparison of analytical results between the proposed automated method and UPLC-FLD method for tetracycline determination. Triplicate results with error bars (\pm SD).

wastewater treatment plant located in Bouc-bel-air, France. The samples were previously analyzed by UPLC-FLD to verify the presence and type of tetracyclines in the samples. In this case, no tetracyclines were detected in the samples. Therefore, the addition-recovery test (at three concentration levels of TC) was performed directly to assess the precision and accuracy of the proposed method. If concentrations of two or more different tetracyclines (TCs) were found in the samples, the total content of TCs would be expressed as TC equivalents ($\mu\text{g L}^{-1}$). The spectrofluorimeter detects a single result that includes the fluorescence contributions of each of the TCs, their potential metabolites and isomers. The instrumental LOD and LOQ for UPLC-FLD method were 1 and $3.5 \mu\text{g L}^{-1}$ TC, respectively.

As given in Table 2, the recoveries for TC were found to be in the range $87\text{--}106\%$. The precision of the procedure, expressed as the relative standard deviation (RSD) for five determinations on the same day (intra-day precision), using wastewater samples with concentrations

Table 3

Analytical characteristics of the proposed method in comparison to the previous reported automated methods for the determination of tetracycline.

Method	Flow technique	Type of sample	Sample volume (mL)	LOD ($\mu\text{g L}^{-1}$)	Linear range ($\mu\text{g L}^{-1}$)	RSD (%)	Sampling frequency (h^{-1})	Ref.
Spectrophotometry	FIA	Pharmaceuticals	0.6	400	1000–40000	0.2–1.5	52	[15]
MSPE-	FIA	Milk	50	10	30–600	1.6–4.3	2	[32]
Spectrophotometry								
SALLME-HPLC-UV	SIA	Urine	1	170	500–20000	4.0–6.0	9	[8]
SPE-HPLC-UV	SIA	Honey	25	8	50–1000	4.1–7.1	4	[24]
Spectrophotometry	SIEMA	Honey	10	130	400–25000	1.0–5.0	60	[33]
SPE-Fluorimetry	MSFIA	Wastewater	50	9	30–1000	0.5–3.9	3	This work

SALLME: Salting out assisted liquid-liquid microextraction, MSPE: Magnetic solid-phase extraction, FIA: Flow injection analysis, SIA: Sequential injection analysis, SIEMA: Simultaneous injection effective mixing analysis, MSFIA: Multisyringe flow injection analysis.

of 50 and 500 $\mu\text{g L}^{-1}$ TC, was below 4 %. The same samples with a concentration of 200 $\mu\text{g L}^{-1}$ TC, were analyzed on five different days to obtain an inter-day precision that ranged from 4.1 to 5.5 %. These data indicate that this method has a good accuracy, repeatability and reproducibility.

In Fig. 4, the results obtained between our automated method and the UPLC-FLD method, were compared. The same concentrations of TC used for the recovery tests in the automated method were used to enrich the samples analyzed by chromatography. The regression line obtained between the results of both methodologies shows a good determination coefficient, a slope close to unity and an intercept close to zero. Therefore, this confirms the good accuracy of our proposed methodology.

3.8. Comparison of the proposed method with other methods

Table 3 shows the analytical characteristics of the proposed method in comparison with other recently reported automated methods for the determination of tetracycline in different sample types. As it can be seen, the proposed method has a LOD, linear range and precision comparable with the methods that use extraction techniques and HPLC-UV. Moreover, it can be observed that SPE-based methods present the highest sensitivity at the expense of high sample consumption and low sampling frequency. Although the Eu(III)-based method for TCs detection is very simple and selective, without prior chromatographic separation, it has the limitation of not distinguishing the individual compounds. Nevertheless, unlike the informed methods, the proposed method avoids the use of expensive instruments such as chromatographs and benchtop spectrometers or fluorimeters. Additionally, it reduces potential environmental damage caused by the disposal of mobile phase solvents used in liquid chromatography. It is also worth mentioning that utilizing an open-air SPE cartridge prevents the fluids overpressure of fluids usually observed in the columns used in closed flow systems. Furthermore, employing an easily replaceable commercial cartridge once its use is depleted, simplifies the set-up of the method.

4. Conclusions

To the best of our knowledge, this study presents the first automation of tetracycline determination using the europium-based method and incorporating preconcentration and extraction steps. A rigorous study of the parameters affecting the extraction and formation of the Eu-TC-Cit complex in a flow system was carried out. The developed method had very good precision and recoveries in wastewater samples. The tested interferents did not have a significant effect on the analytical response. The validation with the reference method (UPLC-FLD) allowed to demonstrate the good accuracy of the method, demonstrating the efficiency of this methodology for the routine analysis in wastewater samples. Advantages of this method include high sensitivity and less analyst intervention. The proposed system represents an economical and environmentally friendly option for the detection of tetracyclines. Future research is expected to explore other ways of performing SPE to increase

the frequency of analysis and reduce sample consumption. In addition, a minicolumn could be included in subsequent assays to carry out the separation of tetracyclines.

CRediT authorship contribution statement

M.A. Vargas-Muñoz: Conceptualization, Investigation, Writing - original draft. **Jean-Luc Boudenne:** Conceptualization, Formal analysis. **Bruno Coulomb:** Formal analysis, Methodology, Visualization. **Fabien Robert-Peillard:** Supervision, Validation, Writing - review & editing. **Edwin Palacio:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2023.125544>.

References

- [1] R. Daghri, P. Drogui, Tetracycline antibiotics in the environment: a review, *Environ. Chem. Lett.* 11 (2013) 209–227, <https://doi.org/10.1007/s10311-013-0404-8>.
- [2] A.K. Vidaver, Uses of Antimicrobials in Plant Agriculture, *Clinical Infectious Diseases*, vol. 34, 2002. S107–S110, https://academic.oup.com/cid/article/34/Supplement_3/S107/292699.
- [3] J. Scaria, K.V. Anupama, P.V. Nidheesh, Tetracyclines in the environment: an overview on the occurrence, fate, toxicity, detection, removal methods, and sludge management, *Sci. Total Environ.* (2021) 771, <https://doi.org/10.1016/j.scitotenv.2021.145291>.
- [4] M. Topal, E.I. Arslan Topal, Occurrence and fate of tetracycline and degradation products in municipal biological wastewater treatment plant and transport of them

- in surface water, *Environ. Monit. Assess.* 187 (2015) 1–9, <https://doi.org/10.1007/s10661-015-4978-4>.
- [5] M. Czatzkowska, M. Harnisz, E. Korzeniewska, I. Koniuszewska, Inhibitors of the methane fermentation process with particular emphasis on the microbiological aspect: a review, *Energy Sci. Eng.* 8 (2020) 1880–1897, <https://doi.org/10.1002/ese3.609>.
- [6] B. Du, Q. Yang, X. Li, W. Yuan, Y. Chen, R. Wang, Impacts of long-term exposure to tetracycline and sulfamethoxazole on the sludge granules in an anoxic-aerobic wastewater treatment system, *Sci. Total Environ.* 684 (2019) 67–77, <https://doi.org/10.1016/j.scitotenv.2019.05.313>.
- [7] M. Czatzkowska, M. Harnisz, E. Korzeniewska, P. Rusanowska, S. Bajkacz, E. Felis, J.P. Jastrzębski, Ł. Paukšto, I. Koniuszewska, The impact of antimicrobials on the efficiency of methane fermentation of sewage sludge, changes in microbial biodiversity and the spread of antibiotic resistance, *J. Hazard Mater.* 416 (2021), <https://doi.org/10.1016/j.jhazmat.2021.125773>.
- [8] K. Cherkashina, C. Vakh, S. Lebedinets, A. Pochivalov, L. Moskvin, A. Lezov, A. Bulatov, An automated salting-out assisted liquid-liquid microextraction approach using 1-octylamine: on-line separation of tetracycline in urine samples followed by HPLC-UV determination, *Talanta* 184 (2018) 122–127, <https://doi.org/10.1016/j.talanta.2018.02.112>.
- [9] S. Babić, D. Ašperger, D. Mutavdžić, A.J.M. Horvat, M. Kaštelan-Macan, Solid phase extraction and HPLC determination of veterinary pharmaceuticals in wastewater, *Talanta* 70 (2006) 732–738, <https://doi.org/10.1016/j.talanta.2006.07.003>.
- [10] K. Škrášková, L.H.M.L.M. Santos, D. Šatínský, A. Pena, M.C.B.S.M. Montenegro, P. Solich, L. Nováková, Fast and sensitive UHPLC methods with fluorescence and tandem mass spectrometry detection for the determination of tetracycline antibiotics in surface waters, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* 927 (2013) 201–208, <https://doi.org/10.1016/j.jchromb.2012.12.032>.
- [11] F. Du, X. Zheng, L. Sun, Q. Qin, L. Guo, G. Ruan, Development and validation of polymerized high internal phase emulsion monoliths coupled with HPLC and fluorescence detection for the determination of trace tetracycline antibiotics in environmental water samples, *J. Separ. Sci.* 38 (2015) 3774–3780, <https://doi.org/10.1002/jssc.201500497>.
- [12] W. Ben, Z. Qiang, C. Adams, H. Zhang, L. Chen, Simultaneous determination of sulfonamides, tetracyclines and tiamulin in swine wastewater by solid-phase extraction and liquid chromatography-mass spectrometry, *J. Chromatogr. A* 1202 (2008) 173–180, <https://doi.org/10.1016/j.chroma.2008.07.014>.
- [13] R. Santiago Valverde, I. Sánchez Pérez, F. Franceschelli, M. Martínez Galera, M. D. Gil García, Determination of photoirradiated tetracyclines in water by high-performance liquid chromatography with chemiluminescence detection based reaction of rhodamine B with cerium (IV), *J. Chromatogr. A* 1167 (2007) 85–94, <https://doi.org/10.1016/j.chroma.2007.08.043>.
- [14] G. Chen, G. Liu, F. Qin, Use of a portable time-resolved fluorometer to determine oxytetracycline residue in four fruit crops, *Food Chem.* 127 (2011) 264–269, <https://doi.org/10.1016/j.foodchem.2010.12.106>.
- [15] M.P. Rodríguez, H.R. Pezza, L. Pezza, Simple and clean determination of tetracyclines by flow injection analysis, *Spectrochim. Acta Mol. Biomol. Spectrosc.* 153 (2016) 386–392, <https://doi.org/10.1016/j.saa.2015.08.048>.
- [16] L.M. Hirschy, E. V. Dose, J.D. Winefordner, Lanthanides-sensitized luminescence for the detection of tetracyclines, *Analytica Chimica Acta* 147 (1983) 311–316.
- [17] L.C. Courrol, R.E. Samad, Applications of europium tetracycline complex: a review, *Curr. Pharmaceut. Anal.* 4 (2008) 238–248.
- [18] G. Chen, M.J. Schneider, A.M. Darwish, S.J. Lehotay, D.W. Freeman, Europium-sensitized luminescence determination of oxytetracycline in catfish muscle, *Talanta* 64 (2004) 252–257, <https://doi.org/10.1016/j.talanta.2004.02.014>.
- [19] V.G. Amelin, Z.A.C. Shogah, D.S. Bol'shakov, Using a smartphone for determining tetracyclines in water and milk by the sensitized solid state fluorescence of europium on its hydroxide, *J. Anal. Chem.* 76 (2021) 1211–1216, <https://doi.org/10.1134/S1061934821080025>.
- [20] F. Hijaz, Y. Nehela, O. Batuman, N. Killiny, Citrate mediated europium-based detection of oxytetracycline in citrus tissues, *Antibiotics* 10 (2021) 1–11, <https://doi.org/10.3390/antibiotics10050566>.
- [21] F. Hijaz, Y. Nehela, P. Gonzalez-blanco, N. Killiny, Development of europium-sensitized fluorescence-based method for sensitive detection of oxytetracycline in citrus tissues, *Antibiotics* 10 (2021) 1–14, <https://doi.org/10.3390/antibiotics10020224>.
- [22] C.R. Anderson, H.S. Rupp, W.H. Wu, Complexities in tetracycline analysis - chemistry, matrix extraction, cleanup, and liquid chromatography, *J. Chromatogr. A* 1075 (2005) 23–32, <https://doi.org/10.1016/j.chroma.2005.04.013>.
- [23] R. López-Serna, S. Pérez, A. Ginebreda, M. Petrović, D. Barceló, Fully automated determination of 74 pharmaceuticals in environmental and waste waters by online solid phase extraction-liquid chromatography- electrospray-tandem mass spectrometry, *Talanta* 83 (2010) 410–424, <https://doi.org/10.1016/j.talanta.2010.09.046>.
- [24] J. Li, L. Chen, X. Wang, H. Jin, L. Ding, K. Zhang, H. Zhang, Determination of tetracyclines residues in honey by on-line solid-phase extraction high-performance liquid chromatography, *Talanta* 75 (2008) 1245–1252, <https://doi.org/10.1016/j.talanta.2008.01.027>.
- [25] D. Barzallo, K. Danchana, V. Cerdà, E. Palacio, Design of an automatic spectrophotometric system, *Talanta* 218 (2020), <https://doi.org/10.1016/j.talanta.2020.121163>.
- [26] M.A. Vargas-Muñoz, K. Danchana, V. Cerdà, E. Palacio, Field-deployable method for iron analysis using a simple preconcentration procedure and a 3D portable spectrophotometric system, *Microchem. J.* 170 (2021), <https://doi.org/10.1016/j.microc.2021.106774>.
- [27] M. Seifrtová, L. Nováková, C. Lino, A. Pena, P. Solich, An overview of analytical methodologies for the determination of antibiotics in environmental waters, *Anal. Chim. Acta* 649 (2009) 158–179, <https://doi.org/10.1016/j.aca.2009.07.031>.
- [28] M. Pérez-Rodríguez, R.G. Pellerano, L. Pezza, H.R. Pezza, An overview of the main foodstuff sample preparation technologies for tetracycline residue determination, *Talanta* 182 (2018) 1–21, <https://doi.org/10.1016/j.talanta.2018.01.058>.
- [29] E. Bečić, J. Mušanović, B. Imamović, F. Bečić, M. Šober, Optimisation of europium-sensitized fluorescence assay for detection of tetracycline antibiotics, *Int. J. Collab. Res. Intern. Med. Public Health* 8 (2018) 483–490, <https://www.researchgate.net/publication/326255263>.
- [30] Z.-Y. Huang, C. Zhen-Hua, Y. Qu-An, R.-S. Shen, Spectrofluorimetric determination of tetracycline using Eu³⁺-chelation and cetyltrimethylammonium bromide", *Wuhan Univ. J. Nat. Sci.* 4 (1999) 222–226.
- [31] H. Tan, Y. Chen, Silver nanoparticle enhanced fluorescence of europium (III) for detection of tetracycline in milk, *Sensor. Actuator. B Chem.* 173 (2012) 262–267, <https://doi.org/10.1016/j.snb.2012.06.090>.
- [32] J.A. Rodríguez, J. Espinosa, K. Aguilar-Arteaga, I.S. Ibarra, J.M. Miranda, Determination of tetracyclines in milk samples by magnetic solid phase extraction flow injection analysis, *Microchim. Acta* 171 (2010) 407–413, <https://doi.org/10.1007/s00604-010-0428-8>.
- [33] C. Saenjum, N. Pattapong, T. Aunsakol, T. Pattananandecha, S. Apichai, H. Murakami, K. Grudpan, N. Teshima, High sensitivity spectrophotometric determination of tetracycline with zirconium chelation by employing simultaneous injection effective mixing analysis (SIEMA): tetracycline residue in honey, *J. Food Compos. Anal.* 105 (2022), <https://doi.org/10.1016/j.jfca.2021.104215>.