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Analyzes of β-lactam antibiotics by direct injection of environmental water samples into a functionalized graphene oxide-silica packed capillary extraction column online coupled to liquid chromatography tandem mass spectrometry

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ABSTRACT

An environmentally friendly and high-throughput method for monitoring β-lactam antibiotics in environmental water samples is presented. In this study, an in-lab synthesized graphene oxide supported onto aminopropyl silica ($GO@SiO_2$) sorbent was packed inside a fused silica capillary to produce a miniaturized extraction column. The main goal was to develop a greener analytical method that requires low amounts of chemicals to be executed and, consequently, generates a reduced volume of toxic waste. To achieve that, the $GO@SiO_2$ extraction column was online coupled with a LC-MS/MS instrument to carry out an automated and miniaturized sample preparation step before analytical quantification. A chemometric optimization was performed considering the variables possibly affecting the analytical performance: sample loading flow, sample loading time, and temperature of analysis. Under optimized conditions, the method reported good analysis times (12 min) and consumed fewer reagents compared to other publications on the same topic. Important validation parameters were evaluated accordingly with the ICH Q2(R1) validation guideline. The method showed appropriate linearity between 1 – 100 μg/L ($R^2 > 0.99$), as well as LODs ($S/N = 3$) in the range of 0.2 – 0.3 μg/L. Accuracy, intra- and inter-day precision (RSD%) were in acceptable ranges between 82.3 – 107.1%, and 0.8 – 20.6%, respectively. Out of ten collected environmental water samples, two reported the presence of cefalexin. Ultimately, the method was demonstrated to be fast, environmentally friendly, and reliable for monitoring the β-lactams. Therefore, it represents an excellent alternative to still widely-used and non-miniaturized methods based on time-consuming sample preparation.

1. Introduction

β-lactam antibiotics are effective pharmaceutical drugs for treating and preventing bacterial infections [1]. Due to their remarkable efficacy and safety profile, those semisynthetic penicillin-derived molecules have been extensively used since the '40s for successfully combating gram-positive and gram-negative bacteria in human and veterinary medicine [2]. Nevertheless, their massive use has led to concerns regarding their role as dangerous water pollutants. Most antibiotics administrated to humans or animals are excreted innately and then

Abbreviation: **C8**, octylsilane; **DAD**, diode-array detection; **DoE**, design of experiments; **d_p**, particle diameter; **EDC**, N-(3-dimethyl aminopropyl)-N′-ethyl carbodiimide; **EDTA**, ethylenediaminetetraacetic acid; **ESI**, electrospray ionization; **FA**, formic acid; **FS**, fused silica; **G**, graphene; **GO**, graphene oxide; **$GO@SiO_2$**, graphene oxide supported on aminopropyl silica; **H_2O_2**, hydrogen peroxide; **H_2SO_4**, sulfuric acid; **HCl**, hydrochloric acid; **ICH**, International Conference on harmonisation; **ID**, internal diameter; **IPA**, isopropanol; **$KMnO_4$**, potassium permanganate; **LC**, liquid chromatography; **MeCN**, acetonitrile; **MEPS**, microextraction by packed sorbent; **MRL**, maximum residue limit; **MS**, mass spectrometry; **MSPE**, magnetic solid-phase extraction; **N_2H_4**, hydrazine; **$NaNO_3$**, sodium nitrate; **NHS**, and hydrochloride/N-hydroxysuccinimide; **RGO**, reduced graphene oxide; **RSD**, relative standard deviation: **Si**, aminopropyl silica; **SPE**, solid-phase extraction; **SPME**, solid-phase microextraction; **SRM**, selected reaction monitoring; **TCF**, turbulent flow chromatography; **THF**, tetrahydrofuran; **UPLC**, ultra-performance liquid chromatography; **UV**, ultraviolet detection; **WWTPs**, wastewater treatment plants.

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poured into the water sources carried by the domestic sewage and runoff from livestock areas. The presence of antibiotics in the environment contributes to the appearance of resistant and more aggressive bacterial strains becoming a world-widely public health problem, primarily because of the potential risks to human health. Therefore, developing new techniques and methods for fast, sensitive, and reliable analysis of these compounds in waste and surface waters is a current analytical demand. In this niche, modern analytical methods can positively contribute to fields such as water quality monitoring and assisting tools for developing efficient remediation technologies.

β-lactams are susceptible to chemical and biological hydrolysis under environmental conditions and are present in waste and surficial waters samples at trace levels (ng/L – μg/L) [3]. Besides, waste and surface waters are complex matrices containing hundreds of other organic and inorganic pollutants, biomolecules, and particulate material. So, an effective quantitative determination of β-lactams by an analytical technique (e.g., liquid chromatography-tandem mass spectrometry) must be supported by an efficient sample preparation procedure, allowing the selective extraction of analytes with high recovery factors. Sample preparation is crucial to remove matrix-interfering compounds and isolate and pre-concentrate target analytes. Additionally, when carried out successfully, this critical step can sometimes improve the chromatographic and mass spectrometric well-functioning, mainly by providing a cleaner extract to be injected into the instrument.

To our knowledge, most reports describing the determination of β-lactam antibiotics in environmental water have been performed using offline and non-miniaturized sample preparation techniques, often employing commercially available sorbents as Lichrolute EN and Lichrolute C18 [4], Carbograph 4 [5], or OASIS HLB [3,6]. Although efficient, those SPE-based methods are laborious, time-consuming, and do not favor analytical throughput at the end. In addition, these traditional methods often involve several steps, demanding a significant amount of reagents and samples, consequently generating large volumes of toxic waste.

New insight into green sample preparation advocates miniaturized and automated microextraction to simplify procedures, reduce environmental impacts, and speed up the analytical throughput [7]. Among the modern sample preparation techniques, approaches based on column-switching outclass as promising alternatives for directly injecting raw samples into a fully automated workflow, minimizing sample handling [8]. Therefore, these methods can take advantage of positive characteristics from miniaturization and automation to create environmentally friendly analytical procedures. Miniaturized column-switching approaches employ a microextraction column online coupled to an LC column, assisted by a set of switching valves. This group of techniques includes online SPE [9], in-tube solid-phase microextraction (in-tube SPME) [10], and turbulent flow chromatography (TFC) [11].

Although initially introduced aiming mainly for the online treatment of biological samples, column-switching approaches have spread and found substantial applicability in other areas, such as food and environmental analysis [12–14]. Much research is currently devoted to developing new microextraction columns exploring alternative formats and emergent sorbent materials, aiming at more selective and faster analytical methods [15,16]. Among solid materials currently explored as alternative sorbents, graphene (G), graphene oxide (GO) and reduced graphene oxide (RGO) have demonstrated excellent recovery capabilities for many molecular structures. They have been used in different sorbent-based microextraction formats, such as SPME (solid-phase microextraction) [10], MEPS (microextraction by packed sorbent) [17], and MSPE (magnetic solid-phase extraction) [18]. In short, graphene is a carbon allotrope composed of monoatomic thickness layers of sp2 - hybridized carbon atoms in a honeycomb-like lattice. This two-dimensional structure harness graphene with a large surface area to interact with potential analytes on both sides of its structure. Also, an extensive delocalized π-electron system promotes effective interactions with molecules containing carbon-based ring structures [19]. This is a great property as several of the most recognized low-molecular weight pollutants possess aromatic-based sites in their structure, including antibiotics, pesticides, cosmetics, and others. Besides, graphene is commonly produced by the chemical exfoliation of graphene oxide derived from stacked layers of graphite. In this case, graphene oxide is a useful precursor as it has several oxygenated functional groups in its structure (e.g., carbonyl, epoxides, hydroxyl), which can be chemically functionalized by the insertion of other structures towards sorbent customization [20].

For column-switching applications, graphene-derived compounds has been used occasionally in open tubular columns attached to the tube wall [21] or mainly being covalently attached to amino silica particles (usually represented as GO@SiO_2), creating packed microextraction columns [20]. GO@SiO_2 and RGO@SiO_2 particles have demonstrated outstanding performance in the online extraction and pre-concentration of several classes of analytes from a wide range of matrices, such as the antidepressants and antiepileptic drugs in urine [22], xanthines in coffee [23], and mycotoxins in beverages [24].

This paper introduces a fully automated and miniaturized setup for the online extraction and determination of four β-lactam antibiotics from wastewater samples. A fused-silica microextraction column packed with GO@SiO_2 particles was online coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS), allowing the direct injection of 50 uL of wastewater raw samples and turning this strategy into a practical analytical procedure for high-throughput analysis of emergent pollutants. Furthermore, we focused on using low volumes of reagents and samples to reduce the volume of toxic waste generated, aiming to develop a more environmentally friendly procedure.

2. Experimental section

2.1. Analytical standards and reagents

High-purity (>99%) analytical standards of benzylpenicillin, cefalexin, cefoperazone, and ceftiofur were acquired from Fluka Analytical (Steinheim, NW, Germany). A stock solution of each target compound was prepared in acetonitrile (MeCN) and ultrapure water 80:20 (v/v) at a concentration of 1000 mg/L. Working solutions of each target compound and a mix of them were prepared in (i) ultrapure water for initial testing and (ii) tap water from one source for optimizing and evaluating figures of merit. LC-grade MeCN, formic acid (FA), isopropanol (IPA), and tetrahydrofuran (THF) were purchased from Tedia (Fairfield, OH, USA). Ultrapure water (H_2O) was supplied by a Milli-Q® Millipore system (Burlington, MA, USA).

For producing the GO@SiO2 sorbent, it was used graphite powder, potassium permanganate ($KMnO_4$), sodium nitrate ($NaNO_3$), N-(3-dimethyl aminopropyl)-N′-ethyl carbodiimide (EDC), and hydrochloride/N-hydroxysuccinimide (NHS), all acquired from Sigma-Aldrich (Sant Louis, MO, USA). Other reagents included sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) from Tedia (Fairfield, OH, USA), hydrogen peroxide (H_2O_2)' and Hydrazine (N_2H_4) from Acros Organics (Morris, NJ, USA). The spherical aminopropyl silica particles (Si) used as support were acquired from Varian (Lake Forest, CA, USA). The GO@SiO_2 packed capillary extraction column was prepared using fused silica (FS) tubing (0.01 in = 254 μm ID x 50 mm of length) from Polymicro Technologies Inc (Phoenix, AZ, USA).

2.2. Microextraction column & packing

The synthesis route for producing graphene oxide from graphite powder is based on Hummer's method with minor modifications [25]. In short, we mixed H_2SO_4, $NaNO_3$, and $KMnO_4$ with graphite powder to promote oxidation of its intercalated layers and subsequently used chemical exfoliation to obtain the separated GO sheets. Next, the extractive phase GO@SiO_2 was synthesized as previously reported [23, 26]. We explored the formation of peptide bonds between oxygen

functional groups from GO and amino-terminated sites in Si for attaching GO to Si. However, instead of using the organic-based coupling reaction, we choose to use the greener aqueous-based route – a combination of EDC and NHS as coupling reagents for promoting the formation of GO@SiO_2. As this work is focused on presenting the greener analytical method developed to analyze the β-lactam antibiotics in environmental water, we will not discuss in depth the GO@SiO_2 synthesis process. Information about it can be found in the previously cited works and following references [27,28].

Aiming to down-scaling the sample preparation step, we produced microextraction columns made of fused silica (FS) capillary. In short, GO@SiO_2 particles dispersed into THF:IPA (1:6) were slurry packed inside the FS capillary with the aid of a Shimadzu LC20A pump (Kyoto, Kansai, JP) and a lab-made packing chamber. Next, both sides of the GO@SiO_2 microextraction column were capped with metallic frits (porosity, 2 μm; ID, 1/16). At last, the microextraction column was connected to a six-port switching valve to work as an extractive device in a multi-step LC-MS/MS platform. Fig. 1 shows a schematic illustration of the apparatus used for manufacturing the extraction column (Fig 1A) and the sample prep-LC-MS/MS experimental setup (Fig 1B).

2.3. Analytical instrumentation and experimental conditions

The automated sample preparation analyses were carried out in the flow-through configuration at room temperature, employing an electrically assisted six-port switching valve from SUPELCO (Bellefonte, PA, USA) [10]. An auxiliary pump, Shimadzu LC20A, purged with acidified ultrapure H_2O (0.1% FA), was responsible for carrying the water samples into the GO@SiO_2 microextraction column at a loading flow rate of 50 μL/min. For each analysis, 50 μL of the non-treated sample was injected by the auto-sampler belonging to the LC instrument. Then, the target β-lactams were eluted in back-flush mode for the subsequent steps by a mixture of ultrapure H_2O and MeCN (0.1% FA). In the back-flush mode, the eluting flow has an inverse direction compared with the sample loading flow. This approach is used to avoid peak broadening as the target compounds travel shorter distances to come out of the extraction column compared to the front-flush mode – in this case, the analytes must percolate a longer pathway, causing band broadening [29]. For more details about the sample preparation conditions and switching valve events, please see Table 1.

The LC-MS/MS analysis was performed by an Acquity UPLC coupled to a Xevo triple quadrupole MS from Waters (Milford, MA, USA). For ionizing the target β-lactams, an Electrospray Ionization source (ESI) operating at positive mode was employed. Chromatographic separation was achieved by an Acquity UPLC BEH C8 analytical column (2.1 mm ID x 50 mm of length; particles of 1.7 μm d_p and pore size of 130 Å). Other analytical conditions included column temperature of 30 °C; mobile phase of ultrapure H_2O and MeCN (0.1% FA), operating under gradient elution starting with H_2O: MeCN (90:10, v:v), at a flow rate of 250 μL/min. Information about the gradient elution profile is shown in Fig. 2.

The ESI-MS detection was achieved using the following conditions: positive ionization; capillary voltage, 30 kV; desolvation temperature, 400 °C; source temperature, 150 °C; desolvation gas flow, 800 L/h; collision gas flow, 150 μL/min. Tandem MS detection was achieved using a selected reaction monitoring mode (SRM) by considering two ion transitions for quantification and identification. These transitions were determined by direct infusion of a mix containing the β-lactams at a concentration of 500 μg/L in a flow rate of 10 μL/min. This step was assisted by the IntelliStart optimization tool embedded in Waters's data-

Table 1
Detailed information on the sample prep-LC-MS/MS events executed during the automated analysis of the target β-lactams.

Event	Time (min)	Flow rate (μL/min)	*Solvent%	Switching valve
Sorption	0:00 – 1:00	50	H_2O	Loading
Elution	2:00 – 7:00	250	MeCN/H_2O (10:90 to 45:55, v/v)	Analytical
Cleaning	7:00 – 10:00	250	MeCN/H_2O (45:55, v/v)	Analytical
UPLC column conditioning	10:00 – 12:00	250	MeCN/H_2O (10:90, v/v)	Loading
GO@SiO_2 column conditioning	10:00 – 12:00	50	H_2O	Loading

* : solvents were acidified with 0.1% formic acid.

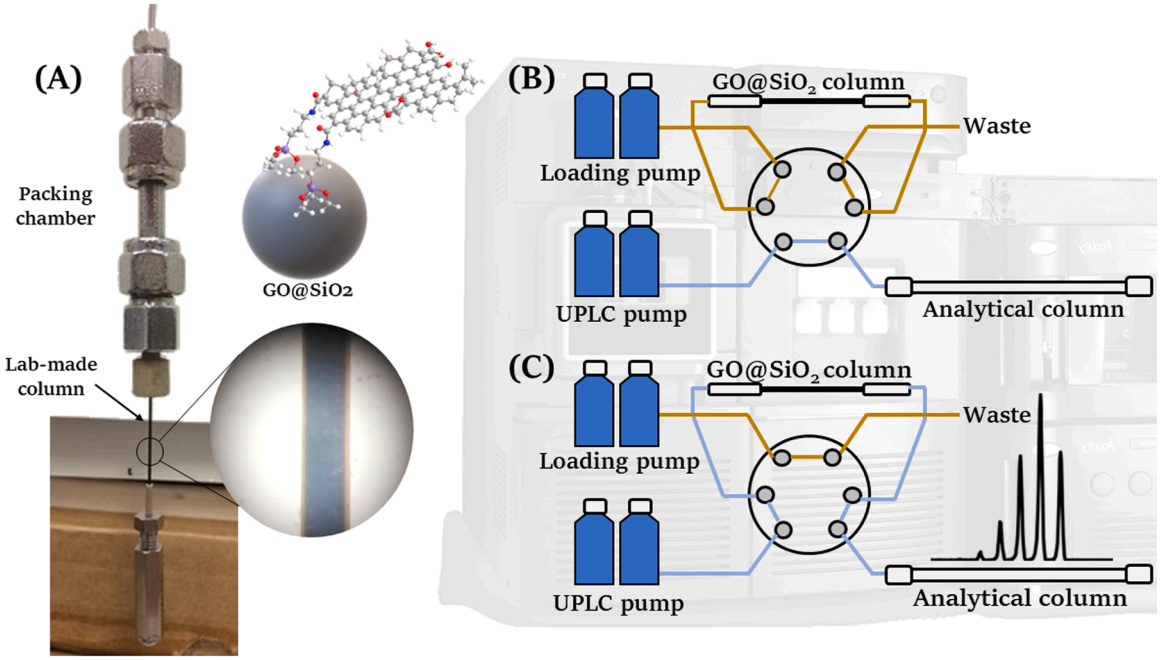

Fig. 1. Schematic image highlighting the instrumental apparatus. (A) Packing system and photos of the GO@SiO_2 microextraction column; (B) Switching valve configuration for loading step; and (C) Switching valve configuration for analytical step.

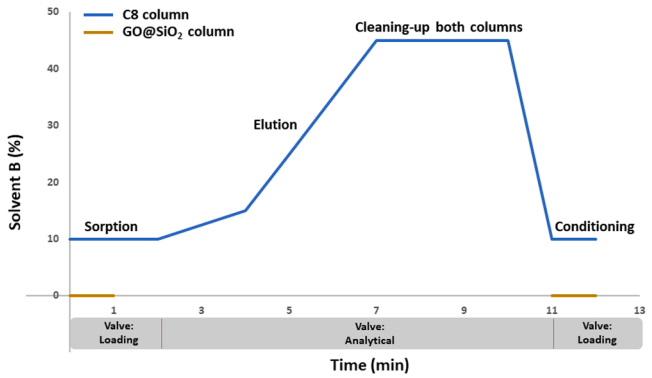

Fig. 2. Gradient elution profile employed during the automated sample prep-LC-MS/MS analysis of the target analytes highlighting time vs. mobile phase composition (A: H_2O and B: MeCN, both acidified). Important: the blue line corresponds to the analytical C8 column; the yellow line to the GO@SiO2 column; and the gray bar below the plot shows the switching valve position at each moment.

processing software MassLynx 4.1. Table 2 shows relevant information about the main parameters selected for the SRM method.

2.4. Multivariate optimization

At first, we did preliminary scouting experiments to find an initial analytical condition and also to glimpse the parameters which could affect the analysis of the target β-lactams. In this case, we evaluated different mobile phases and solvent combinations, taking advantage of the research group's experience in developing online sample preparation approaches employing miniaturized extraction columns [23,24]. After this step, a chemometric optimization approach was performed using a complete factorial design experiment (DoE 2^3) considering the following experimental parameters: sample loading flow, sample loading time, and temperature of analysis. Data from the DoE 2^3 was processed using Statistica 14 from TIBCO Software Inc. (Palo Alto, CA, USA). In short, twenty experiments were carried out using tap water samples spiked at a concentration of 80 μg/L. The levels for each factor were in the range of sample loading flow, 50 – 250 μL/min; sample loading time, 1 – 3 min; and temperature, 30 – 50 °C. For more details about DoE 2^3, please see Tables S1 and S2 in the supplementary material.

2.5. Analytical figures of merit

Even though a comprehensive analytical validation was not the focus here, we evaluated the leading figures of merit accordingly with the ICH Q2(R1) validation guideline to demonstrate the merits of this work [30]. The experiments were performed using spiked tap water samples as we selected to use the matrix-matched calibration approach.

Table 2
Experimental parameters employed in the SRM detection method of each target analyte.

Analyte	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Cone voltage (V)	Collision energy (V)	Retention time (min)
Benzylpenicillin	335	128	34	26	4.88
		159	34	24	
Cefalexin	348	106	14	28	4.64
		174	14	16	
Cefoperazone	646	143	18	34	6.38
		530	18	12	
Ceftiofur	524	125	30	62	7.16
		209	30	22	

First, the method selectivity was evaluated by comparing the chromatographic elution profile of two water samples collected from the same source – one spiked with the target analytes (25 μg/L) and the other without any fortification. We wanted to check if the chosen sample was free-of-βlactams and if there was any interference signal at the characteristic retention time of each analyte. The LOD and LOQ were determined by carrying out a sequence of injections to find the concentration values in which the analytical signal was about 3 and 10 times higher than the signal-to-noise ratio. The linearity tests were performed between 1 – 100 μg/L for the following target β-lactams: benzylpenicillin, cefalexin, cefoperazone, and ceftiofur. The analytical curve was plotted considering six concentration levels (1, 20, 40, 60, 80, 100 μg/L) and repeated three times ($n = 3$). For cefoperazone, the concentration at the first point was 2 instead of 1 μg/L. Accuracy was assessed in three levels (1 or 2, 40, 80 μg/L) by comparing the theoretical concentration (C) with the experimental concentration (C_{exp}) – reported when the chromatographic peak areas for each target β-lactam were inserted into the respective regression equation. Also, the intra- and inter-day precision were assessed through the relative standard deviation (% RSD) of injection from spiked samples (5, 40, 80 μg/L) at three subsequent days. To estimate the method recovery, we compared the signal intensity for each analyte when spiked samples were submitted to the whole procedure (sample prep-LC-MS/MS), with those from samples spiked only after elution from the GO@SiO_2 microextraction column.

2.6. Water samples

The sample prep-LC-MS/MS method was tested in water samples collected from different sources (i.e., tap water, streams, swimming pool, and domestic wastewater) in Sao Carlos, SP, Brazil. They were collected, transported, and kept in the fridge at approximately 4.5 °C for 24 h before executing the analysis batch.

3. Results and discussion

Before discussing the results from the developed analytical method, it is essential to provide some relevant information about the in-house produced capillary extraction column. Although the synthesis and characterization of the employed GO@SiO2 sorbent phase were already discussed deeply in another publication [17], we will highlight the major points. In short, Fourier-transformed infrared spectroscopy and scanning electron microscopy of the particles were performed and confirmed that the GO nanosheets are successfully supported onto the SiO_2 surface. Subsequently, this solid material was packed into an FS capillary and used as sorbent here for extracting the target β-lactams from environmental water samples. The GO@SiO2 capillary extraction column was evaluated first by injections of β-lactams standard solutions, therefore confirming its capability to retain the target analytes. After this preliminary assessment, we proceeded with the chemometric optimization employing water samples.

3.1. Multivariate optimization

The complete factorial design DoE 2^3 was carried out considering the sample loading flow, sample loading time, and analysis temperature. From the Pareto chart (Fig. 3A), it can be seen that the three factors had a significant effect on the analytical performance – similar results were observed to other target β-lactams.

The loading flow was the most influential factor reporting a negative value which is expected as it is directly related to the sample dilution and linear velocity inside the GO@SiO_2 microextraction column. Therefore, low flow rates in this step could avoid unnecessary sample dilution and promote a better interaction between the extractive phase and the target compounds. On the other hand, increasing the values for this factor will interfere negatively by reducing possible interactions in

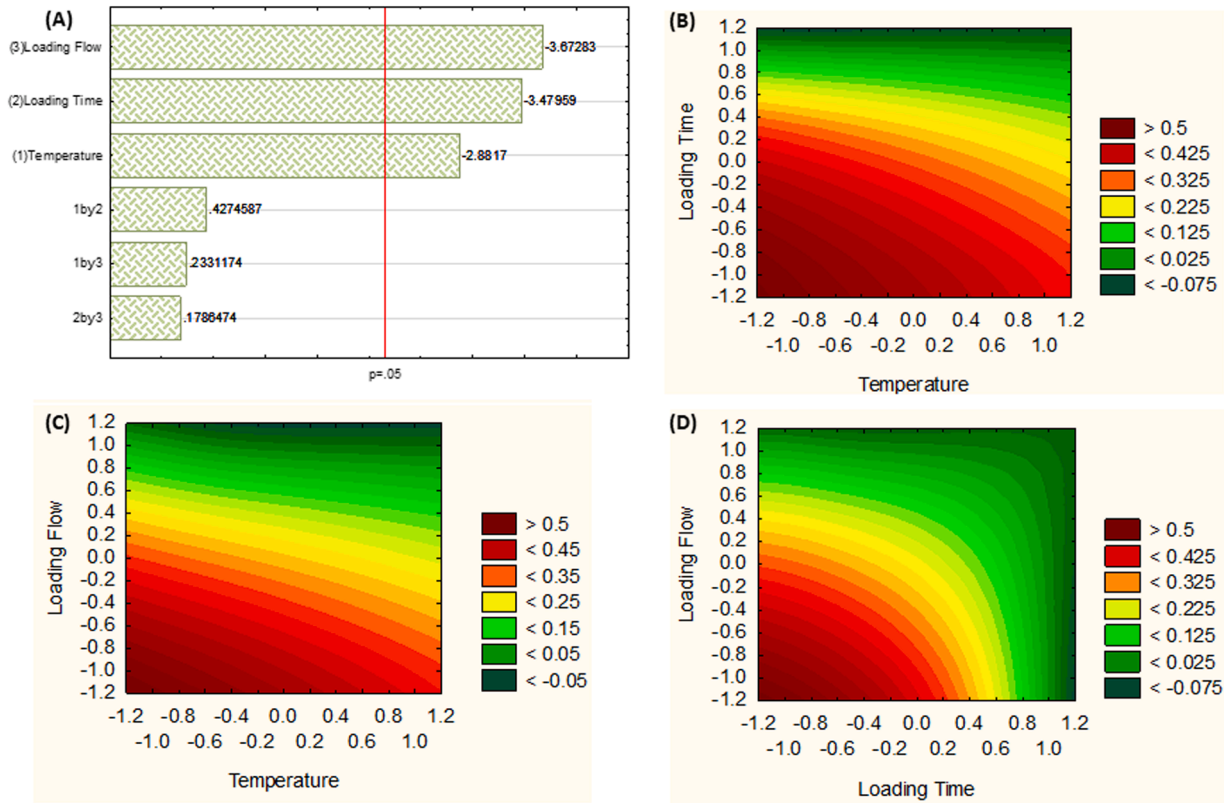

Fig. 3. Informative illustrations achieved through the data collected in the DoE 2^3. (A) Pareto chart showing the influence of each factor alone and combined; (B, C, and D) surface responses highlighting the optimized range of values for two factors simultaneously.

the GO@SiO_2 microextraction column. Therefore, the loading flow was set at 50 μL/min. Also, data from Fig. 3A suggest that loading time should be maintained as short as possible to promote a better analytical signal at the end. This tendency is probably because longer loading times could favor the sorption of some interfering substances from the matrix, diminishing the sites available for β-Lactams interaction. Additionally, longer times can promote loss of target analytes as, at this moment, the switching valve is diverting the flow to the waste and not to the LC-MS/MS.

The temperature factor was assessed by installing the GO@SiO_2 microextraction column inside the same oven where the analytical column was placed. Data from the Pareto chart suggest that lower temperatures positively affect the analytical results. In this case, pressure can positively contribute to the microextraction efficiency of the target molecules as it forces them to interact and penetrate more deeply inside the structure of the sorbent phase. For this reason, the analysis temperature was maintained at 30 °C.

At last, the surface response desirability function to our experiments was generated (Fig. 3B, C, and D) to give a general perspective about the best experimental condition to be used when considering the factors altogether. As can be seen from it, our DoE 2^3 suggests using the lowest possible values. Therefore, we fixed our parameters at loading flow, 50 μL/min; loading time, 1 min; and the temperature of analysis at 30 °C. Therefore, these conditions were applied in the subsequent steps of the work (i.e., figures of merit determination and application).

3.2. Analytical figures of merit

As stated before, a full validation of the developed method was out of the scope of this work, once its focus was on the sorption behavior of GO@SiO_2 for the β-lactams extraction from wastewater and the its evaluation of its environmentally friendly characteristics such as the lower consumption of reagents and samples and, consequently, waste generation. On the other hand, some of the most important figures of merit were studied as we believe they can support our data/discussion, especially compared to other publications (Section 3.4).

Fig. 4 shows a typical chromatographic separation of a spiked tap water sample obtained through the automated sample-prep-LC-MS/MS method (blue line), superimposed with a chromatogram obtained from a non-spiked one (yellow line). As can be seen, there are no interfering peaks at the characteristic retention time of each β-lactam, confirming the method's selectivity. Also, all target compounds were eluted before ca. 7.2 min, resulting in a total time per analysis of around 12 min, which is excellent if we consider the complexity of the sample. The matrix effect was not evaluated as the entire study was already developed using an actual matrix (i.e., tap water). Another important fact to mention is that the samples were injected into the GO@SiO_2 column without any previous step of dilution or centrifugation. The only step required was a filtration through a cellulose membrane of 0.22 μm.

The limits of detection and quantification were in the same range for most analytes, except for cefoperazone, which showed lower overall signal intensity. Therefore, LODs and LOQs were in the range of 0.2 – 0.3 μg/L and 1 – 2 μg/L, respectively. By comparing the LOQs with other publications, it is possible to find even lower values. On the other hand, we describe in this work an automated and miniaturized procedure using a synthesized sorbent material in an underdevelopment stage. Although some downsides was expected, the promising environmentally friendly-related positive characteristics of it encourages us to keep working toward improving its performance. After linear regression, the matrix-matched calibration curves reported coefficients of determination (R^2) above 0.99. The residual dispersion plots for each analytical curve showed random points, suggesting the homoscedasticity of the experimental data. The method reported 82.3 – 107.1% accuracy, which is under the ICH Q(2)R1 acceptance criteria. Similar to accuracy, intra and inter-day precision values covered a range between 0.8 – 20.6% staying under the acceptance criteria.

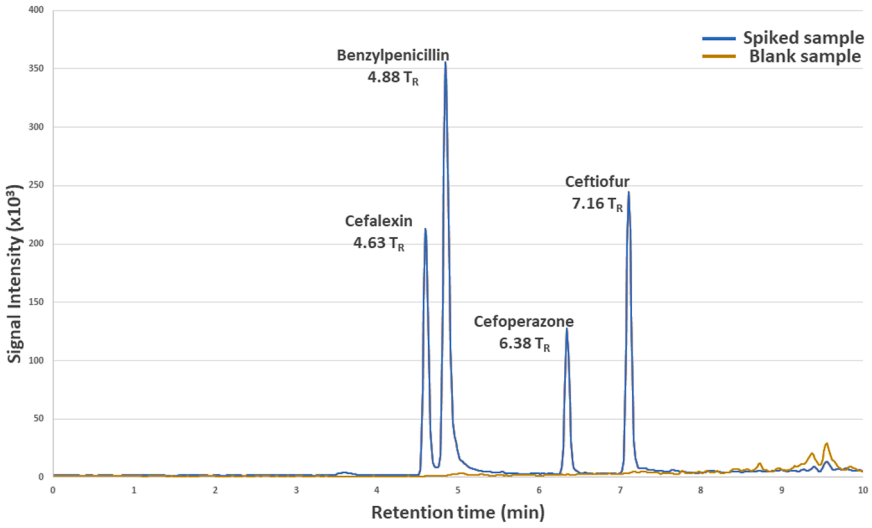

Fig. 4. Comparison between chromatograms obtained after an automated sample prep-LC-MS/MS analysis of a blank tap water sample and the same one spiked with the target antibiotics at a concentration of 20 μg/L.

At last, the method recovery showed values into an interval acceptable by most validation guidelines: 70.4 – 91.6%. Although most of our recovery values were not so close to 100% considered as the best situation, it is important to emphasize that the sample prep-LC-MS/MS method is based on an automated flow-through microextraction approach. This means the water samples are injected into the GO@SiO_2 column once, and the target analytes flow just in one direction to interact with the extractive phase. On one hand, there is the alternative possibility of using an approach termed drawn-and-eject, in which the analytes interact multiple times with the sorbent phase. On the other hand, drawn-and-eject approach comprises multiple steps, higher backpressure rates, and demands more sophisticated instrumentation [10]. Considering the data presented in this work, we concluded that there is no need to replace the simpler flow-through approach once it achieved its goals. Table 3 shows in detail the analytical figures of merit evaluated in this work.

3.3. Application to the analysis of water samples

After the promising results obtained for the leading figures of merit, the sample prep-LC-MS/MS method was applied in the analysis of different water samples to screen the target β-lactams. Ten samples were collected from different sources (i.e., tap water, streams, swimming pool, and domestic wastewater) in Sao Carlos, SP – Brazil. The only previous step performed before injecting raw samples into the analytical platform was a filtration through a cellulose-membrane (0.22 μm). This is a relevant characteristic of this method, as in most cases, a dilution or centrifugation step is necessary for getting rid of some interfering substances. Furthermore, even performing a direct injection into the GO@SiO_2 microextraction column, at least 102 samples were analyzed during this work after which the column continued performing as in the first injections.

Although most samples did not report any signal that could be attributed to our target analytes, two samples from different water streams showed well-defined peaks attributed to cefalexin (Fig. 5). Fortunately, both exhibited concentration values close to the lowest point in the calibration curve (*sample 1*, 2.5 μg/L; *sample 2*, 4.0 μg/L).

As there is no specific regulation for MRLs of cefalexin in natural water sources, we checked on the regulation of other matrices (i.e., bovine) [31]. The MRL in the case of milk and muscle were ca. 100 and 200 μg/L – much higher values than those reported here. Considering that humans widely consume milk and bovine-derived meat, we reasoned that the concentration levels of cefalexin found in samples 1 and 2 are not harmful to humans. However, the frequent use of antibiotics in humans and animals has been raising attention by regulatory agencies worldwide, as these compounds are continuously thrown out into the environment [32]. Considering that most wastewater treatment plants (WWTPs) are not so effective in removing them, this can lead to a persistent problem, possibly affecting the population in the long term. That is why – despite the fact we did not identify dangerous levels in our samples – we would like to raise attention to the topic and highlight the importance of developing methods for analyzing antibiotics present in

Table 3
The analytical figures of merit are evaluated in this work. Note: recovery, accuracy, and precision data were acquired in triplicate ($n = 3$) and are reported as the mean values.

Analytes	Regression equation	R^2	Concentration (μg/L)	Recovery (%)	Accuracy (%)	Precision (% RSD.)		
						Intra day	Day 1	Day 2
Benzylpenicillin	$y = 1571.4x - 3710$	0.9916	5	66.6	102.6	14.3	1.1	19.9
			40	71.0	97.4	3.4	2.0	1.8
			80	89.4	82.5	14.4	2.9	2.2
Cefalexin	$y = 400.32x + 825.44$	0.9965	5	66.4	82.3	1.4	4.6	17.8
			40	72.2	96.4	14.9	6.0	4.0
			80	83.2	103.3	6.2	14.6	20.6
Cefoperazone	$y = 400.82x - 524.03$	0.9966	5	64.4	107.1	14.5	4.8	10.3
			40	69.8	101.4	18.7	1.3	0.8
			80	80.9	106.3	3.3	1.0	2.8
Ceftiofur	$y = 990.02x - 2530.2$	0.9949	5	70.4	101.0	19.0	14.0	14.9
			40	82.6	85.0	2.5	1.5	1.8
			80	91.6	91.0	7.1	0.9	4.19

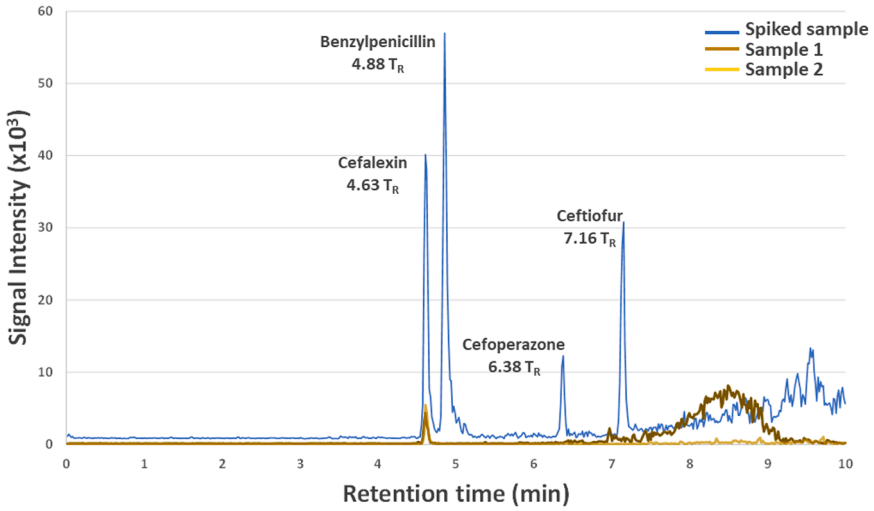

Fig. 5. Chromatograms from two cefalexin-positive water samples (1 and 2) overlaid with one spiked water sample.

water sources.

3.4. Overall performance

This section will present an overall perspective of this work regarding to other approaches reported in the literature on analyzing β-lactams and antibiotics in general. As our focus here was to develop an effective and fast analytical method, at the same time that could generate less toxic waste and consume fewer reagents and samples, we will concentrate the discussion on these characteristics.

First, considering the increasing demand for high-throughput methods for analyzing larger cohorts of samples, the time per analysis is of utmost importance nowadays. Developing analytical methods to process several samples faster with satisfactory performance and robustness is a longing-time goal for many researchers dedicated to the topic. Compared with the literature, the 12 min of the total analysis time required in this work (including sample preparation and LC-MS/MS analysis) is a significant characteristic of the proposed method to favor the analytical frequency. In most cases used for the same or similar purpose, sample preparation is carried out offline before injection into the analytical platform (e.g., LC-UV or LC-MS), and these approaches easily surpass 30 min of analysis [33–37]. Even when an online SPE-LC-DAD method was carried out [38], it required more than 12 min to be executed properly.

Another relevant feature to discuss is the number of chemicals necessary to execute an analytical method directly once this is related to the method's consumption and generation of toxic residues. Considering the sample preparation step and LC-MS/MS analysis, this work consumed ca. 3.6 mL of solvents per analysis. On the other hand, we do not need to execute any solvent-based extraction step before injection. For example, Nascimento and coworkers [39] reported a green analytical method for quantifying cephalothin, an antibiotic similar to our target analytes. In this case, it was necessary to use a more significant amount of solvent than our work (ca. of 7.0 mL per analysis).

On the other hand, they combined water and ethanol as a mobile phase, and this later solvent is known for being greener than MeCN – the one used in this work. That is why in our opinion, their method deserves to be highlighted here. Finally, Zhang et al. [38] presented an online SPE-LC-DAD approach using a lab-made monolithic column, but in their case, it required ca. 30 mL of mobile phase per analysis. We presume this more significant amount of reagent is because the monolithic column has conventional dimensions (50 mm of length x 4.6 mm ID). On the bright side, this method can cope with river water samples directly injected into the instrument without any previous extraction step.

In short, we can confirm that the proposed method showed a better overall analysis time and consumed fewer reagents compared to other publications on the same topic. For example, offline-based methodologies often require previous extraction steps employing additional chemicals such as ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), buffers, and others [35,36,40]. On the other hand, several of the cited papers reported lower LOQs ranges than this work. Commonly LOQs are in the ng/L range compared to μg/L. In our opinion, this is an aspect to be further investigated aiming to enhance sensitivity even more. Even so, it is relevant to remember that our analytical linear range is still inside the MRLs commonly established for these compounds in complex matrices [31,41]. The choice for an automated sample prep-LC-MS/MS approach helped us achieve a reliable, fast, and greener method for analyzing β-lactams in water samples. Most importantly, we hope this might contribute to the field of antibiotic environmental surveillance by showing some environmentally-friendly benefits of using automation and miniaturization combined. However, more research is needed to improve the LOQs of these more straightforward methods compared with labor-intensive but more sensitive conventional analytical methods (e.g., SPE-based procedures).

4. Concluding remarks

From our point of view, the primary goals in this work were successfully reached. The in-lab produced GO@SiO_2 microextraction column reported good performance in extracting and pre-concentrating the target β-lactams from environmental water samples. In addition, the column could be reused for more than 100 analyses without losing its sorbent characteristics, in opposite to SPE cartridges that are used once and discharged. Furthermore, using the column-switching approach to online coupling it with the LC-MS/MS has allowed us to create an environmentally friendly method that consumes fewer reagents and generates less toxic waste than most of the published methods cited here. Also, this work showed one of the best overall times per analysis compared to the literature (ca. 12 min in total). This is a promising feature, as analytical frequency is a very important parameter nowadays when large cohorts of sample must be often analyzed.

Moreover, a multivariate optimization step using full factorial DoE (2^3) was carried out to find the ideal analytical condition for extracting the target compound from water samples with adequate selectivity and performance. After that, the leading validation figures of merit were evaluated according to an international guideline. In short, the method reported a linear interval range between 1 – 100 μg/L with R^2 above 0.99. Accuracy, intra- and inter-day precisions were also in an

acceptable range (82.3 – 107.1% and 0.8 – 20.6% RSD, respectively). The LODs and LOQs also stood in a suitable range when considering the MRLs established by international regulatory documents.

At last, ten samples of environmental water were analyzed by this method, and traces of cefalexin were found in two of them, both at levels considered non-dangerous for humans. Ultimately, this miniaturized and automated sample prep-LC-MS/MS method can be reliably used for monitoring the presence of β-lactams in water samples. At the same time, greener aspects like reduced consumption of reagents and waste generation are evidenced.

CRediT authorship contribution statement

Edvaldo V.S. Maciel: Conceptualization, Methodology, Writing – original draft, Formal analysis, Investigation. **Deyber A. Vargas-Medina:** Conceptualization, Writing – original draft, Writing – review & editing. **Fernando M. Lancas:** Conceptualization, Methodology, Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no competing interests or personal relationships that could have influenced this work.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2023.100185.

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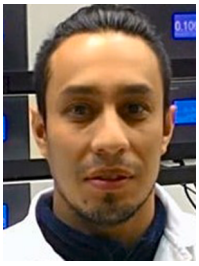

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