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1 **Non conventional biological treatment based on *Trametes versicolor* for**
2 **the elimination of recalcitrant anticancer drugs in hospital wastewater.**

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16

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18 HRMS.

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Abstract

This work presents a study about the elimination of anticancer drugs, a group of pollutants considered recalcitrant during conventional activated sludge wastewater treatment, using a biological treatment based on the fungus *Trametes versicolor.* A 10-L fluidized bed bioreactor inoculated with this fungus was set up in order to evaluate the removal of 10 selected anticancer drugs in real hospital wastewater. Almost all the tested anticancer drugs were completely removed from the wastewater at the end of the batch experiment (8 d) with the exception of Ifosfamide and Tamoxifen. These two recalcitrant compounds, together with Cyclophosphamide, were selected for further studies to test their degradability by *T. versicolor* under optimal growth conditions. Cyclophosphamide and Ifosfamide were inalterable during batch experiments both at high and low concentration, whereas Tamoxifen exhibited a decrease in its concentration along the treatment. Two positional isomers of a hydroxylated form of Tamoxifen were identified during this experiment using a high resolution mass spectrometry based on ultra-high performance chromatography coupled to an Orbitrap detector (LTQ-Velos Orbitrap). Finally the identified transformation products of Tamoxifen were monitored in the bioreactor run with real hospital wastewater.

68
69 ## 1. Introduction

70 Cancer is ranked (year 2012) in the second place (21%) of non-communicable diseases
71 (this means non-infectious and non-transmissible medical conditions) which are causing
72 deaths, after cardiovascular illness (48%) and followed by respiratory diseases (12%)
73 (www.who.int); for that reason the high consumption of the drugs for chemotherapy
74 treatments has became a cause of concern. These specific drugs have been shown to have
75 potent cytotoxic, genotoxic, mutagenic, carcinogenic, endocrine disruptor and/or
76 teratogenic effects in several organisms, since they have been designed to disrupt or
77 prevent cellular proliferation, usually by interfering in DNA synthesis or disrupting the
78 endocrine system. The occurrences of these drugs in the aquatic environment could be
79 especially critical since they are intrinsically hazardous. Several ecotoxicological studies
80 have shown that in some cases such as for the cancer drug 5-Fluorouracil, the lowest
81 observed-effect concentration values (in algal and bacterial assays) were close to the
82 concentration found in sewage effluents (Zounkova et al., 2007) . More recently, studies
83 have revealed that mixtures of anticancer drugs in real samples possess an important
84 toxicological effect comparing with the individual drug (Mater et al., 2014).
85 In general these so-called anticancer drugs can be released to the aquatic environment via
86 hospital or domestic wastewater (Kovalova, 2009; Kosjek and Heath, 2011; Ferrando-
87 Climent et al., 2013; Negreira et al., 2013; Ferrando-Climent et al., 2014) since there is a
88 large number of them not removed from the wastewaters neither by biological
89 conventional treatments (Kümmerer et al., 1997; Ferrando-Climent et al., 2014) nor by
90 advanced technologies studied so far, such membranes bioreactors (Lenz et al., 2007;
91 Kovalova et al., 2012), electrolysis, advanced oxidation processes (ozonization, UV,

92 H_2O_2), etc (Chen et al., 2008; Zhang et al., 2013). Therefore there is a need of
93 development and application of new technological alternatives for wastewater treatment,
94 and for the removal of the anticancer drugs from sewage.

95 In this work the performance of an alternative biological treatment based on white-rot
96 fungi (WRF) was explored to eliminate selected anticancer drugs. *Trametes versicolor*
97 has been already shown to have a special capacity to remove a wide amount of
98 pharmaceutical compounds (Cruz-Morató et al., 2013) including β-blockers, antibiotics,
99 anti-inflammatory and psychiatric drugs and achieving even the mineralization of some
100 compounds such Diclofenac and Ketoprofen (Marco-Urrea et al., 2010a; Marco-Urrea et
101 al., 2010b, 2010c; Marco-Urrea et al., 2010d; Prieto et al., 2011; Jelic et al., 2012;
102 Rodriguez-Rodriguez et al., 2012). WRF has an unspecific oxidative enzymatic system
103 which includes lignin-modifiers enzymes, in particular laccase and peroxidases (extra-
104 cellular enzymes), and also intracellular enzymatic complexes (cytochrome P450)
105 (Asgher et al., 2008). Hydroxylation, formylation, deamination and dehalogenation
106 mechanisms in the anthropogenic pollutants take place during the fungi metabolism
107 (Harms et al., 2011; Cruz-Morató et al., 2012) and enable the degradation of the parent
108 compound. However, detoxification does not necessarily occur since transformation
109 products (TPs) of parent compounds can be in occasions more recalcitrant or even more
110 toxic than the parent compound.

111 The objective of this work was to study the potential ability of WRF *T. versicolor* to
112 eliminate selected anticancer drugs from real hospital effluents. 10 anticancer drugs,
113 selected because of their use, ubiquity, non-biodegradability and also their potential
114 bioaccumulation in the environment (Besse et al., 2012), were monitored along the

experiment performed in a fluidized bed bioreactor. Later, studies for the single degradation of Cyclophosphamide, Ifosfamide and Tamoxifen at high concentration in flasks inoculated with *T. versicolor* were performed. These experiments were done in order to assess their possible degradation by this fungus under optimal growth conditions and the identification of transformation products by high resolution mass spectrometry (HRMS) along the degradation experiments.

2. Materials and methods

2.1. Fungus preparation

T. versicolor (ATCC#42530) was provided by the American Type Culture Collection. It was kept by subculturing on 2 % malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 days. *T. versicolor* was grown in form of pellets as previously described (Blánquez et al., 2004) and subsequently were washed with sterile deionized water before its use.

2.2. Standard preparation and reagents.

Ciprofloxacin HCl, Cyclophosphamide, Ifosfamide, Methotrexate, Azathioprine, Etoposide, Docetaxel, Paclitaxel, Vincristine Sulphate and Tamoxifen Citrate were purchased by European Directorate for the Quality of Medicines and Healthcare (EDQM) Reference Standards (Strasbourg, France). Isotopically labelled compounds, used as internal standards, [^{2}H4]-Cyclophosphamide, [^{13}C6]-Tamoxifen Citrate, [^{2}H3]-Etoposide, [^{2}H3]-Methotrexate, [^{2}H3]-Vincristine Sulphate, [^{13}C4]-Azathioprine were purchased

137 from Toronto Chemical Research Inc. (Canada) and [^{2}H8]-Ciprofloxacin from EDQM
138 Reference Standards (Strasbourg, France). HPLC-grade Water and HPLC-grade
139 acetonitrile and water (LiChrosolv) were supplied by Merck (Darmstadt, Germany).
140 Reagents like Formic acid 98% (HCOOH) were provided by Sharlab (HPLC-grade).
141 Ethylenediaminetetraacetic acid disodium Salt 0.1 M solution (SV) and NH_3 30% was
142 provided by Panreac (Barcelona, Spain).

143 The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL) from
144 Waters Corporation (Milford, MA, USA). Glass fiber filters (1 µm) and nylon membrane
145 filters (0.45 µm) were purchased from Whatman (U.K.). Glucose, ammonium tartrate
146 dibasic and malt extract were purchased from Sigma-Aldrich (Barcelona, Spain).

147 Individual stock standard solutions of each target compound were prepared on a weight
148 basis in methanol at 1mg/mL and kept frozen at -20ºC. A mixture of all pharmaceutical
149 standards was prepared by appropriate dilution of individual stock solutions. Stock
150 solutions of internal standards were also prepared in methanol and were stored at -20ºC.
151 A mixture of these internal standards was also prepared by diluting the individual stock
152 solution in methanol.

153

154 **2.3. Hospital wastewater samples**

155 The main hospital of Girona, Dr. Josep Trueta, was selected for this study. This
156 municipality, which is located in the north of Spain, has approximately 96.000 habitants
157 and the hospital, which counts with around 400 beads, receives the major of the
158 oncologic patients of this area. Two non-consecutive samplings (Sample 1 and 2) were

159 performed at hospital wastewater effluent prior to the connection with the WWTP. In
160 order to isolate the effect of *T. versicolor* onto the pollutants so discarding the activity
161 from the rest of microorganism present in the wastewater, two treatments were tested in
162 the wastewaters: Sample 1 was sterilized while Sample 2 was not sterilized.

163

164 **2.4. Biodegradation experiments**

165 **2.4.1. Degradation of anticancer drugs in bioreactors fed with real**
166 **hospital effluents.**

167 A glass fluidized bed bioreactor with a useful volume of 10 L (Blanquez et al., 2008) was
168 used to carry out both sterile (Sample 1) and non-sterile (Sample 2) hospital wastewater
169 treatment in batch mode. Approximately, 2.0 g dry weight (d.w.) pellets L^{-1} were
170 inoculated in both sterile and non-sterile treatments. Fungal biomass was maintained
171 fluidized by air pulses generated by an electrovalve. The electrovalve was controlled by a
172 cyclic timer (1 second open, 5 seconds close) and the air flow was 12 L h^{-1}. The
173 bioreactor was equipped with a pH controller in order to keep pH at 4.5 and the
174 temperature was maintained at 25 ºC. Glucose and ammonium tartrate were fed
175 continuously from their stock solution (300 g L^{-1} and 675 mg L^{-1}, respectively) at a flow
176 rate to ensure an uptake rate of 0.31 g glucose g^{-1} d.w. pellets d^{-1} and 2 mg ammonium
177 tartrate g^{-1} d.w. pellets d^{-1}. For sterile conditions the bioreactor and the wastewater
178 (Sample 1) were autoclaved at 121 ºC for 30 min. Samples of 250 mL were taken
179 periodically. All the samples were filtered with 0.45 µm filters. 200 mL were stored at -
180 20ºC to be further analyzed by UPLC coupled to a triple quadrupole-ion trap mass

181 spectrometer (QqLIT). 50 mL from each sample were used to measure glucose
182 concentration, COD, N-NH_4^+ and laccase.
183

184 ### 2.4.2. Degradation of Cyclophosphamide, Ifosfamide and Tamoxifen in
185 ### Erlenmeyer flasks.

186 Individual degradation experiments for Cyclophosphamide, Ifosfamide and Tamoxifen
187 were performed in 500 mL Erlenmeyer flask containing appropriate amounts of mycelial
188 pellets (0.6 g d. w.) in a total volume of 100 mL of Kirk medium (pH 4.5) (Kirk et al.,
189 1978). Stock solution of Cyclophosphamide, Ifosfamide and Tamoxifen were prepared in
190 ethanol and they were spiked into the flasks reaching the desired concentration
191 (approximately 10, 10 and 0.3 mg L^{-1} respectively). The concentration selected for
192 Tamoxifen was limited for its solubility into water solutions (maximum 0.3 mg L^{-1}).

193 All these experiments were performed under sterile conditions using autoclave at 121 ºC
194 for 30 min before adding the WRF. The flasks were incubated under darkness in an
195 orbital shaker (135 rpm) at 25 ºC. The whole content of the flasks was sacrificed at times
196 30 min, 6 h, 1 d, 2 d, 3 d, 6 d and 9 d, and filtered through 0.45 µm glass fiber filter GF/A
197 from (Whatman, Spain).

198 In parallel, two types of control experiments were performed. One experimental blank,
199 was prepared with target compound in the same conditions that in the experimental
200 cultures (feed, pH, etc) but without inoculation of *T. versicolor*. This control sample was
201 used to assess the potential photodegradation of the micropollutants as well as the matrix
202 effect onto the contaminants from the experimental conditions. Another control consisted

203 in heat-killed cultures by autoclave (121 ºC for 30 min) under identical conditions to
204 those of the experimental cultures. This control was used to evaluate those potential
205 fungal sorption processes that could be taking place in time-courses degradation
206 experiments. The amount of adsorbed pollutant was determined from the difference in the
207 Cyclophosphamide, Ifosfamide and Tamoxifen concentration between the non-inoculated
208 and heat-killed control.

209 Samples were analyzed by UPLC-QqLiT to evaluate the quantitative degradation of each
210 compound using the analytical method previously developed(Ferrando-Climent et al.,
211 2013). Samples were also analyzed by HRMS technology in order to identify the
212 potential transformation products of parent cancer drugs.

213

214 **2.5. Target analysis of anticancer drugs**

215 Anticancer drugs were quantified in wastewater samples using an analytical methodology
216 previously described (Ferrando-Climent et al., 2013) for 10 target compounds:
217 Azathioprine (AZA), Cyclophosphamide (CY), Ciprofloxacin (CIP), Docetaxel (DOC),
218 Etoposide (ETO), Ifosfamide (IF), Methotrexate (MTX), Paclitaxel (PAC), Tamoxifen
219 (TAM) and Vincristine (VIN).

220 Briefly, samples were filtered through 0.45 μm nylon membrane filters (Whatman, U.K.).
221 A suitable volume of the chelating agent EDTA was added to all of them to a final
222 concentration of 0.1 % (g solute g^{-1} solution), as it is well known that it improves the
223 extraction of some antibiotics such Ciprofloxacin (Cha et al., 2006; Hernandez et al.,
224 2007; Gros et al., 2012). Pre-concentration of samples was performed by solid phase

225 extraction (SPE) by the automatically extract system GX-271 ASPECTM (Gilson,
226 Villiers le Bel, France). 50 mL of each sample was loaded at 1 mL min-1 in the Oasis
227 HLB (200 mg, 6 mL) cartridge previously conditioned using 5 mL of methanol followed
228 by 5 mL 0.1 % formic acid solution at 2 mL min^{-1}. Elution was performed with 10 mL at
229 a flow rate of 2 mL min^{-1} using pure methanol. The extract was evaporated under gentle
230 nitrogen stream using a Reacti-Therm 18824 System (Thermo Scientific) and
231 reconstituted with 500 µL of methanol-water (10:90, v/v). Finally, 5 µL of standard of
232 internal standard mix at 10 ng μL^{-1} was added in the extract for internal standard
233 calibration and to compensate possible matrix effect.

234

235 Chromatographic separation was carried out with a Ultra-Performance liquid
236 chromatography system (Waters Corp. Mildford, MA, USA) equipped with a binary
237 solvent system (Mildford, MA, USA) and a sample manager, using an Acquity HSS T3
238 column (50mm x 2,1mm i.d. 1,7 µm particle size; Waters Corp. Mildford, MA, USA)
239 under positive electrospray ionization (PI). The UPLC instrument was coupled to 5500
240 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied
241 Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. All transitions were
242 recorded by using Multiple Reactive Monitoring Mode (MRM) and the data were
243 acquired and processed using Analyst 2.1 software.

244 **2.6. Non target analysis of TPs of anticancer drugs**

245 Analysis were performed using an UPLC (Accela 1250 chromatograph with autosampler
246 Thermopal PAL AS) coupled to a LTQ-Velos Orbitrap from Thermo Scientific. The MS

247 analysis was performed with an electrospray ionization (ESI) interface in positive
248 ionization mode. Samples were injected in the system either after appropriate dilution (in
249 the case of bench-scale experiments) or after off-line SPE pretreatment (Ferrando-
250 Climent et al., 2013) (in the case of real wastewater samples obtained from bioreactor
251 along the experiment).

252

253 Samples obtained from the bench-scale experiments were injected after dilution in the
254 system and MS full-scan was acquired. Chromatograms obtained were compared in order
255 to identify new chromatographic peaks generated during the biodegradation of the target
256 compounds selected. Only those peaks produced along the experiments, that were not
257 present in control samples, were considered as a TP candidate for further evaluation.
258 Accurate masses of those candidates were selected included in a mass list to use it in a
259 second set of analysis where samples extracts were acquired using data-dependant
260 acquisition where only if the masses included in the candidate mass list were triggering;
261 MS/MS experiments. MS/MS spectra obtained were carefully studied in order to propose
262 a chemical structure. The assignment of fragmentation profile detected in MS/MS spectra
263 to each TP candidate was supported by Mass Frontier (software from Thermo Science)
264 which has enable the theoretical generation of mass fragments based on a proposed
265 chemical structure.

266

267 **2.7. Toxicity assays.**

268 A Microtox bioassay was used to perform toxicity test. This method is based on the
269 percent decrease in the amount of light emitted by the bioluminescent bacterium *V.*
270 *fischeri* upon contact with a filtered sample at pH 7. The effective concentration, EC50,
271 was measured after 15 min. The toxicity of the liquid medium was expressed in
272 percentages of EC50. Toxicity of samples from Erlenmeyer flasks treatments were
273 assessed during the experiments.

274

275 **2.8. Other analysis**

276 Laccase activity was assessed during the experiments using an adapted procedure from a
277 method for the determination of manganese peroxidase (MnP) previously described (Kaal
278 et al., 1993) . The reaction mixture used consisted in 200 µL of 250 mM sodium
279 malonate at pH 4.5, 50 µL of 20 mM 2,6-dimethoxiphenol (DMP) and 600 µL of sample.
280 DMP is oxidized by laccase even in the absence of cofactor. Changes in the absorbance at
281 468 nm were monitored for 2 min on a Varian Cary 3 UV-vis spectrophotometer at 30ºC.
282 One activity unit (U) was defined as the number of micromoles of DMP oxidized per
283 minute. The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1}(Wariishi et al.,
284 1992).

285 Biomass pellets dry weight was determined after vacuum-filtering the cultures through
286 pre-weighed glass-fiber filters (Whatman GF/A, Barcelona, Spain). The filters containing
287 the biomass pellets were dried at 105 ºC to constant weight.

288 Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow
289 Springs Instrument and Co. (Yellow Springs, OH, USA).

290

291 **3. Results**

292 **3.1. Fluidized bed bioreactor treatment of hospital effluent**

293 The method detection limits (MDL) and the initial concentration of the anticancer drugs
294 analyzed in the hospital wastewaters are shown in table 1. The presence of anticancer
295 drugs in the samples collected from the hospital in two occasions was dissimilar since
296 chemotherapy drugs dosed per patient can vary from day to day. Only three anticancer
297 drugs were initially detected in the Sample 1 (after its sterilization), the cytotoxic
298 quinolone Ciprofloxacin, Tamoxifen and Etoposide (below limit of quantification).
299 Concentration found for Ciprofloxacin (7000 ng L^{-1}) was similar of those detected by
300 other authors in similar effluents (Gros et al., 2012; Verlicchi et al., 2012; Ferrando-
301 Climent et al., 2013). Tamoxifen was detected at very high concentration (970 ng L^{-1})
302 much higher than ever found in other wastewater effluents (Figure 1) (Langford and
303 Thomas, 2009; Liu et al., 2010; Kosjek and Heath, 2011; Ferrando-Climent et al., 2013).
304 Removals achieved for Ciprofloxacin and Tamoxifen at the end of the batch degradation
305 experiments (8 d) were quite high, 84 % and 91 % respectively (Table 1).

306

307 In the case of the non-sterile hospital sample, Ifosfamide and Etoposide were also
308 detected besides Ciprofloxacin and Tamoxifen. Concentrations found in Sample 2 were
309 in the range of those detected previously in hospital wastewater (Ferrando-Climent et al.,
310 2013): 54.5 ng L^{-1} (Azathioprine), 2179.4 ng L^{-1} (Ciprofloxacin), 77.2 ng L^{-1}
311 (Ifosfamide), 197.5 ng L^{-1} (Etoposide) and 44.5 ng L^{-1} (Tamoxifen) (Figure 1).

312 In this case, the removals from wastewater at the end of the experiment were also high for
313 Azathioprine (100%), Etoposide (100 %), Ciprofloxacin (97%), Ifosfamide (61 %) and
314 Tamoxifen (48%) (Table 1).

315

316 In both sterile and non-sterile treatments, the highest laccase activity (about 100 U L^{-1})
317 was reached at the end of the experiment. Although glucose was accumulated during the
318 first hours, it was almost completely consumed after 24 h. The glucose and nitrogen
319 consumption and the laccase production observed indicate that *T. versicolor* was active
320 through the experiment (data not shown). Biomass concentration was constant during the
321 treatment (2.3 g d.w. L^{-1} at the end) because the process was carried out at growth-limited
322 conditions.

323 **3.2. Degradation of Cyclophosphamide, Ifosfamide and Tamoxifen in**
324 **flask experiments: Target and non-target analysis**

325 In the light of results obtained from both hospital samples which were treated by the
326 fluidized bed bioreactor, where Ifosfamide and Tamoxifen were partially eliminated from
327 hospital wastewater; three anticancer drugs were selected for further comprehensive
328 study of their degradation by *T. versicolor*: Ifosfamide, Tamoxifen and
329 Cyclophosphamide. Despite Cyclophosphamide was not present in any of hospital
330 wastewater samples analyzed in this work, it was selected for a more detailed study since
331 it is still one of the most important anticancer drugs: it has been detected in wastewaters
332 (both hospitals effluents and influents of WWTPs) (Buerge et al., 2006; Gómez-Canela et
333 al., 2012)(Ferrando-Climent et al., 2013, 2014) and their elimination during conventional

334 activated sludge treatments has been described as negligible (Zhang et al., 2013;
335 Ferrando-Climent et al., 2014).
336
337 Individual experiments with two control samples (non-inoculated and heat-killed) in
338 addition to the inoculated samples spiked with Cyclophosphamide, Ifosfamide or
339 Tamoxifen were performed in triplicate in order to study the degradation of these
340 compounds as well as the formation of their potential TPs. Cyclophosphamide and
341 Ifosfamide showed neither degradation nor sorption by *T. versicolor* at 10 mg L^{-1} (figure
342 2 A and B respectively). Cyclophosphamide showed a similar very slight decrease in both
343 controls and culture, which might be due to some kind of affinity of this compound for
344 the glass material of the recipient. Photodegradation of these compounds is discarded as
345 all the experiments were performed in darkness. These results are in contrast with the
346 results previously described, where Ifosfamide was slowly removed from the hospital
347 effluent in the experiment performed with the reactor at non-sterile conditions after 5
348 days of treatment. Concentration of Ifosfamide in real wastewater was in the range of ng
349 L^{-1} whereas it was spiked at 10 mg L^{-1} in the synthetic water for the individual
350 experiments. The high concentration of the pollutant in the flask experiments could have
351 inhibited WRF degradation potential. In order to discard this possibility, a second set of
352 experiments with Ifosfamide spiked in synthetic samples was performed but using lower
353 concentration (100 µg L^{-1}). No degradation was also observed in this experiment
354 (supplementary material S1) and therefore high removal in real wastewater experiments
355 should be attributed to other processes in the non-sterile reactor such synergic
356 biodegradation pathways by WRF with other microorganisms (bacteria), which are

357 present in the hospital effluent. The elimination of Ifosfamide observed in bioreactor
358 experiments only began after 5 days, although it was not completely removed.

359

360 In the case of Tamoxifen, it showed an elimination of 92% and 99 % after 1 hour and 9
361 days respectively in the experiments performed with synthetic wastewater spiked at 0.3
362 mg L^{-1} and treated with *T. versicolor* (figure 3 and S3). Most of the removal observed
363 can be attributed to sorption processes since the heat-killed experiment showed 83 % and
364 94 % of elimination from the water after 24 h and 9 days respectively (figure 3). In fact,
365 Tamoxifen, has a high hydrophobicity (log Kow = 6.30), and is quite prone to be sorbed
366 onto the fungi surface because of its physical-chemical characteristics. Nevertheless, after
367 the sorption process, biodegradation is likely taking place since two potential TPs, with
368 related chemical structure of Tamoxifen, were found in the liquid phase during the
369 experiment. In particular, two suspected hydroxylated forms of Tamoxifen (TP 388 A
370 and B) were detected after 30 min of fungal treatment and their concentration were
371 increasing along the experiment while Tamoxifen was at very low concentration in the
372 liquid phase (figure 4 and S3). These hydroxylated compounds were not detected in the
373 heat-killed controls. A possible degradation pathway would involve the intracellular
374 transformation of Tamoxifen by the cytochrome P-450 system, which typically yields
375 hydroxylated metabolites as the products, and further excretion to the liquid medium.

376 In order to identify the molecular ions of these TPs, to propose their empirical formula
377 and to elucidate their chemical structure, data-dependent experiments combining full-
378 scan MS data (at 60.000 units of resolutions) with the product ion spectra were acquired

379 using a quadrupole-orbitrap MS instrument. Samples diluted (1:1) from the flask
380 experiments were used for this purpose.

381 Tamoxifen has a m/z of 372.2322 sin the protonated form with a formula of $C_{26}H_{30}ON$.
382 The hydroxylated form of Tamoxifen which is proposed here has the molecular formula
383 of $C_{26}H_{30}O_2N$ (the hydroxylation has an increment of 16 Da) for the protonated form with
384 a m/z of 388.2271 as theoretic mass. As it is shown in figure 5a, two hydroxylated forms
385 are suspected to be present in during the experiment, peak called "A" (RT=2.96min)
386 corresponds with the m/z 388.2268 (TP 388 A) and the peak called "B" (RT=3.08 min)
387 corresponds with the m/z 388.2272 (TP 388 B). The molecular formula for both peaks
388 where proposed based on the calculation of the elemental composition for peak A (m/z
389 388.2269) and peak B (m/z 388.2272). Accurate molecular masses of peak A and B were
390 compared with the theoretical mass (m/z 388.2272) of hidroxytamoxifen and a difference
391 lower than 5 ppm (-0.45 and 0.24 ppm respectively) were found.

392 The next step for the identification of these potential TPs was the evaluation of the
393 MS/MS spectra where the fragmentation profile for both peaks was quite similar.

394 The mass fragments obtained for the TP 388 A were 325, 247 and 205 (figure 5b). The
395 fragment 325 correspond to the loss of the amine side chain (45 Da) through the fragment
396 343 which immediately loss H_2O (18 Da), showing a total loss of 63 Da. The fragment
397 247 correspond to a loss of benzene unit (78 Da) from the fragment 325 with a total loss
398 of 141 Da and finally the fragment 205 correspond with a loss of 42 Da (ketone HC-
399 HC=O) (view supplementary material, S 2). In case of TP 388 B, the mass fragments
400 obtained were, as in the case of TP 388 A, 325, 247 and 205 (figure 5b), but also

401 fragment 370 was detected. This last fragment might be formed due to the different
402 position of the hydroxyl group (-OH) in the molecule proposed for the TP 388 B. Based
403 on the different fragmentation spectra of the TPs, two positional isomers (position of
404 hydroxyl group; -OH) were proposed (figure 5b). In the case of TP388B the suggested
405 position of the –OH may be that place where it is conjugated with the olefin and the two
406 aromatic rings of the molecule (figure 5b). This means that a loss of H_2O (18 Da) may be
407 possible because of the charge generated onto the molecule in this position is stabilized
408 by resonance with olefin and the aromatic rings whose can delocalized this charge.
409 Therefore the fragment of 370 is generated by a loss of 18 Da but only in case of TP 388
410 B. On the contrary, the suggested position of hydroxyl in the TP 388 A is not conjugated
411 with the olefin, and it can not be possible to generate this additional stability which
412 allows the observation of the fragment 370 (see supplementary material S2). Thereby the
413 profile of both MS/MS spectra confirms the presence of two positional isomers with the
414 protonated mass of 388.2271 m/z. The mass fragments obtained in the MS/MS spectra
415 were supported by the same findings obtained using the software Mass Frontier (Thermo
416 Science) (figure S2) which allows simulating the potential mass fragments in the working
417 conditions (ionization source, mode, etc) for the molecule proposed.

418 Based on a semi-quantification approach (using the chromatographic areas)(Rubirola et
419 al., 2014), relative removal percentage values of tamoxifen removal (0.72% at the end)
420 and relative formation percentage of TPs formation (nothing at initial and TP 388 A:
421 14.62 % and TP 388 B: 10.70 % at the end) were calculated. It might be concluded that,
422 at the end of the flask experiments, 25-30 % of the total removal of the target compound
423 is due to the TPs formation (supplementary material S3).

424 In the time-course of the flask experiments, the activity of laccase was measured reaching
425 different values for the three tested compounds. Laccase was around 100 U L^{-1} for
426 Cyclophosphamide and Ifosfamide while it reached values up to 350 U L^{-1} for the
427 Tamoxifen. Both Cyclophosphamide and Ifosfamide are perhaps affecting the enzymatic
428 system of the WRF but not killing the microorganism since the glucose is almost
429 consumed at the end of the experiment for three target compounds. Laccase can play an
430 important role in the biodegradation of different pharmaceuticals such as analgesics
431 (diclofenac and naproxen)(Marco-Urrea et al., 2009; Marco-Urrea et al., 2010) or
432 endocrine disruptors (Jonsson, 1990; Catapane et al., 2013), but here we cannot establish
433 a direct correlation between the degradability of the tested anticancer drugs with this
434 enzyme.

435

436 Finally, the Tamoxifen TPs found at flask experiments were screened in the real hospital
437 samples treated with WRF at the bioreactor. Cytotoxic drugs are found in hospital
438 effluents at low concentrations (table 1) and therefore, previous to the MS analysis, SPE
439 preconcentration of these samples was performed following the methodology described at
440 Ferrando-Climent et al. 2013. The SPE extracts were analyzed employing the same non-
441 target approach mentioned in this section. However it was not possible to detect any of
442 these TPs in the real samples maybe due to the low concentration of Tamoxifen. Taking
443 into account its initial concentration in hospital wastewater (970 and 45 ng L^{-1}) and the
444 relative concentrations of its TPs at flask experiments (10-14 %), the levels of TPs that
445 might be present in these effluents were probably close to limit of detection.
446 Unfortunately, it is not possible to assess the efficiency of the SPE process for the

447 preconcentration of TPs recoveries because of the absence of reference standards to
448 perform recovery studies.

449 **3.3. Toxicity evaluation**

450 The bioassay with bacteria *V. fischeri* (Microtox test) was performed in order to evaluate
451 the feasibility of the treatment as well as the potential toxicity of the TPs generated along
452 the process with WRF. Microtox results can only refer to the total toxicity of the liquid
453 medium contained in the flasks during the batch experiment, without distinguishing
454 individual toxicity of each compound present in the sample. Despite of this, it provides
455 useful information about the toxicity of the process comparing the initial samples, the
456 controls and the liquid medium at the end of the experiment.

457 Abiotic controls of Cyclophosphamide and Ifosfamide as well as the samples taken at
458 initial time and at the end of the flask experiments with *T. versicolor*, showed a EC_{50} (15
459 min) below than 5%. These compounds are intrinsically cytotoxics consequently the
460 value of the EC_{50} exhibited a high toxicity not only at the beginning but also at the end of
461 the experiment where these compounds remained inalterable.

462 In the case of Tamoxifen, abiotic controls and the samples taken during the flask
463 experiment with *T. versicolor* showed a EC_{50} (15 min) around 43%, which means that
464 this compound and also their transformation products have no toxicity for the bacteria *V.*
465 *fischeri* at the concentrations where these experiments were performed. However, it could
466 be interesting in further work, to study other type of effects such endocrine disruptor
467 effects which is more related with the pharmaceutical activity of this anticancer drug
468 (Williams et al., 2007).

469

470 **4. Conclusions**

471 Most of the tested anticancer drugs were removed form hospital wastewater using *T.*
472 *versicolor* in comparison with the conventional biological treatments that are inefficiently
473 eliminating these compounds. This biological treatment has shown not to be useful for
474 the removal of Cyclophosphamide and Ifosfamide which might require the combination
475 of different species of microorganism (bacteria, fungi, etc) or more specific biological
476 systems such other fungi species able to degrade this type of chemical structures.
477 Conversely, Tamoxifen showed a total removal from the wastewater (by combined
478 sorption-biodegradation processes) where two hydroxylated positional isomers were
479 identified as TPs derived from biodegradation of Tamoxifen through the WRF activity.

480

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492

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Table
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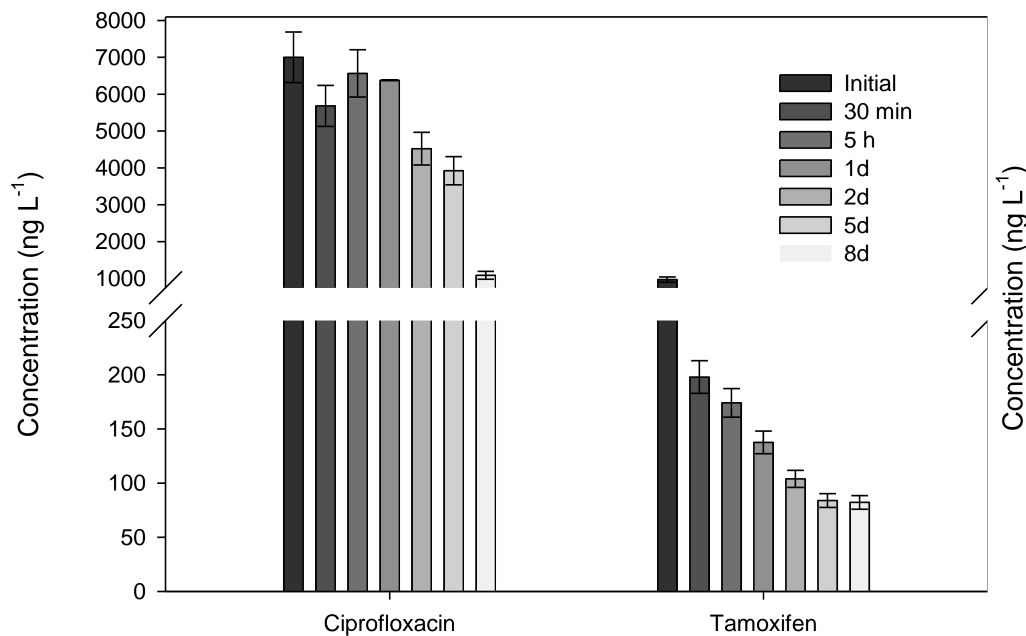
Table 1. Method detection limits (MDL), initial concentration in hospital effluents and removal of anticancer drugs after the batch bioreactor treatment in sterile (sample1) and non sterile (sample 2) conditions (blq= below limit of quantification, ND= non detected)

Type of agent	Compound	MDL (ng L^{-1})	Sample 1 (Sterilized wastewater)		Sample 2 (Non-sterilized wastewater)	
			Concentration (ng L^{-1})	Removal (%)	Concentration (ng L^{-1})	Removal (%)
Cytotoxic quinolone	Ciprofloxacin	0.6	7000 ± 686	84	2179 ± 214	97
Antiestrogenic	Tamoxifen	1.0	970 ± 74	91	45 ± 3	48
Alquilant agent	Ifosfamide	1.7	N.D.	-	77 ± 6	61
	Cyclophosphamide	1.3	N.D.	-	-	-
	Vincristine	9.2	N.D.	-	-	-
Plant alkaloid (antimicrotubule agent)	Docetaxel	0.7	N.D.	-	-	-
	Paclitaxel	5.5	N.D.	-	-	-
Plant alkaloid (topoisomerase II inhibitor)	Etoposide	48	blq	100	198 ± 12	100
Anti-metabolites	Methotrexate	2.1	N.D.	-	-	-
	Azathioprine	3.8	N.D.	-	55 ± 2	100

Figure



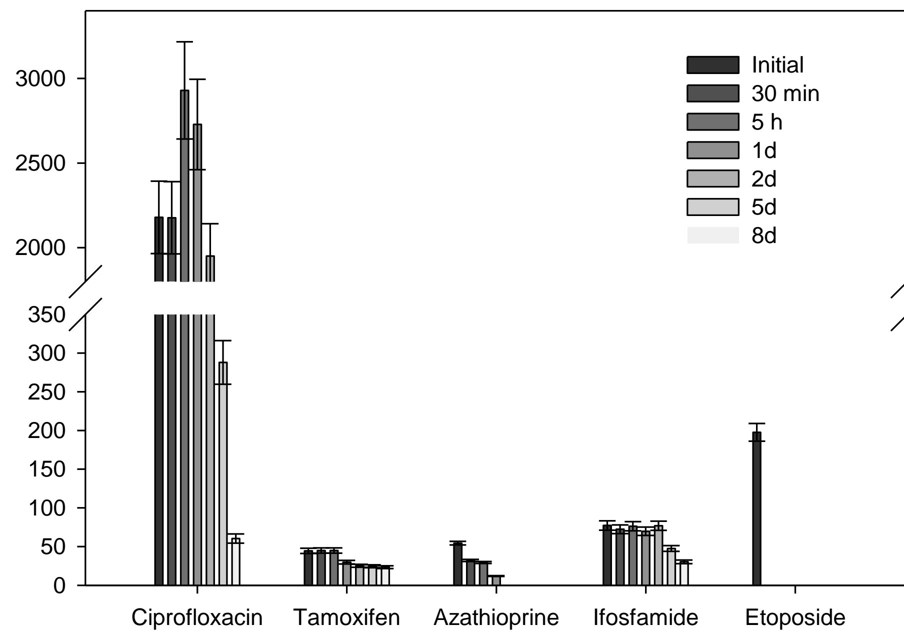


Figure 1. Degradation of anti-cancer drugs present in both hospital wastewater sterile (samples 1) and non-sterile (sample 2) by *T. versicolor* batch fluidized bed reactor.

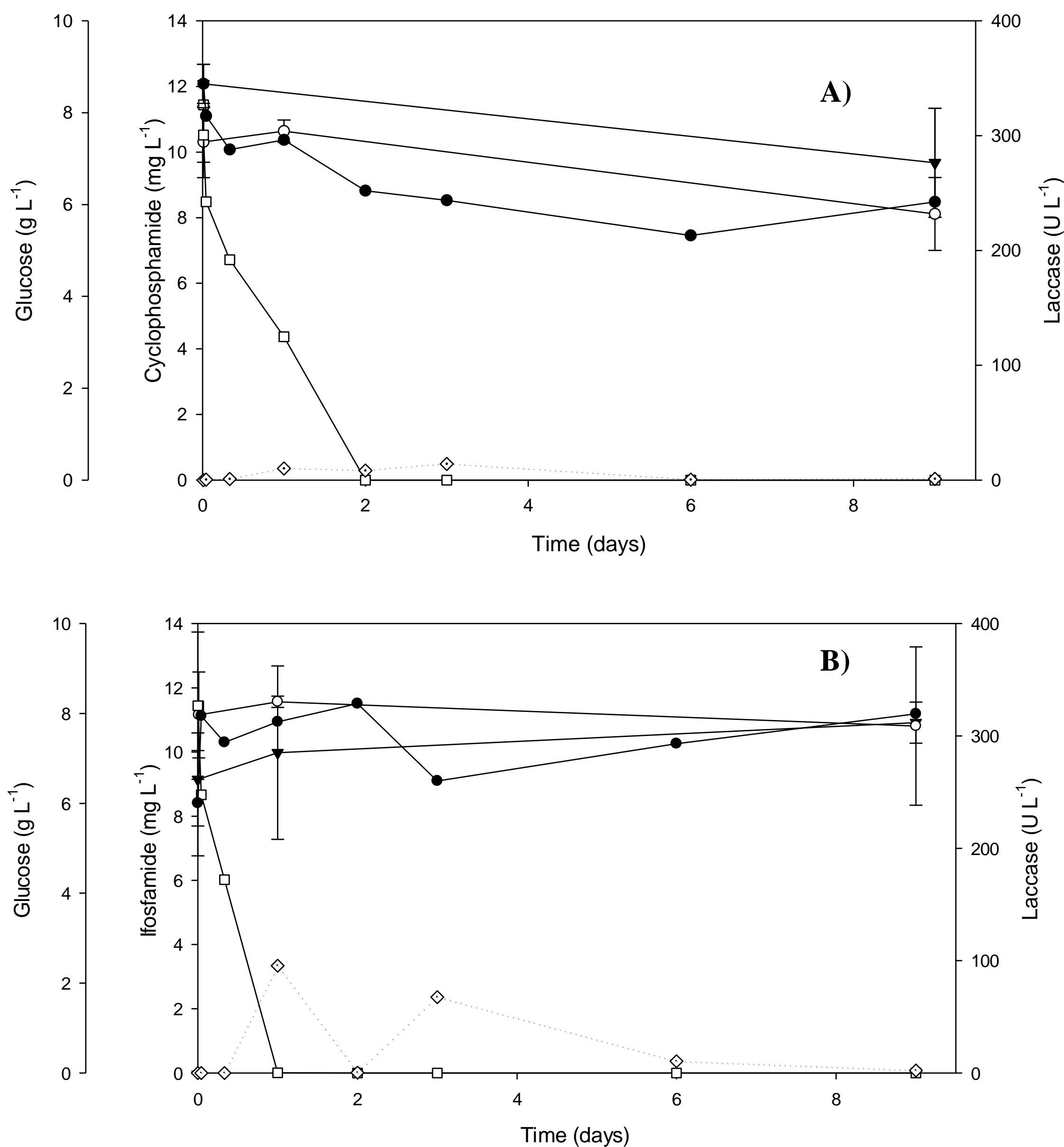


Figure 2. A) Time-course of Cyclophosphamide (CY) degradation spiked at 10 mg L^{-1} by *Trametes versicolor* pellets in Erlenmeyer flask. Uninoculated control (▼), heat-killed experiment (○), inoculated experiment (●). Levels of Glucose (□) and laccase activity (◇). B) Time-course of Ifosfamide (IF) degradation spiked at 10 mg L^{-1} by *Trametes versicolor* pellets in Erlenmeyer flask. Symbology: uninoculated controls (▼) , heat-killed (○), Glucose (□), laccase activity (◇), and experimental cultures (●) .

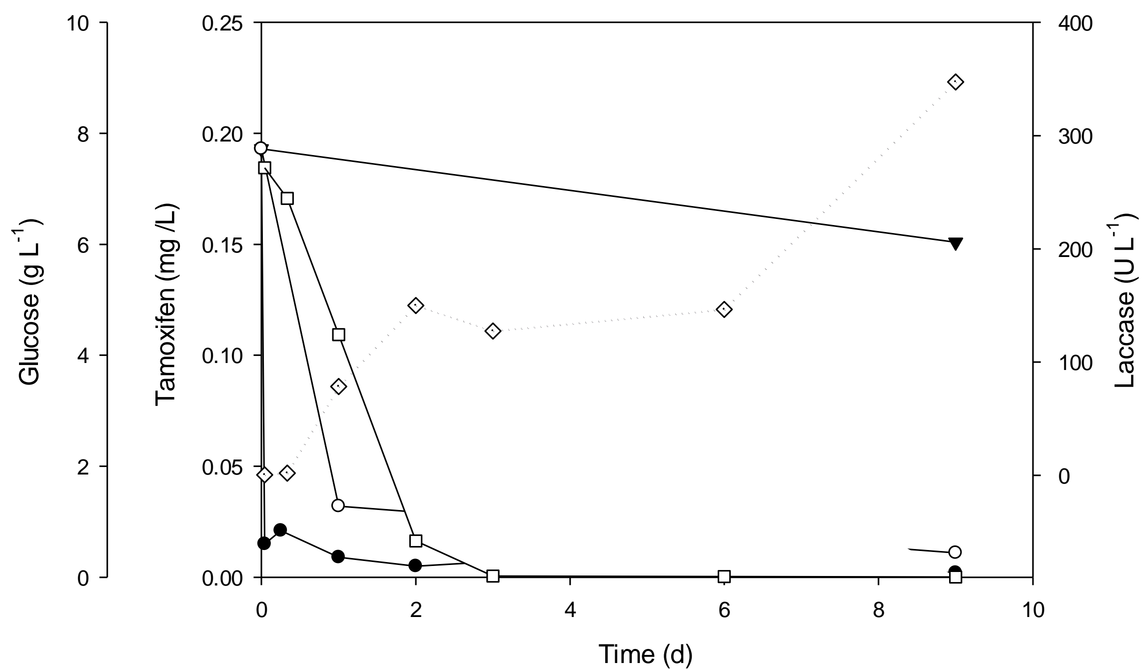
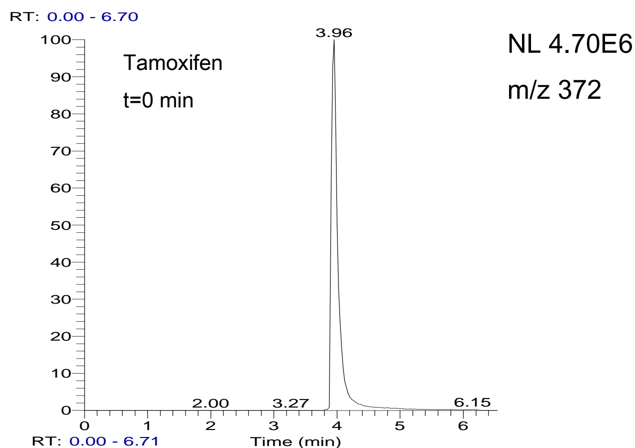
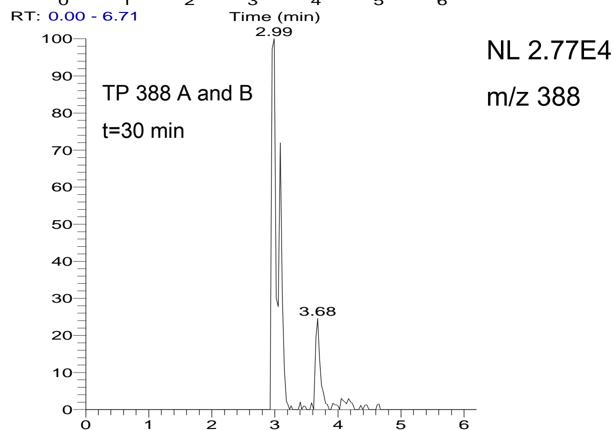
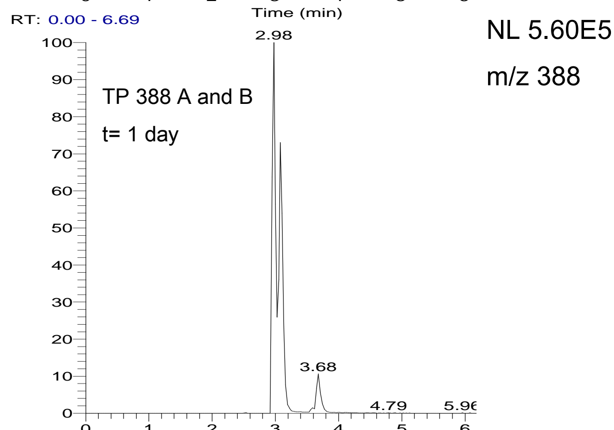


Figure 3. Time-course of Tamoxifen (TAM) degradation spiked at 0.3 mg L^{-1} by *Trametes versicolor* pellets in Erlenmeyer flask. Symbology: uninoculated controls (▼) , heat-killed (○), Glucose (□), laccase activity (◇), and experimental cultures (●).







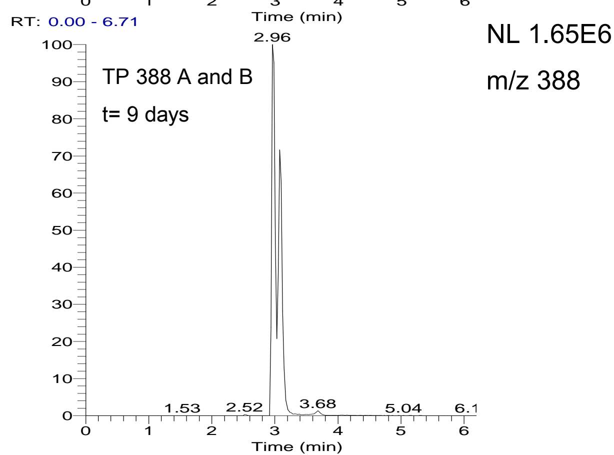
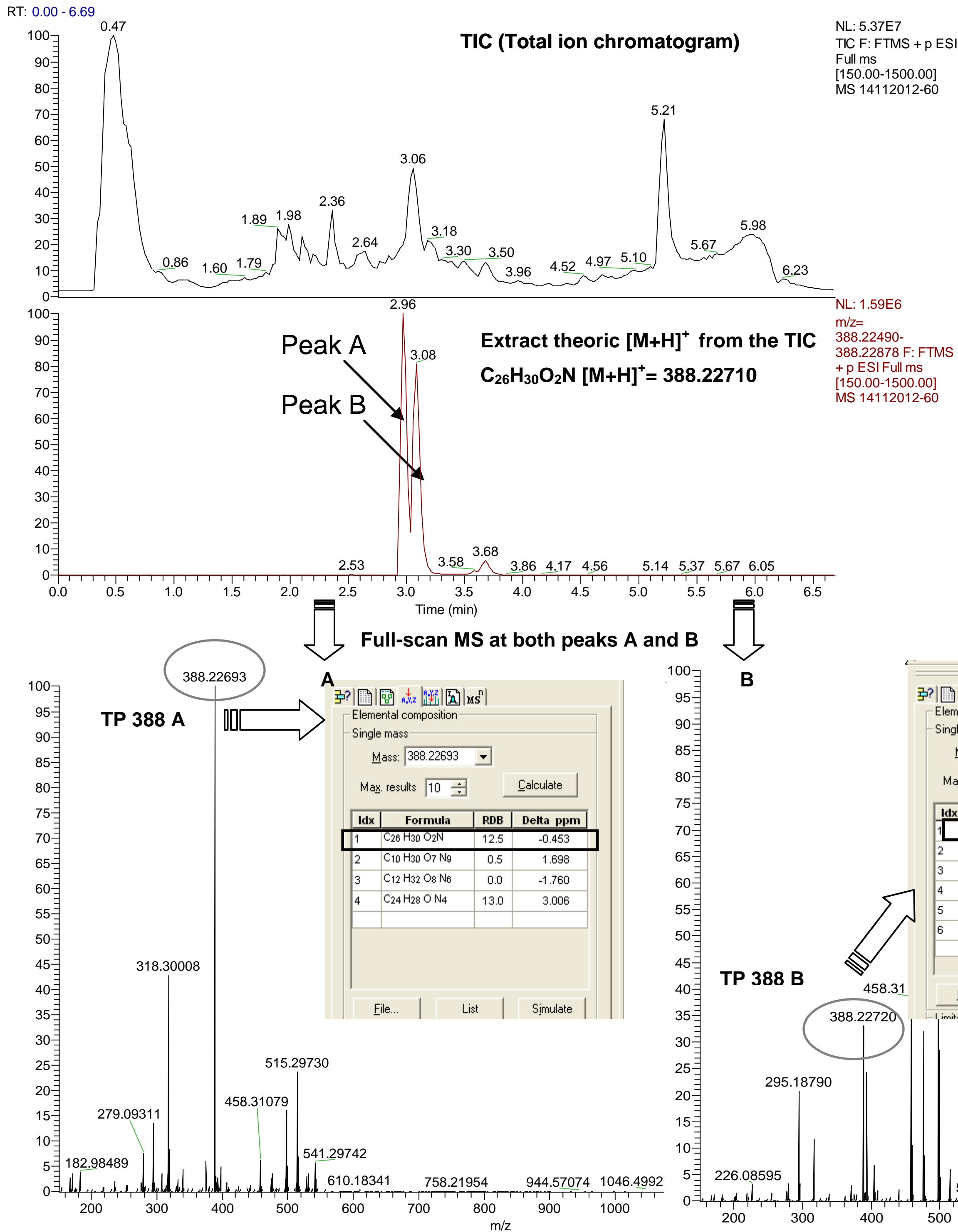


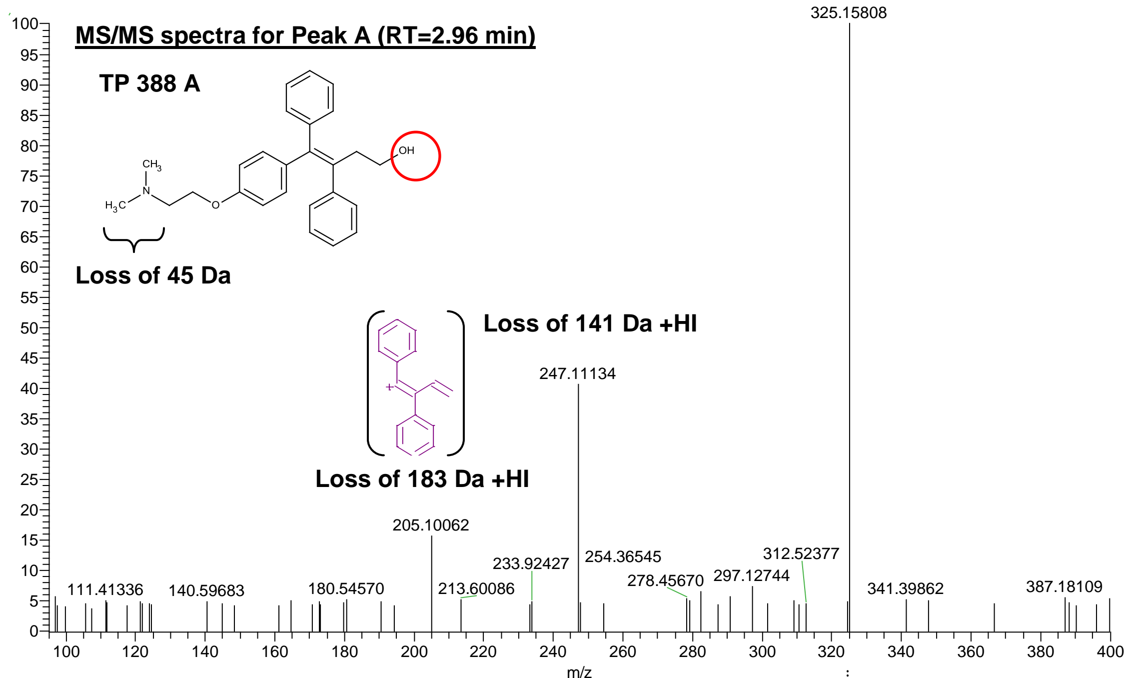
Figure 4. Extract ion chromatograms for the tamoxifen at initial conditions and the two suspected transformation products (TP 388 A and B) which have the theoretical m/z of 388.22710 during the culture experiment (t=0; t=30 min, t=1day and t= 9 days).



Idx	Formula	RDB	Delta ppm
1	$C_{26}H_{30}O_2N$	12.5	-0.453
2	$C_{10}H_{30}O_7N_9$	0.5	1.698
3	$C_{12}H_{32}O_8N_6$	0.0	-1.760
4	$C_{24}H_{28}ON_4$	13.0	3.006

Idx	Formula	RDB	Delta ppm
1	$C_{26}H_{30}O_2N$	12.5	0.243
2	$C_{12}H_{32}O_8N_6$	0.0	-1.065
3	$C_{10}H_{30}O_7N_9$	0.5	2.394
4	$C_{24}H_{28}ON_4$	13.0	3.701
5	$C_{13}H_{28}O_4N_{10}$	5.0	-4.510
6	$C_{14}H_{34}O_9N_3$	-0.5	-4.523

Figure 5a. Identification of TP 388 A (Peak A) and 388 B (Peak B). Extraction of the exact masses $[M+H]^+$ from the TIC, evaluation of full-scan MS at both peaks (A and B) and calculation of the elemental composition at 388.2269 m/z (A)/388.2272 m/z (B) and 388.2271 m/z (theoretical) where the difference between the measured and theoretical exact mass has to be lower than 5 ppm.



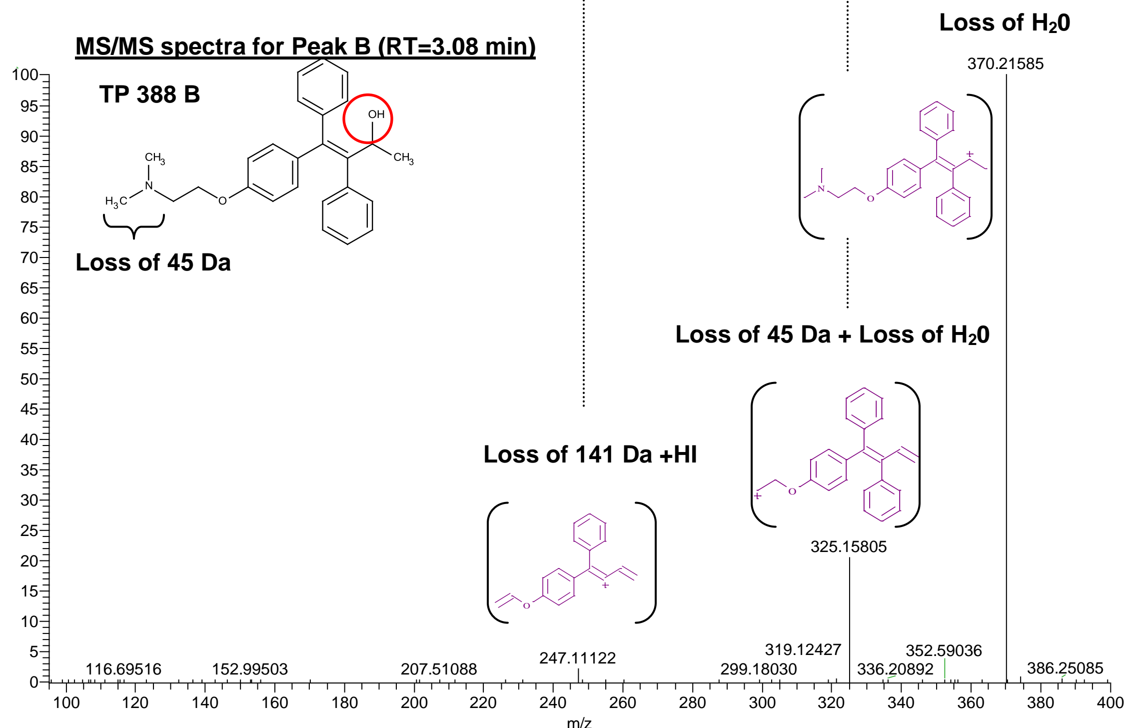


Figure 5b. Evaluation of the MS/MS spectra for the transformation products proposed: TP 388 A (Peak A) and 388 B (Peak B). Both TPs have the same mass $[M+H]^+$ but they shown differences in the abundance and presence of some the fragments due to the different position on the group hydroxyl which is affecting the mechanism of fragmentation.

Supplementary Material
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