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ABSTRACT

The presence of trace levels of organic contaminants in the environment is currently an environmental concern. When these contaminants are subjected to environmental transformations, environmental transformation products (ETPs) are obtained, whose structures often remain unknown. The absence of information concerning these new compounds makes them unavailable and consequently makes their environmental detection as well as their (eco)toxicological study impossible. This report describes a multidisciplinary approach that seeks to both anticipate the fate and evaluate the impact of organic environmental contaminants.

Our approach consists of three steps. First, isolated and fully characterized transformation products (TPs) of the parent molecule are obtained. This step requires classical organic synthesis and focuses on providing standards of the compounds. In the second step, the parent molecule is subjected to environmentally relevant transformations to identify plausible ETPs. The detection of previously characterized TPs allows the concomitant identification of plausible ETPs. The third step is devoted to the toxicological evaluation of the identified plausible ETPs.

Such an approach has recently been applied to furosemide and has allowed the identification of its main TPs. This report now seeks to identify and evaluate toxicologically plausible ETPs of this drug, which is also known as an environmental contaminant.

Keywords: Furosemide, Electro-Fenton, Bioconversion, Toxicity

1. INTRODUCTION

Emerging pollutants are a class of compounds that result from human activity, are detected in the environment and whose impact on ecosystems remains unknown (Levi, 2009). These pollutants consist of compounds that were originally developed as pharmaceuticals and personal care products (PPCPs) (Daughton, 2002; Khetan and Collins, 2007; Fatta-Kassinos et al., 2011a) bactericides or industrial additives (Jurado et al., 2012; Rodil et al., 2012). Regarding the pharmaceuticals, the progress made in trace analysis during the last decade (Buchberger, 2011) has allowed their detection and quantification in the effluents of sewage treatment plants (STPs), rivers, ground water, and sometimes even in drinking water (Heberer, 2002; Kuemmerer, 2004). The presence of pharmaceuticals in the environment mainly occurs after their excretion and results from an incomplete degradation in STPs (Deblonde et al., 2011). Despite their low environmental concentrations, which are generally measured to be less than human therapeutic levels (from ng/L to µg/L), the continuous release of these substances makes them pseudo-persistent, thus presenting a risk of ecotoxicity or chronic toxicity for non-target organisms (Daughton and Ternes, 1999).

Assessing the impact of the presence of drugs (i.e., molecules designed to exert a biological effect) on aquatic biota requires an ecotoxicity or a chronic toxicity to be univocally associated with a perfectly identified chemical structure (or alternatively, to a well-defined mixture of molecules). For this purpose, biological models have been applied to molecules whose presence in the environment had been previously highlighted (Henschel et al., 1997). However, the primary limitation of such studies is that they do not consider the environmental transformation products (ETPs), i.e., compounds stemming from the parent molecule and likely to appear in the environment consecutively to (a)biotic transformations. Attempts to identify ETPs from the analysis of complex environmental matrices, such as soils, sediments or biota, have been demonstrated to be prone to failure (Ibanez et al., 2004; Richardson, 2010). Under these conditions, chromatographic techniques coupled with mass spectrometry in "full-scan" detection mode cannot achieve the detection limits required to characterize these unknown compounds that are present at trace levels. In the absence of characterization, the majority of ETPs remain ill-defined, thus ruling out the evaluation of their (eco)toxicities, which is problematic because the toxicity of ETPs may exceed that of their parent molecules (Dirany et al., 2011; Fatta-Kassinos et al., 2011b; Dirany et al., 2012). In the case of pharmaceuticals, metabolites are a class of ETPs whose structure can be rather well-anticipated (Lohmann and Karst, 2008), and these have already been the subject of

environmental detections (Celiz et al., 2009; Mompelat et al., 2009). Further investigations focusing on the identification of new ETPs and the assessment of their impact has led to the development of various strategies (Escher and Fenner, 2011). With this aim, experiments that mimic the transformations that drugs undergo in the environment have been developed (Ziylan and Ince, 2011). These experiments, which were applied to the parent molecule, resulted in transformation products (TPs) assumed to be real (at least similar to) ETPs. Such experiments typically proceed by electro-Fenton (Oturan et al., 1999), photodegradation (Packer et al., 2003) or biodegradation (Ericson, 2010; Gauthier et al., 2010). Because of such protocols and thanks to the means now available, elucidating the structure of these compounds is possible. Nevertheless, authentication of the structure requires the use of standards either commercially available or synthesized (Schulze et al., 2010; Svanfelt and Kronberg, 2011). In the absence of isolated compounds, (eco)toxicological studies are conducted on mixtures of TPs resulting from the degradation of the parent molecule. Interestingly, examples of (eco)toxicological evaluations of fully characterized TPs obtained and isolated after the degradation of the parent pharmaceutical remain scarce (Isidori et al., 2006).

In this report, we describe a new approach that enables both the identification of plausible ETPs and their toxicological evaluation. Because the main difficulty in studying the fate of drugs in the environment lies in the lack of authentic samples of their ETPs, we propose a new stepped approach. The first step consists of isolating the TPs of a drug that is known to be an environmental contaminant. This step, which requires the tools of organic synthesis, focuses on providing fully characterized standards and the development of methods for their detection in complex mixtures. In the second step, the contaminant is submitted to environmentally relevant transformations, i.e., processes that are representative of the transformations undergone by contaminants in the environment. By highlighting previously characterized TPs, this step allows the identification of plausible ETPs. In the third step, a toxicological study is initiated on each of the TPs that are identified as plausible ETPs.

In a recent work, we described the behavior of furosemide **1** (fig 1) under oxidative conditions (Laurence et al., 2011). This drug, which has been widely used as a diuretic since the nineteen sixties, is one of the forty compounds having the highest risk with a predicted environmental concentration greater than 100 ng/L (Besse and Garric, 2008). Poorly metabolized and eliminated either unmodified or as glucuronide conjugate (Lee et al., 1997) (which releases furosemide after hydrolysis), this drug has been detected at concentration ranges between 61 and 200 ng/L in European rivers (Castiglioni et al., 2006; Khalaf et al.,

2009). During the course of our preliminary study, three TPs of furosemide were identified: furfural **2**, saluamine **3** (both resulting from the oxidation of the amino group) and a zwitterionic pyridinium **4** (resulting from the oxidation of the furan ring). These three TPs, which are obtained by anodic oxidation, have been isolated and fully characterized, thereby allowing their detection by LC-MS in complex mixtures.

This study seeks to confirm whether these three TPs, which were obtained without any environmental relevance, are indeed plausible ETPs. For this purpose, environmentally relevant degradation pathways were applied to furosemide. To be representative of the transformations likely to occur in the environment, two protocols were selected. The first protocol is abiotic and uses an electrochemical advanced oxidation process (electro-Fenton); the second protocol is biotic and requires microorganisms. The TPs identified as plausible ETPs were then subjected to a preliminary toxicological study.

2. MATERIAL AND METHODS

2.1 Chemicals

Furosemide (> 99.0%) was purchased from TCI Europe N.V. Furfural (99%) and 1-Methyl-4-phenylpyridinium iodide (MPP^+) were purchased from Sigma-Aldrich. Saluamine **3** and pyridinium **4** employed for the toxicological evaluation and required as standards for the analysis of complex mixtures were prepared as previously described (Laurence et al., 2011). Dulbecco's Minimal Essential Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA solution and phosphate buffered saline (PBS) were purchased from GibcoBRL (France). The caspase fluorogenic substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC) was purchased from Calbiochem (France). Sodium chloride (NaCl), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), triton X-100, dithiothreitol (DTT), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Germany).

2.2 Analytical procedure

ESI-MS/MS analyses were conducted using a Q-TOF Premier instrument equipped with a Z-spray electrospray source (Waters, Saint Quentin-en-Yvelines, France) operating in the positive mode. For fragmentation studies, solutions were introduced into the electrospray ionization source using a syringe pump at an infusion rate of 10 µL/min. To investigate the degradation products, the sample was analyzed using a 2690 liquid chromatography module from Waters (Saint Quentin-en-Yvelines, France) coupled with a Q-TOF premier mass spectrometer. The employed analytical column was a C18 Atlantis T3, 3 µm, 150 x 2.1 mm (Waters, Saint Quentin-en-Yvelines, France). The HPLC solvents were acetonitrile with 0.1% formic acid (A) and water with 0.1% formic acid (B). The following linear gradient elution program was applied: 20% of A for 11 min and 100% of A from 11.1 to 20 min. Then, before the next injection cycle, the column was reconditioned with 20% of A for 10 min. The effluent was introduced at a rate of 0.2 mL/min into the Z-spray interface for ionization. The following three acquisition modes were used to characterize each compound: (i) full-scan mode in V-mode; (ii) MS/MS of the precursor ion and (iii) full-scan in the W-mode for high resolution. The full-scan acquisition mode allowed the cone voltage to be optimized to obtain maximum intensity for the precursor ion. Several MS/MS spectra were recorded to obtain the decomposition pathways.

The cone voltage was adjusted for each ion of interest that was generated in the ion source. The ion source parameters were adjusted as follows to optimize the ion signals: the cone voltage was set between 20 to 80 V while the capillary voltage was set to 2.6 kV. Typical values for the other source parameters were as follows: extraction cone, 1.7 V; ion guide, 3 V; source and desolvation temperatures were set at 80 °C and 250 °C for direct infusion and at 100 °C and 450 °C for LC-MS studies, respectively. Nitrogen was used as both nebulizing and desolvation gases. The gas flow rates were dependent on the introduction mode; they ranged from 20 L/h to 300 L/h for direct introduction and from 70 L/h to 700 L/h for LC-MS. For LC-MS analysis, the injected volume of sample was 10 µL. Argon was used as a collision gas at a flow of 0.28 mL/min, which corresponds to a pressure of ca. 4 10^{-3} mbar. To record the MS/MS spectra of ions, the collision energies were varied between 2 to 30 eV for each compound to obtain their primary characteristic ions. For accurate mass measurements, the analyses were performed using W-mode and an independent reference spray via the LockSpray interface. Sulfadimethoxine was used as the lock mass compound at a flow rate of 10 µL/min. The LockSpray frequency was set at 10 s, and data for the reference compounds were averaged over 10 spectra/min. Accurate masses and elemental compositions

for all ions were obtained using the MassLynx instrument software. The ion used for the mass correction was m/z 311.0814.

2.3 Electro-Fenton

Experiments were conducted in an open, cylindrical and undivided glass cell of 6 cm diameter and 250 mL capacity. This cell was equipped with a cylindrical Pt mesh (4.5 cm height, 3.1 cm internal diameter) as an anode and a large surface area (14 cm x 4.5 cm each side, 0.5 cm width) carbon felt (Carbon-Lorraine, France) as a cathode. The anode was centered in the electrochemical cell and surrounded by the cathode, which covered the inner wall of the cell. Electrolyses were conducted under a constant current applied by a Hameg HM8040 triple power supply. H_2O_2 was produced from the reduction of O_2 dissolved in the solution. Continuous saturation of this gas at atmospheric pressure was ensured by bubbling compressed air. 220 mL of solutions containing 0.1 to 0.3 mM furosemide and 0.5 mM Fe^{2+} at the optimum pH value of 3.0 were electrolyzed at a constant current (30-350 mA) at room temperature. The decrease in the concentration of furosemide and the formation of oxidation intermediates were followed using a Merck Lachrom HPLC chromatograph composed of a degasser (L-7614), an injection pump (L-7100 equipped with a 20 µL injection loop), a reverse phase RP-18 Purospher column (5 µm, 4.6 mm× 25 cm) placed into a L-7350 oven set to 40 °C and a photodiode array detector (L-4755) selected at 280 nm. The mobile phase consisted of a mixture of methanol and phosphoric acid (55/45 (v/v)) at a pH of 3. Elution was performed in isocratic mode with a flow rate of 0.7 mL/min. Identification of aromatic intermediates was conducted by comparing the retention times with that of authentic compounds and UV-Vis spectra. The analyses were monitored using the EZChrom Elite software package.

2.4 Bioconversion

Microorganisms, including 48 fungi and 24 bacteria, were cultured in liquid medium (25 mL) containing in g/L: yeast extract (Conda) 4, malt extract (Conda) 10, soybean peptones (OrganoTechnie) 5, and glucose 16. The process of selection involved six series of 12 microorganisms. After sufficient growth, the biomasses were harvested by centrifugation (bacteria) or filtration (fungi) and suspended in 12 mL of a 0.1 M sodium citrate buffer (pH 5). For each series, four incubation mixtures (3 fungi and 2 bacteria) were created using the

suspensions (4 mL, total volume of 20 mL). Furosemide was dissolved in *N*,*N*-dimethylformamide (0.066 mg/µL) and 60 µL of this solution were added to the incubation mixtures (final concentration 0.2 mg/ml). Biotransformations were performed under an air atmosphere at 27 °C and 200 rpm and monitored by HPLC and MS. Aliquots (culture medium and biomass; 0.8 mL) were withdrawn every two days, diluted with methanol (200 µL), mixed vigorously and centrifuged at 13,000 g for 5 minutes. The resulting supernatants were micro-filtered (0.45 µm) before analysis.

2.5 Toxicity measurements

2.5.1 MTT Cell Viability Assay

Human neuroblastoma cells (SH-SY5Y) (ATCC CRL2266) were cultured in DMEM (in 75 mL flasks), containing 4.5 g/L glucose and supplemented with 1% penicillin/streptomycin and 10% FBS, at 37 °C in a humidified atmosphere of 5% CO_2. Cell viability was measured using the MTT assay in 96-well plates at a cell density of 8×10^4 cells/mL. The cells were exposed to different compounds (**1**, furfural **2**, **3** and **4**) at various concentrations (from 1 to 1000 µM). All drugs were dissolved in a mixture of DMSO and DMEM (1/1 (v/v)) in order to obtain a 10^{-1} M stock solution. All controls were carried out using the same solvent mixture. The final solution contained no more than 0.5% (v/v) of DMSO. After 24, 48, 72 and 96 h of exposure, MTT (20 µL, 5 mg/mL) was added to each well. Following incubation at 37 °C for 2 h, the supernatants were carefully removed and 200 µL of DMSO were added. The optical density (OD) value of each well was read using a micro-plate reader (TECAN Infinite M1000) at a wavelength of 570 nm. Cell viability was calculated as a percentage of the control.

2.5.2 Caspase-3 activity

The caspase-3 activity was assessed by following the cleavage of the fluorogenic substrates Ac-DEVD-AFC. Treated cells were harvested through trypsinization using 0.05% trypsin and 0.53 mM EDTA at 37 °C in a humidified atmosphere containing 5% CO_2 for 4 min. Trypsinization was terminated by addition of 20% FBS, the cell suspension was then centrifuged, the supernatant discarded and the pellet washed with 1 × PBS ($CaCl_2$-, $MgCl_2$-free) (Gibco, Invitrogen). The cell pellet was gently suspended in a buffer containing 30 mM

HEPES, 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100 and 10 mM DTT and then centrifuged. The supernatant was used for the assay. The fluorogenic substrates were added at a final concentration of 100 mM. The samples were gently mixed and incubated in the dark at 37 °C for 1 h. Fluorescence intensity of the samples was measured at an excitation λ of 400 nm and emission λ of 505 nm using a fluorescence micro-plate reader (TECAN infinite™ M1000).

2.5.4 Data analysis

Data were expressed as the mean ± SEM. Comparisons among groups were conducted using an ANOVA (F-test) test. Bonferroni adjusted t-tests were used for multiple group comparisons and the unpaired t-test was used for single comparisons. A two-tailed $p<0.05$ was selected to indicate a statistically significant difference. All values were calculated using the GraphPad Prism 5.00 software package (GraphPad Software, San Diego, CA).

3. RESULTS

3.1 Electro-Fenton

In the first step, furosemide was subjected to electro-Fenton oxidative degradation. This method is based on the *in situ* and catalytic production of hydroxyl radicals (•OH) from the electrochemically assisted Fenton's reaction (Eqs. 1-3).

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (1)$$

$$Fe^{3+} + e^- \rightarrow Fe^{2+} \quad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + \cdot OH \quad (3)$$

The hydroxyl radical is a very powerful oxidizing agent, and it non-selectively reacts with organic molecules to lead to the formation of abiotic TPs. In the treatment strategy, the operating parameters were established to further oxidize these intermediates until they reached their ultimate degree of oxidation, i.e., their mineralization (Eq. 4) (Oturan et al., 2000; Sires et al., 2007; Brillas et al., 2009). This process has been successfully applied for

the treatment of toxic/persistent pollutants and particularly for the removal of pharmaceutical pollutants from water (Ozcan et al., 2008; Dirany et al., 2010; Sires et al., 2010).

$$\text{•OH + organic pollutants} \rightarrow \text{[abiotic TPs]} \rightarrow \text{mineralization} \quad (4)$$

As stated above, the electro-Fenton process was used to mimic the abiotic transformations that are likely to occur in the environment. Contrary to the abatement (treatment) strategy, the inhibition of oxidation kinetics was consequently required to allow the accumulation of the abiotic TPs. Briefly, this inhibition consisted of applying a small current (for weakening the rate of Eqs. 1 and 2) and using a high concentration of catalyst (Fe^{2+}) to favor the waste reaction between •OH and Fe^{2+} (Eq. 5), thus lowering the concentration of •OH and consequently the oxidation rate of the intermediates.

$$\text{•OH} + Fe^{2+} \rightarrow Fe^{3+} + OH^- \quad (5)$$

This strategy was applied under a pH of 3 at room temperature to a 230 mL aqueous solution of furosemide (0.3 mM) using a current of 50 mA between a Pt anode and a carbon-felt cathode in the presence of Fe^{2+} (0.5 mM) as a catalyst. After 5 min of electrolysis, HPLC analysis of an aliquot revealed the presence of several abiotic TPs. Preparative chromatography on an RP 18 column allowed the isolation of the main compound and its characterization by ^{1}H-NMR. After comparison with the previously obtained data, the aniline derivative **3** (Fig 1) was unambiguously identified. The co-product of this oxidation process, the furfural, was also identified among the other abiotic TPs by comparison with the HPLC chromatogram and UV spectrum of an authentic sample.

Monitoring the electro-Fenton treatment of furosemide by HPLC and analyzing aliquots by LC-MS confirmed the formation of compound **3** with the presence of the m/z 251 protonated molecular ion (see SI §2). Two other compounds with protonated molecular ions at m/z 329 and m/z 345 were also observed. The structure associated with m/z 329 was confirmed by comparison with previously obtained data and unambiguously identified as pyridinium **4** (see SI §4). The CID mass spectrum analysis led to the proposal of structure **A** (see SI §5, 6) for m/z 345 (Fig 2a).

To identify further oxidation products of furosemide, electro-Fenton oxidation was performed on pyridinium **4**. Under these conditions, the compound previously associated with

structure **A** was again observed (see SI §5) as well as the aniline derivative **3** (see SI §3). The oxidation of the pyridinium ring of **4,** which leads to intermediates such as **A**, may explain the formation of the latter. Meanwhile, LC-MS analysis of the reaction mixture allowed the observation of two compounds with pseudo-molecular ions at m/z 317 and 282 for which structures **B1** and **C1** (Fig 2b) have been, respectively proposed (see SI §7-10). The same experiment also revealed the presence of two isomers of **B1** and **C1**. The analysis of the CID mass spectra did not allow their univocal characterization, which leads to the proposal of the regioisomers **B2** or **B3** for m/z 317 and **C2** or **C3** for m/z 282.

3.2 Bioconversion

Furosemide is poorly metabolized by humans; however, this does not exclude the possibility for non-target organisms disposing of the appropriate enzymatic equipment to achieve the transformation of furosemide. One way to fairly accurately identify the spectrum of biotic TPs arising from furosemide consists of subjecting this molecule to microbial transformation (bioconversion). The enzymes that are present in many microbial strains allow access to a wide range of biotransformation products. This approach, which is especially used in the preparation of mammalian metabolites (Smith and Rosazza, 1975; Azerad, 1999; Asha and Vidyavathi, 2009), requires the selection of active microorganisms (Li et al., 2008; Marvalin and Azerad, 2011).

To obtain the biotic TPs of furosemide, a selection of microorganisms among seventy-two strains of bacteria and fungi was performed. To complete the screening using minimal time and assays, we used our recently developed combinatorial approach (Fromentin et al., 2012; Joyeau et al., 2013). This method is based on incubating the substrate in the presence of five microorganisms (three fungi and two bacteria) in a reaction buffer. After furosemide was added to the suspensions, the biotransformations were monitored by HPLC and MS analyses of aliquots collected every forty-eight hours.

This strategy allowed identification of four microorganisms capable of performing the biotransformation of furosemide as well as the observation of two major biotic TPs. Saluamine **3**, which resulted from the hydrolysis of the intermediate hemiaminal, was obtained with a yield of 25-30% after four days of incubation with the bacteria *Agrobacterium tumefaciens (Rhizobium radiobacter* CIP 67.1) and *Arthrobacter ureafaciens* CIP 67.3. This result confirmed the ability of the microorganisms to oxidize the α-position of the amino group of furosemide, as previously observed (Hezari and Davis, 1992). The absence of

furfural may be explained by the ability of some microorganisms to degrade it (Koopman et al., 2010). The fungus *Aspergillus candidus* ATCC 20023 produced pyridinium **4** in moderate yields after eight days (see SI §4). The fungus *Cunninghamella echinulata* var. *elegans* ATCC 9245 fully metabolized furosemide in six days to yield a mixture of both compounds. In some cases, the compound associated with structure **A** was also observed as a minor biotic TP (see SI §5). Further investigations are in progress to optimize the production of this TP to isolate it and confirm its structure.

3.3 Toxicological evaluation of plausible ETPs

The evaluation on the neuroblastoma cell line (SH-SY5Y) of furosemide **1** revealed no activity on the tested cells after 24 and 48 h. However, after 72 h, a slight inhibition of approximately 20% of the cell survival was observed, which is possibly explained by the action of furosemide on the regulation of the cytosolic pH (Rakonczay et al., 2008). An extended exposure to furosemide for 96 h did not reveal any increased inhibition.

In the same test, the saluamine **3** was completely inactive up to 96 h at all investigated concentrations. Exhibiting no toxicity after 24 and 48 h, furfural **2** reduced the cell viability by approximately 20% after 72 h at the maximal concentration of 1000 µM. As observed with the furosemide, no increased inhibition was observed after extended exposure. Interestingly, pyridinium **4** also began to have an effect after 72 h of exposure. The effect of pyridinium **4** on the cell mortality was increased from 40 to 60% between 72 to 96 h. The concentration at which 50% of the cells survived (EC_{50}) was estimated to be 973 µM (Fig 3a).

The induction of cell death by exposure to pyridinium **4** was confirmed after evaluation of the caspase-3 activity (Fig 3b). The increased activity (175%) observed after 96 h for the treated cells compared to the untreated ones (100%) allowed the previously observed cell death to be clearly associated with an apoptotic process.

4. DISCUSSION

Anticipating the fate of emerging pollutants often leads to complexity in investigating and researching ETPs, which for the most part remain unknown. By detecting after degradation of a contaminant by environmentally relevant transformations, compounds that have been fully characterized in a previous step, our approach has demonstrated its ability to univocally identify plausible ETPs of organic pollutants. Interestingly, the alternative

approach consisting of applying environmentally relevant transformations to a contaminant to obtain, isolate and characterize its plausible ETPs in a single step might seem intuitively quicker and more appropriate. However, the results of this study suggest that such an approach would have failed to detect plausible ETPs exhibiting significantly different physicochemical properties from those of the parent compound, as in the case of pyridinium **4**. Note that saluamine **3** has already been identified as a biotic TP of furosemide after bioconversion with the fungus *Cunninghamella elegans* (Hezari and Davis, 1992). The fact that pyridinium **4** was not observed in the same study may be explained by the unavailability of any standard. However, Burka observed the formation of pyridinium **4** after the liver microsomal incubation of furosemide (Chen and Burka, 2007), but this was consecutive to its NMR characterization after the oxidation of furosemide with dimethyldioxirane in deuterated acetone.

Because electro-Fenton degradation and bioconversion are representative of the transformations that are likely to occur in the environment, the saluamine **3** and the pyridinium **4**, which both result from these two degradation processes, can consequently be considered as plausible ETPs of furosemide. With the help of available standards, the implementation of protocols for the environmental detection of these ETPs can now be initiated. A possible co-product of the oxidation leading to aniline **3**, furfural, is therefore also a plausible ETPs of furosemide. The abundant use of this compound stemming from the biomass for industrial purposes makes it nevertheless an unreliable marker of the presence of furosemide in the environment. Additionally, while the growth-inhibiting properties of furfural have already been mentioned (Almeida et al., 2009), it appears that the concentrations at which furosemide is detected in the environment make the risk associated to its ability to release furfural not significant (Wierckx et al., 2011).

By the observation of compounds **A**, **B** or **C** and the collection of their analytical data, our approach has also allowed the identification of new plausible ETPs, including their detection in any complex mixtures. To complete their characterization, their structure proposed on the basis of CID mass spectrum analysis and structural similarities with provided standards, has to be confirmed or refined (which is why they were indicated with letters, not numbers). This confirmation process requires either their isolation from degradation mixtures or their total synthesis (this work is currently underway). Note that each new compound that is isolated and authenticated as a plausible ETP can be subsequently subjected to environmentally relevant transformations and toxicologically evaluated. Such iterative protocol appears appropriate to anticipate further oxidation states of contaminants and thus, to

accurately predict their environmental fate, either on a structural or on a toxicological basis. Interestingly, anticipating such further oxidation states of contaminants allowed the environmental fate of furosemide to be reconsidered. Indeed, whereas the anodic oxidation, which is focused on the earlier oxidation states, suggested the aforementioned two distinct degradation pathways (proceeding by oxidation of either the amino group or the furan ring), this study revealed the ability of **4** to release saluamine **3** after over-oxidation of its pyridinium ring. Such information may be of interest for investigating the kinetics of the appearance and disappearance of toxicity associated with the transformation of contaminants in the environment.

The final part of this study was devoted to the toxicological evaluation of plausible ETPs, and it highlighted the difference in terms of toxicity between the parent molecules and their ETPs. The present study has consequently confirmed the necessity of developing new methodologies to anticipate the fate of environmental contaminants. Furthermore, this study has also demonstrated the need to provide suitable samples for biological tests, i.e., obtained on preparative scale and isolated. This latter point was demonstrated to be crucial for univocally associating toxicity to a chemical structure. In the case of furosemide, note that if a genotoxicity has already been mentioned, no molecule has yet been clearly associated with it (Rocco et al., 2010; Mondal et al., 2012). To date, only a reactive intermediate in the form of an epoxide has been proposed to explain the cytotoxicity of this drug (Williams et al., 2007). The present work may consequently help the mechanism for the toxicity of furosemide to be reconsidered in relation with one of its TPs.

In summary, our multidisciplinary approach for anticipating the fate of environmental contaminants allowed identifying three plausible ETPs of furosemide, and five other ETPs were observed whose chemical structures must be confirmed or refined. The availability of standards now enables the environmental investigations and, possibly, the quantification of aniline **3** and pyridinium **4,** which appear as relevant markers of the presence of furosemide in the environment.

The biological results also revealed a significant toxicity for the pyridinium **4**. Following this work, assessment of the risk associated with the presence of furosemide in the environment requires a particular focus on this plausible ETP, whose mechanism of action remains unknown and whose ecotoxicity remains to be investigated.

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465

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621
622

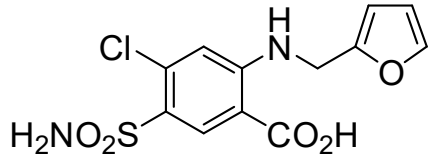
Figure captions

Figure 1: Furosemide 1 and its TPs

Figure 2: Structures proposed for a) m/z 345, b) m/z 317 and 282

Figure 3: Cytotoxic evaluation of pyridinium **4**. A) Concentration-dependant survival of neuroblastoma cells (SH-SY5Y) exposed for 96 h to pyridinium **4**. Percentage survival was calculated using values obtained for untreated cells in the MTT assays as a reference. The calculated EC_{50} was 973±46 µM. B) The caspase-3 activity was measured after 96 h of treatment with pyridinium **4** (1 mM). Data shown are the mean ± S.E.M. of 3 independent experiments with duplicate wells per experiment,* $p<0.05$ compared with control.

Figure 1



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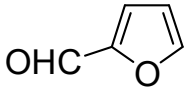

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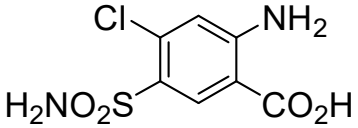

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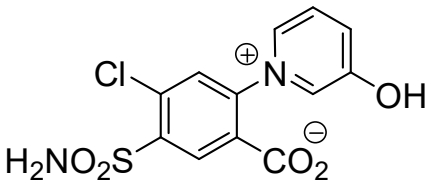

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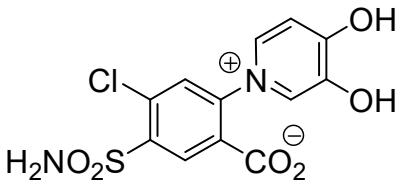
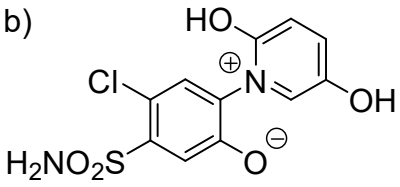
Figure 2

a)

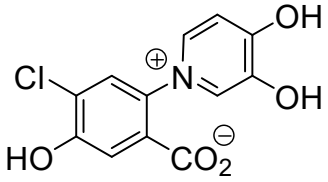


A

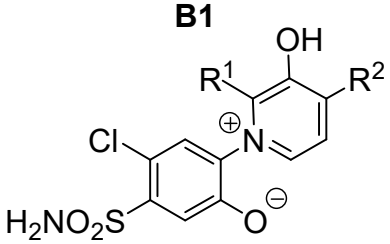
b)



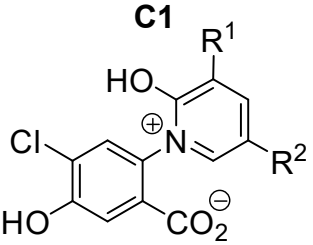
B1



C1



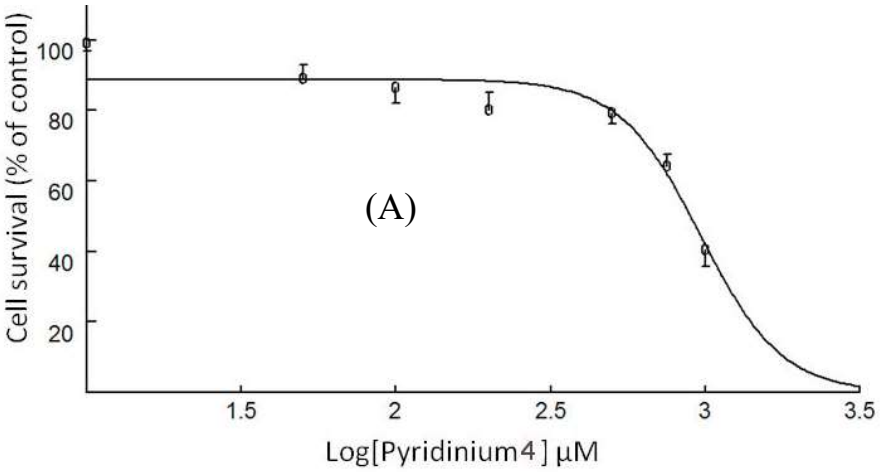
B2,3

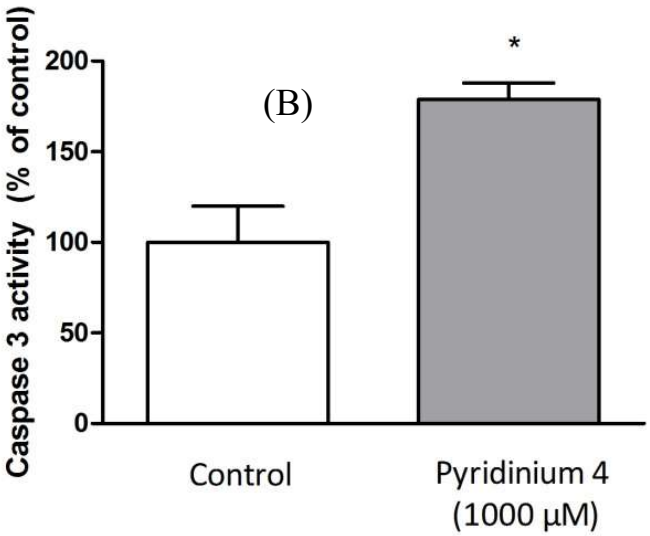


C2,3

2: R^1 = H, R^2 = OH; **3**: R^1 = OH, R^2 = H.

Figure 3


Cell survival (% of control)
100
80
60
40
20
(A)
1.5
2
2.5
3
3.5
Log[Pyridinium 4] µM


Caspase 3 activity (% of control)
200
150
100
50
0
(B)
*
Control
Pyridinium 4
(1000 µM)

Supplementary Materials

Anticipating the Fate and Impact of Organic Environmental Contaminants: a New Approach Applied to the Pharmaceutical Furosemide.

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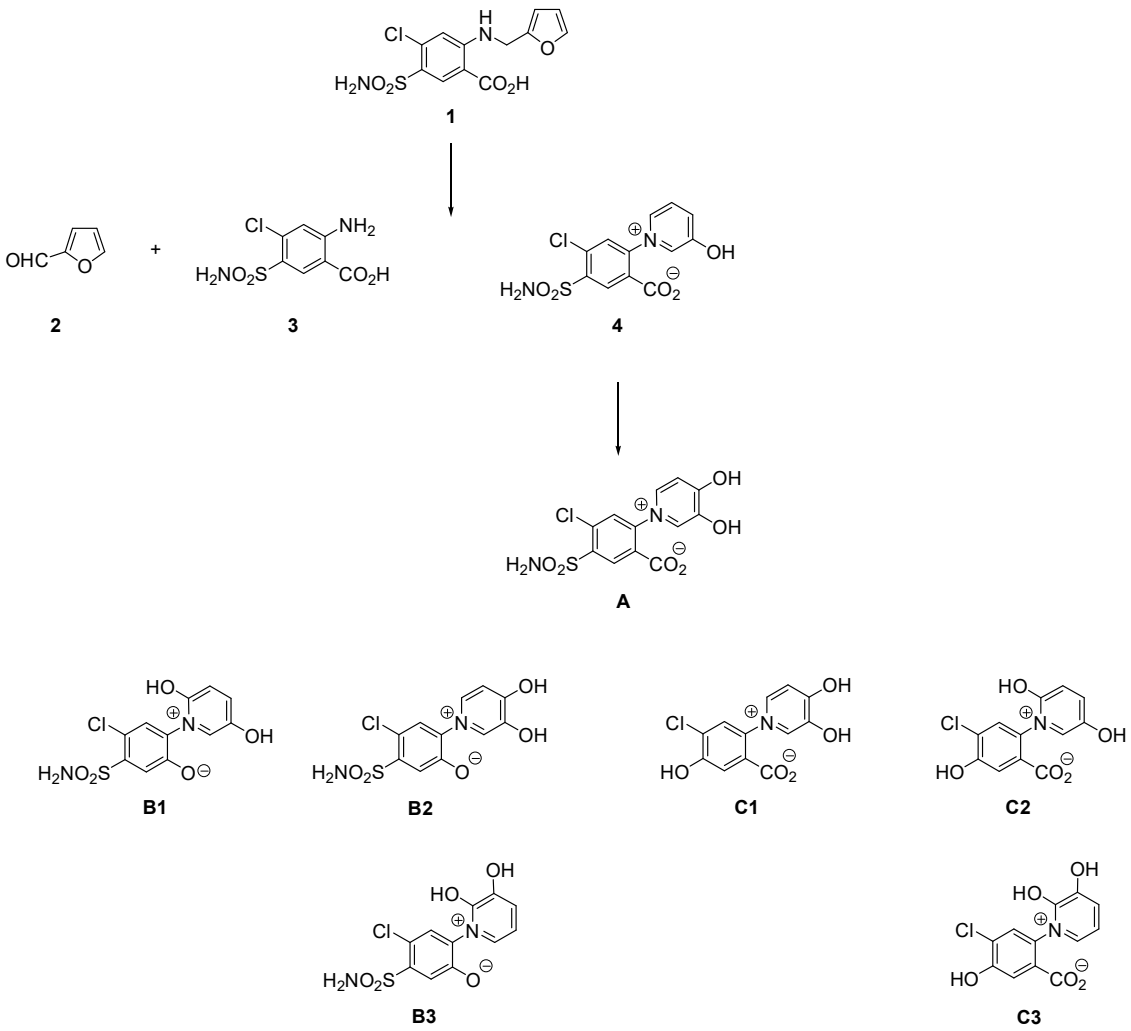
1. Furosemide 1 and its oxidation products



Oxidation products	RT (min)	$M H^+$ m/z	Exp. mass	Theo. mass	m Da	p ppm	DBE[1]	Elemental composition	Detection[2] a)	b)	c)
3	5.251	251	250.9906	250.9893	1.3	5.2	4.5	$C_7H_8ClN_2O_4S$			
4	2.29	329	328.9999	328.9999	0	0	8.5	$C_{12}H_{10}ClN_2O_5S$			
A	5.24	345	344.9948	344.9948	0	0	8.5	$C_{12}H_{10}ClN_2O_6S$			
B1	7	317	317.	317.	-	-	7	$C_{11}H_{10}ClN$			

	.0	17	0005	0024	1.9	6	.5	$_2O_5S$
B2or	4	3	316.	317.	-	-	7	$C_{11}H_{10}ClN$
3	.0	17	9997	9999	0.2	0.6	.5	$_2O_5S$
C1	1	2	282.	282.	0	2	8	$C_{12}H_9ClN$
	0.4	82	0176	0169	.7	.5	.5	O_5
C2or	4	2	282.	282.	-	-	8	$C_{12}H_9ClN$
3	.2	82	0168	0169	0.1	0.4	.5	O_5

[1] DBE: Double Bond Equivalency. [2] Detection (+) during: (a) electro-Fenton oxidation of furosemide **1**, (b) electro-Fenton oxidation of pyridinium **4**, (c) bioconversion of furosemide **1**.

2. Detection of 3 after electro-Fenton oxidation of 1

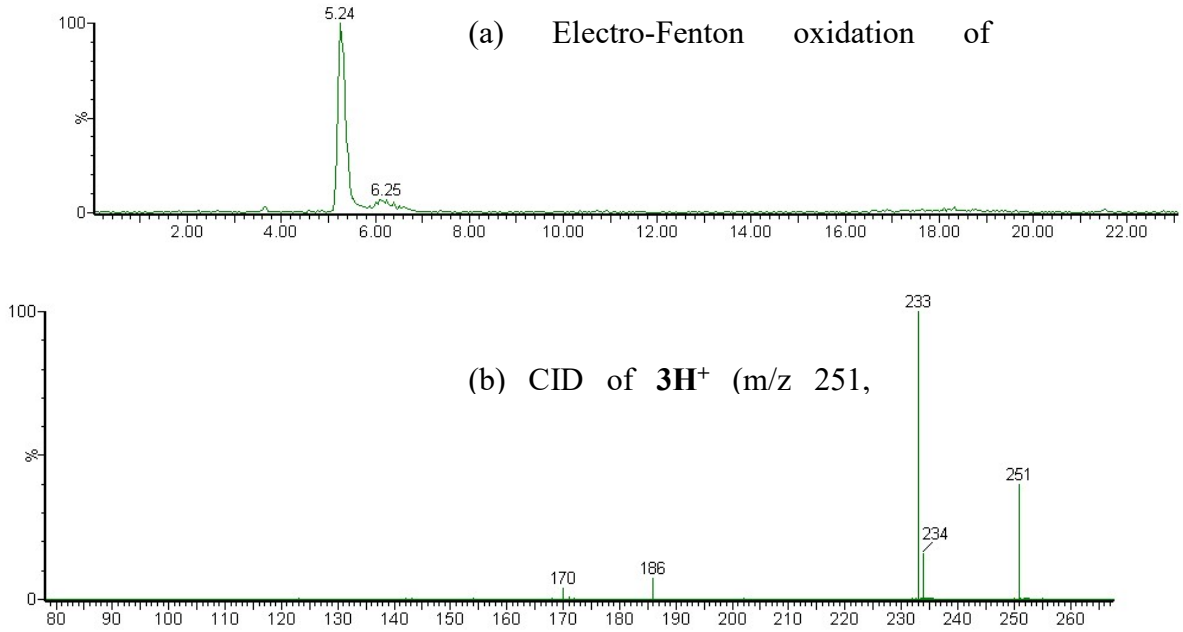
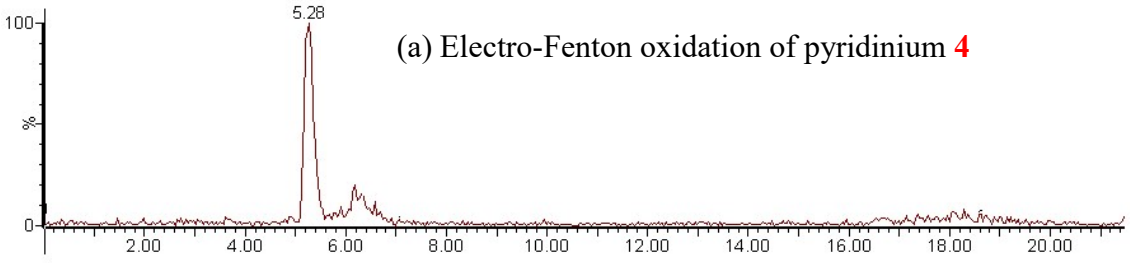


Fig. SM-1. (a) Extracted ion chromatogram (positive ion current) of m/z 251 for electro-Fenton oxidation of furosemide **1**. (b) CID mass spectrum of protonated molecular ion of compound **3** (MH^+ m/z 251), cone =25 V, Ecol = 10 eV. The CID spectrum of the ion at m/z 251 (RT=5.2 min) is the same as that obtained for the MH^+ of compound **3**.

3. Detection of 3 after electro-Fenton oxidation of 4



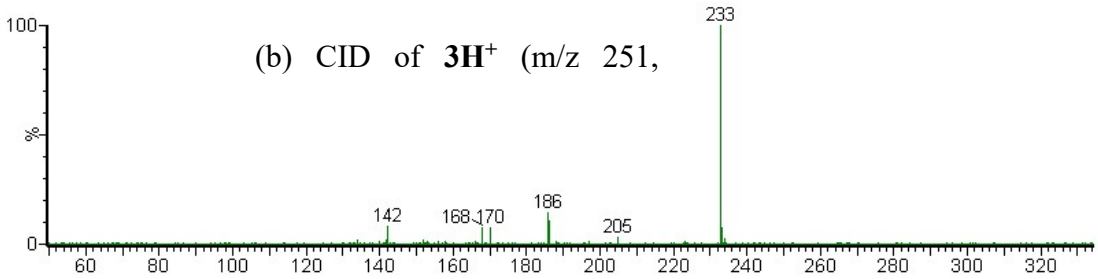
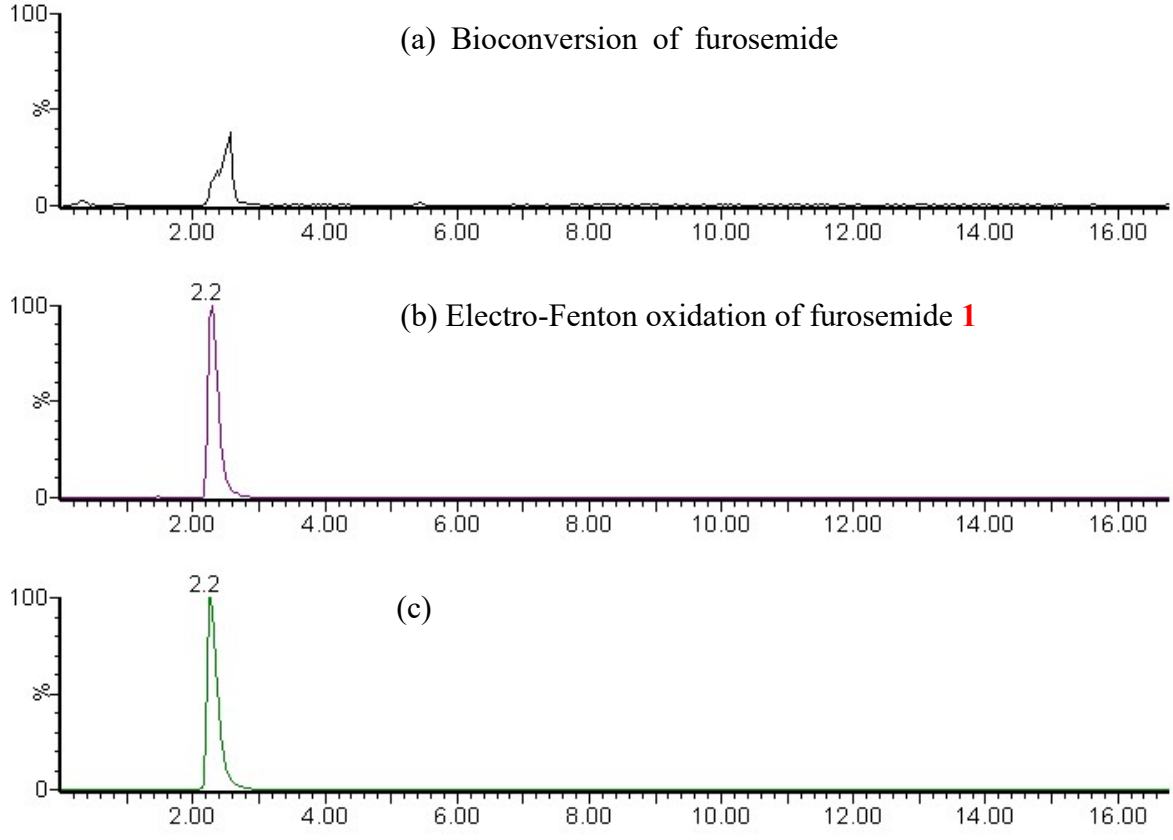


Fig. SM-2. (a) Extracted ion chromatogram (positive ion current) of m/z 251 for electro-Fenton oxidation of pyridinium **4**. (b) CID mass spectra of ion at m/z 251 at RT=5.2 min. Cone=25 V, Ecol = 15 eV.

4. Detection of 4 after electro-Fenton oxidation and bioconversion of 1



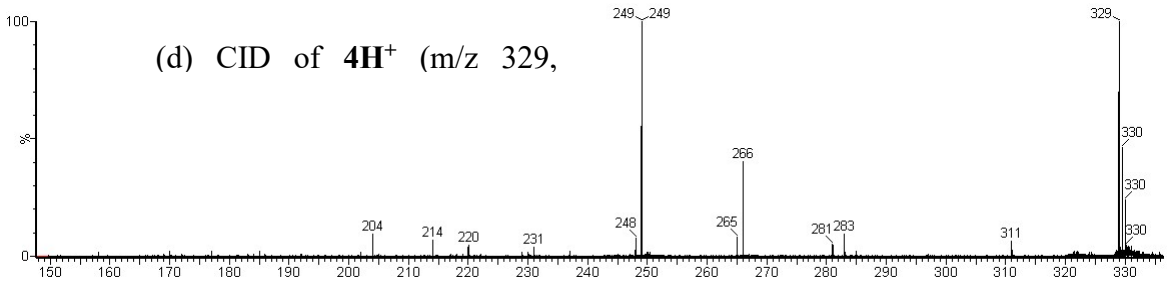


Fig. SM-3. Extracted ion chromatograms (positive ion current) of m/z 329 for (a), (b) and (c) samples. (d) CID mass spectrum of m/z 329 ion at RT=2.2 min. Cone =25 V, Ecol=10 eV. This CID mass spectrum is the same for both samples (a) and (b) and corresponds to that of the pyridinium **4**.

5. Detection of A after electro-Fenton oxidation and bioconversion of 1 and electro-Fenton oxidation of 4

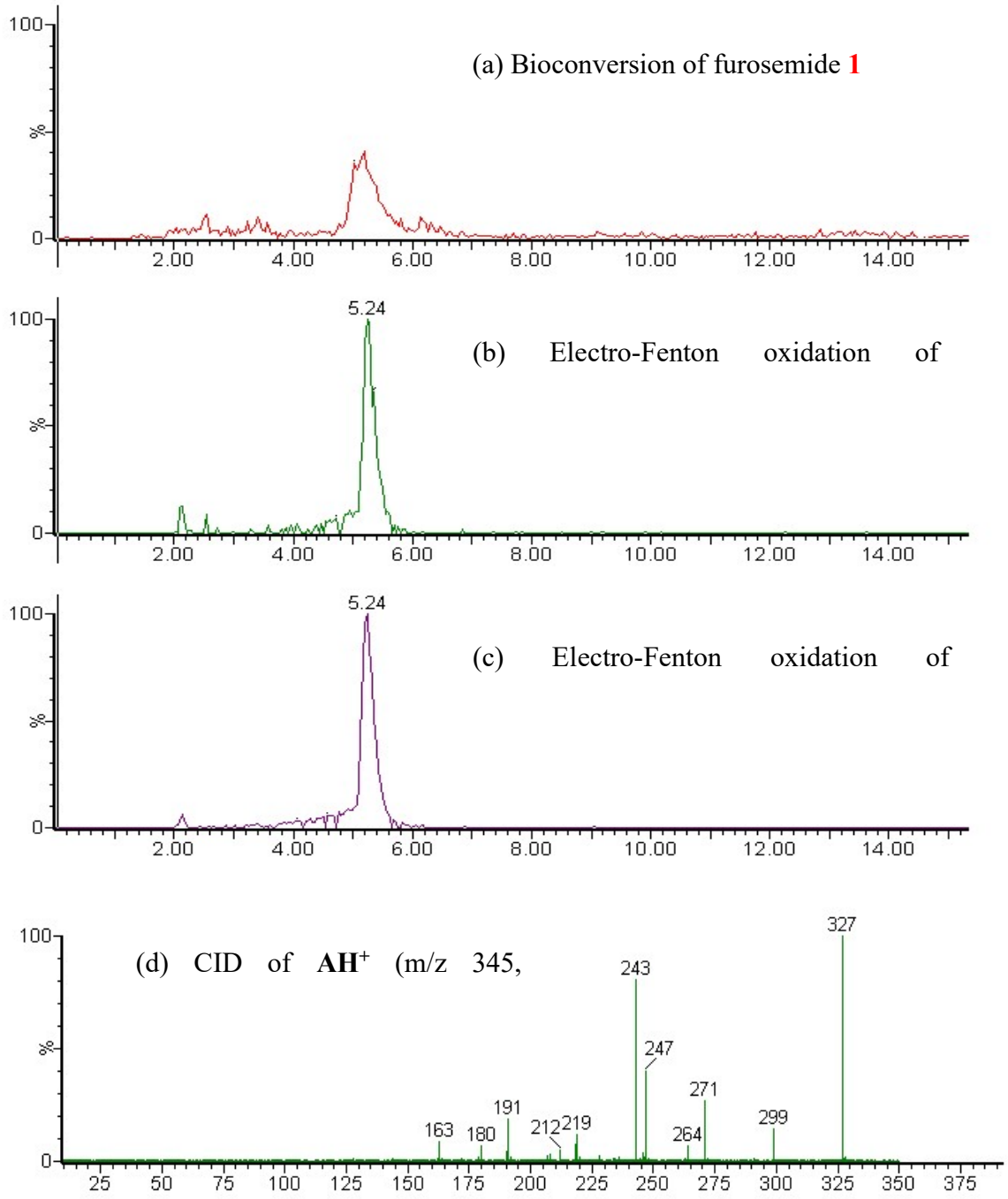
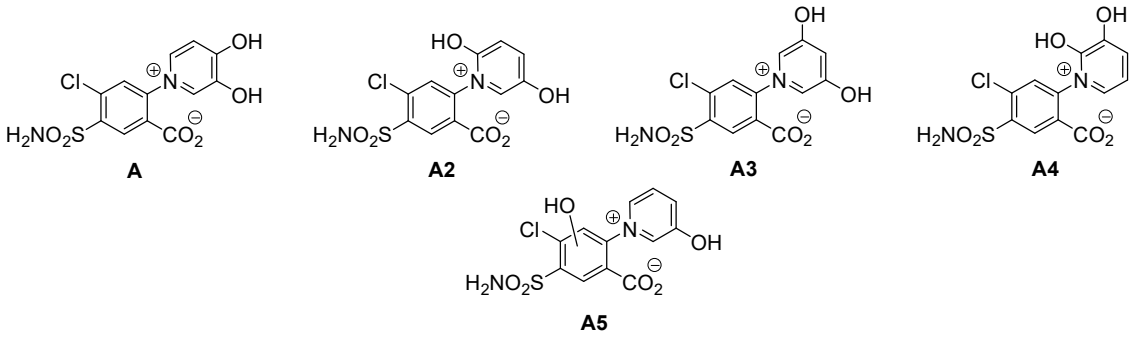


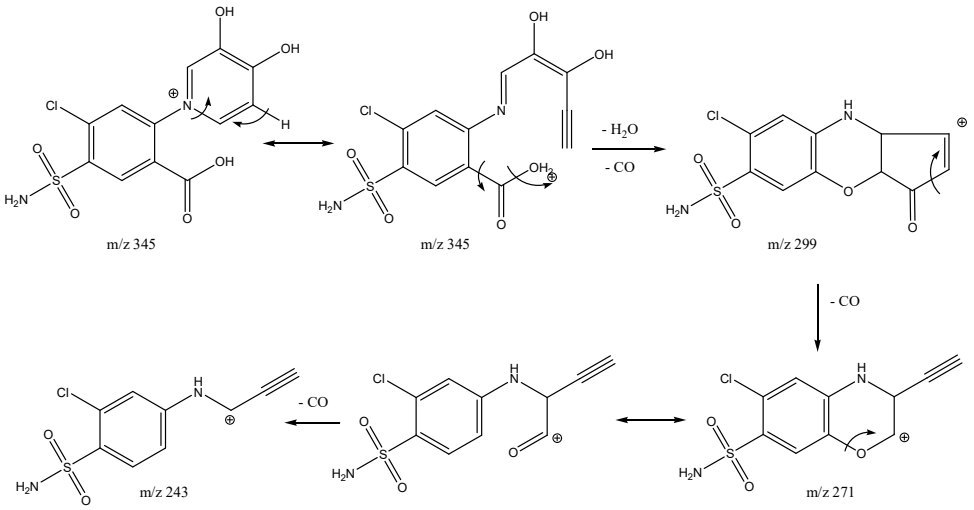
Fig. SM-4. Extracted ion chromatograms (positive ion current) of m/z 345 for (a), (b) and (c) samples. (d) CID mass spectra of MH^+ of compounds at m/z 345 (at RT=5.24 min). Cone=25 V, Ecol=15 eV. The selection of MH^+ is made only on ^{35}Cl isotope atom. Ions at m/z 327, 299, 271, 243 have preserved chlorine atom.

6. Discussion on the structure of m/z 345, RT=5.24 min

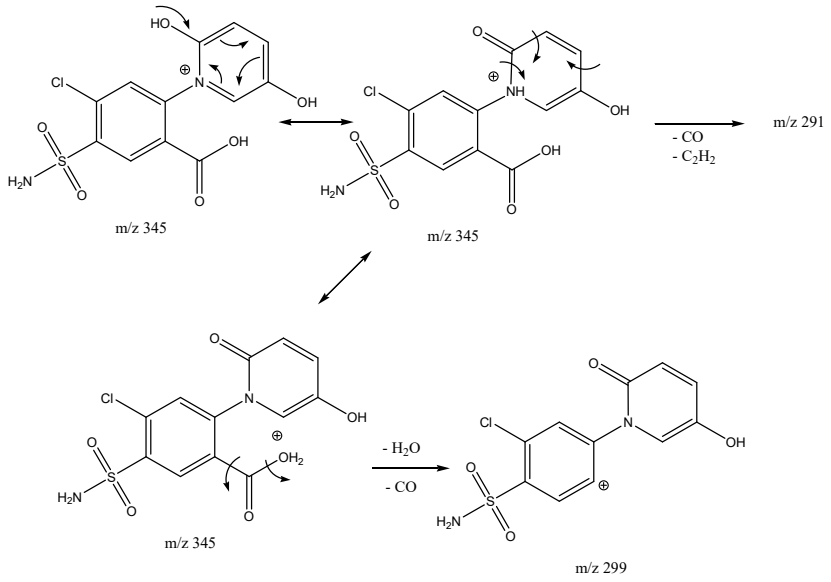
The elemental composition of the protonated molecular ion, eluted at RT=5.24 min, is $C_{12}H_{10}ClN_2O_6S$. This compound is corresponding to an oxidized form of pyridinium **4**. The protonated molecular ion loses one water molecule and one, two or three molecules of carbon monoxide to give ions at m/z 299, m/z 271 or m/z 243 respectively. Among the five possible structures **A**, **A2**, **A3**, **A4** and **A5**, only **A** revealed to be in agreement with the fragmentations observed.



6.1 Expected fragmentations of MH^+ deriving from A

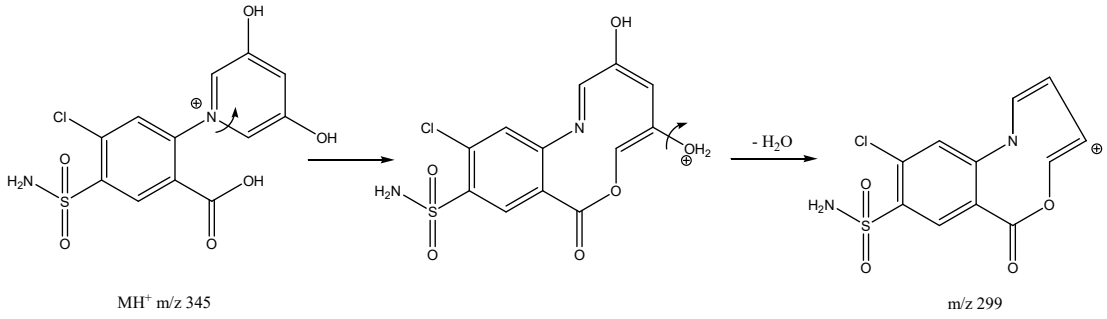


6.2 Expected fragmentations of MH^+ deriving from A2



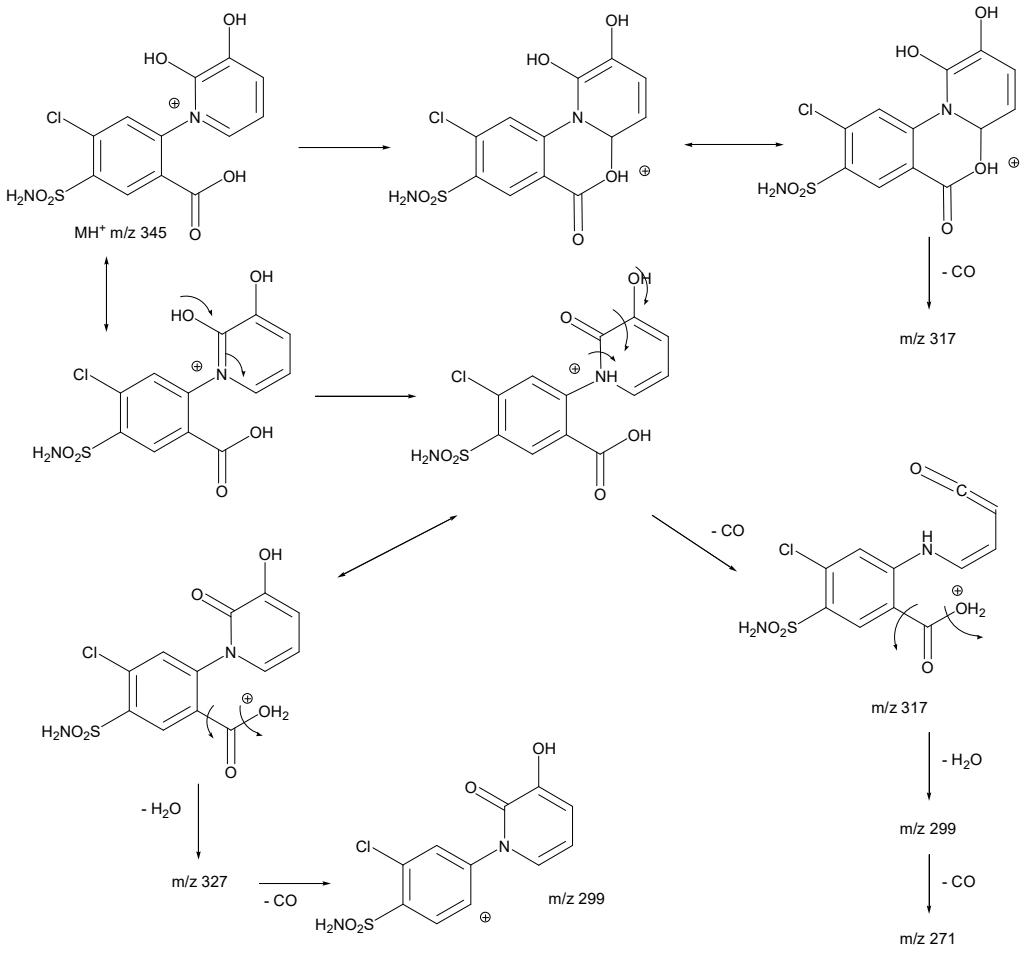
Ion at m/z 291 is not observed and m/z 299 does not allow the formation of ions observed at m/z 271 and m/z 243.

6.3 Expected fragmentations of MH^+ deriving from A3



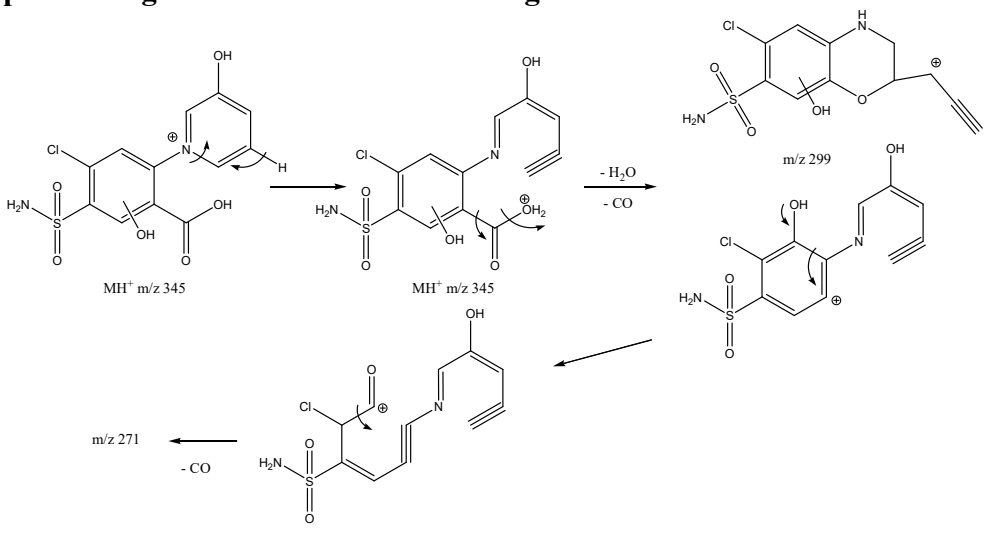
Ion at m/z 299 does not allow the formation of ion m/z 271.

6.4 Expected fragmentations of MH^+ deriving from A4



Ions at m/z 299 and m/z 271 do not allow the formation of ion at m/z 243. Ion at m/z 317 is not observed.

6.5 Expected fragmentations of MH^+ deriving from A5



Ion at m/z 271 does not allow the formation of ion at m/z 243.

7. Detection of B1 and B2 or B3 after electro-Fenton oxidation of 1 and 4

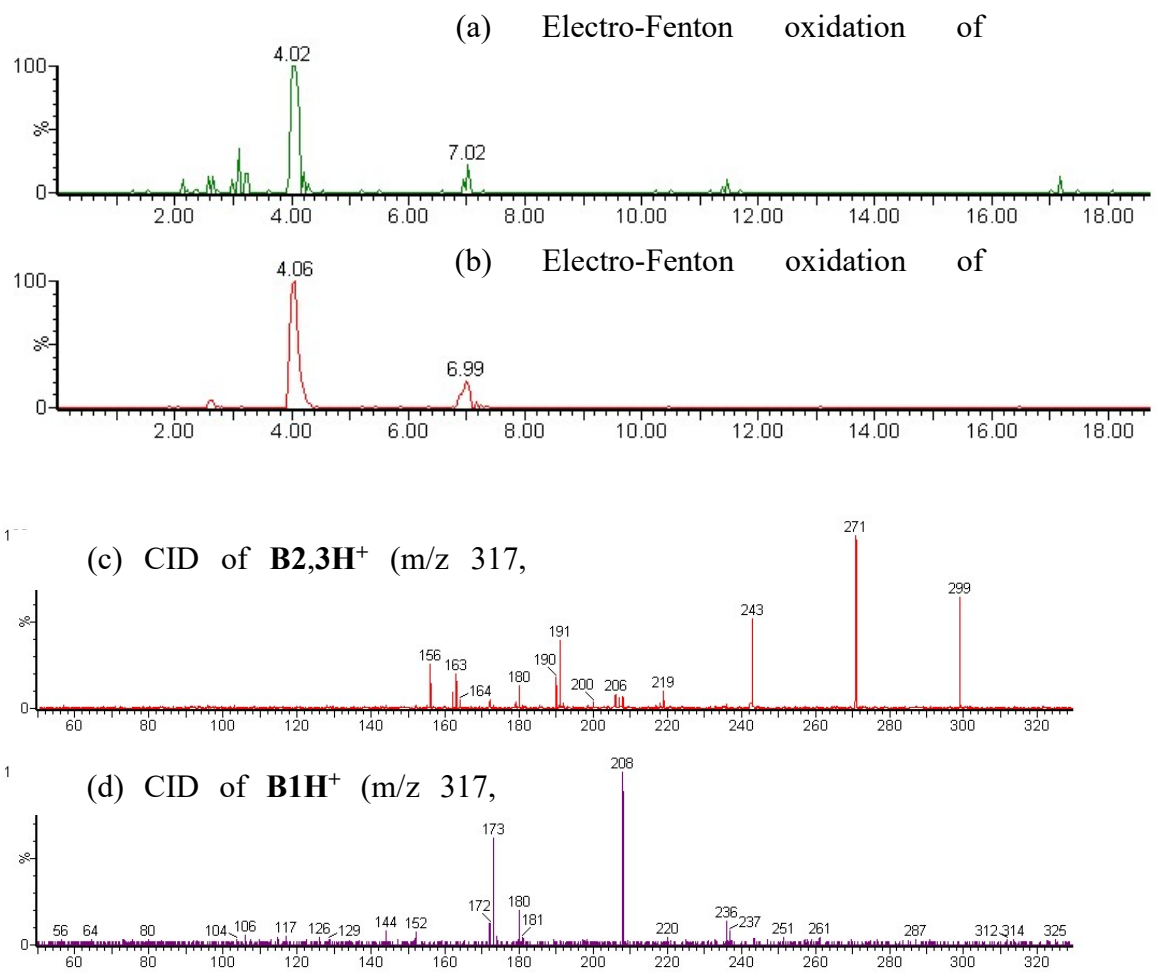
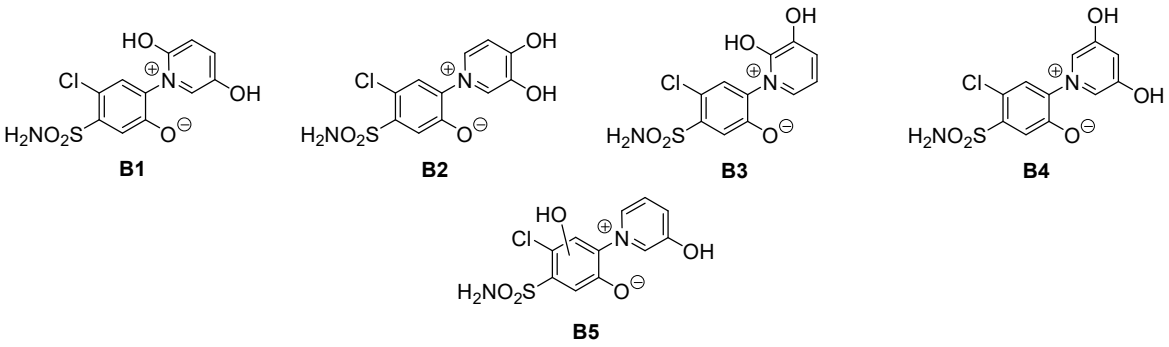


Fig. SM-5. Extracted ion chromatograms (positive ion current) of m/z 317 for electro-Fenton oxidation of (a) furosemide **1** and (b) electro-Fenton oxidation of pyridinium **4**. CID mass spectra of ion at m/z 317 at (c) RT=4.0 min and (d) RT=7.0 min. Cone=25 V, Ecol=15 eV.

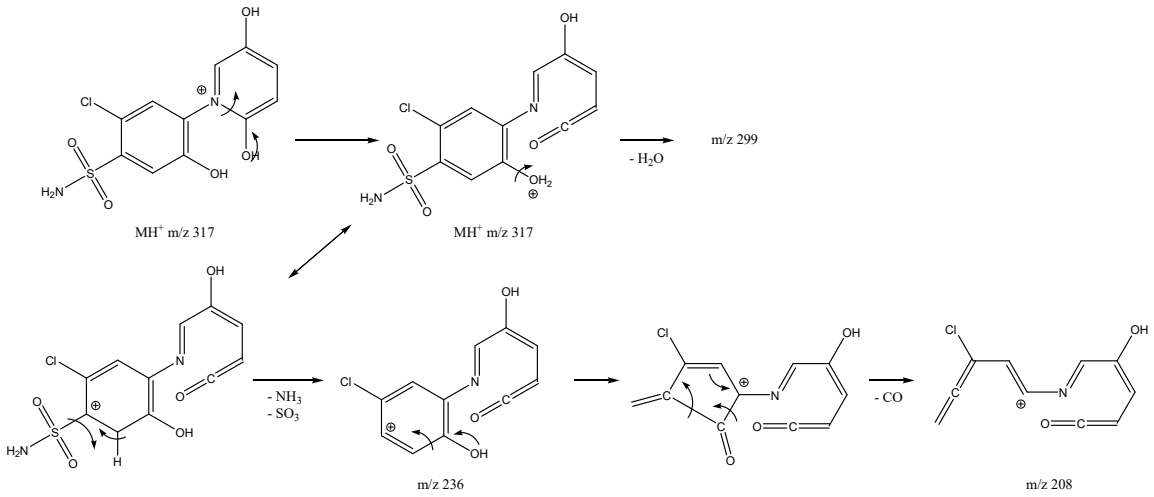
8. Discussion on the structures of m/z 317, RT=4.0 and 7.0 min

The elemental composition of both protonated molecular ions at m/z 317 is $C_{11}H_{10}ClN_2O_5S$. They lost a carbon and an oxygen atom relative to the pyridinium **4**. We assume that the CO of carboxylic function was lost. Five structures **B1**, **B2**, **B3**, **B4** and **B5** have been considered. **B1** is in agreement with the CID mass spectrum of MH^+ obtained for

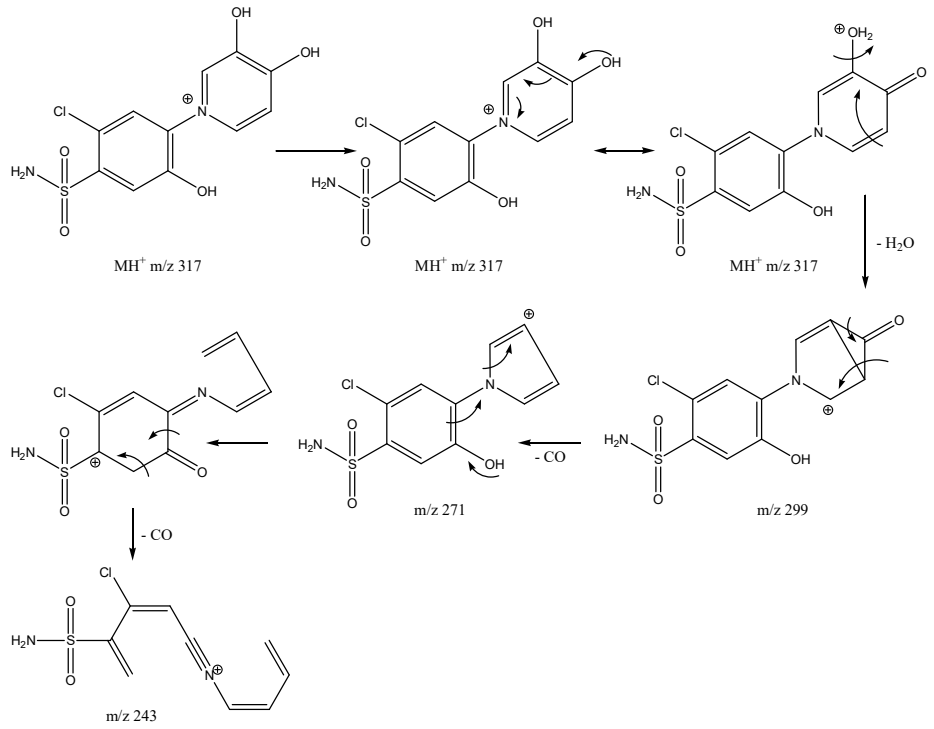
the compound eluted at 7 min. **B2** and **B3** both allow the formation of ions observed for the decomposition of MH^+ eluted at 4.0 min.



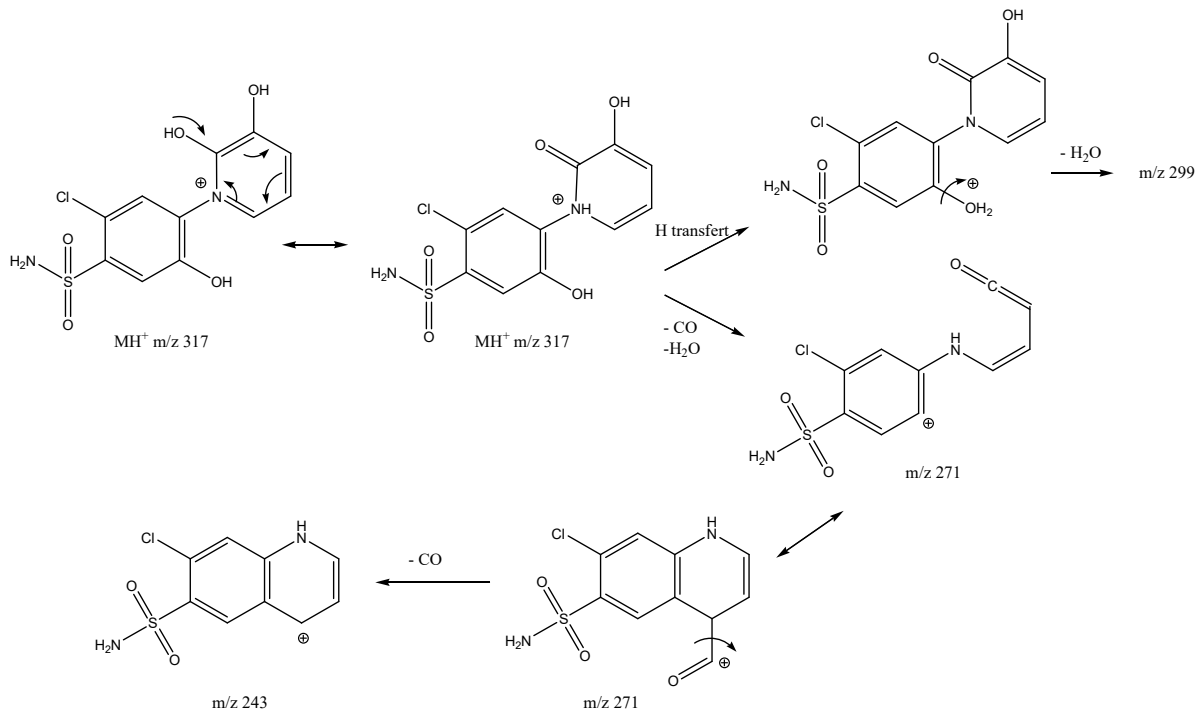
8.1 Expected fragmentations of MH^+ deriving from B1



8.2 Expected fragmentations of MH^+ deriving from B2



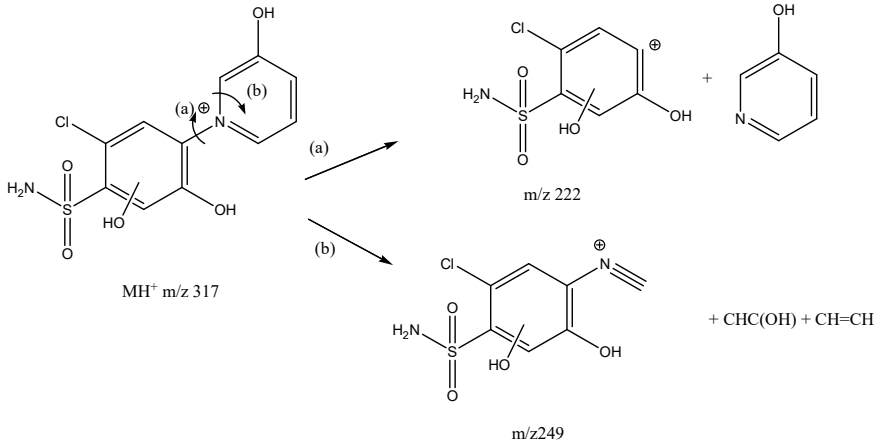
8.3 Expected fragmentations of MH^+ deriving from B3



8.4 Expected fragmentations of MH^+ deriving from B4

The fragmentation of **B4H$^+$** leads to the elimination of one or two molecules of CHCOH to form the ions at m/z 275 and m/z 233 respectively. These ions are unobserved on CID mass spectra of m/z 317 (see 10.3).

8.5 Expected fragmentations of MH$^+$ deriving from B5



The proposed fragmentation of **B5H$^+$** leads to the formation of two ions unobserved on CID mass spectra of m/z 317.

9. Detection of C1 and C2 or C3 after electro-Fenton oxidation and bioconversion of 1 and electro-Fenton oxidation of 4

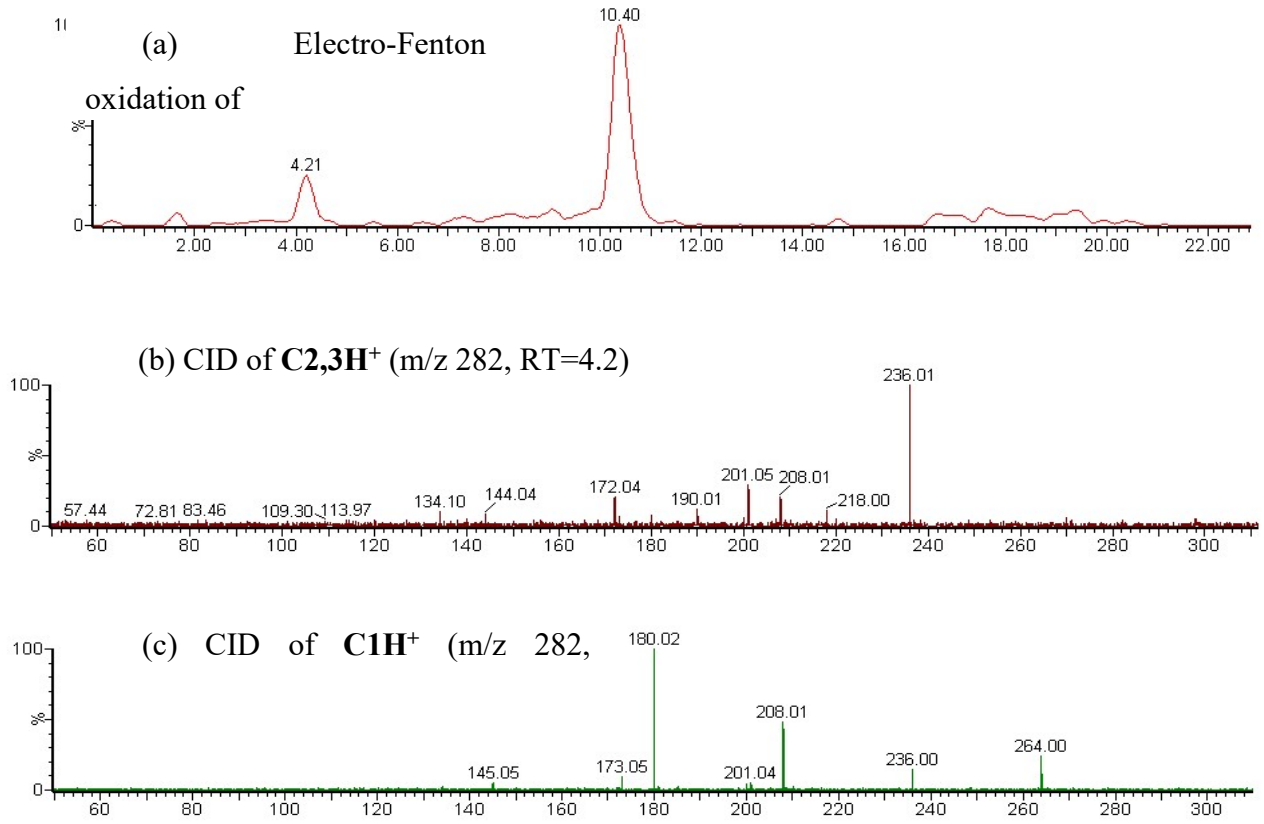
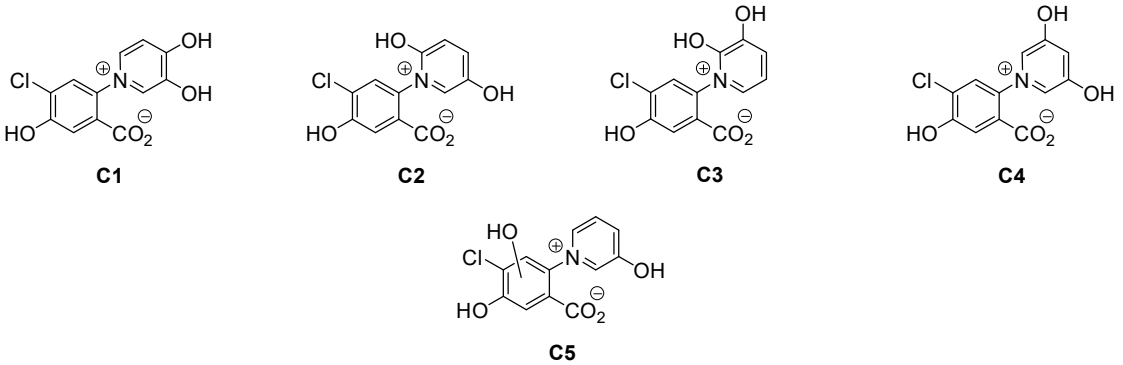


Fig. SM-6. (a) Extracted ion chromatogram (positive ion current) of m/z 282 for electro-Fenton oxidation of pyridinium **4**. CID mass spectra of ion at m/z 282 at (b) RT=4.2 min and (c) RT=10.4 min. Cone=25 V, Ecol=15 eV.

10. Discussion on the structures of m/z 282, RT=4.2 and 10.4 min

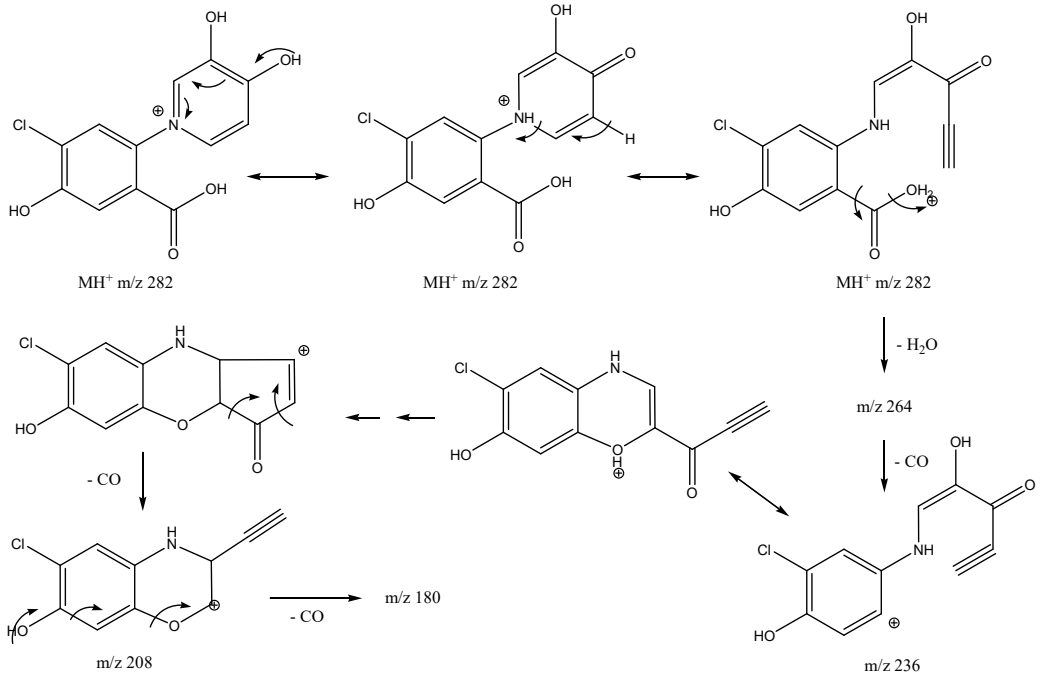
Structural characterization led to focus on the five compounds **C1**, **C2**, **C3**, **C4** and **C5**. **C1** is in agreement with fragmentations observed for RT = 10.4 min. Structures **C2** and **C3** both allow the formation of ions observed for the decomposition of MH^+ eluted at 4.2 min.



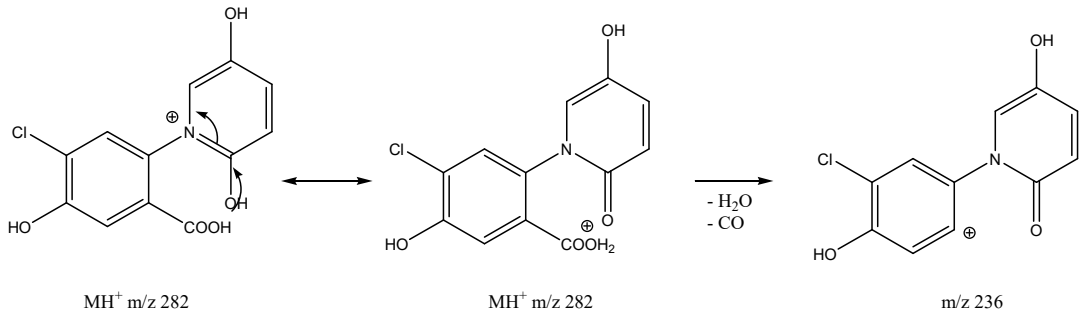
C1 **C2** **C3** **C4**

C5

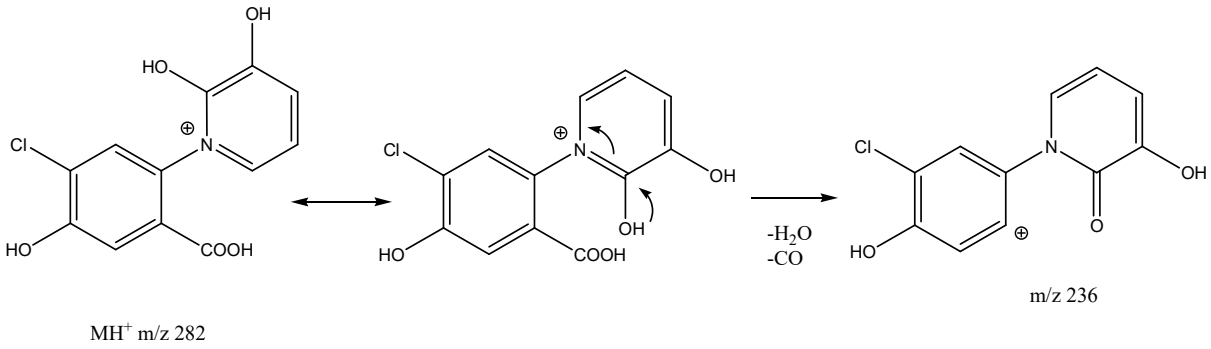
10.1 Expected fragmentations of MH^+ deriving from C1



10.2 Expected fragmentations of MH^+ deriving from C2

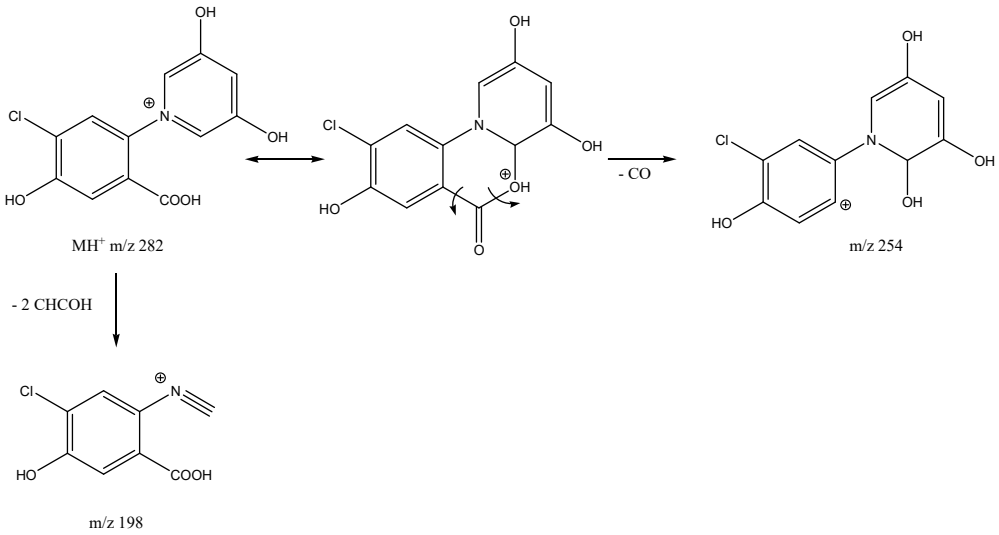


10.3 Expected fragmentations of MH^+ deriving from C3



MH^+ m/z 282

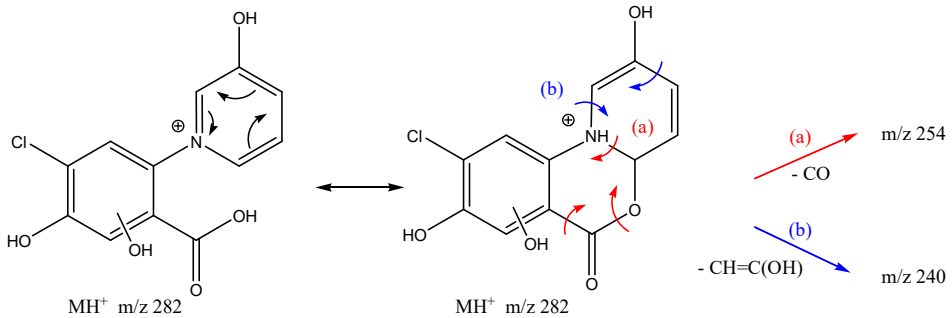
10.4 Expected fragmentations of MH^+ deriving from C4



MH^+ m/z 282

The fragmentation of **C4H$^+$** leads to the ions at m/z 254 and m/z 198 unobserved on CID mass spectra of m/z 282.

10.5 Expected fragmentations of MH^+ deriving from C5



MH^+ m/z 282 MH^+ m/z 282