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MCM-41 based dispersive micro-solid phase extraction of selected cephalosporin antibiotic residues from water samples prior to liquid chromatographic quantification

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ABSTRACT

The occurrence of antibiotics in water bodies is of concern owing to the serious risk they pose to the environment, water security, aquatic organisms, and human health. In this study, mesoporous silica (MCM-41) nanostructured material was synthesised and characterized by various analytical techniques to determine its morphology and dimension, functional groups, surface charge, and textural properties. The nanostructured MCM-41 was used as an adsorbent in ultrasound-assisted dispersive micro solid-phase extraction (UA-D-μ-SPE) of cefadroxil (CFDX) and cephalexin (CPLX) in water samples. The concentration of the analytes in aqueous solution and real water samples was determined using high-performance liquid chromatography coupled with diode array detection (HPLC-DAD). The developed method for CFDX and CPLX in various water samples exhibited relative linear ranges of 0.1–600 μg/L and 0.1–550 μg/L, respectively, and correlation coefficients ranging from 0.9923–0.9993. Under optimum condition, the UA-D-μ-SPE/HPLC-DAD method displayed low limits of detection (LOD) and quantification (LOQ), with values ranging from 0.02–0.16 μg/L and 0.067–0.53 μg/L, respectively. The investigated intraday and interday accuracy in spiked water samples showed acceptable extraction efficiencies with a range of 73.5–98.1% and a relative standard deviation less than 6.0%. Moreover, the performance of MCM-41 was also assessed for the removal of cephalosporin antibiotics aqueous solutions. The kinetic and isotherm studies revealed that the adsorption process followed pseudo-second order and the Langmuir model, respectively. Furthermore, the maximum adsorption capacities for CFDX and CPLX were 43.2 and 47.8 mg/g. Lastly, the UA-D-μ-SPE/HPLC-DAD method was successfully applied in extracting, preconcentrating, separating and determining CFDX and CPLX in wastewater and surface water samples. These results demonstrated that MCM-41 can be applied in the removal of cephalosporin antibiotics from aquatic environments.

1. Introduction

Antibiotics are emerging pollutants that pose a serious environmental concern due to their widespread use, continuous release, and persistence in various environmental settings [1]. The main sources of antibiotics include households (discharge of expired and unused antibiotics), pharmaceutical industry, wastewater treatment plants (hospital and domestic), and human and animal excreta [2]. The discharge of effluents from these sources leads to elevated concentrations of antibiotic residues in surface water, thus posing notable ecological and public health risks [3]. Furthermore, environmental pollution by antibiotics leads to increasing broad-spectrum antibacterial drug resistance and risk of allergies, and subsequently causing significant negative economic and health impacts [4]. The World Health Organization (WHO) conducted an antibiotic resistance surveillance campaign because of the increasing concentrations of antibiotics in the

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environment. The results revealed that the most frequently used drugs from antibiotic classes such as carbapenems, cephalosporins, fluoroquinolones, and penicillins have developed bacterial resistance [4,5]. Cephalosporins are β-lactam antibiotics commonly used for the treatment of Gram-positive and negative bacterial diseases [4,6]. In case of allergic reactions or drug resistance, cephalosporins are mostly prescribed to complement penicillins [4]. As a result, cephalosporins are extensive use by both human and veterinary medicine, and their occurrence has been reported in various aquatic environments [1,6–10].

The determination of cephalosporins in various complex matrices has been achieved using chromatographic techniques such as high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) [11,12]. These chromatographic analyses have been used in tandem with sample preparation to increase the concentration of cephalosporins since they are often found in low concentrations which makes it difficult to detect and quantify. Solid phase extraction (SPE) has been extensively used as a sample preparation method for cephalosporins in complex sample matrices, owing to its high recovery, ease of process operation and use of different adsorbents. Recently, the dispersive micro-solid phase extraction (D-μ-SPE) has gained popularity over the traditional cartridge-based SPE method due to its simplicity, rapidity, and effectiveness [13,14]. In D-μ-SPE, the adsorbent material is dispersed in the sample to extract the analyte of interest, which is followed by the separation the adsorbent from the sample solution using techniques such as centrifugation [13]. The main advantages of using D-μ-SPE include short analysis time and enhanced interaction between the analytes and adsorbent due to increased surface contact [15,16].

The D-μ-SPE method is usually assisted by external energies such as ultrasonic radiation to facilitate fast and better extraction of analytes. Ultrasound-assisted DSPE increases mass transfer of analytes to the adsorbent and breaks the affinity between analytes and solution, which leads to enhanced extraction efficiency in a short time span [15,17]. The choice and characteristics of the adsorbent play a principal role in the effectiveness of D-μ-SPE. Therefore, it is not surprising that nano-adsorbents have been extensively employed as adsorbents due to their remarkable properties, such as large surface area and pore volume, high chemical activity, and low diffusional resistance. These properties lead to increased extraction efficiency and quick extraction dynamics. Various nanomaterials have been used as adsorbents in D-μ-SPE methods [13,18,19], but mesoporous silica nanoparticles (MSNs) such as MCM-41 and SBA-15 have drawn greater interest. This phenomenon is attributed to their large surface area, highly ordered structure, tuneable pore size, good thermal and chemical stability [20]. The highly organized and orderly structures of MSNs allow selective adsorption of various analytes when they are used as adsorbents in sample preparation methods [21–25].

Previous studies have used MSNs and their composites for extracting and removing cephalosporins from various matrices including wastewater [2,26–28]. MSNs were applied to remove cefadroxil, cefprozil, and cefradine from industrial wastewater and displayed high percentage removal ranging from 95.2–102% [2]. Another study also employed MCM-41 to remove cephalexin from simulated wastewater [27]. On the other hand, Magnetic porous silica microspheres were explored for magnetic solid-phase extraction of cephalosporins in wastewater before analysis by HPLC [26]. This method proved to have a relatively high sensitivity because of low detection limits (0.045–0.11 μg/L) which may be partly attributed to the large adsorptive sites of the magnetic porous silica microspheres [26]. Furthermore, many studies utilised MSNs as adsorbents in preconcentration or removal of other classes of antibiotics from water [23,28–31]. However, the application of MSNs in sample preparation and remediation of contaminants from real water still needs to be explored to improve sensitivity and efficiency.

Therefore, this work aimed to develop ultrasound-assisted D-μ-SPE (UA-D-μ-SPE) utilizing MCM-41 as an adsorbent to preconcentrate cefadroxil (CFDX) and cephalexin (CPLX) from environmental matrices (surface water and wastewater). The single factor, fractional factorial design, and response surface methodology (RSM) approaches were employed to optimise experimental parameters affecting the efficiency of UA-D-μ-SPE. The HPLC coupled with a diode array detector (HPLC-DAD) was selected for the separation and quantification of analytes. This study compared the performance of MCM-41 to other adsorbents used for preconcentrating and removing cephalosporins, and it showed better performance. This study further evaluated the feasibility and effectiveness of MCM-41 as an adsorbent in D-μ-SPE and removal of cephalosporins from real water samples. Our results demonstrated that MCM-41 is a promising adsorbent for trace analysis of cephalosporins and their adsorptive removal from real water.

2. Experimental

2.1. Reagents and materials

Ethylene glycol, Hexadecyltrimethylammonium bromide (CTAB, ≥98%), ammonium nitrate, ethanol (EtOH) and HPLC-grade methanol (MeOH) were used as purchased from Sigma-Aldrich. Tetraethyl orthosilicate (TEOS, 99.999%) and sodium hydroxide (NaOH) were bought from Alfa Aesar and PanReact, respectively. All reagents were used as acquired, without any further purification, and all solutions, unless otherwise indicated. Two first-generation cephalosporin antibiotics, cefadroxil and cephalexin, were employed for analysis and they were sourced from Sigma-Aldrich. Fresh working solutions were prepared before each analysis to ensure accurate and consistent concentration throughout the experiments. This involved diluting the initial stock solutions with ultrapure water produced by the Direct-Q® 3UV-R purification system from Millipore (Merck, Germany).

2.2. Instrumentation and chromatographic conditions

Chromatographic analysis employed an Agilent 1200 Infinity HPLC system with a photodiode array detector from Agilent Technologies (Waldbronn, Germany). A chromatography method was developed using a C18 column and an isocratic elution method that consisted of methanol and water in specific proportions (50:50, v/v) flowing at the rate of 1.00 mL/min; 10 μL sample volume was injected. Various wavelengths (214, 254, 270, and 300 nm) were tested, and it was found that 254 nm offered a favourable combination of strong chromatographic response and high sensitivity. The calibration curves for cefadroxil and cephalexin were created by preparing eight standard solutions of a mixture in a concentration range of 10–1000 μg/ml. This was achieved by diluting the working solution with an appropriate amount of methanol.

Nanoparticle size distributions and zeta potential were measured using a dynamic light scattering Malvern Nano Zetasizer, with a 633 nm laser diode, from the PROTEOMASS-BIOSCOPE facility (Caparica, Portugal). Pore size distribution and surface areas were determined by N_2 adsorption/desorption at 77 K in a Micromeritics ASAP 2010 (Micrometrics Instrument Corporation, Norcross, USA) (Accelerated Surface Area and Porosimetry), at the Laboratory of Analysis LAQV-REQUIMTE at the FCT—UNL. Specific surface areas (SBET) and pore volume were estimated using Brunauer Emmett and Teller (BET) and Barrett-Joyner-Halenda (BJH) methods. Fourier Transform infrared spectra (FT-IR) were collected on an Alpha II (Bruker, Massachusetts, USA). Transmission electron microscopy (TEM) images were recorded using a JEOL JEM 2100 80–200 kV at the i3S – Instituto de Investigação e Inovação em Saúde at the University of Porto facility (Portugal).

2.3. Synthesis of mesoporous silica nanoparticles (MSNs)

MCM-41 type mesoporous silica nanoparticles were synthesized using the Stöber method with some modifications. Briefly, 150 mg of CTAB was dissolved in 30 mL Milli-Q H_2O at 50 °C under constant stirring. After 10 min, 10 mL of ethylene glycol and 700 µL of 1.0 mol/L NaOH were added, and the mixture was left under stirring at 80 °C for 30 min. Afterwards, 750 µL of TEOS was added dropwise to the reaction mixture, and it was left under constant stirring at 80 °C for 2 h and further left under stirring at room temperature for 12 h. The mixture was removed from stirring and washed twice with MeOH (2 × 10 mL). For template removal, the MSNs were resuspended in 20 mL of a 30 mg/mL ammonium nitrate methanolic solution and stirred for 1 h at 60 °C, followed by centrifugation and washing in MeOH. This procedure was repeated twice and confirmed by FTIR-ATR spectroscopy.

2.4. *Ultrasound-assisted dispersive micro-solid phase extraction (UA-D-µ-SPE) procedure*

The UA-D-µ-SPE of analytes was carried out by placing an appropriate mass (10–30 mg) of MCM-41 adsorbent on 50 mL amber sample bottle, followed by adding of 20 mL simulated sample (adjusted to pH 2–9) containing 100 µg/L of CFDX and CPLX. This mixture was placed into an ultrasonic bath and sonicated for 5–30 min and then centrifuged at 3500 rpm for 5 min. The supernatant was discarded, and the analytes were eluted by adding 500–1000 µL of an appropriate eluent solution and sonicating the mixture for 5–15 min. Subsequently, the eluent and the adsorbent were separated by centrifuging the mixture at 3500 rpm for 5 min. Eventually, the eluent was filtered through a 0.22 µm PVDF membrane filter prior to analysis by HPLC-DAD.

2.5. *Design of experiments*

The optimization study in this research employed a combination of univariate and multivariate optimization approaches to identify the optimal conditions for the adsorption and extraction of cephalosporin antibiotics using MCM-41 adsorbent. The univariate optimization approach was initially used to investigate the influence of pH on the performance of the adsorbent. This method allowed for a systematic evaluation of how pH affects the adsorption efficiency, revealing its significant impact on the extraction process. Once the importance of pH was established, multivariate optimization approaches, specifically fractional factorial design (FrFD) and central composite design (CCD), were implemented to screen and optimize the most influential factors. FrFD was used to identify significant factors among sample pH, eluent volume, mass of adsorbent, extraction time, and elution time. This method efficiently narrowed down the critical parameters affecting the extraction efficiency. Subsequently, CCD was employed to further refine the optimal conditions for the selected factors, providing a more comprehensive understanding of their interactions and effects on the adsorption process. The combination of both these methos (OVAT and DOE) allowed for a systematic and efficient optimization of the UA-D-µ-SPE method, resulting in the identification of the optimal conditions for quantitative extraction and recovery of cephalosporin antibiotics.

2.5.1. *Univariate optimization*

Univariate optimization method was carried using the method described in Section 2.4. This approach was used to assess the performance of the adsorbent at different pH values (5, 7 and 9) while the mass of adsorbents, eluent type, eluent volume, extraction and elution times were 15 mg, methanol, 1000 µL, 30 min and 10 min, respectively. In addition, the selection of suitable eluent types, including MeOH, ACN, ACN-MeOH (50:50, v/v), MeOH-0.1%FA (45:55, v/v), ACN-0.1%FA (45:55, v/v) and MeOH-CAN-0.1%FA (25:25:50, v/v) was evaluated using OFAT approach. In these experiments, the sample pH, mass of adsorbents, extraction time, eluent volume and elution time were fixed at 5, 15 mg, 30 min, 1000 µL and 10 min, respectively.

2.5.2. *Screening of significant variables affecting the performance of MCM-41 based UA-D-µ-SPE using fractional factorial design*

The fractional factorial design (FrFD) (2^{5-2}) was used to investigate the effects of the five independent variables, such as sample pH, mass of adsorbents, extraction time, eluent volume and elution time, on the extraction and preconcentration of cefadroxil and cephalexin. Eleven experiments were generated using FrFD and were performed in triplicates in a random manner. The independent variable levels and actual values are presented in Table S1.

2.5.3. *Optimisation of MCM-41 based UA-D-µ-SPE method using central composite design*

The most significant experimental conditions for the UA-D-µ-SPE method were optimised using a central composite design (CCD). The significant independent variables were the sample pH, the extraction time and the eluent volume. Sixten experiments were generated using CCD and were carried out randomly in triplicates. The independent variable levels and the experimental domain are shown in Table S2.

2.6. *Adsorption isotherm and adsorption kinetics studies*

Under optimum conditions, the absorption efficiency of MCM-41 adsorbent was investigated by adding 20 mL of cefadroxil and cephalexin solution at different concentration levels (1–10 mg/L) into sample bottles with 20 mg of the adsorbent and sonicating the mixture for 17.5 min. The residual analyte concentrations in the solution were determined using the HPLC-DAD method after the separation and elution step using sonication and centrifugation, sequentially. The kinetics studies were performed by dispersing 20 mg MCM-41 adsorbent in 20 mL of cefadroxil and cephalexin solution (10 mg/L). The samples were sonicated at different time intervals (5–60 min). The adsorbent and the sample solution were separated via centrifugation. The concentration of the analytes in the solution was determined using the HPLC-DAD method. Eqs. (1) and (2) were used to calculate the amount of cefadroxil and cephalexin adsorbed onto MCM-41 adsorbent.

$$q_e = \frac{(C_0 - C_e)V}{m} \tag{1}$$

Where q_e (mg/g), V (L), and m (g) were the adsorption capacity, the volume of the solution, and the mass of MCM-41, respectively, C_0 and C_e were the initial and equilibrium concentrations of cefadroxil and cephalexin.

$$q_t = \frac{(C_0 - C_t)V}{m} \tag{2}$$

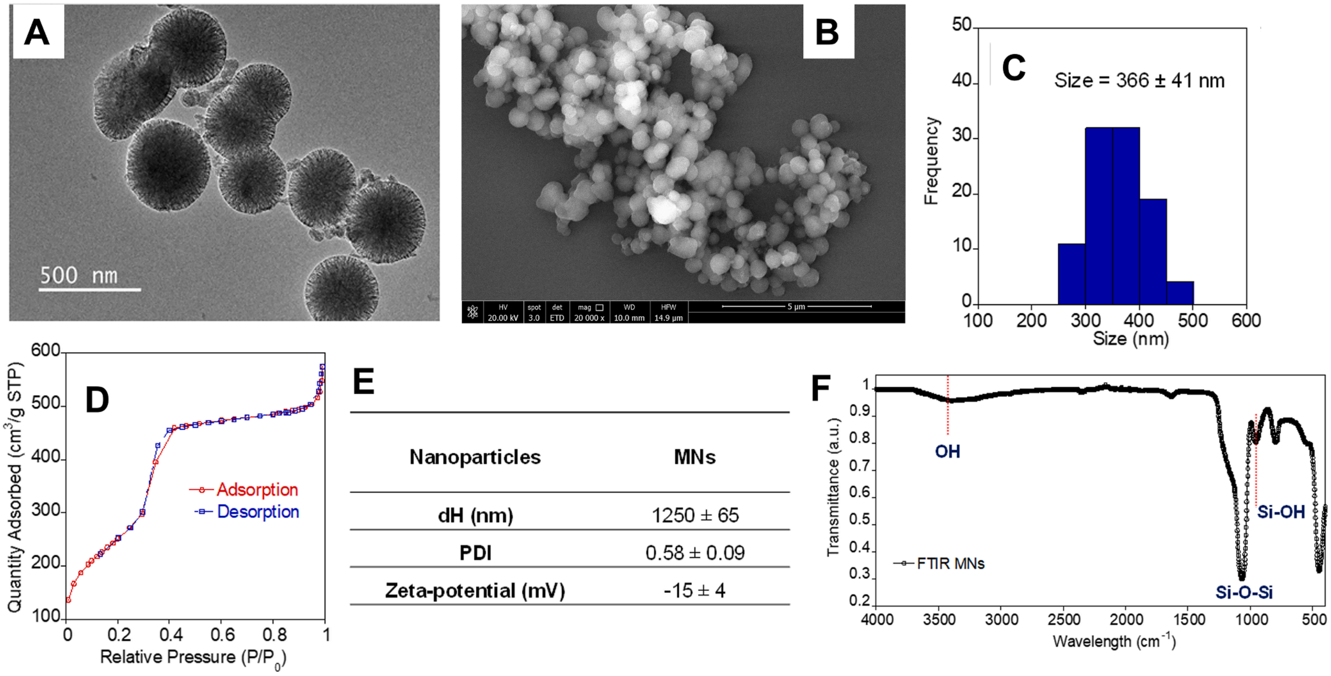
Where qt (mg/g) and C_t were the adsorption capacity and equilibrium concentration at time t.

2.7. *Method validation*

The validity and applicability of the optimized UA-D-µ-SPE/HPLC-DAD method was evaluated in terms of specificity, linearity, limits of detection (LOD), accuracy, limits of quantitation (LOQ), matrix effect (ME) and precision.

2.7.1. *Linearity, LODs, LOQs, specificity and matrix effect (ME)*

The linearity of the method was evaluated by spiking a series of blank surface and tap water samples (free of cefadroxil and cephalexin) with different concentrations (1–1000 µg/L) of cefadroxil and cephalexin, and each concentration level was analysed in triplicates. The calibration curve for each analyte was constructed by plotting the corresponding peak area obtained after preconcentration against concentration at six levels. The specificity of the developed UA-D-µ-SPE/HPLC-DAD method was determined by analysing the blank solvent, spiked ultrapure, tap and surface water samples. The selectivity of the UA-D-µ-SPE/HPLC-DAD method was evaluated by comparing the retention times of analytes on the chromatogram produced from spiked ultrapure water and spiked surface water samples. On the other hand, the ME of the method was determined by comparing the slopes of the calibration



Nanoparticles	MNs
dH (nm)	1250 ± 65
PDI	0.58 ± 0.09
Zeta-potential (mV)	-15 ± 4

Fig. 1. TEM image (A), SEM image (B), Size histogram (C), N_2 Isotherms (D), Size and Zeta Potential (E), and FTIR spectrum (F) of MSNs particles.

curves made in ultrapure water and the matrix, which were constructed by spiking the samples with known antibiotic concentrations (1–500 μg/L) followed by analysis using the optimised method. The ME was calculated according to the previous studies [32]. The LODs and LOQs of the UA-D-μ-SPE/HPLC-DAD method were assessed by extracting and preconcentrating water samples spiked at 0.1 μg/L ($n = 10$). Thereafter, LOD and LOQ were calculated based on 3 and 10 times the standard deviation of the signal (peak area) of 1 μg/L of CFDX and CPLX.

The detection limits for each analyte were calculated by multiplying the minimum detectable concentration by 3 and 10 times the standard deviation.

2.7.2. *Precision and accuracy*

The accuracy and precision of the UA-D-μ-SPE/HPLC-DAD method were evaluated by spiking surface water and wastewater (influent and effluent) at three levels with known standard concentrations (1.0, 2.5 and 5.0 μg/L) of CFDX and CPLX. The accuracy and precision were expressed as percentage extraction efficiency (%EE) and relative standard deviation (%RSD). For intra-day accuracy and precision, ten measurements of real water samples spiked at three concentration levels were analyzed on the same day. On the other hand, the inter-day accuracy and precision were evaluated by analysing spiked real water samples in triplicate for five consecutive days.

2.7.3. *Quality assurance/quality control (QA/QC) procedure*

The QA/QC was conducted to guarantee the reliability of the results obtained using the UA-D-μ-SPE/HPLC-DAD method. To avoid cross-contamination, all the glassware used in this study was pre-cleaned with ultrapure water and methanol. The stability of the HPLC-DAD method was assessed by analysing spiked tap water samples (50 μg/L) after a batch of 5 or 10 samples, depending on the number of samples to be analysed. Furthermore, the procedure blank samples, processed the same way as samples, were analysed before and after every batch of samples. Procedure blank samples were analysed before and after each batch of samples. This was done to assess whether the samples were contaminated during sample pretreatment and analysis.

2.8. *Application to real samples*

The real environmental samples, surface water (upstream and downstream), tap water and wastewater (influent and effluent), were used to evaluate the applicability of the optimised UA-D-μ-SPE/HPLC-DAD method. The wastewater and receiving surface water samples were collected from Gauteng (Pretoria) and KwaZulu Natal (Durban and Pietermaritzburg) Provinces in South Africa. Tap water was collected from one of the laboratories at the Department of Chemical Sciences, University of Johannesburg (Johannesburg, South Africa). The real environmental samples were filtered through 0.22 μm PVDF syringe membrane and kept in amber sample glass containers at 4 °C for subsequent UA-D-μ-SPE analysis.

3. Results and discussion

3.1. *Characterization of mesoporous silica nanoparticles*

Mesoporous silica nanoparticles were synthesized using an adapted version of the well-established and reported Stöber method, also known as the sol-gel method [33–35]. In this process, tetraethyl orthosilicate (TEOS) was used as the silica source, cetyltrimethylammonium bromide (CTAB) as a cationic templating surfactant, ethylene glycol as a stabilizer, and sodium hydroxide (NaOH) as a reducing and morphological agent. The resulting MSNs powder was characterized using various techniques, including dynamic light scattering (DLS), zeta potential analysis, Fourier-transform infrared spectroscopy (FTIR-ATR), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and nitrogen adsorption-desorption isotherms. (Fig. 1).

TEM and SEM images shown in Fig. 1(a&b) confirmed the spherical shape of the MSNs particles and revealed the presence of pores within the silica matrix. Fig. 1(c) shows the average size of the MSNs which was determined to be 366 ± 41 nm from a TEM image. Furthermore, TEM also reveals that the particles are dispersed which mean during the extraction process the entire surface is accessible for the sorption of CFDX and CPLX. Nitrogen adsorption/desorption analysis further confirmed the porosity of the MSNs, showing a type IV isotherm characteristic of mesoporous materials, Fig. 1(d). The calculated pore size

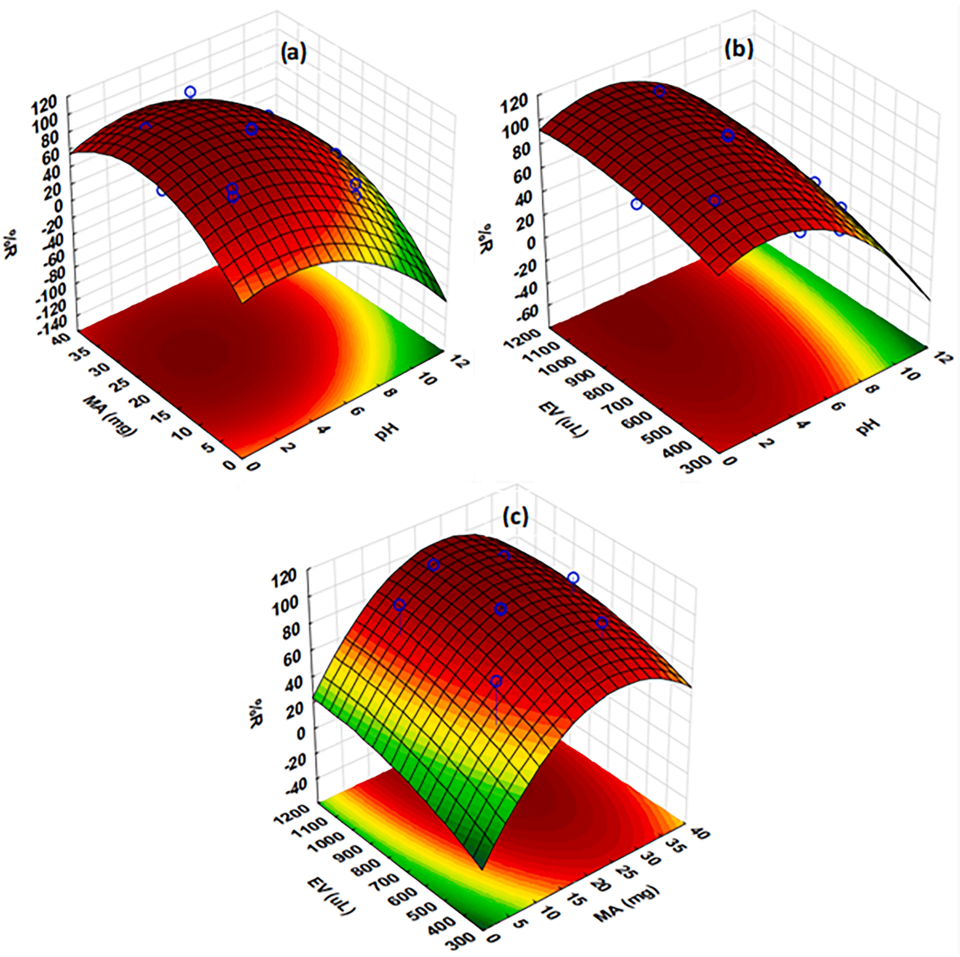


Fig. 2. 3D response surface plots from CCD for extraction and preconcentration of cephalexin and cefadroxil. Interactive effects of (a) extraction time (ET) and sample pH, (b) eluent volume (EV) and pH, as well as (b) ET and EV on analytical response.

from these curves was 3.4 nm, falling within the typical range for MCM-41-type MSNs. The surface area of the MSNs was estimated using the BET model, yielding a relatively high value of approximately 943 m^2/g, which aligns with the mesoporous structure of the nanoparticles. The mesoporosity will ensure there is fast diffusion and large surface area will provide abundant sorptive sites for CFDX and CPLX during D-μ-SPE.

The hydrodynamic diameter and zeta potential of the particles were measured as 1250 ± 65 nm and -15 ± 4 mV, respectively, with a polydispersity index of 0.58 ± 0.09. These values indicate some particle instability, as they tend to aggregate over time, as seen in Fig. 1(e). The negative charge observed is attributed to the presence of silanol groups on the particle surface, as confirmed by FTIR-ATR spectroscopy. The FTIR spectrum (Fig. 1(f) exhibited characteristic peaks associated with the MSNs, including a peak at 3409 cm^{-1} corresponding to O-H vibration, peaks at 1069 cm^{-1} and 960 cm^{-1} related to Si-O-Si and Si-O-H vibrations, respectively, and a peak at 801 cm^{-1} corresponding to Si-O-Si symmetric stretching vibrations. These peaks indicate the purity of the nanoparticles and the presence of silanol and siloxane groups in their composition. The absence of peaks in the 2800–2900 cm^{-1} range confirms the successful removal of the template used during synthesis.

3.2. Optimization of UA-D-μ-SPE conditions

3.2.1. Performance of the adsorbent at different pH values

The pH of the solution can influence the performance of an adsorbent for adsorption of cefadroxil and cephalexin. This is because sample pH tends to affect the surface charge of the adsorbent and the ionization of the functional groups of the analytes. The results showing the performance of the MCM-41 adsorbent on the adsorption of CFDX and CPLX at different pH values are presented in Fig. 2. The adsorption efficiency of MCM-41 adsorbent was highest at pH 5, followed by a decrease in extraction efficiency at pH values higher than 5.0. These results suggest that the adsorption of CFDX and CPLX onto MCM-41 is strongly dependent on the pH of the solution. MCM-41 is a mesoporous silica material with a negatively charged surface due to silanol groups, and this charge is influenced by the solution's pH. The analytes, CFDX and CPLX, are amphoteric, existing as cations at lower pH and shifting to neutral or anionic forms at higher pH. At low pH (e.g., pH 5), the negatively charged MCM-41 attracts the cationic forms of these analytes, enhancing adsorption efficiency. However, as pH increases, the competition for adsorption sites arises from the neutral or anionic forms of the analytes and other ions in solution. Additionally, pH affects hydration levels, which can influence adsorption interactions. To optimize adsorption, it is important to maintain a stable pH around 5, use buffering solutions to prevent fluctuations, and control ionic strength to minimize competitive interactions. The effect of pH on the extraction efficiency of the adsorbent is discussed in more detail in Section 3.2.4.

3.2.2. Selection of eluent solvent

To obtain a satisfactory analyte recovery or extraction efficiency, eluent type and composition plays a significant role in desorbing the adsorbed cephalosporins onto the surface of the MSN adsorbent. In addition, it was observed in Section 3.2.1 that the adsorption of the analytes on the surface of the adsorbent was pH-dependent. Therefore,

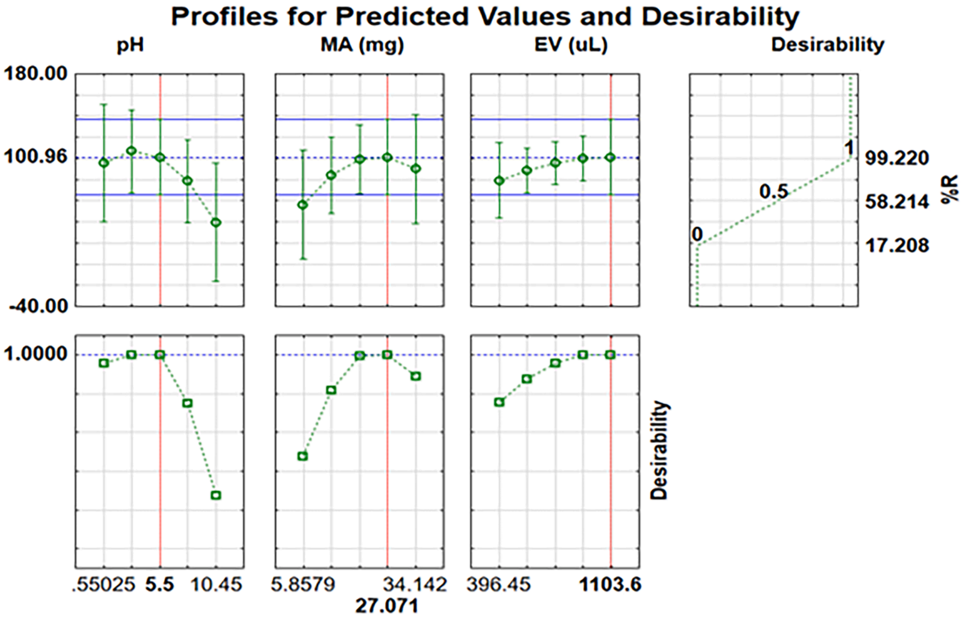


Fig. 3. Profiles for predicated values and desirability for preconcentration of selected cephalosporin antibiotic residues using UA-D-μ-SPE method.

the elution of the CFDX and CPLX was investigated by changing the eluent pH. In this study, the capabilities of a series of elution solvents, including methanol, acetonitrile, acetonitrile-methanol (50:50, v/v), methanol-water (45:55, v/v), acetonitrile-water (45:55, v/v), methanol-%formic acid (45:55, v/v), and acetonitrile-%formic acid (45:55, v/v), were investigated. As shown in Figure S2, pure solvents and their combination were not suitable for the desorption of the analytes. However, the addition of 0.1% formic acid to either ACN, MeOH or MeOH-ACN significantly improved the elution of the target analytes. These results suggest that the acidified desorption solvent was able to penetrate the pores of MCM-41 and destroy the strong interactions between the analytes and adsorbent. Furthermore, the combination of the strong elution strength of ACN and the high dissolution ability of MeOH towards the analytes led to high desorption efficiency. The results in Figure S2 indicated that MeOH-ACN- 0.1%FA (25:25:50, v/v) mixture as an elution solvent yielded high and satisfactory recoveries of the target analytes. Therefore, MeOH-ACN-0.1%FA (25:25:50, v/v) was selected for further experiments.

3.2.3. *Fractional factorial design*

A two-level FrFD comprising twelve (2^{5-2}) experiments was used to assess the importance of five independent variables on UA-D-μ-SPE performance. To minimise the uncontrollable parameters during the design of experiments, they were carried out in a randomized manner. The design matrix and respective analytical response values obtained for all experiments are shown in Table S3. The experimental error was evaluated by adding four central points. The experimental data in Table S3 were assessed using the software Statistica software version 14. The significance of the independent variables considered in the screening step using FrFD is assessed using analysis of variance (ANOVA) illustrated in the form of Pareto charts of standardized effects. As seen in Figure S3, the sample pH had a significant negative effect on the analytical response. This might be explained by considering that the sample pH influences the interactions between the adsorbent and analytes. The results suggest that decreasing the sample pH increases the extraction efficiency of the adsorbent, thus increasing the analytical response. In contrast, increasing eluent volume proved to have a positive influence on the analytical response. These observations suggest that quantitative recovery of the analytes required high eluent volumes. Figure S3(b) shows that increasing the mass of adsorbent has positive effects on the adsorption efficiency of the analytes. This might be due to the increase in the adsorption active sites on the surface of the adsorbent, thus enhancing the interactions between the adsorbent and the analytes. However, this mass of adsorbent was not significant for the adsorption of CPLX because it had slightly different chemical properties compared to CFDX, which could result in favourable extraction toward MCM-41. Other factors, such as extraction and elution times, were not significant at a 95% confidence level. Therefore, mass of adsorbent, sample pH and eluent volume were significant parameters for UA-D-μ-SPE of CFDX and CPLX and their optimum conditions were further investigated using the response surface methodology (RSM) based on the CCD optimization approach. Extraction and elution times were fixed at their central point values, which are 17.5 min and 10 min.

3.2.4. *Central composite design for optimization of most influential factors*

The central composite design was used to understand better the effect of the significant factors that influence the performance of the UA-D-μ-SPE method. Additionally, the RSM based on CCD enabled the exploration of the interactive effects, quadratic effects, and main effects on the extraction and preconcentration of cephalosporin antibiotics. As discussed earlier, the three most influential factors were the mass of adsorbent, sample pH and eluent volume while fixing extraction and elution times at values determined according to the sign of their main effect (Figure S3). Table S4 shows the CCD matrix for the significant variables selected from the screening step and their respective analytical response of each experimental run.

The 3D response surface plots were used for the assessment of the main and interactive effects of the independent variables on the analytical response (Fig. 2). Fig. 2 (a) shows that the interaction between sample pH and mass of adsorbent played an important role in the adsorption and extraction of the analytes. As seen, the recovery of cephalosporin antibiotic residues increased with increasing sample pH and the mass of the adsorbent. Increasing mass of adsorbent increases the number of adsorption active sites that are available for interaction with analytes in the sample solution (Fig. 2 (a&c)).

Fig. 2 (a&b) presents the effect of pH on the extraction of CFDX and CPLX from the sample solution. The results show that the increase in sample pH up to 7 led to better CFDX and CPLX recoveries. Sample pH influences the surface charge of the adsorbent and the chemical speciation of the analytes. According to Fig. 1 (e), the adsorbent had a net

Table 1
Analytical performances of the developed UA-D-μ-SPE/HPLC-DAD method for the determination of selected cephalosporin antibiotic residues in water (n = 3).

Analytes	Sample matrix	Linear range (μg/L)	Regression equation	R^2	LODs (μg/L)	LOQs(μg/L)	%ME
CFDX	Ultrapure water	0.1–500	$Y = 1.943x + 4.123$	0.9991	0.020	0.067	-
	Tap water	0.3–450	$y = 1.756x + 12.05$	0.9978	0.087	0.29	-9.62
	Surface water	0.5–600	$y = 1.569x + 15.16$	0.9923	0.15	0.50	-19.4
CPLX	Ultrapure water	0.1–550	$y = 2.418x + 12.16$	0.9993	0.024	0.080	-
	Tap water	0.3–500	$y = 2.105x + 10.21$	0.9967	0.079	0.26	-12.9
	Surface water	0.5–450	$y = 1.987x + 17.06$	0.9934	0.14	0.47	-17.8

negative charge attributed to the presence of silanol groups, suggesting that changing the pH of the solution from lowest to highest does not affect the surface charge of MCM-41; it remains negatively charged. The CFDX and CPLX have more than one pKa value. For instance, CFDX has three pKa values of 3.45, 7.22 and 9.48 assigned to carboxylic acid, phenylglycine amino and phenolic groups, respectively [36].

On the other hand, CPLX has two pKa values of 2.56 and 6.88, corresponding to the amine and carboxyl group [37]. The CFDX and CPLX molecules exist in the anionic forms at pH values higher than their respective pKa values. In contrast, the amino/amine groups of CFDX and CPLX are protonated at pH values between 0.5 and 7, leading to high extraction efficiency (high recoveries) due to electrostatic attraction between the analytes and the negatively charged adsorbent. Relatively lower recoveries at more acidic conditions were due to high concentrations of hydronium ions, which competed with protonated species of the analytes for the adsorption sites. With pH values higher than 7, the recoveries decreased drastically due to the electrostatic repulsion anionic species of the analytes and the negatively charged surface of the adsorbent. The combined interactive effects of the mass of adsorbent and eluent volume on the %R showed that the analytical response increased with increasing eluent volume (Fig. 2(c)). This suggests that higher eluent volumes allow the dispersibility of adsorbent, thus enhancing the recovery of the analytes from the surface of the adsorbent. Low eluent volumes led to low recoveries, suggesting there was incomplete elution of the analytes.

Based on the discussion above, the possible mechanisms of interaction between MCM-41 and CFDX and CPLX involves electrostatic interactions and hydrogen bonding. MCM-41 possesses negatively charged surface due to the presence of silanol group ((Si-OH), which then can attract the cationic forms of cephalexin and cefadroxil, which predominate at lower pH values. This electrostatic interaction is likely the primary driving force for adsorption. Furthermore, functional groups like amide and hydroxyl groups that are present on both the sorbent and analyte surfaces might promote hydrogen bonding, which will improve the contact and adsorption even more.

The profiles of the predicted values and desirability for preconcentration of CFDX and CPLX using the UA-D-μ-SPE method are presented in Fig. 3. The desirability score of 1 was used to estimate the optimum conditions of the UA-D-μ-SPE method. Considering the results in Fig. 3, quantitative extraction, preconcentration and recovery of the analytes by the developed method was achieved at pH of 5.5, 27 mg mass of sorbent and 1000 μL of MeOH-ACN-0.1% FA (25:25:50, v/v) mixture. Therefore, the overall optimal conditions for UA-D-μ-SPE adsorbent, extraction time, eluent volume and elution times, respectively. At these optimum conditions, the predicted percentage recoveries of the target analytes were 101% and 99.7% for CFDX and CPLX, respectively. Under similar optimum conditions, six replicate experiments were performed to verify the reliability of the optimization strategy. The experimental % R values were found to be 98.7±3.4 % and 99.1±3.1% for CFDX and CPLX. The obtained experimental values were closely related to predicted %R values, revealing that the RSM optimization approach was reliable.

3.3. Adsorption isotherm and adsorption kinetics studies

The adsorption of CFDX and CPLX on MCM-41 adsorbent was studied using different initial concentrations of the analytes. This was performed to assess the maximum adsorption capacity of the adsorbent

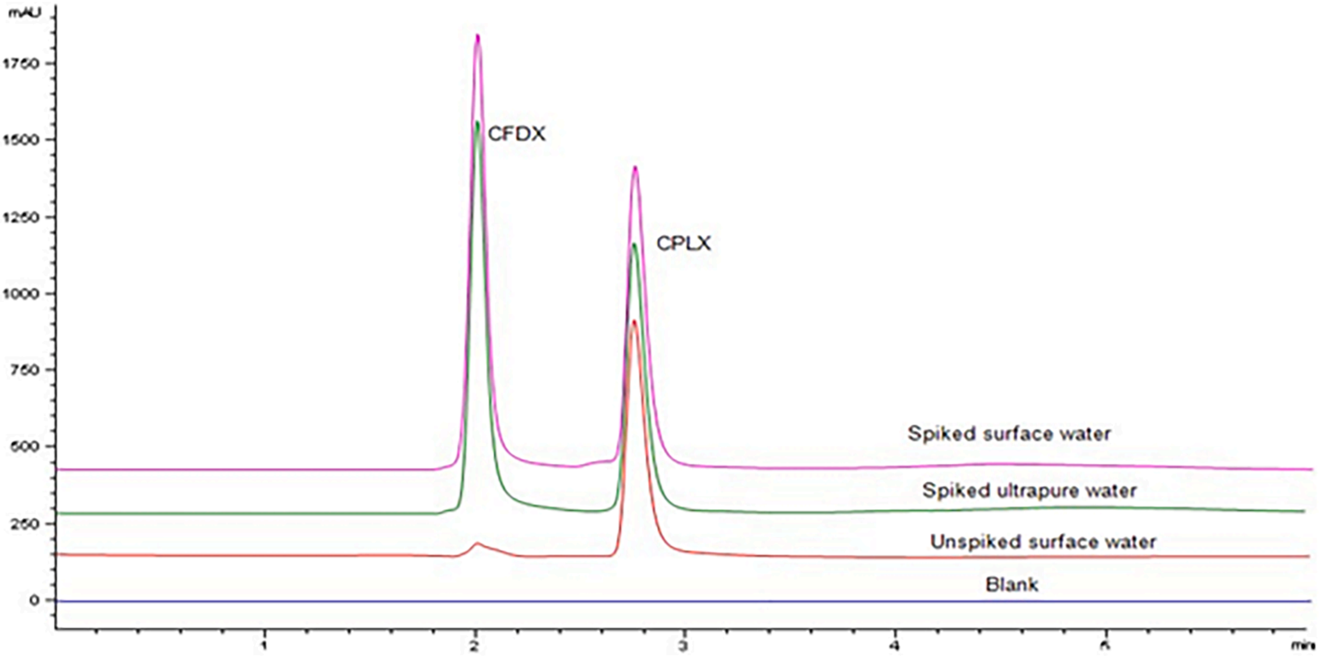


Fig. 4. Chromatograms obtained for preconcentration of cephalosporin antibiotics using UA-D-μ-SPE: Evaluation of the specificity of the developed method.

(Figure S4A), and the adsorption isotherm data was fitted to Langmuir and Freundlich models (Table S5). Using the correlation of determination (R^2), the results in Table S5 clearly show that the Langmuir model describes the adsorption process better than the Freundlich model. These findings suggest that the adsorption of the CFDX and CPLX onto MCM-41 surface was dominated by homogeneous monolayer adsorption. The maximum adsorption capacities of the adsorbent estimated using the Langmuir model were 43.2 and 47.8 mg/g for CFDX and CPLX.

The kinetics of CFDX and CPLX on MCM-41 adsorbent were studied to investigate the adsorption rate. Figure S4 shows that a fast adsorption rate was observed between 5 and 15 min, followed by a slow rate towards reaching the equilibrium. The adsorption data in Figure S4 was fitted to two kinetics models (pseudo-first order and pseudo-second order). The pseudo-first-order and pseudo-second-order parameters are shown in Table S5. As shown, the kinetics data for both analytes were better explained by pseudo-second-order model (0.9994–0.9997) because it had a higher correlation of determination compared to the pseudo-first-order model (0.6173–0.6440). Moreover, the calculated sorption capacity attained from the pseudo-second-order regression was closer to the experimental adsorption capacities (Table S5). These results suggest that the adsorption process of cephalosporin antibiotics on mesoporous adsorbent was governed by chemisorption.

3.4. Method validation

The feasibility of the developed UA-D-μ-SPE/HPLC-DAD method was validated by evaluating various relevant analytical performance parameters described in Section 2.7. Under optimum conditions, linearity, precision, specificity, LODs, LOQs, accuracy and matrix effects were evaluated for each analyte.

3.4.1. Linearity, LODs and LOQs, specificity and matrix effects

The linearity, LODs and LOQs examined using different sample matrices are presented in Table 1. As shown, LODs and LOQs differ slightly depending on the sample matrix. Therefore, the sensitivity of the developed UA-D-μ-SPE/HPLC-DAD method for preconcentration and quantification of CFDX and CPLX in different sample matrices in terms of LODs and LOQs ranged from 0.02–0.087 μg/L and 0.079–0.53 μg/L, respectively. In addition, the linearity parameters such as linear range, regression equation and coefficients of determination were comparable across different sample matrices, suggesting that the UA-D-μ-SPE method served as a sample clean-up step by eliminating possible interfering ions. In general, the linear range of the target analytes was 0.1–600 μg/L, with R^2 values ranging from 0.9923–0.9993, demonstrating suitable linearity.

Fig. 4 presents the typical chromatograms of spiked surface water samples superimposed with unspiked surface water, spiked ultrapure and procedure blank samples obtained via the UA-D-μ-SPE/HPLC-DAD method. As shown in Fig. 4, the UA-D-μ-SPE/HPLC-DAD method had a satisfactory specificity since there were no peaks belonging to interfering ions detected within the ±3% retention time range of the target analytes. In addition, the variation of retention times of the CFDX and CPLX in surface water samples was not greater than ±5% when compared to the retention times of the analytes in ultrapure water. These findings suggest that the D-μ-SPE/HPLC-DAD method had good specificity, and it could be concluded that the analytical procedure could differentiate between the target analytes and interfering substances.

The matrix effect (%ME) obtained by comparing the slopes obtained using ultrapure water and in spiked tap and surface water samples are presented in Table 1. As seen, %ME ranged from -9.62% to -12.9% and 17.8% to -19.4% in tap and surface water. It should be noted that negative values are indicative of signal suppression. The %ME were within ± 20%, suggesting that the developed D-μ-SPE procedure could eliminate the interferences. In addition, these results suggest that HPLC-DAD could differentiate between the co-eluted interfering substances and the target analytes.

Table 2
Concentration (μg/L) of CFDX and CPLX in environmental water samples using UA-D-μ-SPE/HPLC-DAD method.

Samples	UA-D-μ-SPE/HPLC-DAD	
	CFDX	CPLX
Surface water 1 upstream	<LOQ	<LOQ
Surface water 1 downstream	0.87±0.03	1.2±0.1
WWTP Effluent 1	1.4±0.1	4.7±0.2
WWTP influent 1	3.9±0.2	6.8±0.4
Surface water 2 upstream	<LOQ	<LOQ
Surface water 2 downstream	<LOQ	1.7±0.1
WWTP Effluent 2	0.78±0.05	5.5±0.3
WWTP influent 2	1.5±0.1	7.3±0.5

LOQ= 0.5 μg/L.

Table 3
Concentration (μg/L) of cefadroxil and cephalexin in environmental water bodies from other countries in the world.

Country	Analytes	WWTP influent	WWTP effluent	Surface water	Refs
Iran	Cephalexin	0.122–0.9777	0- 0.02893	0- 0.1844	[9]
Iran	Cephalexin	29.7–655.2	4.24–116.4	-	[38]
China	Cephalexin	670–1200	240–980	-	[39]
China (Hong Kong)	Cephalexin	0–64.0	0–5.1	0–0.182	[40]
Australia	Cephalexin	55–315	ND-40	-	[41]
Australia	Cephalexin	0–2 μg/L	0–0.078	-	[42]
United States	Cephalexin	ND-13.8	ND-5.62	-	[43]
South Africa	Cefadroxil, cephalexin	1.54–3.88, 6.78–7.33	0.78–1.37, 4.66–5.45	<LOD-0.87, <LOQ-1.69	This work

3.4.2. Precision and accuracy

The precision and accuracy of the UA-D-μ-SPE/HPLC-DAD method was evaluated based on the %RSD and %EE in intraday and interday investigations. As seen in Table S6, the interday and interday %EE from 73.5–98.1%. Lowest but acceptable extraction efficiencies were obtained when the samples were spiked with the lowest concentration of the analytes. Intraday and interday %RSDs ranged from 2.5–4.7% and 4.1–5.6%, suggesting that the UA-D-μ-SPE/HPLC-DAD method could be used for routine analysis. Therefore, these results affirm that the UA-D-μ-SPE/HPLC-DAD method was trustworthy and suitable for the determination of CFDX and CPLX in complex matrices.

3.5. Application to water samples

The UA-D-μ-SPE/HPLC-DAD method was applied for the quantification of CFDX and CPLX in surface water and influent and effluent wastewater samples. The concentrations (n = 4 for each sample determination) of the target analytes in the samples are summarized in Table 2. As seen, CFDX was detected in all the wastewater samples, and its concentrations were < LOD in surface water samples except in one sampling site located downstream of the WWTP (Table 2). Moreover, CPLX residues were detected in wastewater and surface water samples located downstream of the WWTPs. The occurrence of CPLX residues in surface water suggests that WWTP effluents were the main source of antibiotics in receiving river water systems. These results revealed that the UA-D-μ-SPE/HPLC-DAD method was reliable, precise, accurate, highly sensitive and suitable for the determination of CFDX and CPLX in real samples.

Table 4

Comparison of the developed D-μ-SPE /HPLC-DAD procedure with other methods for the detection of cefadroxil and cephalexin.

Sample	Analytes	Method	Linearity (μg/L)	LODs(μg/L)	%EEs	%RSD	Refs
Wastewater	CFDX, CPLX	SPE-LC-ESI-MS/MS	0.08–250	0.0016- 0.0038	82–118	0.64–10.5	[6]
Surface water	CPLX	Syringe filter-SPE-HPLC-PDA	35–800	10	95–105	4.1–5.0	[44]
Surface water	CPLX	Fe_3O_4@N-3DFC-based MSPE/HPLC-DAD	0.5–100	0.2	81.6–101	0.11–5.69	[45]
Surface water	CPLX	CZE-LIF	-	0.00486- 0.015	87.7 - 104.6	4.82 - 6.1	[40]
River water	CPLX	MIPSBME-HPLC	15–240	3.0–10.0	86.5–97.8	-	[46]
Ultrapure, tap and surface water	CFDX and CPLX	D-μ-SPE /HPLC-DAD	0.1–550	0.020–0.16	73.5–98.1	2.5–5.6	This work

%EE= percentage extraction efficiency.

3.6. Global concentration of cefadroxil and cephalexin in environmental water bodies

Table 3 presents data on the occurrence of cephalexin, an antibiotic, in wastewater (influent and effluent) and surface water across various countries, including South Africa. The concentrations in influent were generally higher than in effluent, indicating partial removal during the wastewater treatment process. There is significant variability in cephalexin concentrations across the countries and even within the same country; this could be due to variations in antibiotic consumption practices, differences in wastewater efficiency, and environmental factors like dilution and degradation (Table 3). Iran shows the highest influent concentrations (29.7–655.2 μg/L), possibly due to higher antibiotic consumption or less efficient treatment systems. China shows high influent (670–1200 μg/L) and effluent (240–980 μg/L) concentrations, further highlighting the issue of antibiotic prevalence in wastewater. Lower levels are observed in Australia, potentially due to stricter regulations or different treatment practices. The concentration of the target analytes detected in South African wastewater systems was lower than those detected in other countries [31–34] and lower than those reported elsewhere [10,35]. However, the concentrations of the antibiotics were comparable to those reported by researchers in the United States [36].

3.7. Comparison of the developed method with previous studies

The effectiveness of the currently used method (UA-D-μ-SPE/HPLC-DAD) was compared with those that were previously reported for the extraction and preconcentration of cephalexin and cefadroxil antibiotics. Table 4 summarizes the parameters of both the currently used method and the methods that have already been published. The developed method showed lower LODs and RSDs compared to the recently published techniques. This indicates the high adsorption affinity of MCM-41 towards CFDX and CPLX at trace levels. However, the lowest LODs are achieved by SPE-LC-ESI-MS/MS and CZE-LIF compared to the developed method. Overall, D-μ-SPE/HPLC-DAD appears to be a promising approach for the analysis of CFDX and CPLX in various water samples. It offers advantages such as good linearity, low LODs, high extraction efficiencies, and acceptable RSDs. Additionally, it is a relatively simple and cost-effective method.

4. Conclusion

This study reported the application of MCM-41 as an adsorbent for UA-D-μ-SPE of CFDX and CPLX in water samples before HPLC-DAD analysis and removal of these antibiotics in aqueous solutions. Pseudo-second-order kinetic and Langmuir isotherm models described the adsorption process better than pseudo-first order and Freundlich models. The electrostatic attraction between the analytes and the negatively charged adsorbent enhanced the extraction efficiency of the MCM-41 sorbent. According to the Langmuir model, the maximum sorption capacities of MCM-41 for CFDX and CPLX were 43.2 mg/g and 47.8 mg/g. The UA-D-μ-SPE coupled with HPLC-DAD displayed low LODs, high precision (<6%) and acceptable specificity and accuracy (satisfactory %EE > 70%). Finally, the UA-D-μ-SPE/HPLC-DAD was successfully applied for the determination of CFDX and CPLX in real environmental water (wastewater and surface water) samples. Furthermore, the MCM-41 proved to be a promising adsorbent for trace analysis and wastewater remediation.

CRediT authorship contribution statement

Andisiwe Bangani: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mthokozisi Mnguni:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Thollwana Andretta Makhetha:** Writing – review & editing, Visualization, Validation, Supervision, Conceptualization. **Elisabete Oliveira:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **José Luis Capelo-Martínez:** Writing – review & editing, Visualization, Validation, Resources, Formal analysis, Data curation, Conceptualization. **Carlos Lodeiro:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Philiswa Nosizo Nomngongo:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

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Ethical Approval

We declare that we have no human participants, human data, or human tissues.

Consent to participate

Not applicable

Consent to Publish

Not applicable

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Supplementary materials

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Data availability

Data will be made available on request.

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