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## Anticipating the fate and impact of organic environmental contaminants: A new approach applied to the pharmaceutical furosemide

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1           **Anticipating the Fate and Impact of Organic Environmental**  
2           **Contaminants: A New Approach Applied to the Pharmaceutical**  
3                           **Furosemide**

4  
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39 **ABSTRACT**

40

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

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

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

59

60 Keywords: Furosemide, Electro-Fenton, Bioconversion, Toxicity

61

## 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; Kiemmerer, 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

96 environmental detections (Celiz et al., 2009; Mompelat et al., 2009). Further investigations  
97 focusing on the identification of new ETPs and the assessment of their impact has led to the  
98 development of various strategies (Escher and Fenner, 2011). With this aim, experiments that  
99 mimic the transformations that drugs undergo in the environment have been developed  
100 (Ziylan and Ince, 2011). These experiments, which were applied to the parent molecule,  
101 resulted in transformation products (TPs) assumed to be real (at least similar to) ETPs. Such  
102 experiments typically proceed by electro-Fenton (Oturán et al., 1999), photodegradation  
103 (Packer et al., 2003) or biodegradation (Ericson, 2010; Gauthier et al., 2010). Because of such  
104 protocols and thanks to the means now available, elucidating the structure of these  
105 compounds is possible. Nevertheless, authentication of the structure requires the use of  
106 standards either commercially available or synthesized (Schulze et al., 2010; Svanfelt and  
107 Kronberg, 2011). In the absence of isolated compounds, (eco)toxicological studies are  
108 conducted on mixtures of TPs resulting from the degradation of the parent molecule.  
109 Interestingly, examples of (eco)toxicological evaluations of fully characterized TPs obtained  
110 and isolated after the degradation of the parent pharmaceutical remain scarce (Isidori et al.,  
111 2006).

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

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

130 2009). During the course of our preliminary study, three TPs of furosemide were identified:  
131 furfural **2**, saluamine **3** (both resulting from the oxidation of the amino group) and a  
132 zwitterionic pyridinium **4** (resulting from the oxidation of the furan ring). These three TPs,  
133 which are obtained by anodic oxidation, have been isolated and fully characterized, thereby  
134 allowing their detection by LC-MS in complex mixtures.

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

142

## 143 **2. MATERIAL AND METHODS**

144

### 145 **2.1 Chemicals**

146

147 Furosemide (> 99.0%) was purchased from TCI Europe N.V. Furfural (99%) and 1-Methyl-  
148 4-phenylpyridinium iodide (MPP<sup>+</sup>) were purchased from Sigma-Aldrich. Saluamine **3** and  
149 pyridinium **4** employed for the toxicological evaluation and required as standards for the  
150 analysis of complex mixtures were prepared as previously described (Laurence et al., 2011).  
151 Dulbecco's Minimal Essential Medium (DMEM), fetal bovine serum (FBS),  
152 penicillin/streptomycin, trypsin-EDTA solution and phosphate buffered saline (PBS) were  
153 purchased from GibcoBRL (France). The caspase fluorogenic substrate N-Acetyl-Asp-Glu-  
154 Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC) was purchased from  
155 Calbiochem (France). Sodium chloride (NaCl), 4-(2-hydroxyethyl)-1-  
156 piperazineethanesulfonic acid (HEPES), triton X-100, dithiothreitol (DTT), and 3-(4,5-  
157 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-  
158 Aldrich (Germany).

159

### 160 **2.2 Analytical procedure**

161

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

179 The cone voltage was adjusted for each ion of interest that was generated in the ion  
180 source. The ion source parameters were adjusted as follows to optimize the ion signals: the  
181 cone voltage was set between 20 to 80 V while the capillary voltage was set to 2.6 kV.  
182 Typical values for the other source parameters were as follows: extraction cone, 1.7 V; ion  
183 guide, 3 V; source and desolvation temperatures were set at 80  $^{\circ}\text{C}$  and 250  $^{\circ}\text{C}$  for direct  
184 infusion and at 100  $^{\circ}\text{C}$  and 450  $^{\circ}\text{C}$  for LC-MS studies, respectively. Nitrogen was used as  
185 both nebulizing and desolvation gases. The gas flow rates were dependent on the introduction  
186 mode; they ranged from 20 L/h to 300 L/h for direct introduction and from 70 L/h to 700 L/h  
187 for LC-MS. For LC-MS analysis, the injected volume of sample was 10  $\mu\text{L}$ . Argon was used  
188 as a collision gas at a flow of 0.28 mL/min, which corresponds to a pressure of ca.  $4 \cdot 10^{-3}$   
189 mbar. To record the MS/MS spectra of ions, the collision energies were varied between 2 to  
190 30 eV for each compound to obtain their primary characteristic ions. For accurate mass  
191 measurements, the analyses were performed using W-mode and an independent reference  
192 spray via the LockSpray interface. Sulfadimethoxine was used as the lock mass compound at  
193 a flow rate of 10  $\mu\text{L}/\text{min}$ . The LockSpray frequency was set at 10 s, and data for the reference  
194 compounds were averaged over 10 spectra/min. Accurate masses and elemental compositions

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

197

### 198 **2.3 Electro-Fenton**

199

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

220

### 221 **2.4 Bioconversion**

222

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



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

236

## 237 **2.5 Toxicity measurements**

238

### 239 *2.5.1 MTT Cell Viability Assay*

240

241 Human neuroblastoma cells (SH-SY5Y) (ATCC CRL2266) were cultured in DMEM (in 75  
242 mL flasks), containing 4.5 g/L glucose and supplemented with 1% penicillin/streptomycin and  
243 10% FBS, at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Cell viability was measured using  
244 the MTT assay in 96-well plates at a cell density of  $8 \times 10^4$  cells/mL. The cells were exposed  
245 to different compounds (**1**, furfural **2**, **3** and **4**) at various concentrations (from 1 to 1000  $\mu$ M).  
246 All drugs were dissolved in a mixture of DMSO and DMEM (1/1 (v/v)) in order to obtain a  
247  $10^{-1}$  M stock solution. All controls were carried out using the same solvent mixture. The final  
248 solution contained no more than 0.5% (v/v) of DMSO. After 24, 48, 72 and 96 h of exposure,  
249 MTT (20  $\mu$ L, 5 mg/mL) was added to each well. Following incubation at 37 °C for 2 h, the  
250 supernatants were carefully removed and 200  $\mu$ L of DMSO were added. The optical density  
251 (OD) value of each well was read using a micro-plate reader (TECAN Infinite M1000) at a  
252 wavelength of 570 nm. Cell viability was calculated as a percentage of the control.

253

### 254 *2.5.2 Caspase-3 activity*

255

256 The caspase-3 activity was assessed by following the cleavage of the fluorogenic  
257 substrates Ac-DEVD-AFC. Treated cells were harvested through trypsinization using 0.05%  
258 trypsin and 0.53 mM EDTA at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> for 4  
259 min. Trypsinization was terminated by addition of 20% FBS, the cell suspension was then  
260 centrifuged, the supernatant discarded and the pellet washed with  $1 \times$  PBS (CaCl<sub>2</sub>-, MgCl<sub>2</sub>-  
261 free) (Gibco, Invitrogen). The cell pellet was gently suspended in a buffer containing 30 mM

262 HEPES, 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100 and 10 mM DTT and then  
263 centrifuged. The supernatant was used for the assay. The fluorogenic substrates were added at  
264 a final concentration of 100 mM. The samples were gently mixed and incubated in the dark at  
265 37 °C for 1 h. Fluorescence intensity of the samples was measured at an excitation  $\lambda$  of 400  
266 nm and emission  $\lambda$  of 505 nm using a fluorescence micro-plate reader (TECAN infinite™  
267 M1000).

268

#### 269 2.5.4 Data analysis

270

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

276

### 277 3. RESULTS

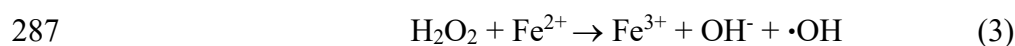
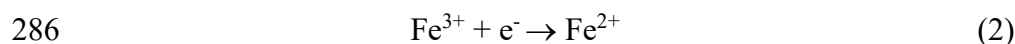
278

#### 279 3.1 Electro-Fenton

280

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

284

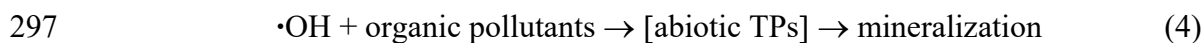


288

289 The hydroxyl radical is a very powerful oxidizing agent, and it non-selectively reacts  
290 with organic molecules to lead to the formation of abiotic TPs. In the treatment strategy, the  
291 operating parameters were established to further oxidize these intermediates until they  
292 reached their ultimate degree of oxidation, i.e., their mineralization (Eq. 4) (Oturán et al.,  
293 2000; Sires et al., 2007; Brillas et al., 2009). This process has been successfully applied for

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

296



298

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

306



308

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

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

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

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

335

### 336 **3.2 Bioconversion**

337

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

347 To obtain the biotic TPs of furosemide, a selection of microorganisms among seventy-  
348 two strains of bacteria and fungi was performed. To complete the screening using minimal  
349 time and assays, we used our recently developed combinatorial approach (Fromentin et al.,  
350 2012; Joyeau et al., 2013). This method is based on incubating the substrate in the presence of  
351 five microorganisms (three fungi and two bacteria) in a reaction buffer. After furosemide was  
352 added to the suspensions, the biotransformations were monitored by HPLC and MS analyses  
353 of aliquots collected every forty-eight hours.

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

361 furfural may be explained by the ability of some microorganisms to degrade it (Koopman et  
362 al., 2010). The fungus *Aspergillus candidus* ATCC 20023 produced pyridinium **4** in moderate  
363 yields after eight days (see SI §4). The fungus *Cunninghamella echinulata* var. *elegans* ATCC  
364 9245 fully metabolized furosemide in six days to yield a mixture of both compounds. In some  
365 cases, the compound associated with structure **A** was also observed as a minor biotic TP (see  
366 SI §5). Further investigations are in progress to optimize the production of this TP to isolate it  
367 and confirm its structure.

368

### 369 **3.3 Toxicological evaluation of plausible ETPs**

370

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

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

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

387

## 388 **4. DISCUSSION**

389

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

395 approach consisting of applying environmentally relevant transformations to a contaminant to  
396 obtain, isolate and characterize its plausible ETPs in a single step might seem intuitively  
397 quicker and more appropriate. However, the results of this study suggest that such an  
398 approach would have failed to detect plausible ETPs exhibiting significantly different  
399 physicochemical properties from those of the parent compound, as in the case of pyridinium  
400 **4**. Note that saluamine **3** has already been identified as a biotic TP of furosemide after  
401 bioconversion with the fungus *Cunninghamella elegans* (Hezari and Davis, 1992). The fact  
402 that pyridinium **4** was not observed in the same study may be explained by the unavailability  
403 of any standard. However, Burka observed the formation of pyridinium **4** after the liver  
404 microsomal incubation of furosemide (Chen and Burka, 2007), but this was consecutive to its  
405 NMR characterization after the oxidation of furosemide with dimethyldioxirane in deuterated  
406 acetone.

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

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

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

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

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

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

460

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462

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465



466

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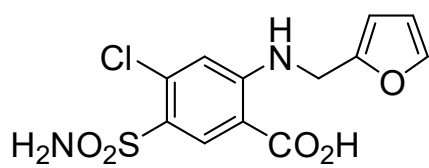
## Figure captions

**Figure 1:** Furosemide 1 and its TPs

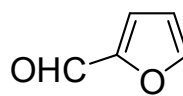
**Figure 2:** Structures proposed for a) m/z 345, b) m/z 317 and 282

**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  $973 \pm 46 \mu\text{M}$ . B) The caspase-3 activity was measured after 96 h of treatment with pyridinium 4 (1 mM). Data shown are the mean  $\pm$  S.E.M. of 3 independent experiments with duplicate wells per experiment, \*  $p < 0.05$  compared with control.

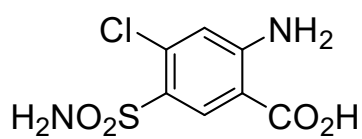
Figure 1



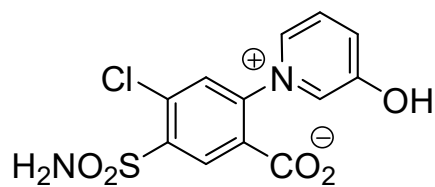
**1**



**2**



**3**



**4**

Figure 2

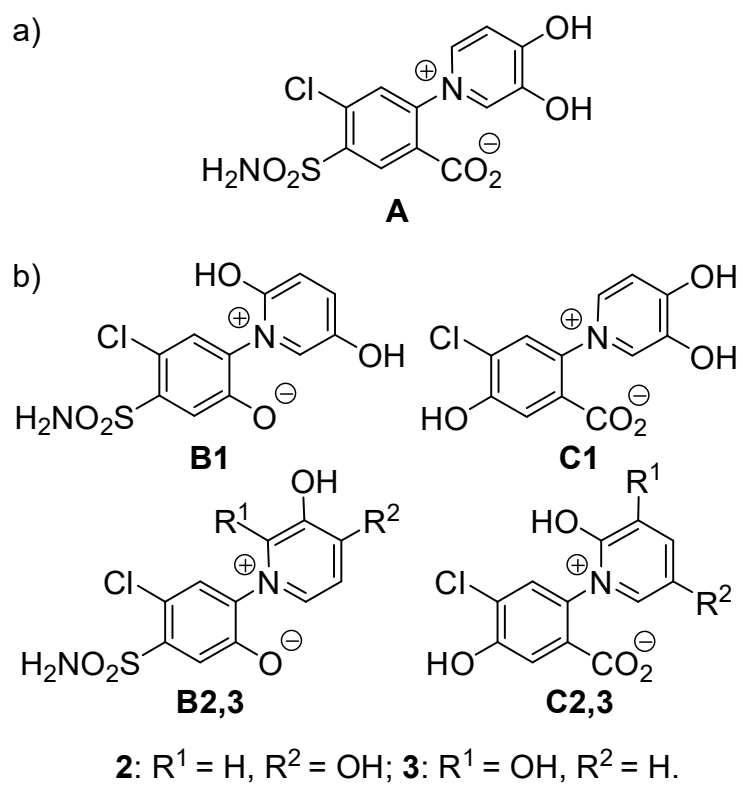
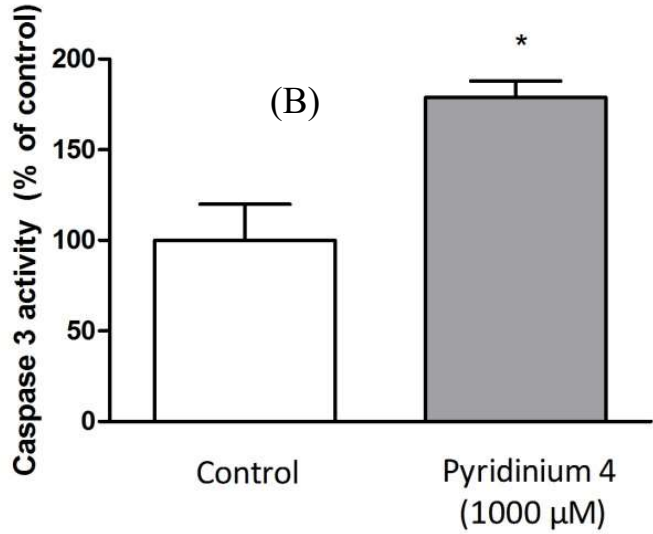
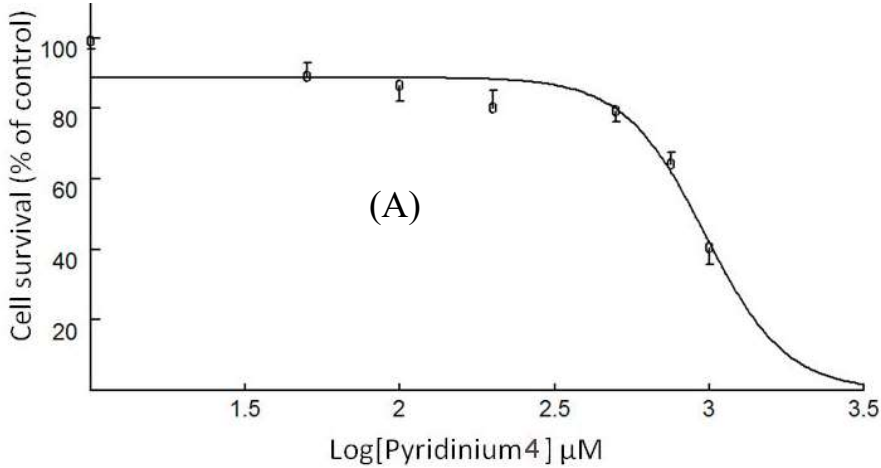




Figure 3



## Supplementary Materials

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

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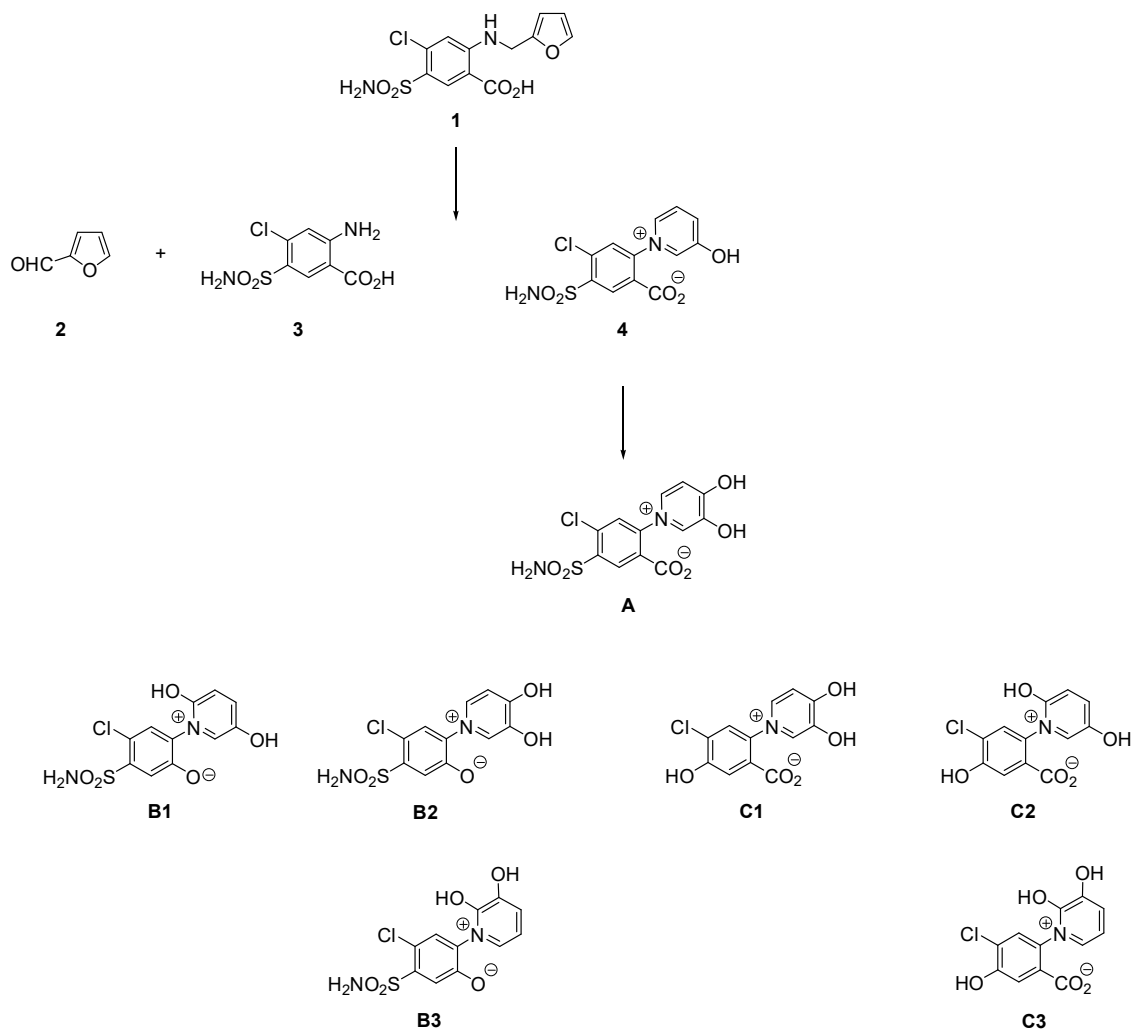
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# 1. Furoseamide **1** and its oxidation products



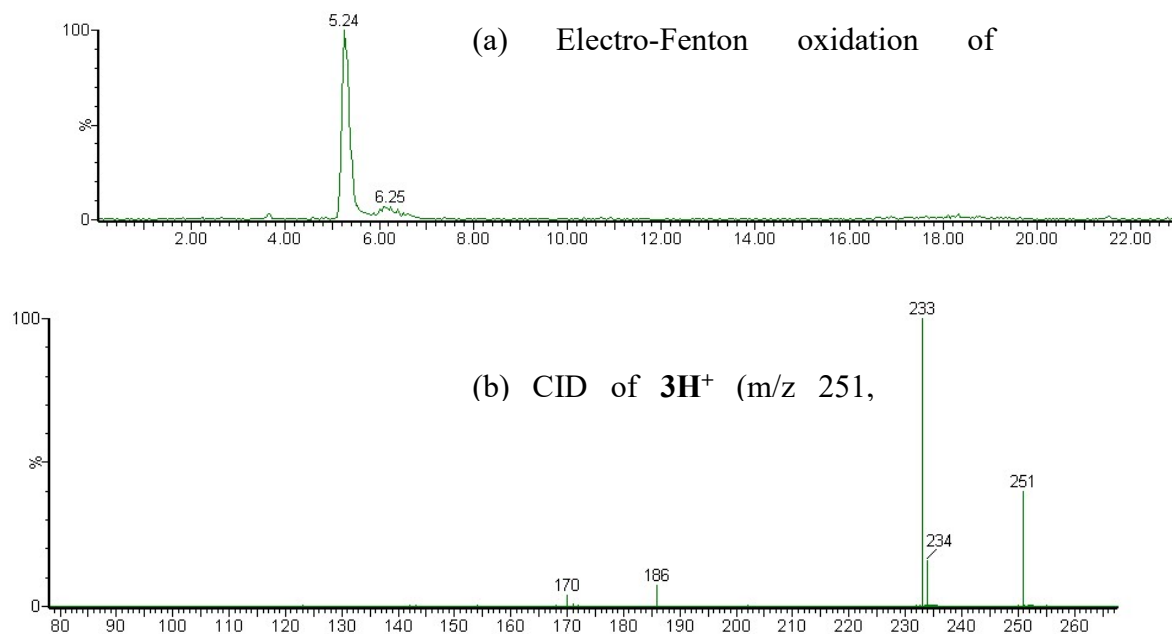
Oxidation products	Retention Time (min)	Molecular Weight (m/z)	Experimental Mass	Theoretical Mass	m/z	p	D BE <sup>1</sup>	Elemental composition	Detection		
									a)	b)	c)
<b>3</b>	5.2	251	250.	250.	1.3	5.2	4.5	C <sub>7</sub> H <sub>8</sub> ClN <sub>2</sub> O <sub>4</sub> S			
<b>4</b>	2.2	329	328.	328.	0	0	8.5	C <sub>12</sub> H <sub>10</sub> ClN 2O <sub>5</sub> S			
<b>A</b>	5.24	345	344.	344.	0	0	8.5	C <sub>12</sub> H <sub>10</sub> ClN 2O <sub>6</sub> S			
<b>B1</b>	7	317	317.	317.	-	-	7	C <sub>11</sub> H <sub>10</sub> ClN			

	.0	17	0005	0024	1.9	6	.5	$_2\text{O}_5\text{S}$
<b>B2or</b>	4	3	316.	317.	-	-	7	$\text{C}_{11}\text{H}_{10}\text{ClN}$
<b>3</b>	.0	17	9997	9999	0.2	0.6	.5	$_2\text{O}_5\text{S}$
<b>C1</b>	1	2	282.	282.	0	2	8	$\text{C}_{12}\text{H}_9\text{ClN}$
	0.4	82	0176	0169	.7	.5	.5	$\text{O}_5$
<b>C2or</b>	4	2	282.	282.	-	-	8	$\text{C}_{12}\text{H}_9\text{ClN}$
<b>3</b>	.2	82	0168	0169	0.1	0.4	.5	$\text{O}_5$

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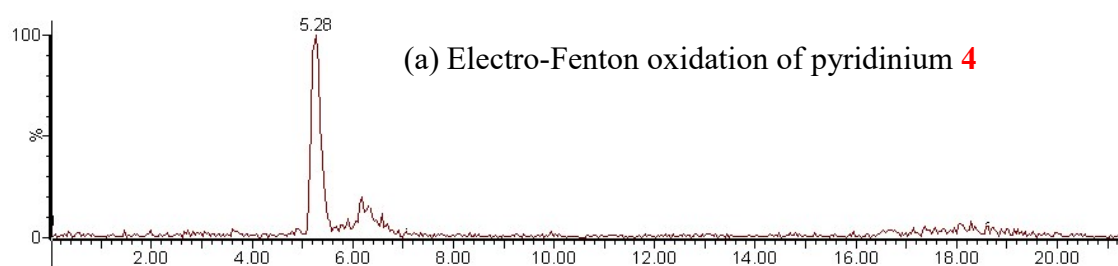
<sup>1</sup>DBE: Double Bond Equivalency. <sup>2</sup>Detection (+) during: (a) electro-Fenton oxidation of furosemide **1**, (b) electro-Fenton oxidation of pyridinium **4**, (c) bioconversion of furosemide **1**.

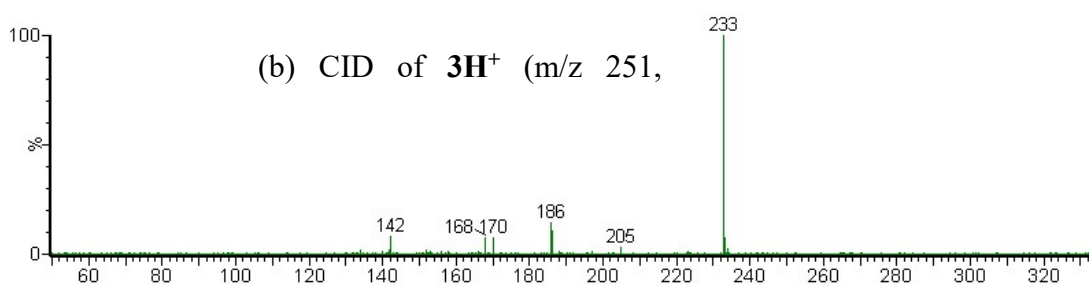
## 2. Detection of **3** after electro-Fenton oxidation of **1**



**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** ( $\text{MH}^+$  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  $\text{MH}^+$  of compound **3**.

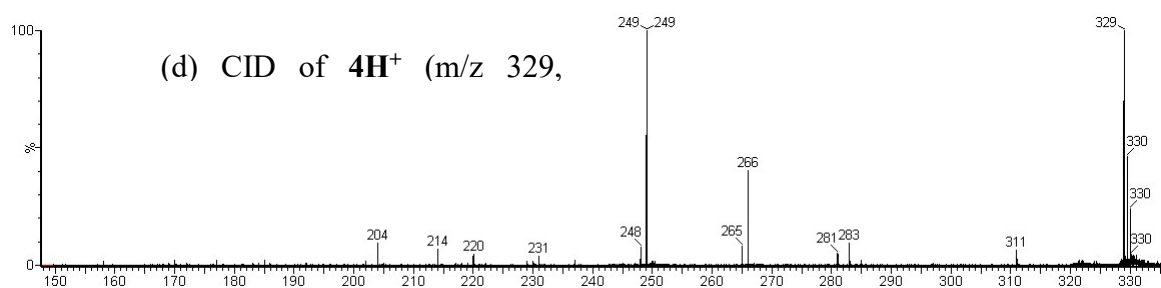
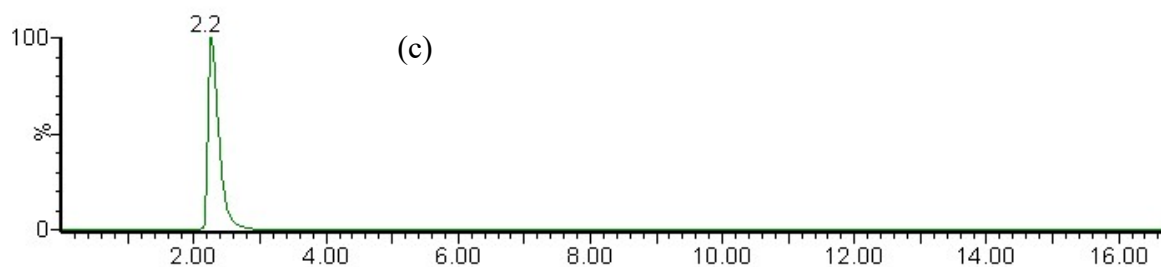
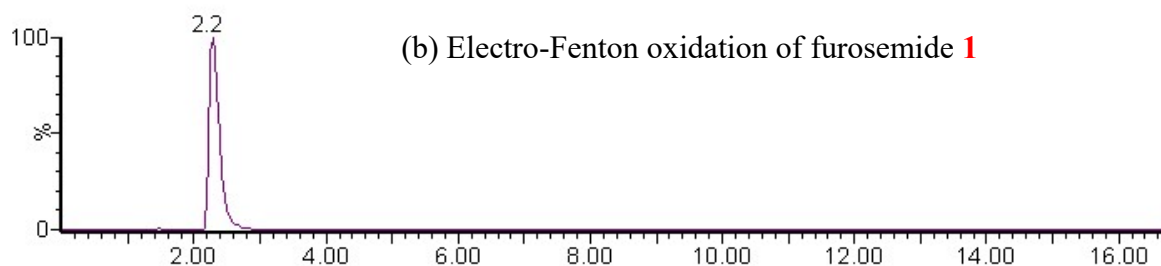
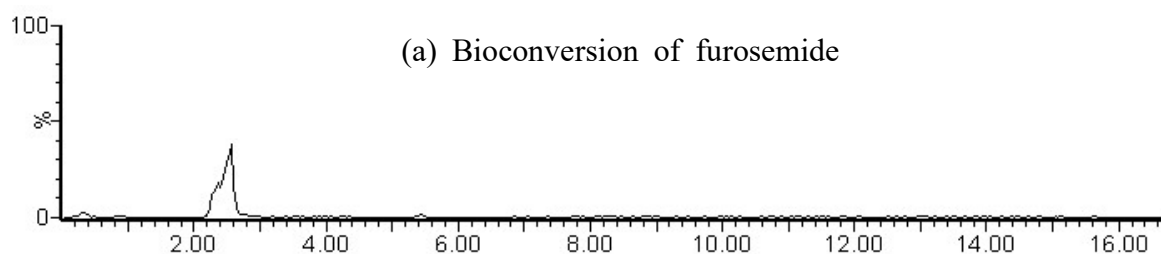
## 3. Detection of **3** after electro-Fenton oxidation of **4**





**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  $\text{RT}=5.2$  min. Cone=25 V, Ecol = 15 eV.

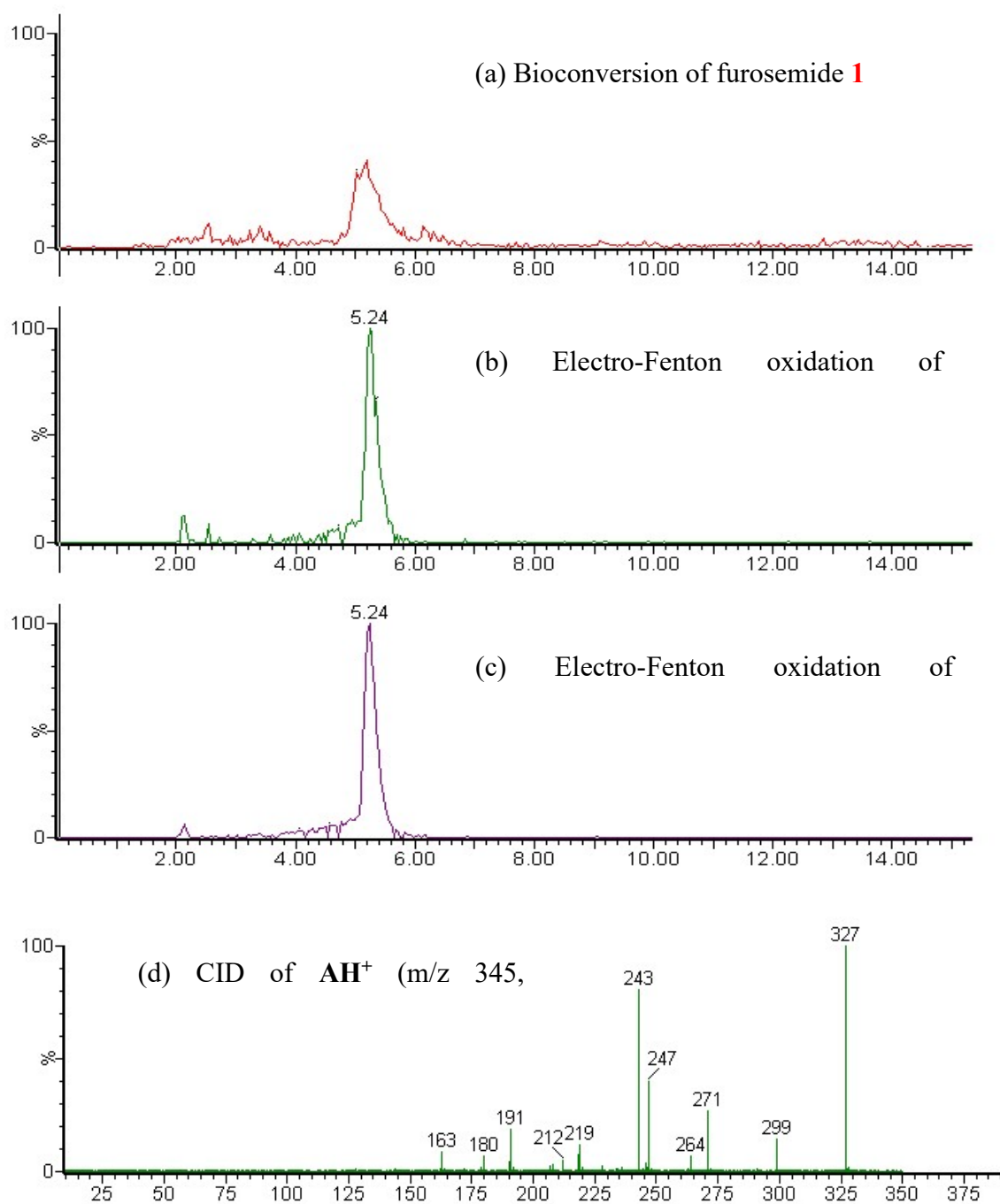
#### 4. Detection of **4** after electro-Fenton oxidation and bioconversion of **1**





**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,  $E_{col}=10$  eV. This CID mass spectrum is the same for both samples (a) and (b) and corresponds to that of the pyridinium **4**.

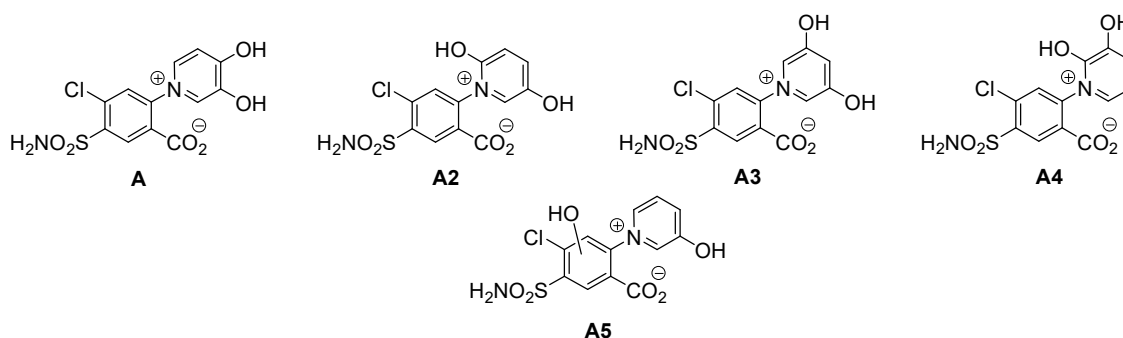
5. Detection of A after electro-Fenton oxidation and bioconversion of **1** and electro-Fenton oxidation of **4**



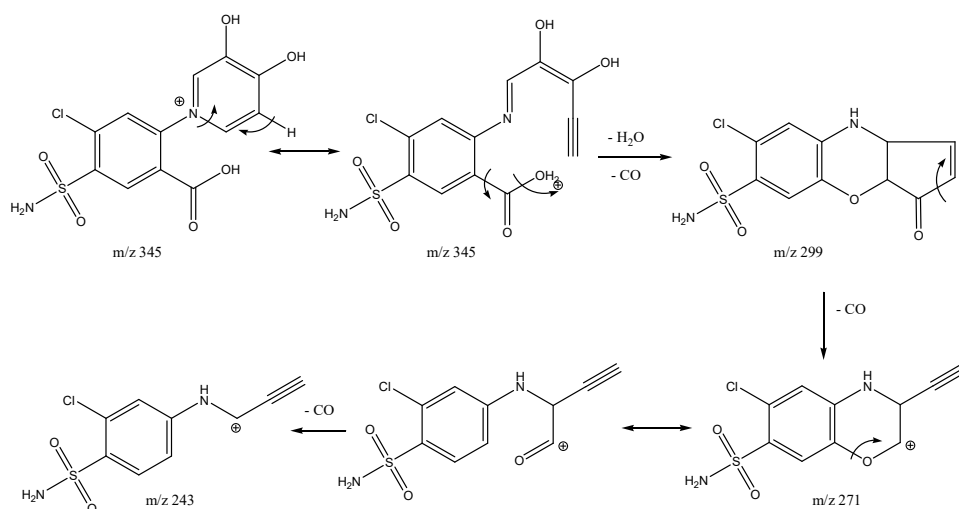
**Fig. SM-4.** Extracted ion chromatograms (positive ion current) of  $m/z$  345 for (a), (b) and (c) samples. (d) CID mass spectra of  $MH^+$  of compounds at  $m/z$  345 (at  $RT=5.24$  min).  $Cone=25$  V,  $Ecol=15$  eV. The selection of  $MH^+$  is made only on  $^{35}Cl$  isotope atom. Ions at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

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

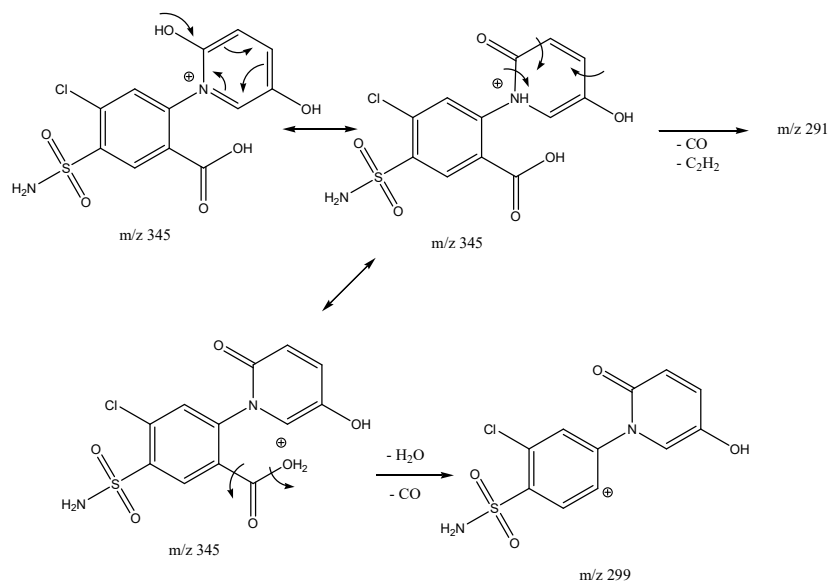
The elemental composition of the protonated molecular ion, eluted at  $RT=5.24$  min, is  $C_{12}H_{10}ClN_2O_6S$ . This compound is corresponding to an oxidized form of pyridinium **4**. The protonated molecular ion loses one water molecule and one, two or three molecules of carbon monoxide to give 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**

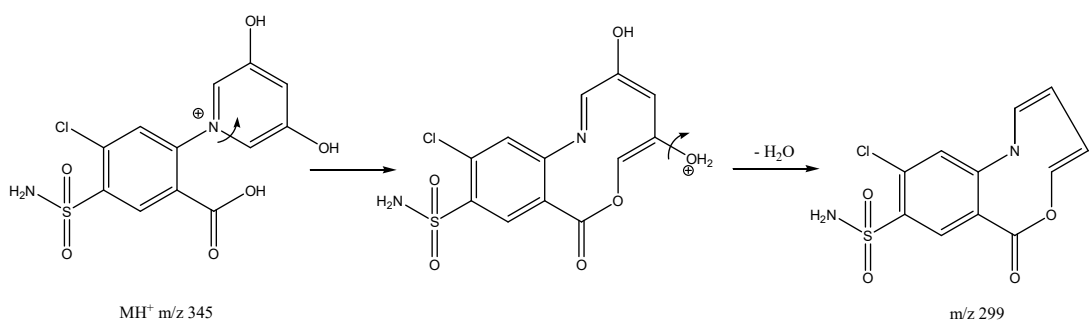


### 6.2 Expected fragmentations of $MH^+$ deriving from **A2**



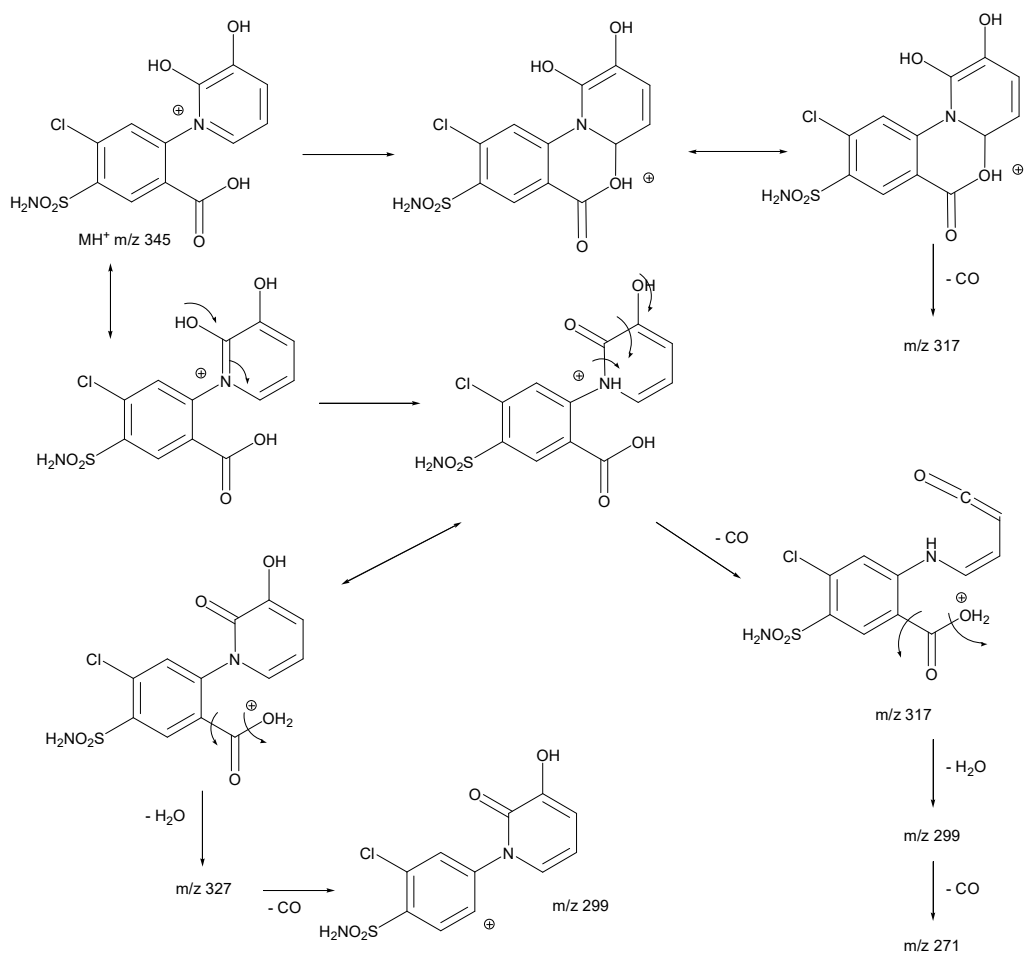
Ion at  $m/z$  291 is not observed and  $m/z$  299 does not allow the formation of ions observed at  $m/z$  271 and  $m/z$  243.

### 6.3 Expected fragmentations of $MH^+$ deriving from A3



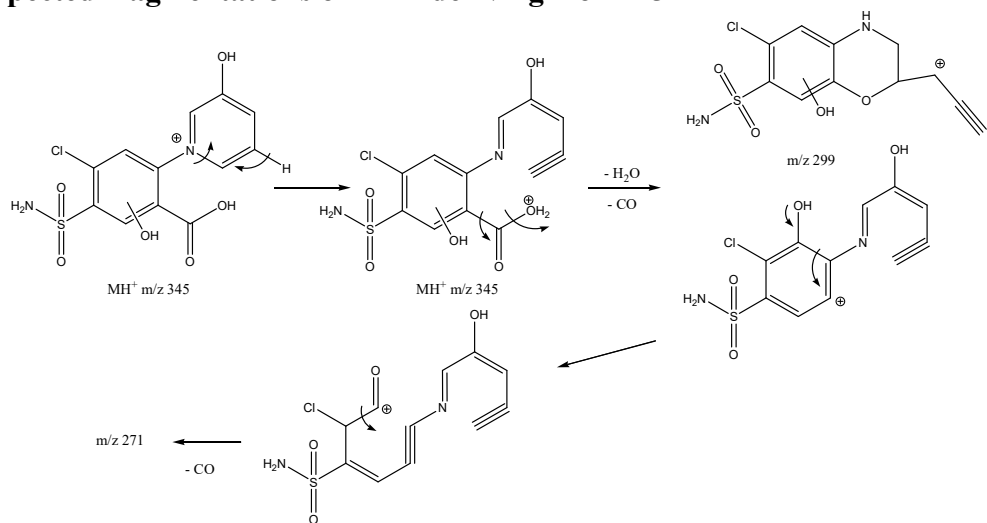
Ion at  $m/z$  299 does not allow the formation of ion  $m/z$  271.

### 6.4 Expected fragmentations of $MH^+$ deriving from A4



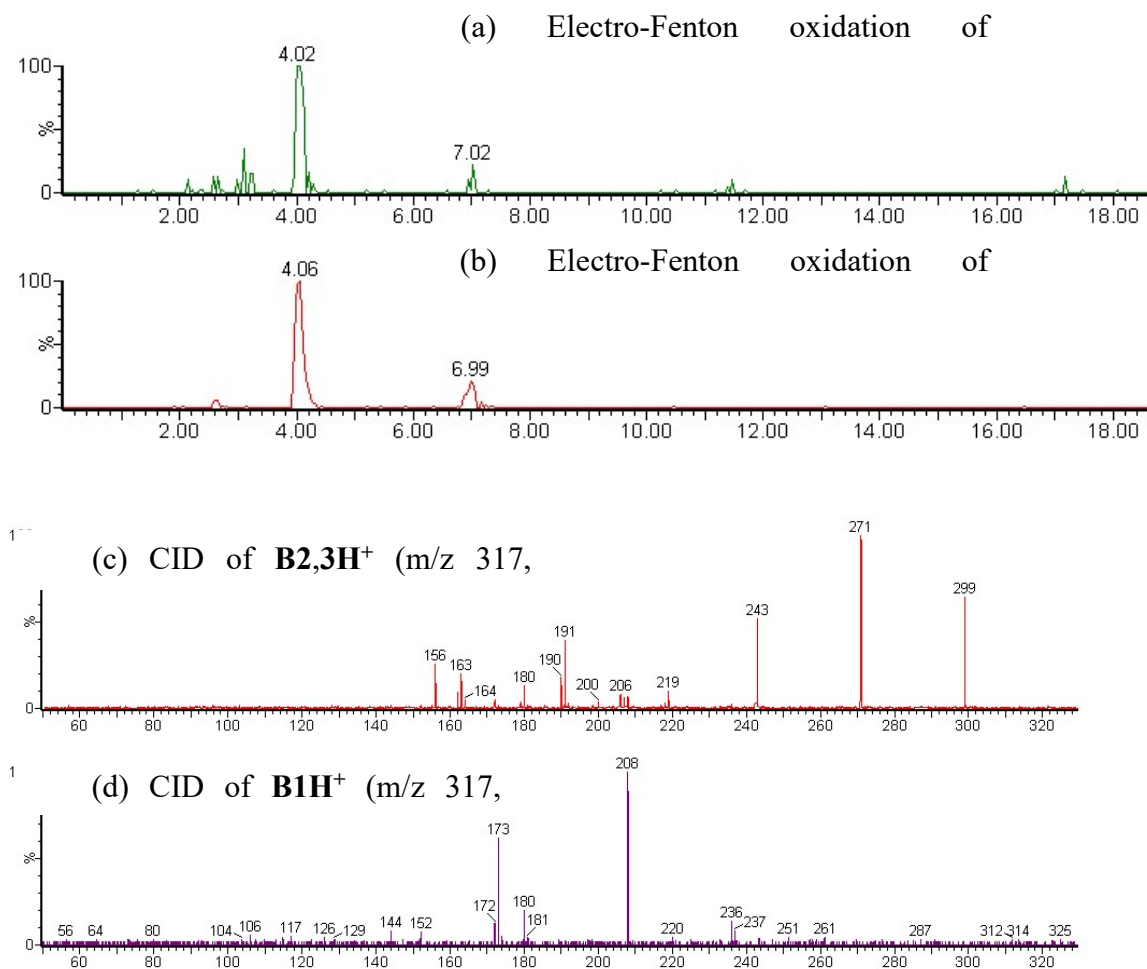
Ions at  $m/z$  299 and  $m/z$  271 do not allow the formation of ion at  $m/z$  243. Ion at  $m/z$  317 is not observed.

### 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**

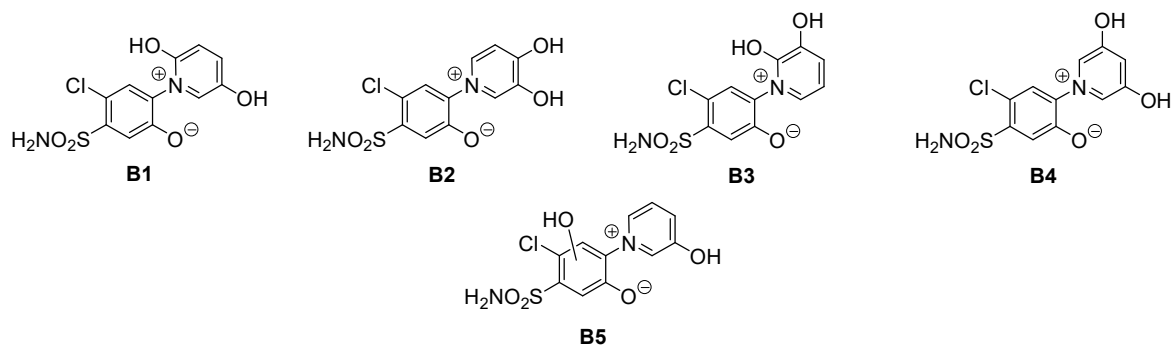


**Fig. SM-5.** Extracted ion chromatograms (positive ion current) of m/z 317 for electro-Fenton oxidation of (a) furosemide **1** and (b) electro-Fenton oxidation of pyridinium **4**. CID mass spectra of ion at m/z 317 at (c) RT=4.0 min and (d) RT=7.0 min. Cone=25 V, Ecol=15 eV.

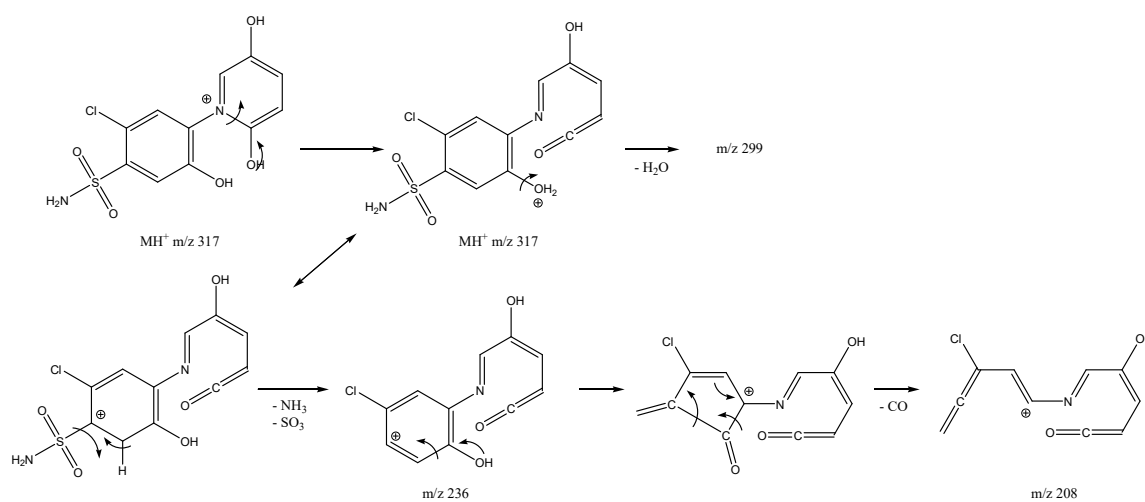
## 8. Discussion on the structures of m/z 317, RT=4.0 and 7.0 min

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

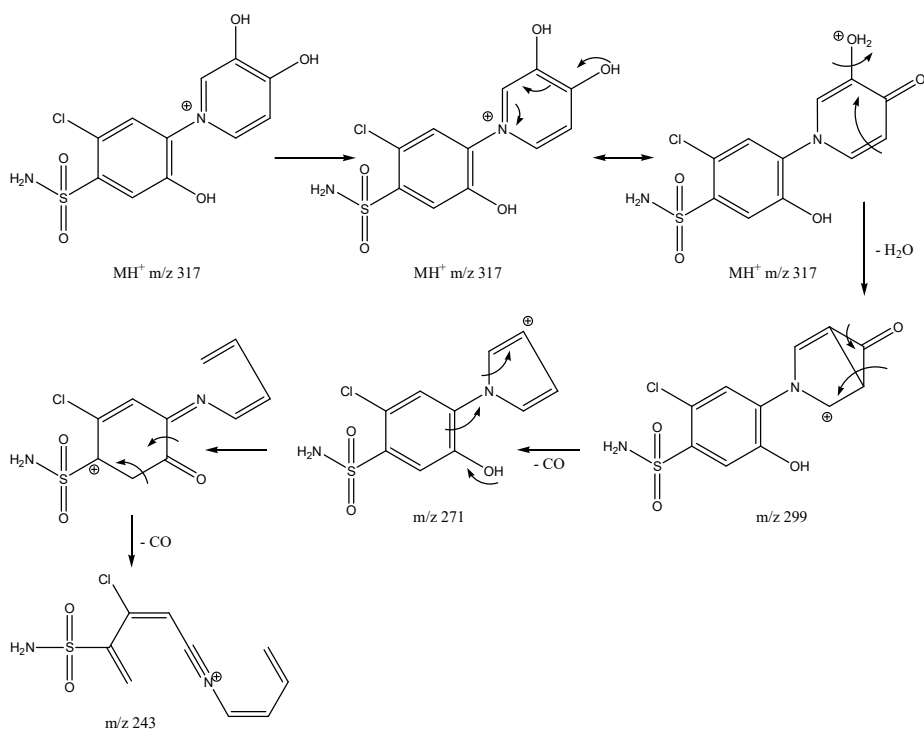
the compound eluted at 7 min. **B2** and **B3** both allow the formation of ions observed for the decomposition of  $MH^+$  eluted at 4.0 min.



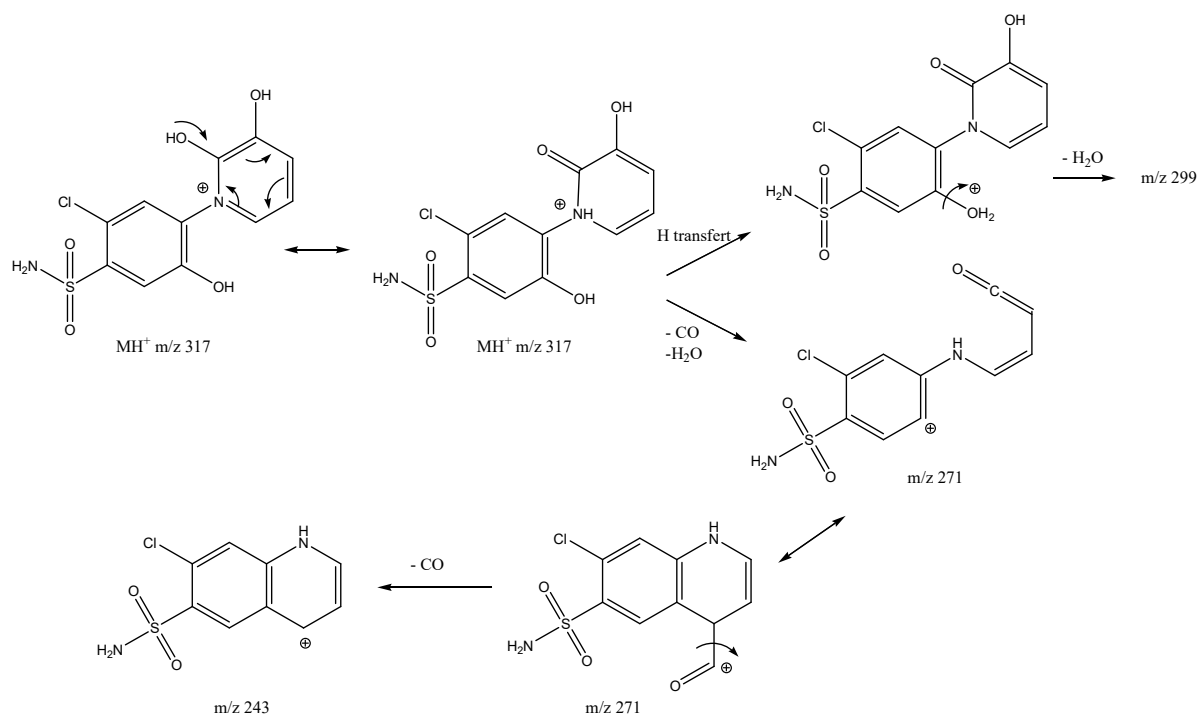
### 8.1 Expected fragmentations of $MH^+$ deriving from B1



### 8.2 Expected fragmentations of $MH^+$ deriving from B2



### 8.3 Expected fragmentations of $MH^+$ deriving from B3

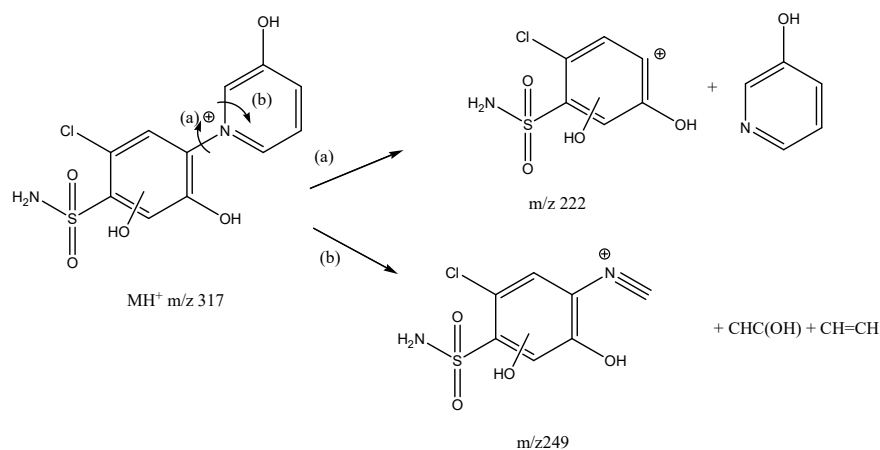


### 8.4 Expected fragmentations of $MH^+$ deriving from B4



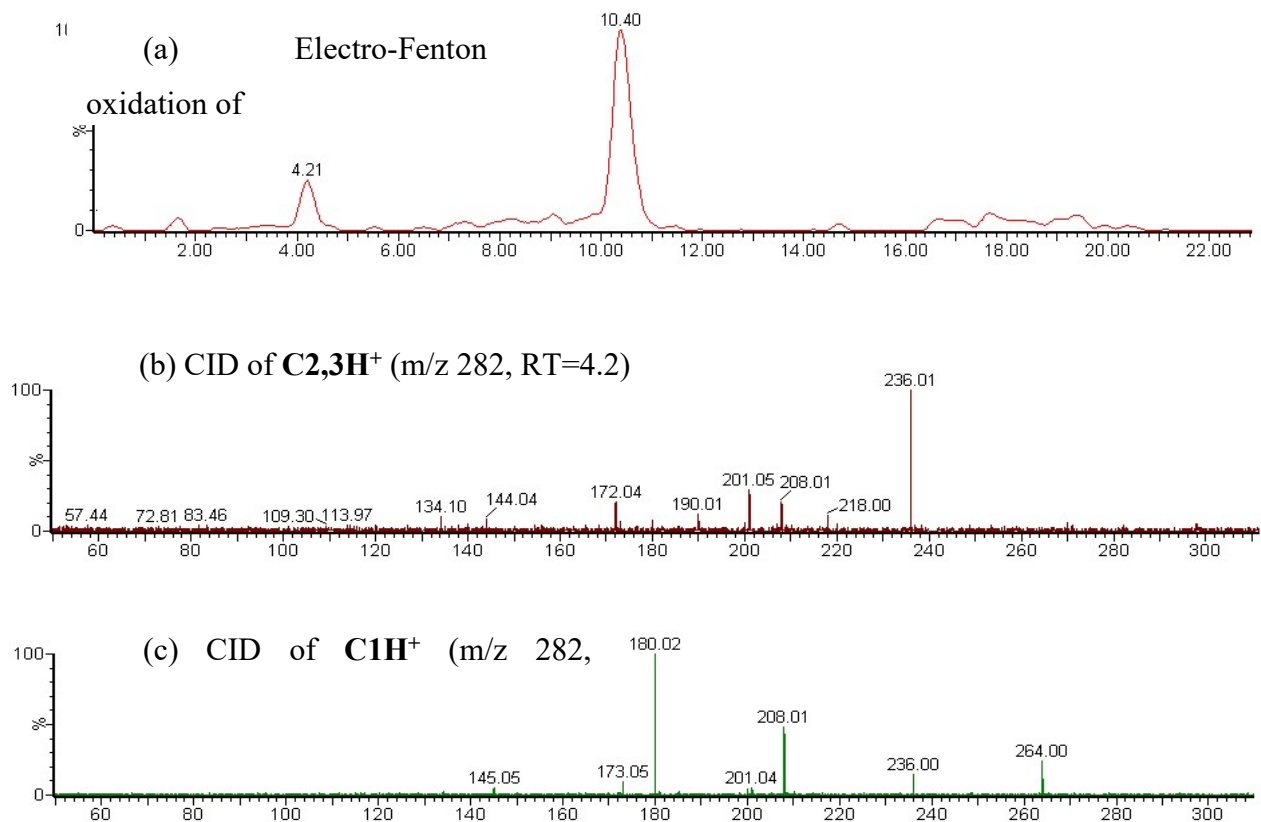
The fragmentation of **B4H<sup>+</sup>** leads to the elimination of one or two molecules of CHCOH to form the ions at  $m/z$  275 and  $m/z$  233 respectively. These ions are unobserved on CID mass spectra of  $m/z$  317 (see 10.3).

### 8.5 Expected fragmentations of **MH<sup>+</sup>** deriving from **B5**



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

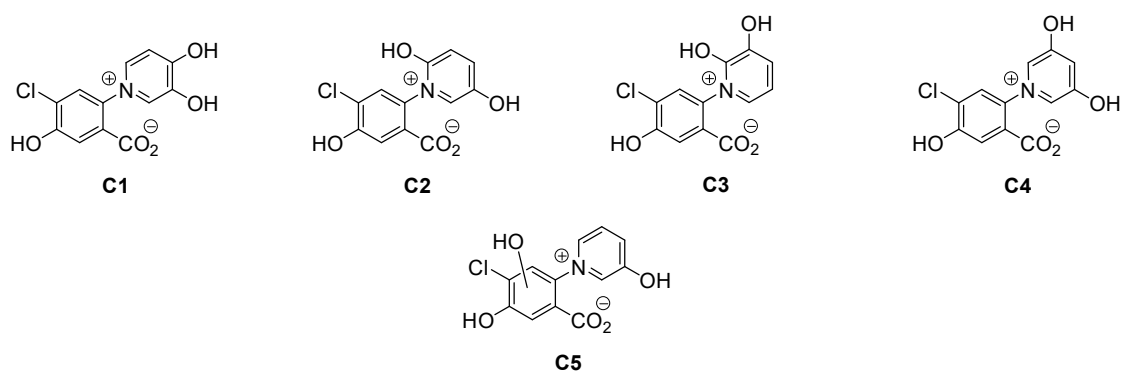
## 9. Detection of C1 and C2 or C3 after electro-Fenton oxidation and bioconversion of **1** and electro-Fenton oxidation of **4**



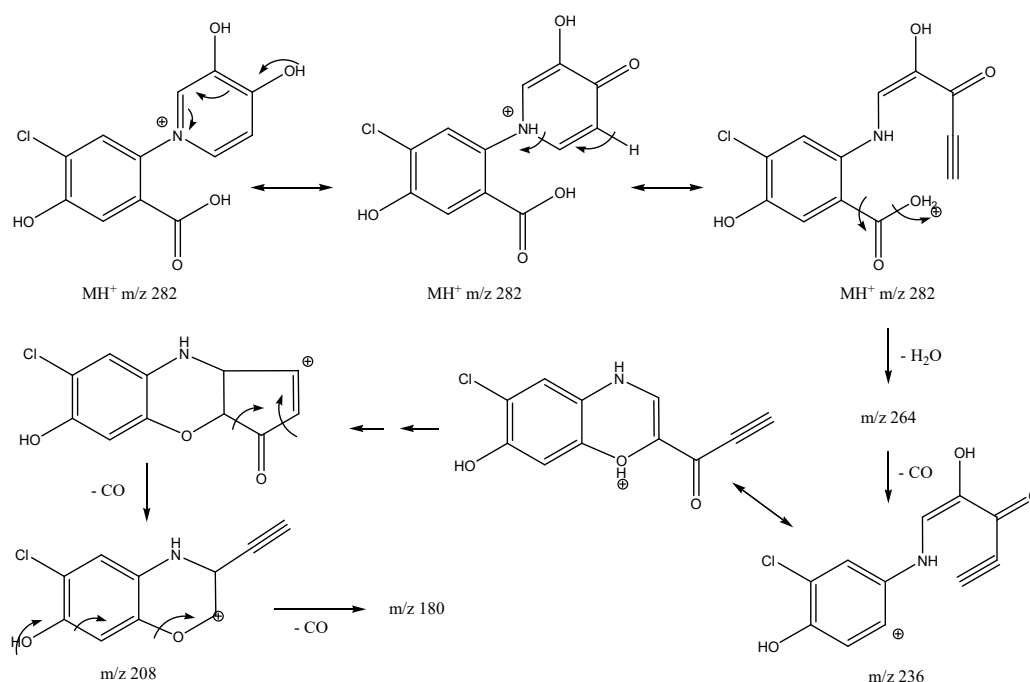
**Fig. SM-6.** (a) Extracted ion chromatogram (positive ion current) of  $m/z$  282 for electro-Fenton oxidation of pyridinium **4**. CID mass spectra of ion at  $m/z$  282 at (b)  $RT=4.2$  min and (c)  $RT=10.4$  min. Cone=25 V, Ecol=15 eV.

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

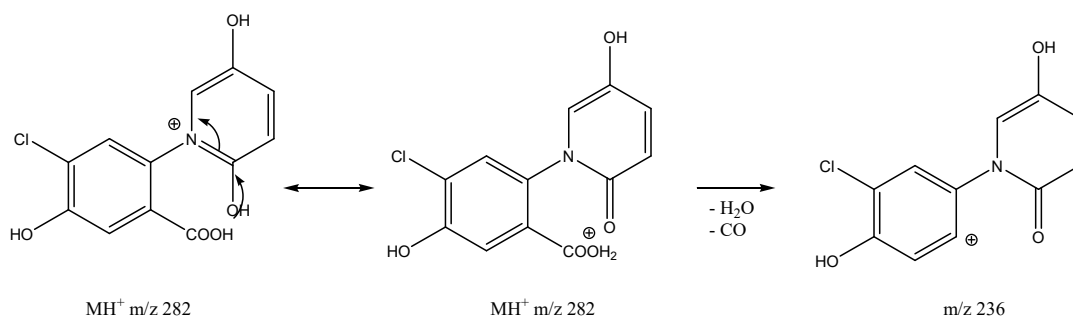
Structural characterization led to focus on the five compounds **C1**, **C2**, **C3**, **C4** and **C5**. **C1** is in agreement with fragmentations observed for  $RT = 10.4$  min. Structures **C2** and **C3** both allow the formation of ions observed for the decomposition of  $MH^+$  eluted at 4.2 min.



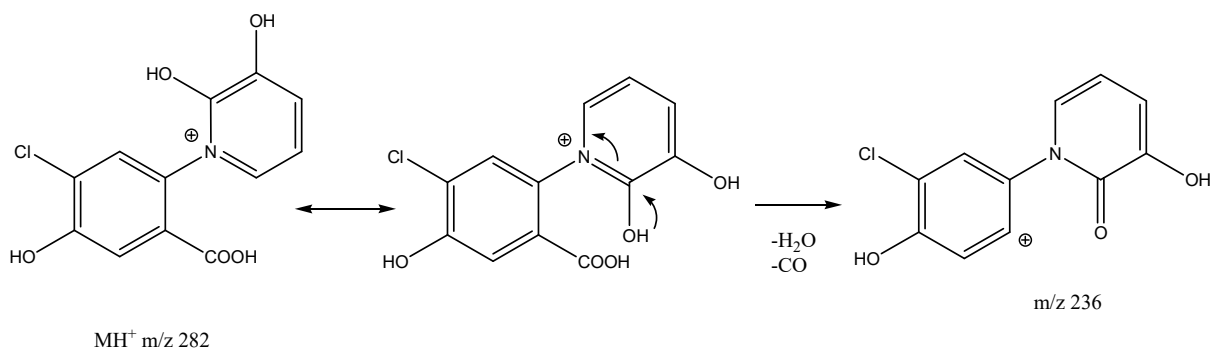
### 10.1 Expected fragmentations of MH<sup>+</sup> deriving from C1



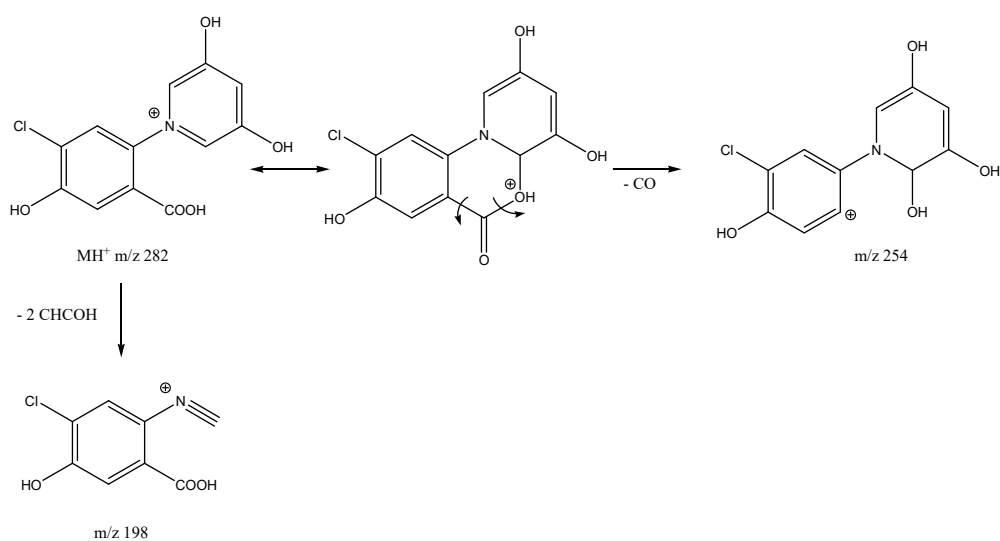
### 10.2 Expected fragmentations of MH<sup>+</sup> deriving from C2



### 10.3 Expected fragmentations of MH<sup>+</sup> deriving from C3



#### 10.4 Expected fragmentations of $\text{MH}^+$ deriving from C4



The fragmentation of  $\text{C4H}^+$  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 $\text{MH}^+$ deriving from C5

