1 **IDENTIFICATION OF NEW OMEPRAZOLE METABOLITES IN**

2 **WASTEWATERS AND SURFACE WATERS**

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ABSTRACT

Omeprazole is one of the world-wide most consumed pharmaceuticals for treatment of gastric diseases. As opposed to other frequently used pharmaceuticals, omeprazole is scarcely detected in urban wastewaters and environmental waters. This was corroborated in a previous research, where parent omeprazole was not detected while four transformation products (TPs), mainly resulting from hydrolysis, were found in effluent wastewaters and surface waters. However, the low abundance of omeprazole TPs in the water samples together with the fact that omeprazole suffers an extensive metabolism, with a wide range of excretion rates (between 0.01-30%), suggests that human urinary metabolites should be investigated in the water environment. In this work, the results obtained in excretion tests after administration of a 40 mg omeprazole dose in three healthy volunteers are reported. Analysis by liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF MS) reported low concentrations of omeprazole in urine. Up to twenty-four omeprazole metabolites (OMs) were detected and tentatively elucidated. The most relevant OM was an omeprazole isomer, which obviously presented the same exact mass (*m/z* 346.1225), but also shared a major common fragment at *m/z* 198.0589. Subsequent analyses of surface water and effluent wastewater samples by both LC-QTOF MS and LC-MS/MS with triple quadrupole revealed that this metabolite (named as OM10) was the compound most frequently detected in water samples, followed by OM14a and OM14b. Up to our knowledge, OM10 had not been used before as urinary biomarker of omeprazole in waters. On the contrary, parent omeprazole was never detected in any of the water samples. After this research, it seems clear that monitoring the presence of omeprazole in the aquatic environment should be focused on the OMs suggested in this article instead of the parent compound.

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1. INTRODUCTION

Environmental contamination by pharmaceuticals (both human and veterinary medicines) is an issue of general concern. They are emerging pollutants widely distributed in the environment, which can enter through different routes (Zuccato et al. 2005). Once a pharmaceutical is administered, it can be excreted unchanged or as metabolites in the urine or faeces, reaching the aquatic environment commonly throughout sewage waters (Besse et al. 2012; González Alonso et al. 2010; Ortiz de García et al. 2013).

Omeprazole is one of the most frequently prescribed and administered pharmaceuticals in humans for proton pump inhibition (Andersson et al. 1993; Bruni and Ferreira. 2008; José Gómez et al. 2007; Ortiz de García et al. 2013). As an example, 51,874,630 packages, under prescription, were dispensed in Spain in 2010 (http://www.msssi.gob.es/biblioPublic/publicaciones/recursos_propios/infMedic/docs/S ubgruposATCvol35n4.pdf). It is known to act by irreversibly blocking the terminal stage of gastric acid secretion in the gut. This compound is reported to be metabolized by the enzyme CYP2C19 to form the 5-hydroxy metabolite whereas CYP3A4 catalyzes the sulfone formation (Kanazawa et al. 2002; Rost et al. 1995). About 80% of orally administered omeprazole dose is excreted in urine as metabolites, whereas the remainder is excreted in the faeces, mainly from biliary secretion (Andersson et al. 1993). Different percentages of omeprazole excretion (as intact parent) can be found in the literature, ranging from 0.01% (Besse et al. 2008) to 5% (Hernando et al. 2007), or even up to 30% (Ortiz de García et al. 2013). This variation has been justified based on the different enzymatic activity of each individual. Sulfonated and 5-hydroxylated compound are the major omeprazole metabolites (OMs) found in plasma (Espinosa Bosch et al. 2007; Song and Naidong. 2006), whereas in urine the 5-hydroxylated OM

76 is the predominant one (Petsalo et al. 2008) (**Figure 1**). The concentration of

77 omeprazole sulfide, another OM reported in the literature, is usually too low to be

78 determined in plasma, and it is also negligible in urine (Rezk et al. 2006).

79   Several analytical methods have been reported for determination of omeprazole in

80 plasma (Kanazawa et al. 2002; Macek et al. 2007; Rost et al. 1995; Song and Naidong.

81 2006) while only a few articles deal with analysis of urine samples. Petsalo et al.

82 (Petsalo et al. 2008), focused on the determination of the 3-hydroxy-, 5-hydroxy-,

83 demethyl-, and sulfone-OMs and omeprazole itself in urine. It was not possible to detect

84 3-hydroxy OM, and the concentrations of omeprazole and its sulfone OM were very

85 low. Chung et al. (Chung et al. 2004), reported the detection of four unconjugated and

86 two conjugated OMs in horse urine by LC-MS.

87   The available literature on omeprazole determination highlights the application of

88 liquid chromatography (LC) as the most appropriate analytical tool for this compound

89 (Kanazawa et al. 2002; Petsalo et al. 2008; Song and Naidong. 2006; Ternes et al.

90 2001). Although some methods have made use of UV as detection technique (Rezk et

91 al. 2006), currently mass spectrometry (MS) is the technique of choice for

92 determination of omeprazole, particularly LC coupled to tandem MS (MS/MS), the

93 advantages of which, short analytical run time as well as excellent selectivity and

94 sensitivity, are widely recognized (Espinosa Bosch et al. 2007). While LC-MS/MS with

95 triple quadrupole (QqQ) analyzer is the workhorse for quantitative analysis of

96 pharmaceuticals, omeprazole included, in the aquatic environment (Castiglioni et al.

97 2004; Gracia-Lor et al. 2010; Van Nuijs et al. 2010; Zuccato et al. 2005), LC coupled to

98 high resolution mass spectrometry (HRMS) such as Orbitrap (Calza et al. 2012; Thevis

99 et al. 2011), FTMS (Awasthi et al. 2012) or time-of-flight MS (Ibáñez et al. 2004;

100 Ibáñez et al. 2006) is a powerful analytical tool for investigation of metabolites and/or

101 transformation products (TPs) in water. These HR MS techniques are also appropriate

102 to perform metabolism studies of pharmaceuticals within the biomedical field (Corcoran

103 et al. 2000; Hopfgartner et al. 1999) due to the accurate-mass full-spectrum acquisitions

104 provided by these analyzers.

105   Considering the high consumption of omeprazole and the reported excretion rates of

106 up to 20% as intact omeprazole, one might expect to find this compound in urban

107 wastewater, or even in environmental waters. Nevertheless, its detection in water

108 samples is rarely reported. Additionally, in our previous study on omeprazole

109 degradation (Boix et al. 2013), only four low-abundant TPs were rarely found in water

110 samples, with omeprazole sulfide the most frequently detected. The initial hypothesis on

111 a possible degradation of omeprazole in waters was thus discarded and a detailed study

112 on human urinary metabolites of omeprazole was initiated. This paper pursues the

113 detection and elucidation of urinary OMs making use of LC-QTOF MS. Subsequently,

114 27 surface water (SW) and 25 wastewater (WW) samples have been analyzed by LC-

115 QTOF MS and LC-MS/MS QqQ to investigate the presence of OMs.

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117 **2. EXPERIMENTAL**

118   2.1 <u>Reagents and chemicals</u>

119   See Supplementary Information (SI).

120   2.2 <u>Instrumentation</u>

121   <u>UHPLC-QTOF MS</u>

122   A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to

123 a hybrid quadrupole–orthogonal acceleration-TOF mass spectrometer (Q-oaTOF

124 Premier, Waters Micromass, Manchester, UK), using an orthogonal Z-spray

125 electrospray ionization (ESI) interface operating in positive and negative ion modes

126 (For further details, see SI).

127 QTOF data were acquired under MS^E mode, an approach that enables the

128 simultaneous acquisition of both parent protonated molecules and fragment ions in a

129 single injection. So, two acquisition functions with different collision energies were

130 created. The first one, the low energy (LE) function, selecting a collision energy of 4

131 eV, and the second one, the high energy (HE) function, with a collision energy ramp

132 ranging from 15 eV to 40 eV (Díaz et al. 2011; Hernández et al. 2011a; Hernández et al.

133 2011b; Plumb et al. 2006).

134 UHPLC-MS/MS QqQ

135 A Waters Acquity UPLC system was interfaced to a triple quadrupole mass

136 spectrometer (TQD, Waters) with an orthogonal Z-spray-ESI interface. Capillary

137 voltages of 3.5 and -3.0 kV were used in positive and negative ionization mode,

138 respectively (For further details, see SI).

139 2.3 Analysis of metabolites excreted in urine

140 Urine samples were collected from three healthy volunteers with different gender (1

141 male, 2 females) and origins (2 Europeans and 1 Latin-American, all living in Europe).

142 Each volunteer ingested an oral dose of 40 mg omeprazole (i.e., two 20 mg capsules).

143 Urine samples were collected before ingestion of the drug (control sample), and after 15

144 minutes, 1, 3.5, 6.5, 9, 12 and 24 hours of drug administration. Polyethylene bottles

145 were used for collecting samples, which were immediately stored at −18 ºC until

146 analysis. The study protocol was approved by an ethical committee (University Jaume I,

147 Spain).

148   For analysis of urine, 1 mL of sample previously centrifuged (10,000 r.p.m., 10

149   minutes), was two-fold diluted with Milli-Q water. After that, 50 µL were directly

150   injected in the UHPLC-QTOF MS system.

151   For the determination of metabolites released from glucuronide conjugates, 1 mL of

152   centrifuged urine was buffered with 50 µL acetic acid/ammonium acetate (pH 5.5).

153   After being hydrolyzed overnight with 200 units of β-D-glucuronidase at 37 ℃, 50 µL

154   of the hydrolyzed mixture were injected in the UHPLC-QTOF MS system (Hernández

155   et al. 2004).

156   2.4 Retrospective QTOF MS analysis of water samples

157   25 wastewater samples (15 influents and 10 effluents) were collected from three

158   different wastewater treatment plants (WWTPs) of the Valencian region (Eastern

159   Spain), whose anonymity must be respected, from June 2008 to December 2010. 27

160   surface waters were also sampled from several points located in the same area in

161   October 2010. All these samples had been used previously in different studies

162   performed at our lab using UHPLC-QTOF MS for their analysis.

163   2.5 Data processing

164   QTOF MS data were processed using MetaboLynx XS and ChromaLynx XS

165   application managers (Waters Micromass vs 4.1). Regarding data from triple

166   quadrupole, TargetLynx software (also from Waters) was used (More details in ref.

167   (Boix et al. 2013)).

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169   **3. RESULTS AND DISCUSSION**

170   3.1 Urinary excretion of parent omeprazole

171   Omeprazole was detected at low concentration levels (not quantified) in non-

172 hydrolyzed urine from the two European volunteers approximately between 1 and 3

173 hours after administration of the drug. In the third volunteer (Latin American female),

174 the drug was not detected in any of the urine samples collected. This would be in

175 accordance with the scientific literature where different levels of excretion for

176 omeprazole have been reported (Besse et al. 2008; Hernando et al. 2007; Ortiz de

177 García et al. 2013; Zuccato et al. 2005).

178   Interestingly, the narrow window eXtracted Ion Chromatogram (nw-XIC, 0.02Da) at

179 the exact mass of omeprazole ([M+H]$^+$ *m/z* 346.1225), showed a highly abundant peak

180 at different retention time (5.88 min for omeprazole, 4.91 min for this compound,

181 OM11a) (**Figure S1a**). Although HE MS spectra of both compounds were quite

182 different (e.g. specific fragment ions: *m/z* 136 and 151 for omeprazole; *m/z* 138 and 149

183 for OM11a, **Figures S1b,c**), they shared an important common fragment at *m/z*

184 198.0589. This fact cannot be neglected, as it might lead to a false positive of

185 omeprazole even by LC-MS/MS if only the transition 346.1>198.1 (the most commonly

186 reported in the literature) is acquired (Petsalo et al. 2008; Macek et al. 2007; Song and

187 Naidong. 2006), without sufficient chromatographic separation. This is of relevance

188 taking into account the high abundance of this metabolite.

189   3.2 <u>Elucidation of metabolites</u>

190   **Table 1** shows the elemental composition, mass errors of the (de)protonated

191 molecule and fragments ions, and retention time for omeprazole and twenty-four OMs

192 detected in hydrolyzed and non-hydrolyzed urine. **Figure 1** shows the structures

193 suggested for the main fragment ions together with the omeprazole metabolites reported

194 in the literature. Tentative structure proposals for OMs identified in this work are given

195 in **Figure 2**. All metabolites were detected in positive-ion mode except for the OM12,

196 which was only detected in negative-ion mode.

197 Different conjugated metabolites, as cysteine (OM1), glucuronides (OM2a, OM2b,

198 and OM2c) and sulfates (OM5 and OM12) were directly detected (**Figure 2**) in the non-

199 hydrolyzed urine. After hydrolysis, a decrease of cysteine and glucuronide conjugates

200 was observed, as well as the corresponding increase in abundance of the OM11a and

201 OM7d. Additionally, a new peak appeared, corresponding to $[C_{17}H_{20}N_3O_3S]^+$ (OM11b)

202 (*m/z* 346.1225, Δ = 1.5 mDa), with the same elemental composition as omeprazole and

203 its isomeric metabolite OM11a, but eluting even earlier (4.52 min). With these data, it

204 seems that OM7d and OM11a are partly conjugated while OM11b is fully conjugated

205 prior to urinary excretion. Based on similarities in their fragmentation, OM11a and

206 OM11b would correspond to the free forms of OM2(b,c) and OM2a, respectively.

207 The compound OM4 $[C_{16}H_{18}N_3O_3S]^+$, *m/z* 332.1069 (Δ = 0.7 mDa; 3.73 min), has

208 the same exact mass as the reported demethyl-OM (Kanazawa et al. 2002; Petsalo et al.

209 2008; Rost et al. 1995). However, OM4 was not considered to match demethyl-OM, as

210 the spectrum of OM4 shows the specific loss of *m/z* 32.9799 ($[^\bullet SH]$) related to the

211 reduction of the sulfoxide group.

212 The loss of the thiol radical $[^\bullet SH]$ from the protonated molecule was observed in

213 ESI positive for all OMs when the sulfoxide moiety was converted into a sulfide.

214 Initially, cyclation of omeprazole molecule rendering a terminal thiol group was

215 considered, as reported to occur under acidic conditions (Bruni and Ferreira. 2008;

216 DellaGreca et al. 2006; Weidolf and Castagnoli. 2001). However, in the MS/MS

217 spectrum of the 4-hydroxy omeprazole sulfide reference standard ($C_{16}H_{18}N_3O_2S^+$, *m/z*

218 316.1120), the loss of the thiol radical $[^\bullet SH]$ was also observed (**Figure S2a**).

219 Therefore, the initial hypothesis of cyclisation was rejected. The $[^\bullet SH]$ loss (32.9799

220 Da) from the thioether group could be explained from a homolytic cleavage and

221 intermediate binding to the hydrogen at the imidazolyl-N to form [•SH] **(Figure S2b)**.

222 Although the mass error was relatively high (around 4 mDa), other elemental

223 compositions could not be assigned. A subsequent homolytic cleavage, involving the

224 loss of a methyl radical [•CH$_3$] is observed as well (see **Figure S2c**). The [•SH] loss

225 observed for many OMs suggested the reduction of the sulfoxide group to sulfide.

226 Regarding the position at which hydroxylation has occurred (for OM4 but also for

227 OM3, OM7abe, OM8abc, OM10 and OM11a), it was justified according to the

228 fragment ions observed in their spectra. So, two fragments ions at m/z 149.0715

229 (C$_8$H$_9$N$_2$O$^+$) and 135.0558 (C$_7$H$_7$N$_2$O$^+$) (see **Table 1** and **Figure 1a**) correspond to the

230 benzimidazole ring of the original omeprazole molecule and to its demethylated

231 fragment ion, respectively. The presence of one of these fragment ions would therefore

232 imply that hydroxylation has taken place in the other side of the molecule, i.e., in the

233 pyridine ring.

234 In the case of OM6 (and also OM11b), the hydroxylation was located in the

235 benzimidazole ring based on the presence of the fragment ions at m/z 195.0770

236 (C$_9$H$_{11}$N$_2$O$_3$$^+$) and 150.0919 (C$_9H_{12}NO^+$), respectively (see **Table 1** and **Figure 1a**).

237 Five di-oxygenated OM7s [C$_{17}$H$_{20}$N$_3$O$_5$S]$^+$ (m/z 378.1124) and three mono-

238 oxygenated OM8s [C$_{17}$H$_{20}$N$_3$O$_4$S]$^+$ (m/z 362.1175) were also observed (**Figure 2**).

239 However, it was not possible to predict at which position the hydroxylations occurred,

240 as the fragment ions did not provide enough information. The SO$_2$ loss observed for

241 OM7b-7e indicates the presence of the sulfone group in the molecule. Regarding OM7a,

242 the sulfoxide group was maintained (SO$_2$ loss not observed) therefore suggesting a

243 double hydroxylation. A previously searched metabolite (omeprazole sulfone-N-oxide,

244 [C$_{17}$H$_{20}$N$_3$O$_5$S]$^+$) (Hernández et al. 2011b) might also be one of the OM7s. However, no

245   characteristic fragmentation from an N-oxide (involving losses of O, OH$^\bullet$ and H_2O) was

246   observed (Chen et al. 2005).

247   Other reported OMs, like 5-hydroxy omeprazole (Hernández et al. 2011b; Kanazawa

248   et al. 2002; Petsalo et al. 2008; Rezk et al. 2006; Rost et al. 1995; Song and Naidong.

249   2006), 3-hydroxy omeprazole (Kanazawa et al. 2002; Petsalo et al. 2008) and

250   omeprazole sulfone (Kanazawa et al. 2002; Petsalo et al. 2008; Rezk et al. 2006; Rost et

251   al. 1995) matched with the exact mass of metabolites 8 (a, b,c). Based on the structure

252   proposed for these metabolites according to the observed fragmentation, none of them

253   could be assigned to omeprazole-sulfone (OM8a,b are sulfides and OM8c is a

254   sulfoxide). Moreover, 3- and 5- hydroxy omeprazole could only be related with OM8c.

255   However, after injecting the 5-hydroxy omeprazole reference standard, its retention time

256   and mass spectra were not in agreement with OM8c. Therefore, omeprazole-sulfone and

257   5-hydroxy omeprazole were discarded as candidates for the group of OM8 metabolites,

258   while 3-hydroxy omeprazole could still be a plausible candidate for OM8c.

259   It is worth to mention the detection of OM13 $[C_{16}H_{16}N_3O_4S]^+$ (*m/z* 346.0862,

260   ΔmDa= 1.3). This compound shared the same nominal mass than omeprazole (346) and

261   its isomers OM11a and OM11b, but it had different exact mass and retention time. The

262   potential of HR MS allowed differentiating these compounds with different elemental

263   compositions. In this case, the elemental composition for OM13 suggested a

264   demethylation (fragment ion at *m/z* 135.0558), sulfoxide-reduction (loss of *m/z* 32.9799,

265   [$^\bullet$SH]) and subsequent hydroxylation and oxidation (fragment ions at *m/z* 212.0381 and

266   150.0555) from parent omeprazole (**Table 1, Figure 2**).

267   Two isomers, OM14a and OM14b ($[C_{16}H_{18}N_3O_2S]^+$, *m/z* 316.1220), eluting at 4.52

268   and 5.01 min, respectively, are the result of sulfoxide reduction (loss of *m/z* 32.9799,

269   [$^\bullet$SH]) and demethylation reactions in the omeprazole structure. OM14b presented two

270 fragment ions at *m/z* 136.0762 ($C_8H_{11}NO^+$) and 149.0715 ($C_8H_9N_2O^+$) showing that

271 demethylathion occurred in the pyridine ring. The metabolite OM14b corresponds to the

272 already reported 4-hydroxy omeprazole sulfide (Hernández et al. 2011b), and the

273 identity was confirmed with the reference standard available at our laboratory.

274 Regarding the metabolite OM14a, the fragment ions at *m/z* 135.0558 ($C_7H_7N_2O^+$) and

275 150.0919 ($C_9H_{12}NO^+$) indicated that the compound was demethylated at the

276 benzimidazole group.

277   As illustrative example, the elucidation process for the two most abundant

278 metabolites, OM10 and OM11a, is discussed in detail in SI.

279   Up to our knowledge, some of the metabolites reported in this paper have never

280 been investigated in water samples. Reference standards are not commercially available

281 for most of these compounds and therefore their identity, although strongly supported

282 by our QTOF MS accurate-mass data, could not be unequivocally confirmed.

283   3.3 Retrospective analysis of water samples by UHPLC-QTOF MS

284   After the study performed on urinary metabolites of omeprazole, the presence of the

285 24 identified metabolites was investigated in water samples (**Table S1**). To this aim, a

286 total of 52 samples (15 influent wastewater (IWW), 10 effluent wastewater (EWW) and

287 27 surface water (SW)) previously analyzed by UHPLC-QTOF MS were

288 retrospectively re-examined, this is, without the need of additional sample injections.

289   Retrospective analysis was made by performing eXtract Ion Chromatograms using

290 narrow mass windows (± 10 mDa) at the metabolites exact *m/z*-values (nwXICs).

291 Confirmation of the identity of the compounds detected was based on the accurate *m/z*

292 of the (de)protonated molecule and at least two fragment ions, together with the

293 agreement in retention time (deviation lower than ±2.5%). Both the fragment ions and

retention times, used as references were derived from the metabolism experiments. Up to nine OMs were detected in the water samples, oppositely to parent omeprazole that was not found in any of the samples (**Table 2**). OM10 was the most frequently detected compound, as it was present in 21 out of 25 WW, and in 11 out of 27 SW samples analyzed. OM7c, OM7d, OM11a, OM13, OM14a, and OM14b were also found in all types of water matrices, while OM5 was only detected in SW (21%) and EWW (10%) samples. Finally, OM7e was found in 3 SW samples (11%).

3.4 <u>UHPLC-MS/MS analysis</u>

In order to confirm the presence of OMs in the water samples, the same water extracts were re-analyzed by UHPLC-(ESI)-MS/MS with triple quadrupole, applying the same LC conditions used in the UHPLC-QTOF measurements in metabolism experiments. The higher sensitivity of triple quadrupole was expected to facilitate the detection of those OMs that were present at low concentrations in the water samples. The 24 OMs resulting from the metabolism study and parent omeprazole were included in the method. For each compound, two transitions were selected based on fragment ions observed in the QTOF experiments (**Table S1**). Apart from the presence of a chromatographic peak at the two transitions acquired, the identity of the findings was confirmed by calculating the peak area ratio between the quantification "Q" and the confirmation "q" transitions, which was compared with that of the "reference compound" (urine vials with the highest concentration of the OM). A finding was considered positive when experimental ion ratios were within the tolerance range (Commission Decision 2002). The agreement in retention time (deviation lower than ±2.5%) with the "reference compound" was also required.

Up to 14 OMs were detected and their identity confirmed by QqQ (**Table 2**). Eleven of these OMs (OM4, OM5, OM7c, OM7d, OM7e, OM8b, OM10, OM11a, OM13,

OM14a, and OM14b) were found in all types of water matrices analyzed. Similar to QTOF analysis, unchanged omeprazole was not found in any sample. Nevertheless, it is interesting to note that one of its isomers, OM11a, was detected in 80% IWW, 90% EWW and 30% SW samples. This fact reveals the importance of good chromatographic separation to avoid confusion between these two compounds as they share one of the transitions (346.1 > 198.1). Therefore, in order to reduce false positives of omeprazole the acquisition of additional specific transitions for each compound (see **Table S1**) and satisfactory chromatographic separation is of high relevance. This compound might be the same detected in wastewater by (Gomez-Ramos et al. 2011), after performing a XIC at the exact mass of omeprazole. OM10 was the most abundant in terms of MS arbitrary units, being consistent with the results of QTOF analysis. This compound was the most frequently detected metabolite in WW (100%) and SW (48%). Five of the OMs (OM3, OM4, OM8a, OM8b, and OM8c) were only found by LC-MS/MS analysis, due to its higher sensitivity compared to QTOF.

It is noteworthy that up to eight OMs were detected in 90-100% of effluent wastewater samples. These OMs were also present in influent wastewater, although some of them at lower frequency. Surely, the higher complexity and strong signal suppression due to matrix effects in the influent made the detection of OMs more problematic in comparison with effluent wastewater. It is also relevant the detection of up to seven OMs in around 30% of the surface water samples.

As an illustrative example, **Figure 3** shows selected UHPLC-MS/MS chromatograms for the most abundant OMs (OM5, OM10, OM7c,d,e, OM14a,b) detected in an effluent wastewater. As can be seen, experimental ion ratios did not exceed the maximum deviation allowed for any of the OMs detected, with all deviations being below 15%. OM10 and OM14b presented the highest responses, with average

344 areas of 10,000 and 8,000 a.u., respectively. This might reveal that they were the most

345 relevant compounds in terms of concentration, but however quantification could not be

346 performed due to the lack of reference standards.

347

348 **CONCLUSIONS**

349   In this work, urinary omeprazole metabolites have been investigated by UHPLC-

350 QTOF MS. A total of twenty-four OMs were identified in urine samples of three

351 volunteers who participated in this study, while parent omeprazole was present only at

352 very low concentrations. OM11a was the most abundant compound. This OM is an

353 omeprazole isomer and shares the fragment ion at m/z 198.0589 commonly used for the

354 determination of omeprazole in LC-MS/MS methods. The loss of [$^{\bullet}$SH] radical

355 deduced by TOF MS spectra in most of the OMs detected, has been crucial for

356 justifying and suggesting possible chemical structures.

357   After UHPLC-QTOF MS analysis of 52 water samples, nine OMs were detected in

358 surface water and wastewater samples, with OM10 being the most frequently found in

359 wastewater (84% of the samples) and in surface water (41%). The results were

360 confirmed by UHPLC-MS/MS analysis using a triple quadrupole analyzer, which

361 superior sensitivity allowed to detect up to fourteen OMs. Unchanged omeprazole was

362 not found in any sample; nevertheless, its isomer OM11a was detected in several

363 samples.

364   In the light of the results obtained in the present work, it seems evident that

365 monitoring unchanged omeprazole is not the best option to investigate the impact of this

366 widely consumed pharmaceutical in the aquatic ecosystem. Instead, it is recommended

367 to focus the research on the most abundant OMs identified in this work, i.e., those

368   named as OM5, OM7c, OM7d, OM10, OM11a, OM14a, and OM14b, when monitoring

369   omeprazole in urban wastewater and also in surface water. Obviously, it would be

370   necessary to perform absolute configuration on the relevant compounds by NMR to

371   subsequently enable synthesis of reference compounds to perform quantitative studies,

372   but that is beyond the present study. In addition to the OMs proposed in this paper,

373   omeprazole sulfide -a transformation product resulting from hydrolysis of omeprazole

374   that has been previously reported (Boix et al. 2013)- should also be included to have a

375   realistic overview of the omeprazole impact on the aquatic ecosystem.

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FIGURE CAPTIONS

526 **Figure 1.** (a) Structure of omeprazole and some important fragment ions (b)

527 Omeprazole metabolites reported in the literature

528 **Figure 2.** Suggested structures for urinary OMs detected by UHPLC-QTOF MS after

529 omeprazole oral administration

530 **Figure 3.** UHPLC-MS/MS chromatograms for the omeprazole metabolites (a) OM5, (b)

531 OM10, (c) OM7c-7e, (d) OM14a, and (e) OM14b, in effluent wastewater

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