

Degradation of micropollutant cephalexin by ultraviolet (UV) and assessment of residual antimicrobial activity of transformation products

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ABSTRACT

Cephalexin (CEX) is an antibiotic commonly used to treat bacterial infections in humans and animals. However, it is also a micropollutant. Thus, this study evaluated the degradation of CEX using ultraviolet irradiation (UV-C) and analyzed the by-products as well as their residual antimicrobial activity. A reactor with a mercury vapor lamp was used for the degradation. Irradiated CEX solutions were collected over a period of 4 hours and analyzed using high-performance liquid chromatography coupled with mass spectrometry. For the residual antimicrobial activity the susceptibility test was performed using *Staphylococcus aureus* and *Escherichia coli* microorganisms by broth microdilution. It was found that CEX, after treatment, generated a metabolite with a mass of 150 m/z in 15 min. A four- and eightfold increase in the minimum inhibitory concentration of the drug against *S. aureus* and *E. coli* could be observed, respectively, after 20 min. Therefore, this treatment proved to be effective in the degradation of CEX, being able to degrade 81% of the initial molecule of the drug in 20 min. Furthermore, the antimicrobial activity of the CEX solution decreased as the irradiation time increased, indicating loss of antimicrobial function of the initial CEX molecule and the resulting by-products.

Key words | cephalexin, micropollutants, minimum inhibitory concentration, UV-C treatment

HIGHLIGHTS

- Degradation of micropollutant cephalexin.
- Metabolites analyzed and residual antimicrobial activity.
- Water treatment with irradiation.
- Antimicrobial activity using susceptibility test.

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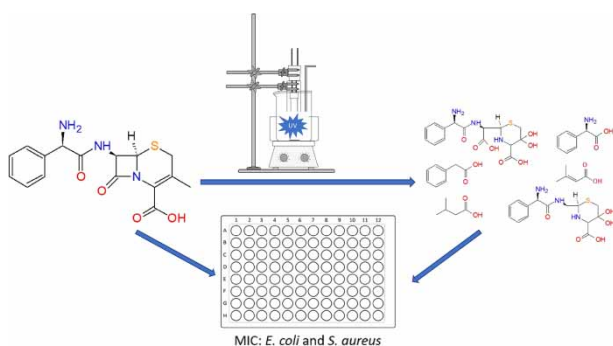
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GRAPHICAL ABSTRACT



INTRODUCTION

In the last decades, industrial expansion and population growth have directly contributed to the increase in the pollution of the aquatic environment. Consequently, studies have reported a new class of emerging contaminants called micropollutants (Harb *et al.* 2019). They are found in very low concentrations in nature (microgram/litre – $\mu\text{g.L}^{-1}$ and nanogram/litre – ng.L^{-1}), and their effects on the environment and human health are still poorly understood. Among the micropollutants, pesticides, heavy metals, antibiotics, hormones, and cosmetics are the most prominent (Wardenier *et al.* 2019).

Antibiotics are drugs capable of stopping or inhibiting the growth of many microorganisms and occupy a significant portion of the total drugs available for commercialization. Moreover, they are very frequently prescribed to treat infections in humans, and are also routinely used in and livestock rearing (Guzmán-Trampe *et al.* 2017). These contaminants have become a growing environmental problem, alarming researchers because of their increased ability to perpetuate and spread genes of antimicrobial-resistant bacteria (Kumar *et al.* 2019), besides causing environmental imbalance and anomalies to several aquatic species (Wang *et al.* 2019).

Accelerated and indiscriminate use of antibiotics, along with a very low percentage of metabolization of these drugs in the body, cause the release into water bodies of a large fraction of effluents containing this type of contaminant (Nielsen *et al.* 2018). These compounds have low biodegradability due to their stable chemical structure, and because they are a relatively new class of pollutants, they are still not commonly treated by conventional water and effluent treatment systems, which makes the situation even more critical. Antibiotics do not have a maximum concentration

limit that certifies their potability, causing the accumulation of several types of antibiotic in the ecosystem (Li *et al.* 2018).

More specifically, cephalixin (CEX) is an antibiotic belonging to the class of first-generation cephalosporins (Figure 1). It has a heteroatomic ring in its chemical structure, consisting of three carbon atoms and one nitrogen atom, characterizing cephalixin as a β -lactam. This drug is commonly prescribed to treat several respiratory and urinary tract infections as it has a broad spectrum of antimicrobial action, making it one of the most consumed antibiotics in the world, with a large scale of production worldwide (Ajoudanian & Nezamzadeh-Ejhieh 2015).

Studies have shown that only 10% of cephalixin consumed is metabolized by the organism, with approximately 90% of the administered dose being excreted in the urine and feces in its unchanged form along with its metabolites, ending up in aquatic matrices (Bansal & Verma 2017). The accumulation of this contaminant in the environment has been related to the emergence of multidrug-resistant pathogens, causing an immeasurable impact on the environment and its organisms (Gulkowska *et al.* 2007; Yan *et al.* 2013; Al-Gheethi *et al.* 2017; Sabri *et al.* 2018).

Divya & Hatha (2019) found that approximately 64% of three hundred *Escherichia coli* strains isolated from a river in India were resistant to at least one of the 15 antibiotics studied and at least 37% of the strains were resistant to more than one antibiotic, with the gene that codifies for the production of β -lactamase being the most frequently found resistance gene. Thus, as an attempt to avoid even worse environmental and public health damage, methodologies have been proposed to remediate and reduce concentrations of CEX released into nature (Wu *et al.* 2017), such as reverse osmosis (Nazari *et al.* 2016b), adsorption onto activated

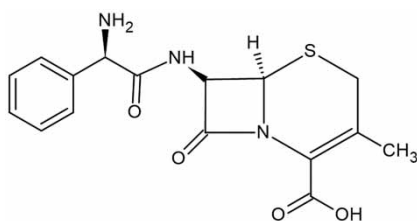


Figure 1 | Chemical structure of cephalixin.

carbon or other adsorbents (Nazari *et al.* 2016a), photocatalysis, and advanced oxidative processes (AOPs), such as treatment in association with ultraviolet light irradiation and hydrogen peroxide (UV-C/H₂O₂) (Bansal & Verma 2017). However, all of these treatments have high operational costs, as well as costs related to the final disposal of the effluent and possible treatments to eliminate residual catalysts added during treatment (Wang & Wang 2016).

Photo-oxidation treatment, also known as photolysis, has advantages in its use to degrade the micropollutant using only irradiation as its source, for example, using lamps that emit ultraviolet (UV-C) irradiation (Kim *et al.* 2009). Direct photo-oxidation occurs through the interaction of the UV radiation emitted by the lamp with the antibiotic molecule, which absorbs photons and excites electrons to their maximum electron energy, breaking the molecule and degrading it through generation of free radicals (Paniagua *et al.* 2019). This degradation has a good cost-benefit ratio for degrading the micropollutant using only the emitted light as an oxidizer, not requiring any other catalyst or oxidant and not requiring further treatment of the residual oxidant that may remain in the effluent (Duan *et al.* 2017). Indirect photolysis, on the other hand, involves reactions with photo-induced reactive species, such as hydroxyl radicals, singlet oxygen, and the triplet excited state of chromophoric dissolved organic matter formed in natural waters after light absorption by different species (nitrite, nitrate, dissolved organic matter, iron complexes) (Lastre-Acosta *et al.* 2019).

It is critical to ensure that the CEX molecule has been degraded and no longer has antibiotic action. For this, the determination of residual antimicrobial activity through the analysis of susceptibility to antibiotics is necessary. This methodology consists of the exposure of one or more strains of the microorganism to different concentrations of the antibiotic (Franje *et al.* 2010). Different methods can be used when evaluating microbial susceptibility to antibiotics, such as disk diffusion, broth macro- and microdilution, and automated methods. In Brazil, the most commonly used method for qualitative assays is the disk diffusion method, and for quantitative assays, the minimum

inhibitory concentration (MIC) assays are commonly used, which provide reliable results regarding the evaluation of antimicrobial agents. The MIC test corresponds to the lowest concentration of the antimicrobial necessary to inhibit 100% of microbial growth of a given species (Arslan *et al.* 2017).

Therefore, this study aims to investigate the degradation of CEX by photolysis, to verify the metabolites formed and to measure the residual antimicrobial activity of the antibiotic by susceptibility test against *Staphylococcus aureus* and *Escherichia coli*.

MATERIAL AND METHODS

Sample preparation

In all tests, the antibiotic cephalixin monohydrate (CEX, LAB Confiança, batch: 838.922, CAS Number: 15686-71-2) was used. For the irradiation test, a stock solution of the antibiotic at a concentration of 512 mg·L⁻¹ was prepared using ultra-purified water (Milli-Q, Millipore, USA). This initial concentration was chosen because it is sufficiently high to allow the analysis of degradation kinetics and the monitoring of possible metabolites, besides facilitating serial dilutions for the MIC tests in accordance to the Clinical & Laboratory Standards Institute (CLSI 2012, 2018).

Photo-oxidation test

The photo-oxidation test was adapted from Sarkar *et al.* (2014), and was performed in a bench-top reactor with a capacity of 250 mL. Into this, a 125 W mercury vapor lamp (Osram) was inserted 5 cm from the bottom of the reactor, surrounded by a quartz tube (14 cm long and 4 cm diameter) that was used as a source of UV_{254nm} radiation. Low pressure (LP) mercury lamps emit monochromatic radiation in wavelengths of less than 254 nm, with extensive UV-C flux, so they are often used in AOPs, because this wavelength range is responsible for the production of ·OH and ·H radicals, which enhance the decomposition of organic contaminants (He *et al.* 2014; Lastre-Acosta *et al.* 2019; Paniagua *et al.* 2019) The reactor (Figure 2) was kept in an ice bath during analysis in order to cool the system and avoid analyte degradation by sources other than radiation.

Volumes of 200 mL of the stock solution were inserted into the reactor shortly after preparation, and were subjected to irradiation for a total period of 4 hours. Samples

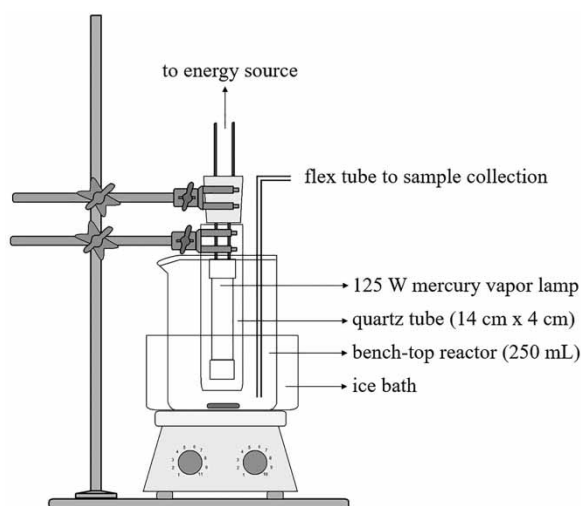


Figure 2 | Bench-top reactor used to irradiation CEX solution.

of 1 mL were collected before irradiation and every 5 min throughout irradiation during the first hour and every 30 min for the next 3 hours, giving a total of 19 aliquots. Analysis was performed in triplicate.

Determination of CEX degradation

In order to determine the degradation level of CEX, all samples were analyzed by high-performance liquid chromatography (HPLC) with detection by ion trap mass spectrometry (MS). The procedure was adapted from [Li *et al.* \(2018\)](#) and performed using a Shimadzu liquid chromatograph, Nexera-XR LC-20 AD pumps, Nexera-XR SIL-20A autosampler, CTO 20-A column oven, and CBM-20A controller. A kinetex 1.7 μ , C18, 100A (50 \times 2.1 mm) chromatographic column and an amaZon Speed ET (Bruker Daltonics) mass spectrometer were used.

Ultrapure water and HPLC-grade acetonitrile were used as the mobile phase, both acidified in 0.1% formic acid *p.a.* (Synth). The flow was 0.2 mL.min⁻¹ and initial gradient was 5% acetonitrile up to 2 min, increasing to 95%, which was maintained for 6 to 7 min, and then returning to 5%, which was maintained until the end of flow in 10 min. The injection volume was 5 μ L and the oven temperature was 40 °C. Ion trap MS/MS operated at a pressure of 40 psi, with nitrogen (N₂) as the drying gas at 9 L.min⁻¹, a gas temperature of 300 °C, helium used as the collision gas (He₂), positive mode electrospray ionization source, a scan range of 100–600 *m/z*, an auxiliary amplitude of 40%, and the capillary voltage at 4,500 V, multiple reaction monitoring (MRM). For the control of the HPLC system HyStar 3.2 software

(Bruker) was used, and for the amaZon ion trap, TrapControl 7.2 software (Bruker) was used. Data Analysis 4.2 software (Bruker) was used to convert the MS/MS spectra with the highest signal intensity to MGF format. The resulting MGF files were merged and converted to mzXML with msconvert. The limit of detection (LOD) and limit of quantification (LOQ) calculations of the HPLC were carried out in accordance with procedures followed by [Liu *et al.* \(2018\)](#). It is worth noting that, with this setup, along with the quantification of CEX, the detection of only the metabolites 100, 102, 150, 386, 326, and 138 *m/z* compounds was possible.

Verification of residual antimicrobial activity

Microbial cultures

For antimicrobial susceptibility testing, two distinct microorganisms which are often found in aquatic matrices were used, one Gram-positive and one Gram-negative. The strains of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were obtained from the University of Vale do Taquari (Univates), and were stored in a freezer at –20 °C.

To reactivate the microorganisms, they were inoculated in a Müeller-Hinton agar culture medium (MHA, Oxoid) and incubated at 37 °C for 24 hours in a bacteriological incubator (Prolab).

Determination of minimum inhibitory concentration (MIC) of the irradiated solutions

MIC was performed according to the 96-well plate broth microdilution technique with serial dilutions according to the M07-A9 and M100 methods of the Clinical & Laboratory Standards Institute ([CLSI 2012, 2018](#)) using a 96-well plate (Ciencor). Bacterial suspensions were prepared in 0.9% saline solution from colonies grown in Müeller-Hinton agar, being standardized in a spectrophotometer in the range of 0.08 to 0.13 absorbance units at 625 nm (Thermo Scientific, model G10S-UV-Vis), representing 1.0–2.0 $\times 10^8$ CFU/mL. Afterwards, a 1:150 dilution was performed in Müeller-Hinton broth, obtaining a final concentration in the wells ranging from 3 to 7 $\times 10^5$ CFU/mL.

In the 96-well plate, 200 μ L of the CEX samples collected during photodegradation was inserted into the first column and 100 μ L of Müeller-Hinton broth was inserted into the others (columns 2 to 10). Serial microdilution was performed for 10 different concentrations, transferring 100 μ L from the first column to the second, homogenizing and transferring again until the 10th column, resulting in a

drug solution concentration of 256 $\mu\text{g}/\text{mL}$ in the first column and 0.5 $\mu\text{g}/\text{mL}$ in the 10th column. At the end, 100 μL of the standardized inoculum was added to all wells in the first and 10th columns. For the negative control (NC), the culture medium was used, and for the positive control (PC), the culture medium and the standardized inoculum were used. Figure 3 illustrates the plate used and the solutions pipetted. A blank was also made containing culture medium and the irradiated CEX solution. The plates were incubated at 37 °C for 16–20 hours and revealed with 0.02% resazurin. The absorbances were observed in a molecular absorption spectrophotometer (SpectraMax i3, Molecular Devices) at 625 nm and the determination of the MIC was stipulated as the lowest solution concentration capable of visually inhibiting 100% of bacterial growth.

All 19 irradiation times (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 90, 120, 150, 180, 210 and 240 min) were

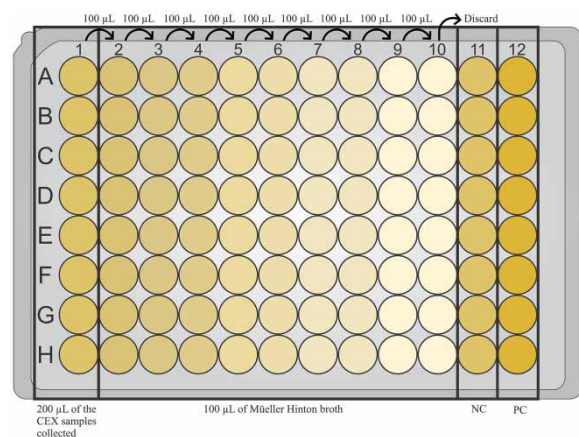


Figure 3 | 96-well plate and pipetted solution volumes for determining the MIC.

tested, including time zero – in which the sample had not been yet exposed to photo-oxidation.

RESULTS AND DISCUSSION

CEX degradation experiments

For analysis of the CEX and its metabolites in the HPLC, the LOD obtained was 0.078 $\mu\text{g L}^{-1}$ and the LOQ was 0.40 $\mu\text{g L}^{-1}$. The results were similar to those of Liu *et al.* (2018).

Ultraviolet irradiation ($\text{UV}_{254\text{nm}}$) can degrade innumerable organic pollutants through direct photolysis, as some of these compounds are capable of absorbing photons, which leads to high molecular excitation and subsequent cleavage of bonds and chemical transformation (Carena *et al.* 2020). The photodegradation kinetics of organic compounds is determined by the molar capacity of absorption and the quantum yield of substances (internal properties). Some organic compounds can increase molar absorptivity at $\lambda = 254 \text{ nm}$, and consequently have increased degradation capacity from direct photolysis because it is the effect of all processes of UV-C irradiation. The coefficient of molar absorption ϵ_{254} of CEX was calculated according to the Beer-Lambert law $\epsilon_{254} = A/c l$, where A corresponds to the absorbance of CEX at the stipulated wavelength, c is the reagent concentration in mol L^{-1} , and l is the optical path of the cuvette used in the measurement, in this case being 1.00 cm. The ϵ_{254} of CEX was 347.4 $\text{M}^{-1} \text{cm}^{-1}$, a high value, similar to that obtained by He *et al.* (2014) for this antibiotic (Stefan & Bolton 2002).

The degradation capacity of the CEX antibiotic was tested against the influence of $\text{UV}_{254\text{nm}}$ irradiation. Figure 4

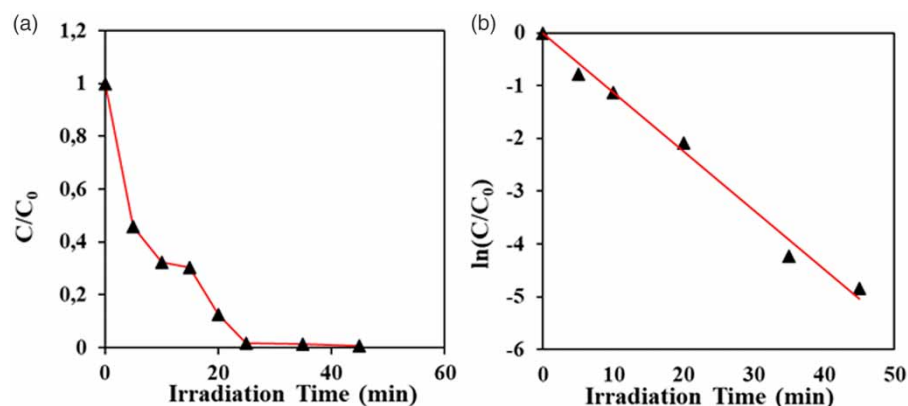


Figure 4 | (a) Photodegradation of CEX by $\text{UV}_{254\text{nm}}$. (b) Kinetic adjustment for the pseudo-first-order model for CEX degradation.

shows the (A) degradation profile of the CEX antibiotic and (B) the process kinetics, which was adjusted to the pseudo-first-order model, resulting in an adequate correlation coefficient ($R^2 > 0.99$), which suggests that the process follows this kinetic model. It was observed that the initial concentration of was reduced by approximately 81.1% after 20 min of treatment, reaching trace levels, below the LOD of the method, at 25 min. The efficiency of the process, based on the kinetic modeling of the first order curve, resulted in a kinetic constant of $k = 0.1119 \text{ min}^{-1}$, which represents the reaction rate, indicating that the chemical structure is susceptible to degradation by the action of $\text{UV}_{254\text{nm}}$. Other similar work, by [Gawande *et al.* \(2017\)](#) for example, present a similar rate constant. According to the literature, CEX has degradable regions located closer to the sulfur and nitrogen atoms, besides regions with the presence of π systems. This is due to the formation of benzylic and allylic radicals or cations, (which are stabilized by resonance), especially in the β -lactam and dihydrothiazine rings, as these regions are the most likely to absorb light ([Challis *et al.* 2014](#); [Serna-Galvis *et al.* 2017b](#); [He *et al.* 2019](#)).

It is worth mentioning that the present work regarding susceptibility testing is preliminary, and CEX degradation was only studied in ultrapure water, in the absence of a sample matrix to evaluate the formation of possible by-products. In the case of studies performed with actual sample matrices, all of their parameters must be reevaluated.

According to [Wang & Lin \(2012\)](#), the degradation rate of CEX is widely influenced by the matrix, possibly reaching superior degradation indices in less time, as demonstrated by their results, in which solutions that were prepared from natural matrices resulted in a kinetic constant k four times higher. The authors suggest that the main process of degradation, in the case of natural matrices, is indirect photolysis, in which irradiation interacts with compounds present in the water, producing highly reactive free radicals, thus degrading the organic substances.

Cephalixin degradation pathway

Low mineralization, as well as the appearance of a yellow color in the solution over the irradiation time, indicated the formation of by-products of the original compound ([De Souza Santos *et al.* 2015](#)). Thus, analysis via high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) was performed to determine the possible intermediates formed in the $\text{UV}_{254\text{nm}}$ direct photolysis experiment, based on the by-products already identified by [Li *et al.* \(2018\)](#) and [Bansal & Verma \(2017\)](#), which were 100, 102, 150, 386, 326 and 138 m/z ([Figure 5](#)). According to the literature, the original CEX molecule ($m/z = 347.77$) is attacked by active compounds (H^+ or $\cdot\text{OH}$), leading to the formation of by-products C1 (unsaturated acid) and C3 (azo heterocyclic compound) through fragmentation ([Li *et al.*](#)

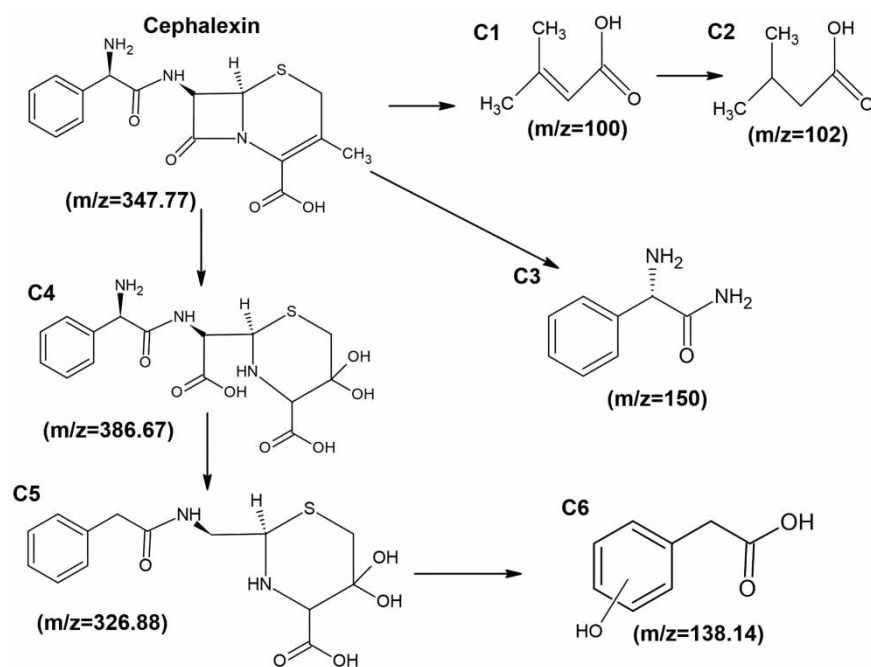


Figure 5 | Proposed degradation pathway of cephalixin.

2018). The compound C4 is obtained by breaking the β -lactam ring through hydroxylation, which leads to the formation of two other compounds (C5 and C6) (He *et al.* 2019). The C1 compound can undergo electron reduction through attack against the C=C bond by addition reaction, leading to the formation of the C2 by-product (Li *et al.* 2018).

In the present study, through the extracted ion chromatogram (EIC) of the selected molecules, only the formation of the C3 compound ($m/z = 150$) was observed at 15 min of irradiation, intensifying at 35 min and reaching peak identification at 75 min of irradiation.

Besides the aforementioned molecules (Figure 5), the need to explore more compounds is emphasized, as both CEX and its degradation products can easily be oxidized or reduced to smaller molecules. Bansal & Verma (2017) studied the degradation of CEX by photocatalysis under a longer period of exposure (4 hours), and investigation of the by-products was performed via gas chromatography coupled to mass spectrometry (GC-MS); they found that the metabolites had been reduced to nitrogen and sulfur. However, in this current work, assays were performed with less irradiation time in order to propose a faster treatment method, but we were unable to determine if the final compounds were recalcitrant or not, since the metabolites were found to be below the LOD employed. For future testing, solutions with higher CEX concentrations must be prepared so that mineralization can be investigated, or a more sensitive method, such as GC-MS, should be employed.

Verification of residual antimicrobial activity

The results obtained for the MIC of CEX solutions irradiated against *S. aureus* and *E. coli* are shown in Table 1. After 50 min of exposure, all MICs were equal to or higher than

Table 1 | Minimal inhibitory concentration of cephalixin before and during photo-oxidation

Exposure time (min)	MIC 100% of inhibition ($\mu\text{g/mL}$)	
	<i>Staphylococcus aureus</i> ATCC 25923	<i>Escherichia coli</i> ATCC 25922
0	4	8
10	4	32
20	16	64
30	32	>256
40	64	>256
50	256	>256

Source: The authors (2020).

256 $\mu\text{g/mL}$. However, it should be noted that these concentrations are not the real concentration of CEX in the irradiated solutions, given that part of the drug molecules had already been degraded. Thus, an MIC greater than or equal to 256 $\mu\text{g/mL}$ in 50 min is a hypothetical concentration based on the non-irradiated solution.

First, it can be observed that *S. aureus* is more susceptible to CEX than *E. coli*, a fact that can already be observed in the MIC at time zero, where a result of 4 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$ was observed for each microorganism, respectively. This is because Gram-negative bacteria are relatively more resistant to antibiotics than Gram-positive bacteria. This is due to the outer layer of lipopolysaccharides in the cell walls of Gram-negative bacteria, which restrict the uptake of many molecules and their movement to openings, called porins (Tortora *et al.* 2017). A lower MIC indicates that a smaller amount of antibiotic is needed to inhibit 100% of microbial growth, and consequently, that the microorganism is less resistant to the antibiotic in question. For the microorganisms tested, both were sensitive to CEX, according to the breakpoints established for the methodology by CLSI.

After irradiation of the CEX solution, it was found that during an irradiation time of 10 min there were no changes in the MIC compared to time zero, remaining at 4 $\mu\text{g/mL}$, indicating that the CEX molecules still present in solution were able to completely inhibit the growth of this microorganism. For *E. coli*, there was a fourfold increase in the MIC compared to the zero time zero, from 8 $\mu\text{g/mL}$ to 32 $\mu\text{g/mL}$. This indicates that although there were still sufficient molecules for the inhibition of *S. aureus*, the antibiotic molecules were partially degraded, reducing the presence of molecules that were still integral and active. With a decrease in the content of active antibiotic molecules in the same aliquot of solution and *E. coli* being more resistant against CEX than *S. aureus*, there was an increase in the MIC required for 100% growth inhibition of the microorganisms.

After irradiation of the CEX solution for 20 min, where 81.1% of the antibiotic molecules had already been degraded, the MIC for *S. aureus* increased fourfold and for *E. coli* increased eightfold compared to the MIC of the non-irradiated solution (time 0). After 30 min of irradiation, there was an eightfold increase in the MIC for *S. aureus* and at least a 32-fold increase for *E. coli* compared to time 0. At this irradiation time and in the following irradiation times (40 and 50 min), the MIC observed for *E. coli* was higher than the highest hypothetical concentration tested (256 $\mu\text{g/mL}$). For *S. aureus*, an MIC of 256 $\mu\text{g/mL}$ was obtained only after the irradiation of the solution for 50 min. These results show

that CEX molecules were degraded over time and the residual antimicrobial activity decreased as the irradiation time increased.

Thus, for inactivation of CEX and its metabolites for 100% inhibition of *E. coli* growth, an irradiation time of 30 min is sufficient. However, for *S. aureus*, a degradation time longer than 50 min is necessary, and possibly the use of a UV-C lamp at a higher power or the use of more than one lamp, to generate more intense irradiation in the same period of time, should be able to achieve even better results than those obtained in the present study. However, to confirm this, new degradation tests and verification of the generated by-products are required.

Evaluation of residual antimicrobial activity is of great importance for indicating that the antibiotic molecule has been degraded and that the resulting by-products do not possess antimicrobial activity. Studies that evaluated CEX degradation through sonochemical or electrochemical processes and the residual antimicrobial activity against *S. aureus* observed loss of antibiotic efficacy as molecules were degraded, resulting in an increased MIC, as in this study, indicating that the fragments generated do not have antimicrobial activity (Serna-Galvis *et al.* 2017a; Serna-Galvis *et al.* 2019).

CONCLUSION

From the results obtained, it is concluded that the treatment using a UV-C lamp is effective in the degradation of cephalixin, degrading 81% of the antibiotic molecules in 20 min. Regarding the formation of by-products, the only one detectable was the $m/z = 150$ compound, and it was observed at 15 min of degradation. The products of the degraded molecules did not show antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. The molecule degradation increased along with the MIC against microorganisms due to the longer treatment time, i.e., antimicrobial compounds decreased, allowing microbial growth. The tests proposed in this study are extremely important for monitoring the formation of by-products to evaluate antimicrobial activity and ensure water quality after UV-C irradiation.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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