Removal of estrogenic compounds from filtered secondary wastewater effluent in a continuous enzymatic membrane reactor. Identification of biotransformation products

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ABSTRACT

In the present study, a novel and efficient technology based on the use of an oxidative enzyme was developed to perform the continuous removal of estrogenic compounds from polluted wastewaters. A 2-L enzymatic membrane reactor (EMR) was successfully operated for 100 h with minimal requirements of laccase for the transformation of estrone, 17β-estradiol and 17α-ethinylestradiol from both buffer solution and real wastewater (filtered secondary effluent). When the experiments were performed at high and low concentrations of the target compounds, 4 mg/L and 100 μg/L, not only high removal yields (80-100%) but also outstanding reduction of estrogenicity (about 84-95%) were attained. When the EMR was applied for the treatment of municipal wastewaters with real environmental concentrations of the different compounds (0.29-1.52 ng/L), excellent results were also achieved, indicating the high efficiency and potential of the enzymatic reactor system. A second goal of this study relied on the identification of the transformation products to elucidate the catalytic mechanism of estrogens transformation by laccase. The formation of dimers and trimers of E1, E2 and EE2, as well as the decomposition of E2 into E1 by laccase-catalyzed treatment has been demonstrated by liquid chromatography atmospheric pressure chemical ionization (LC-APCI) analysis and confirmed by determination of accurate masses through liquid chromatography electrospray time-of-flight mass spectrometry (LC-ESI-TOF). Dimeric products of E2 and EE2 were found even when operating at environmental concentrations. Moreover, the reaction pathways of laccase-catalyzed transformation of E2 were proposed.

Keywords: Laccase; Bioremediation; Continuous bioreactor; Wastewaters; Transformation products

INTRODUCTION

Over the past decades public concern about the environmental impact of steroid estrogens has grown due to their negative effects associated to the disruption of the endocrine system in humans and animals.[1,2] The presence of this type of compounds in the environment has been mainly attributed to their incomplete removal by conventional biological and physicochemical processes in wastewaters treatment plants (WWTP),[1] being the natural compounds estrone (E1) and 17β-estradiol (E2) and the synthetic compound 17α-ethinylestradiol (EE2) the major contributors to the estrogenic activity detected in the WWTP effluents.[3] Therefore, recent works have been focused on the development of advanced oxidation processes such as ozonation and chlorination. These alternatives provide satisfactory removal yields although they present important disadvantages such as high costs and formation of by-products with unknown estrogenicity, which may be even higher than that of the original compound.[4-6] Hence, an effective method is required to fulfill the challenge of their removal.

In this way, the enzymatic treatment is a potential alternative for the removal of estrogens due to its low energy requirements and easy control and operation.[7] Indeed, several previous researchers have studied the transformation of E1, E2 and EE2 by ligninolytic enzymes, being laccase (EC 1.10.3.2, benzenodiol: oxygen oxidoreductase) the most widely applied biocatalyst due to its potential ability to degrade recalcitrant compounds and its simple catalytic mechanism using oxygen as a final electron acceptor.[8] Previous works reported significant removal yields of both the target compounds and their estrogenic activities.[1,4,7,9-14] Nevertheless, most of those experiments were conducted at relatively high concentrations in synthetic media, while only a few investigations assessed the removal of estrogens in real wastewaters.[1] Furthermore, research has been focused in the operation in batch mode, whilst relative low effort has been devoted for its implementation in a continuous process. Thus, the

71 design and operation of a novel technology for the continuous application of enzymatic
72 remediation under more realistic conditions is still a pending objective.
73 We have previously developed a lab-scale enzymatic membrane reactor (EMR) based on a
74 continuous stirred tank reactor coupled to an ultrafiltration membrane for the continuous
75 elimination of E1 and E2 at high initial concentrations from a buffer solution by high doses of
76 laccase obtaining promising results.[6] Besides, this technology presents several advantages
77 such as reuse of the biocatalyst, easy addition of fresh enzyme in case of deactivation, high
78 flow rates, reduced energy requirements, simple operation and control and straightforward
79 scale-up.[15,16] Thus, this enzymatic reactor can be particularly useful when performing the
80 continuous treatment of real wastewaters by laccase at larger-scale (2-L EMR) and much
81 lower initial concentrations of estrogens and enzyme. Indeed, integrate systems including
82 filtration are considered nowadays one of the most promising technologies used for the
83 advanced treatment of secondary effluents, being both micro and ultrafiltration membranes
84 techniques widely used as tertiary treatments.[17-19]
85 Furthermore, an evident lack of knowledge exists with regard to the identification of laccase-
86 catalyzed transformation products of estrogens, in spite of being this enzyme so extensively
87 tested over the past years. The elimination of estrogens has been assumed to occur by means
88 of polymerization reactions.[1,13,14] However, only few works supported this hypothesis by
89 means of the corresponding experimental assays and analytical techniques: Nicotra et al. and
90 Tanaka et al. reported the formation of dimers of E2 and EE2, respectively;[11,12] whilst no
91 other degradation products were identified and by-products resulting from laccase-catalyzed
92 oxidation of E1 have not been characterized up to the date.
93 To sum up, the key objective of this research was to establish and operate an efficient laccase-
94 based bioreactor for the continuous removal of estrogenic compounds present in real
95 wastewaters. The EMR was applied for the treatment of both buffer solution and real

wastewater (filtered secondary effluent) at high and low as well as at environmental conditions. A second goal relied on the identification of transformation products and the proposal of reaction pathways to elucidate the catalytic mechanism of estrogens transformation by laccase.

EXPERIMENTAL SECTION

Chemicals

All chemicals were of analytical grade. Estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol, were obtained from Sigma-Aldrich (USA). Deuterated 17β-estradiol (E2-d_4), deuterium was introduced in positions 2, 4 and 16, was purchased from Cambridge Isotope Laboratories (USA). 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was supplied from Fluka (USA). Stock solutions of E1, E2, EE2 and E2-d4 were prepared in methanol (J.T. Baker, HPLC grade, 99.8%). Commercial laccase from *Myceliophthora thermophila* (56 kDa) was supplied by Novozymes (Denmark).

Reactor design and Experimental procedure

A continuous enzymatic membrane reactor (EMR) was operated for the enzymatic removal of natural (E1 and E2) and synthetic (EE2) estrogens. The system was based on a previous design and consisted of a tank reactor coupled to an ultrafiltration polyethersulfone membrane (Prep/Scale-TFF Millipore) with a nominal molecular weight cutoff of 10 kDa.[6] The influent containing the mixture of the estrogens was continuously fed into the tank by a peristaltic pump, while the laccase was added in a single initial pulse. An additional pump was used to circulate the reaction solution from the tank to the membrane, where the enzyme was retained and continuously recycled to the reactor at a recycling:feed flow ratio 12:1. A valve located in the membrane module permitted the control of both effluent and recycling flow rates. PTFE

121 tubing was used to avoid the adsorption of the target compounds to the inner surface of the
122 tubing.
123 Once the feasibility of a lab-scale reactor of 250 mL of volumen has been previously
124 demonstrated,[6] a larger-scale bioreactor (2 L) was used in the current work (Biostat® MD, B.
125 Braun-Biotech International), which was equipped with pH, temperature and pO_2 sensors, as
126 well as with mechanical stirring. An electrovalve located at the end of a flexible membrane
127 tube controlled by a cyclic timer was used to inject oxygen with a pulsing flow of 1 bar for 30
128 s each 30 min of operation time. Temperature was maintained at 26ºC by circulating
129 thermostated water through the reactor chamber and the reaction mixture was continuously
130 stirred at 250 rpm. A scheme of the bioreactor is shown in Figure S1 (Supporting
131 Information).
132 The hydraulic residence time (HRT) was 4 h, which meant an inlet flow rate of 8.3 mL/min.
133 Besides, an initial laccase activity of 100 U/L was assayed. The reactor was operated for 100
134 h in order to demonstrate the viability of the technology and the stability of both the
135 membrane and the biocatalyst. Samples were withdrawn at different periods from the reaction
136 vessel in order to measure laccase activity, as well as from the effluent to determine both the
137 estrogens concentration and the estrogenic activity. The performed experiments and the
138 corresponding operational conditions are presented in Table 1.
139 The performance of the technology was evaluated in terms of the removal percentage (%) of
140 both target compounds and estrogenicity, as well as by the estrogens removal rates (mg, μg or
141 ng degraded per volume of reactor and time). Corresponding controls lacking laccase were
142 performed under the conditions examined in each removal experiment.
143 The real wastewater used was collected from the outlet of the secondary clarifier of the
144 municipal wastewater treatment plant of Calo-Milladoiro (Ames, Spain). This water was
145 filtered (0.45 μm) to remove particulate matter and suspended solids which could contain

146 bacteria and other microorganisms, to avoid undefined biological transformation or even
147 adsorption of the target compounds, and thus examining the sole role of the laccase, and then
148 it was stored at 4ºC until its use. The wastewater was analyzed according to Standard
149 Methods[20] and the main characteristics are summarized in Table S1.
150
151 **Enzymatic activity**
152 Laccase activity was determined by measuring the oxidation of 5 mM ABTS to its cation
153 radical ($ABTS^{+}$) at 436 nm in 100 mM sodium acetate buffer, pH 5 and 30 ºC (ε_{436} =
154 29,300$M^{-1}\cdot cm^{-1}$) using a Shimadzu UV-1603 spectrophotometer. One unit (U) of activity was
155 defined as the amount of enzyme forming 1 μmol of $ABTS^{+}$ per min.
156
157 **Evaluation of estrogenic activity**
158 The estrogenic activities of the inlet and outlet streams of the reactor were measured by LYES
159 (lyticase yeast estrogen screen assay) using recombinant yeast *Saccharomyces cerevisae*. This
160 method has been adapted from one developed by Schultis and Metzger and described
161 elsewhere.[6,21]
162
163 **Estrogens analysis**
164 All the analytical methods applied are detailed in Supporting Information.
165 The determination of the estrogens concentrations in the Experiment 1 (high initial estrogens
166 concentration in buffered solution) was carried out by high performance liquid
167 chromatography (HPLC) using a diode array detector.
168 The samples withdrawn during the Experiments 2 and 3 (low initial concentrations in buffer
169 solution or real wastewater) were analyzed by gas chromatography-mass spectrometry (GC-
170 MS). First, samples of 20 mL were acidified at pH 2, diluted in distilled water (pH 2 adjusted

with HCl) until a final volume of 100 mL and filtered (0.45 μm). Then, the solid phase extraction (SPE) was carried out with 60 mg OASIS HLB cartridges (Water closet, USA) previously conditioned with 3 mL of ethyl acetate, 3 mL of methanol and 3 mL of distilled water (pH 2). The cartridges were then dried with nitrogen for 45 min and eluted with 3 mL of ethyl acetate. BSTFA (N,O-bis(trimethylsisyl)trifluoroacetamide) was added for the derivatization of the species. Afterwards, the resulting samples were analyzed by GC-MS.

The concentrations of estrogens present in the real wastewater samples used as well as in the samples withdrawn during the Experiment 4 (environmental concentrations) were determined by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS-MS). Aiming to deeply concentrate the samples, volumes of 2 L (wastewater samples) or 5 L (samples after laccase-catalyzed treatment) were collected for their subsequent extraction and concentration to 3 mL by SPE as described above. Then, 2 mL of the resulting samples were evaporated under gentle stream of nitrogen prior to resuspension in methanol; finally, further concentration by nitrogen was conducted until obtaining 50 μL-samples. Concentrations of estrogens quantified in these samples were corroborated by using E2-d_4 as internal standard aiming to correct for matrix effects, as indicated in Supporting Information.

Identification of laccase-catalyzed reaction products

With the objective of identifying the oxidation products and establishing the transformation pathways, experiments were conducted in batch reactors of 250 mL. Each reactor contained 100 mL of 5 mg/L of each estrogen in distilled water. An initial laccase activity of 2,000 U/L was used aiming to ensure the degradation of the compounds and the consequent formation of reaction products at significant concentrations. Each estrogen was assessed separately in order

to identify the corresponding transformation products. The reaction was conducted for 24 h and then the mixture was acidified to a final pH of 2 to inactivate the enzyme.

These acidified samples as well as acidified samples corresponding to time 0 (before the enzyme addition and analogous to samples after 24 h of controls lacking enzyme) and a blank with enzyme (after 24 of incubation in buffer solution) were analyzed by the LC-APCI method used for the quantification of estrogens. However, for this study the mass spectrometer was used in the full scan mode rather than in the tandem MS-MS mode, and the range selected for the mass spectrometer full scan was between m/z ratios of 50 and 900.

Furthermore, samples were analyzed by liquid chromatography electrospray time-of-flight mass spectrometry (LC-ESI-TOF) in negative mode by the method detailed in Supporting Information, aiming to determine accurate masses of the biotransformation products to confirm their proposed structures.

RESULTS AND DISCUSSION

Continuous removal of estrogens in an enzymatic membrane reactor

A 2-L EMR was proposed for the continuous transformation of estrogens by free laccase, once the feasibility of a similar 250-mL bioreactor for the removal of E1 and E2 by 500 U/L of laccase had been previously reported.[6] In the present work, the removal of not only these natural compounds but also a synthetic estrogen (EE2) was attempted, and initial laccase activity was decreased to 100 U/L to reduce the enzyme requirements. The experimental conditions of the different performed assays are detailed in Table 1.

First, the EMR was fed with a buffer solution (phosphate buffer solution, pH 7) containing high estrogens concentration (4 mg/L each) (Experiment 1) to study the effect of the change of scale and the reduction of laccase activity. Removal percentages of 80, 87 and 85% of E1, E2 and EE2, respectively, were attained under steady-state conditions, which implied

degradation rates in the range of 0.80-0.87 mg/(L·h), as shown in Figure S2. The use of a much lower laccase activity slightly decreased the removal yields by 15%: degradation percentages between 96 and 98% were previously found using 500 U/L.[6] Moreover, no biocatalyst inactivation was detected over the 100 h of operation and the estrogenicity was reduced by 84%. Results of the corresponding control assay verified that removal of the target compounds was caused only by laccase action: the average of the estrogens outlet rate (determined as the concentration in the effluent per residence time) matched with the feed addition rate, as also occurred in the following experiments.

Experiment 2 was conducted with 100 µg/L (each) of the estrogens in buffer solution as an attempt to work at lower concentrations and closer to environmental levels. The results evidenced the capability of the enzymatic technology to remove these pollutants even at such low concentration, obtaining higher efficiencies than in the previous experiment: E1 was eliminated by 88% (removal rate 22 µg/(L·h)) and E2 and EE2 were not detected (below detection limits), which means removal percentages up to 99 and 94%, respectively (Figure S3). Moreover, a reduction of 95% of estrogenic activity was found and the biocatalyst retained its total initial activity after 100 h.

With the goal of studying the matrix effect to investigate the possibility of the real implementation of this technology, Experiment 3 was performed with real wastewater (filtered secondary effluent) previously spiked with 100 µg/L of the estrogens. The results of the variables monitored during the operation are shown in Figure 1. As it can be observed, despite a partial inactivation of laccase (20%) during the first hours, probably due to the constituents of the wastewater, an activity close to 80 U/L was constantly maintained after 100 h of operation until the bioreactor was stopped. Furthermore, high removal percentages of the estrogens were achieved: 80-92%, which implies degradation rates of 20-23 µg/(L·h), under steady-state conditions. This loss of removal efficiencies observed when comparing

245 both assays with buffer solution and real wastewater may be caused by the slight biocatalyst
246 inactivation and/or by the presence of other compounds which could compete with the target
247 compounds for the enzyme. As expected, the lower oxidation yields led to diminished
248 estrogenicity reduction, although still a significant decrease was detected (90%).
249 Consequently, the potential detrimental constituents of the real matrix (colloidal particles,
250 organic matter, nitrogen-based compounds, etc.) exerted low impact on the removal yields
251 and on the enzyme activity. Promising performance, stability and catalytic efficiency of this
252 technology were demonstrated, evidencing its noticeable applicability on the treatment of real
253 wastewaters. Indeed, although composite fouling (i.e., a combination of biofouling and
254 inorganic fouling) often occurs in membrane systems because of the nature of wastewaters,[22]
255 neither fouling nor changes in the membrane pressure (1-1.5 bar) was detected here.
256 In Experiment 4, the capability of the system to remove the estrogens from wastewaters at
257 real environmental concentrations was assessed. In this case, the bioreactor was fed with real
258 non-spiked municipal wastewater containing the estrogens at real environmental
259 concentrations of 0.29-1.52 ng/L (Table 1). Under these conditions, E1 was removed by 98%
260 (degradation rate 0.37 ng/(L·h)), while E2 and EE2 were not detected in the effluent, which
261 corresponds to oxidation percentages up to 97 and 99%, respectively (removal rates 0.07 and
262 0.18 ng/(L·h)). Thus, excellent results were found in spite of the possible presence of
263 compounds which also may act as laccase substrates, such as phenols.
264 Few authors reported the oxidation of E2 under abiotic conditions by various mechanisms,
265 such as by manganese oxides or through nitration in the presence of high nitrate
266 concentrations.[23,24] Also, Marfil-Vega et al. demonstrated the abiotic transformation of E1, E2
267 and EE2 in the presence of model vegetable matter under and the important influence of the
268 molecular oxygen during that mechanism. [25-27] Anyhow, control assays corroborated the only

implication of the laccase on the estrogenic compounds removal even when working with real wastewaters at environmental concentrations and under oxygenated conditions.

Some previous works have been also focused on the enzymatic removal of estrogens from real wastewaters.[1,10] However, those investigations used spiked wastewaters while the capability of the laccase system to remove environmental concentration of estrogens was still an important challenge. Furthermore, those previous works only tested the efficiency of the enzymatic system in batch operation while scarce references dealing with enzymatic continuous process are available. Moreover, a relatively low initial enzyme activity was used in the current research (100 U/L) in comparison to those previously needed to attain similar results. For instance, Auriol et al. reported the total transformation of the pollutants using 20,000 U/L of laccase or alternatively 5,000 U/L in the presence of 1-hydroxybenzotriazole (HBT) as mediator.[1]

Overall, the results reported here are encouraging as they present an innovative technology to remove natural and synthetic estrogens found in sewage effluents and thus demonstrated the potential implementation of this novel technology as an alternative advanced oxidation process in conventional treatment plants. As far as we know, this is the first time that an enzymatic bioreactor was successfully developed and operated in continuous mode for the treatment of real municipal wastewaters at both high and low concentrations as well as under environmentally-realistic conditions.

Identification of biotransformation products

Although we have recently demonstrated the formation of metabolites by the laccase-catalyzed oxidation of E1 and E2 using GC-MS, the identification of these reaction products was not possible.[6] On the other hand, some authors suggested that the removal of estrogens could occur by polymerization since these compounds possess a para-substituted phenol

294 structure and the enzyme may catalyze the oxidative coupling of phenolic compounds.[7,11,12]
295 This assumption has been stated in several investigations dealing with laccase-catalyzed
296 treatment of estrogens to explain the disappearance of the target compounds and the
297 difficulties in characterizing the reaction products. Nonetheless, only few works demonstrated
298 this hypothesis by experimental assays and the appropriate analytical methods. For instance,
299 the formation of dimers of E2 by laccase action has been previously reported by Nicotra et al.
300 and Tanaka et al. reported the formation of a single dimer of EE2 by laccase-mediated
301 treatment.[11-12]
302 With the goal of verifying the estrogens radical coupling and characterizing the laccase-
303 catalyzed reaction products of E1, E2 and EE2, a further study was conducted in the present
304 work using a LC-APCI system coupled to a tandem mass spectrometer operated in the full
305 scan mode, which allowed the detection of products with high m/z ratio. A concentration of 5
306 mg/L was selected for the batch assays to favor the extent of the reaction in order to establish
307 the complete laccase-catalyzed transformation pathways.
308 All precursor ions in APCI positive were the results of a simple protonation. Moreover, in the
309 case of E2 and EE2 the analytes underwent a loss of water in the source, as reported by
310 Vanderford et al. when optimizing the analytical method to determine estrogens.[28] Thus, the
311 compounds based on E2 and EE2 were seen as [M+H-H_2O] in the first quadrupole of the
312 mass spectrometer (Q_1), and [M+H] in the case of E1. The use of the Q_1 under full scan
313 monitoring in the range m/z 50-900 revealed new peaks in total ion chromatograms (TIC) of
314 the samples treated with laccase for 24 h. Although the identification of some of the products
315 was not possible, most of them were characterized by their molecular weights (MW) as
316 follow and as indicated in Table 2.
317 In the case of E1, some new peaks were detected in the TIC corresponding to 24 h of laccase-
318 catalyzed transformation (Figure S4-A). The first peaks displayed in the TIC (no indicated by

their corresponding retention times) were also observed in the blank or time 0 samples and thus, were not considered as reaction products. Therefore, only the peaks marked in the figures are supposed to be reaction products and were identified as shown in Table 2. A new product was observed at retention time 13.7 min and showed a m/z of 539. This compound could be an E1 dimer with MW 538: mass of E1 270 x2 -2H = 538, and after protonation the molecular ion would be 539, as shown in the corresponding spectrum (Figure S4-B). Another compound with a molecular ion at m/z 269 was detected at a retention time of 8.1 min, although its identification was not possible.

Regarding E2, different new peaks were observed in the TIC after subtracting the signal of the blank and time 0 samples (Figure S5-A) and were identified as indicated in Table 2. First, the results revealed a new compound with m/z 271 at a retention time of 7.9 min. This compound was presumed to be E1 formed upon oxidation of E2, since its spectrum corresponded to that of E1 standard. It could explain the apparent lower degradation of E1 in comparison to E2 and EE2 when a mixture of the three compounds was treated by laccase in the EMR. Other authors also reported the transformation of E2 to E1 by other different treatments such as activated sludge from sewage treatment plants or nitrifying activated sludge.[29] Nevertheless, this is the first time that E1 is characterized as E2 transformation product by laccase. Furthermore, it is known that transformation of E2 into E1 is a quite unspecific oxidation which can be carried out by many bacteria.[30-32] However, although these assays were not run under sterile conditions, the corresponding controls lacking laccase discarded bacterial transformation of the target compounds.

Moreover, three new peaks were observed at retention times of 9.7, 10.9 and 12.2 min. These products were identified as dimers of E2 due to their molecular ions: dimer of E2 would have a MW of 542 (mass of E2 272 x2 -2H = 542), however the compounds are seen as [M+H-H_2O] in the Q_1, therefore the molecular ion would be 525, as appeared in the spectrum of the

E2 dimer I (Figure S5-B). Also, two trimers of E2 (MW 812) appeared at retention times 13.0 and 13.6 min and presented a molecular ion at a m/z of 795 (Figure S5-C). These results are in agreement with those reported by Mao et al., who demonstrated the formation of dimers and trimers, as well as E1, after the enzyme-mediated transformation of E2 using lignin peroxidase.[33-35]

The formation of dimers and trimers was also demonstrated by analyzing the new peaks in the TIC corresponding to EE2 (Figure S6-A). Two dimers were observed at retention times of 8.9 and 9.6 min, which have MW of 590 (mass of EE2 296 x2 -2H = 590) although presented a molecular ion of 573 (590+H-H_2O) as observed in the spectrum of the EE2 dimer I (Figure S6-B). Besides, two trimers of EE2 (MW 884) were found at 10.9 and 11.8 min of retention time and presented a molecular ion at m/z of 867 (Figure S6-C).

A new peak was also detected at a retention time of 7.7 min with a molecular ion of 295 (Table 2). Previous authors reported hydroxylation reactions from EE2 by fungi and algae, and in some cases a subsequent methoxylation of the hydroxyl derivate.[36] In this way, this compound might also correspond to a hydroxylated product of EE2: its MW would be 312 (mass of EE2 296 –H +OH = 312), and it would be seen as 295 (312+H-H_2O). However, a further study should be conducted to ensure this premise.

Afterwards, samples were analyzed by LC-ESI-TOF in order to obtain accurate masses information of the biotransformation products. Considering the chemical formula of E1, E2 and EE2: $C_{18}H_{22}O_2$, $C_{18}H_{24}O_2$ and $C_{20}H_{24}O_2$, respectively, the dimers and trimers of these target compounds would be: $C_{36}H_{42}O_4$ and $C_{54}H_{62}O_6$, $C_{36}H_{46}O_4$ and $C_{54}H_{68}O_6$, and $C_{40}H_{46}O_4$ and $C_{60}H_{68}O_6$, respectively. Once ESI was used as ionization source in negative mode, parent compounds were deprotonated and seen as M-H. As observed in Table S5, chemical formula of the detected biotransformation products matched with deprotonated dimers and trimers of the estrogens, and also E1 was detected as an E2 product. Furthermore, experimental accurate

masses were in agreement with the calculated ones with errors varying from -5.2 to 4.1 ppm: two E1 dimers were found to have accurate masses of 537.3037 and 537.3038, and E2 and EE2 dimers and trimers had masses in the ranges 541.3315-541.3334 and 811.4942-811.4954 and 589.3312-589.3330 and 883.4949-883.4963, respectively (deprotonated compounds). Also, these values fitted those found in literature.[37,38] A larger number of dimeric and trimeric compounds were detected by LC-ESI-TOF in comparison to LC-APCI analysis, probably because a higher sensitivity of the method.

The novelty of this investigation relies on the successful characterization of different dimers not only of E2 and EE2, but also of E1. Additionally, the formation of E2 and EE2 trimers as well as the transformation of E2 into E1 by laccase-catalyzed treatment was demonstrated for the first time.

The formation of dimers would be also expected after the treatment of environmental concentrations considering the findings of previous investigations.[33,38] Although the identification by LC-APCI of coupling products in the samples collected from the EMR effluent fed with secondary effluent aiming to verify that assumption was not possible, probably due to the detection limits of the instrument, dimers of E2 and EE2 were successfully identified by LC-ESI-TOF: products with accurate masses of 541.3343 and 541.3342 ($C_{36}H_{45}O_4$) and 589.3330 ($C_{40}H_{45}O_4$), respectively, were found with relative errors from -3.7 to -1.2 ppm. Although the possibility of other reaction mechanisms could not be discarded, these results indicated that laccase-catalyzed radical coupling reactions occur even at such low concentrations.

Proposed reaction pathways

As mentioned, the formation of dimers and trimers suggests the elimination via radical coupling reactions by laccase-catalyzed oxidation of the substrates to generate free radicals,

394 which may couple covalently to each other subsequently. In fact, the products followed the
395 pattern of nMW-2(n-1), where n is the number of monomers and MW the mass of the parent
396 compound. However, it is interesting to highlight that in the case of E1 and E2, other species
397 having smaller MW than the initial compounds were found, which may indicate that radical
398 coupling was not the only transformation via, but also different degradation mechanisms are
399 involved.
400 Reaction pathways were proposed for E2 since all the products detected by the applied
401 analytical methods after 24 h of laccase-catalyzed treatment of that compound were identified.
402 The suggested products structures and reactions pathways are schematized in Figure 2.
403 Regarding the oxidative radical-radical coupling, the reaction is initiated by the laccase-
404 catalyzed formation of the primary oxidation product, by abstracting one electron from the –
405 OH group of the original molecule. Thus, the free radical is formed and the unpaired electron
406 may delocalize through resonance to the respective conjugated positions (E2 radical
407 intermediates: compounds 1-3). Thereafter, the subsequent covalent bonding between radical
408 intermediates could occur through C-C or C-O bond formation. Mao et al. reported that
409 oxygen atoms have higher charges and lower spin density than carbon atoms, making bond
410 formation at these sites kinetically less favorable.[35] Anyhow, both possibilities were
411 considered and the possible structures of the dimers are indicated as C-O and C-C dimeric
412 products in Figure 2 (compounds 4-7).
413 Due to the remaining laccase activity and that the coupling products are still substrates of the
414 enzyme due to their phenolic groups, radical coupling reaction can be further performed.
415 Thus, the abstraction of other electron from one of the –OH groups of the dimeric products
416 would occur. Once different dimeric products may have been formed and they present various
417 –OH groups, there exist several possibilities of forming dimer radical intermediates. For
418 instance, it is indicated in Figure 2 the radical intermediates formed from the C-O (compound

4) and C-C dimer (compound 6) products resulted from the abstraction of one electron of the –OH groups indicated (marked with asterisks), to form the corresponding oxygen radicals than can delocalize to carbon-located radicals (intermediates 8 and 9 for the C-O dimer and intermediates 10 and 11 for C-C dimer). Afterwards, the presence of radical intermediates of E2 in the reaction medium could lead to the formation of E2 trimers via radical-radical coupling mechanism. As example of possible E2 trimers formed, the products resulted from the covalent bonding between the second radical form of each pair of dimer radical intermediates (compounds 9 and 11) and both radical forms 1 and 2 of E2 are shown (compounds 12-15).

To our knowledge, the reaction pathways of laccase-catalyzed transformation of an estrogen including dimers, trimers and E1 as a product of E2 degradation have been proposed and demonstrated in this study for the first time.

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SUPPORTING INFORMATION AVAILABLE

1) Scheme of the EMR used; 2) Characteristics of the filtered secondary effluent used; 3) HPLC method for the analysis of estrogens at high concentration; 4) GC-MS method for the analysis of estrogens at low concentrations; 5) LC-APCI-MS-MS method for the analysis of estrogens at environmental concentrations; 6) Results corresponding to Experiments 1-2; 7) TIC of 24-h samples and mass spectra of transformation products detected by LC-APCI; 8)

444 Determination of accurate masses by LC-ESI-TOF. This information is available free of
445 charge via the Internet at http://pubs.acs.org/.
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Table 1. Sequence of experiments performed by operating continuously the EMR with a HRT of 4 h and an initial laccase activity of 100 U/L.

	Matrix	Estrogens concentration	Feed addition rate
Experiment 1	Phosphate buffer solution (0.1 M, pH 7)	4 mg/L	1 mg/(L·h)
Experiment 2	Phosphate buffer solution (0.1 M, pH 7)	100 µg/L	25 µg/(L·h)
Experiment 3	Spiked real wastewater	100 µg/L	25 µg/(L·h)
Experiment 4	Real wastewater	Environmental concentrations: 0.29-1.52 ng/L	0.07-0.38 ng/(L·h)

Table 2. Characterization of the products detected by LC-APCI formed after 24 h of E1, E2 or EE2 laccase-catalyzed transformation, observed in the corresponding TIC after subtracting the signals of the blank and time 0 samples.

Parent compound	Retention time (min)	Molecular ion	Molecular weight	Suggested product
E1	8.1	269	268	not identified
	13.7	539	538	E1 dimer
E2	7.9	271	270	E1
	9.7	525	542	E2 dimer I
	10.9	525	542	E2 dimer II
	12.2	525	542	E2 dimer III
	13.0	795	812	E2 trimer I
	13.6	795	812	E2 trimer II
EE2	7.7	295	312	not identified
	8.9	573	590	EE2 dimer I
	9.6	573	590	EE2 dimer II
	10.9	867	884	EE2 trimer I
	11.8	867	884	EE2 trimer II

CAPTION TO FIGURES

Figure 1. Time course of E1, E2 and EE2 degradation rates, laccase activity and reduction of estrogenicity during the operation of the EMR for the treatment of real wastewater containing 100 µg/L of each estrogen (feed addition rate 25 µg/(L·h)) by 100 U/L of initial laccase activity (Experiment 3), and average of estrogens outlet rate during the corresponding control assay lacking laccase.

Figure 2. Reaction products structures and proposed reaction pathways of laccase-catalyzed transformation of E2.

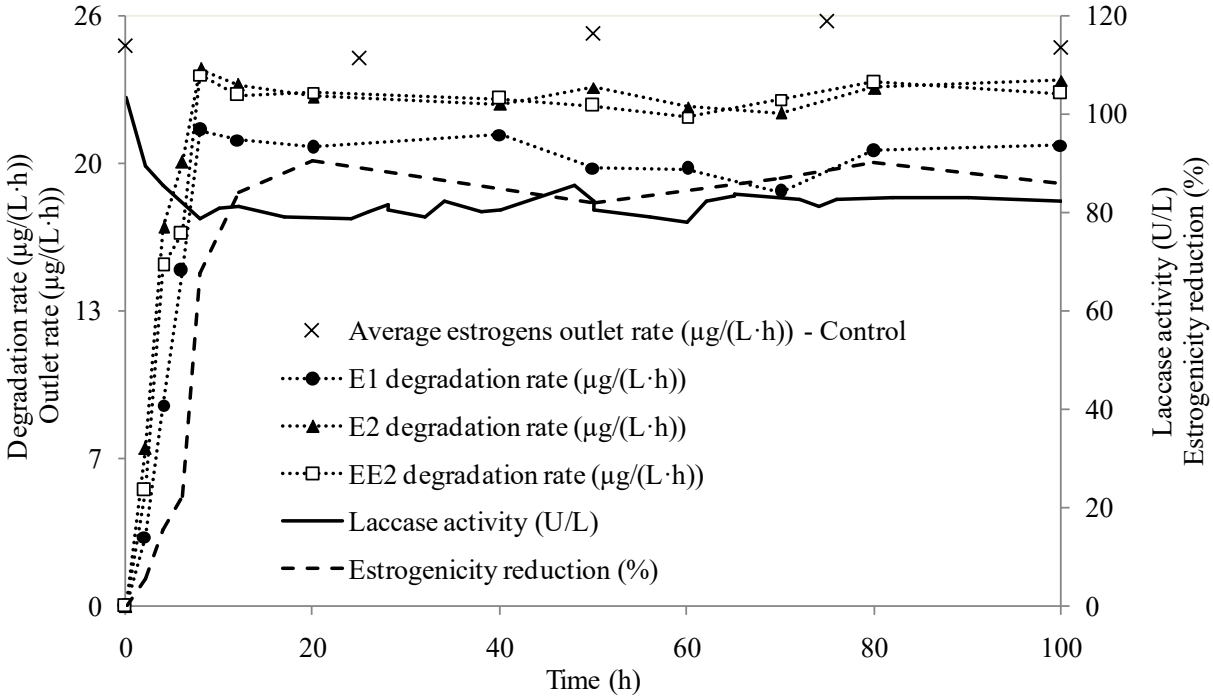

Degradation rate (µg/(L·h))
Outlet rate (µg/(L·h))
Laccase activity (U/L)
Estrogenicity reduction (%)
Time (h)
Average estrogens outlet rate (µg/(L·h)) - Control
E1 degradation rate (µg/(L·h))
E2 degradation rate (µg/(L·h))
EE2 degradation rate (µg/(L·h))
Laccase activity (U/L)
Estrogenicity reduction (%)
0
7
13
20
26
0
20
40
60
80
100
120

FIGURE 1

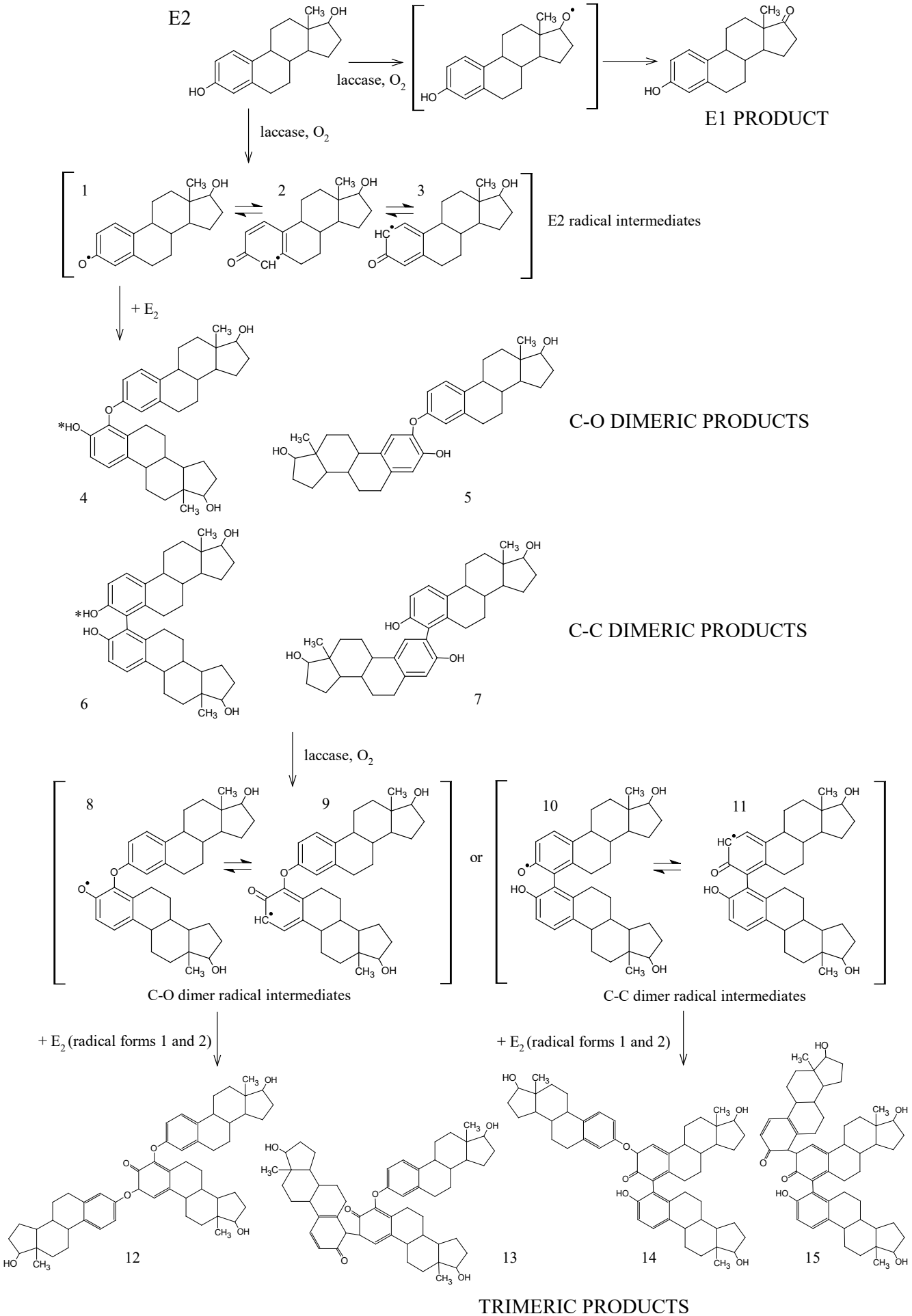
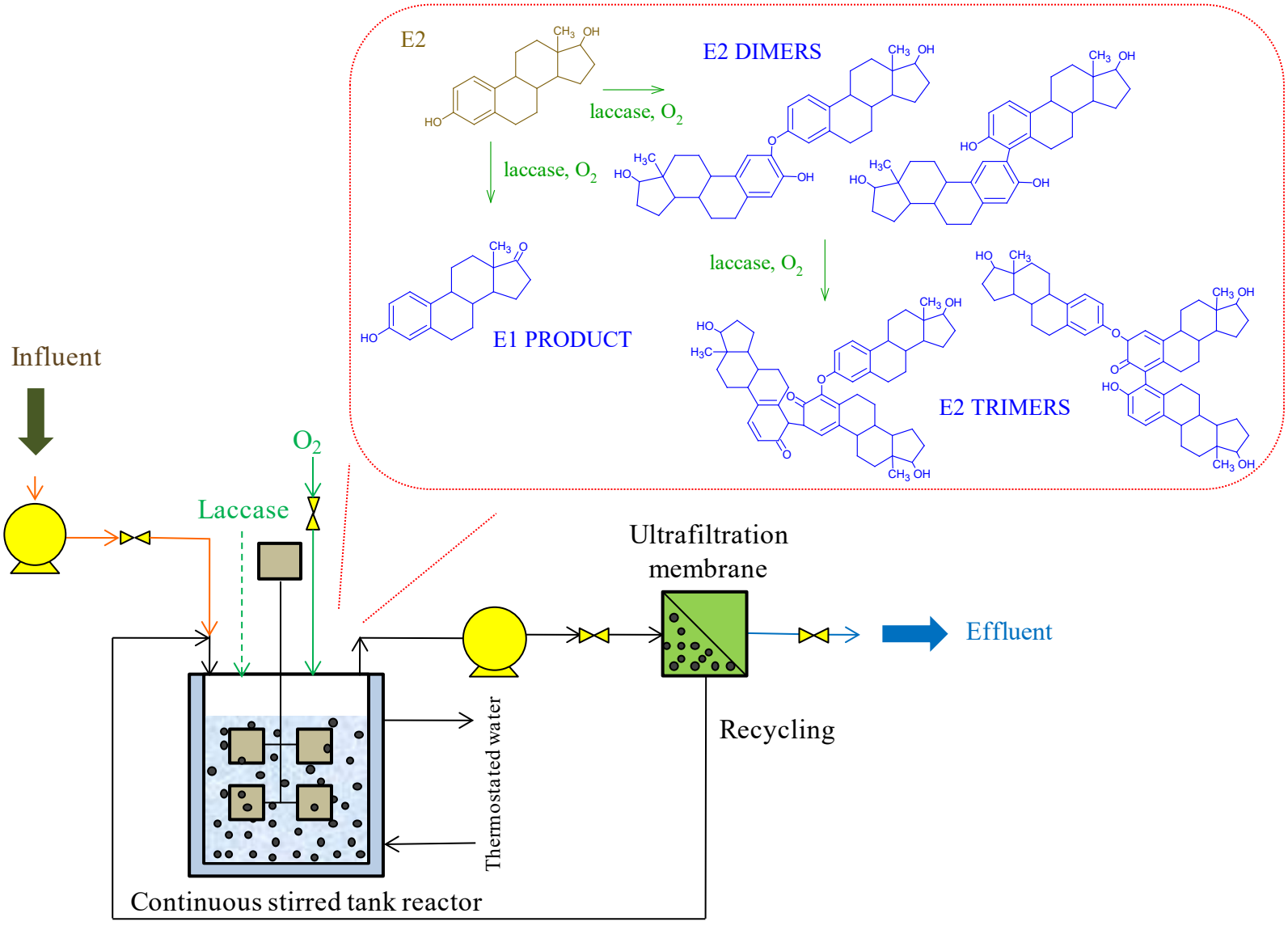

E2
laccase, O_2
E1 PRODUCT
laccase, O_2
1
2
3
E2 radical intermediates
+ E_2
4
5
C-O DIMERIC PRODUCTS
6
7
C-C DIMERIC PRODUCTS
laccase, O_2
8
9
or
10
11
C-O dimer radical intermediates
C-C dimer radical intermediates
+ E_2 (radical forms 1 and 2)
+ E_2 (radical forms 1 and 2)
12
13
14
15
TRIMERIC PRODUCTS

FIGURE 2



ABSTRACT ART

SUPPORTING INFORMATION

Removal of estrogenic compounds from filtered secondary wastewater effluent in a continuous enzymatic membrane reactor. Identification of biotransformation products

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Pages: 11

Figures: 6

Tables: 5

1. Scheme of the enzymatic membrane reactor (EMR) used for the continuous laccase-catalyzed transformation of estrogenic compounds

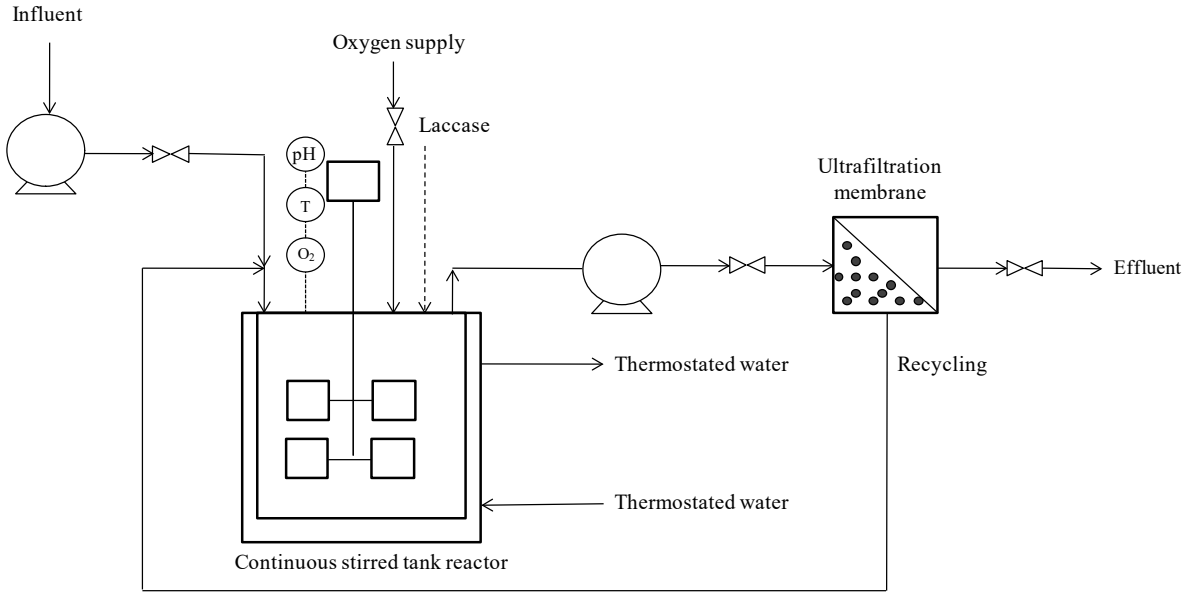

Figure S1. EMR used for the continuous laccase-catalyzed transformation of estrogens.

2. Characteristics of the filtered secondary effluent used for Experiments 3-4.

The real wastewater collected from the outlet of the secondary clarifier of the municipal wastewater treatment plant of Calo-Milladoiro (Ames, Spain) was analyzed according to Standard Methods to determine the main characteristics as well as by LC-APCI-MS-MS to measure the estrogens concentrations. The results are summarized in Table S1.

Table S1. Characteristics of filtered secondary effluent.

Micropollutants	**ng/L**	**Carbon and nitrogen content**	**Concentration (mg/L)**
E1	1.52	inorganic carbon	5.6
E2	0.29	total organic carbon	5.6
EE2	0.74	total nitrogen	7.8
COD (mgO_2/l)	33.80	inorganic nitrogen	3.0
pH	6.85	total Kjendahl nitrogen	4.8

Anions	**Concentration (mg/L)**	**Cations**	**Concentration (mg/L)**
NO^{2-}	0.1	Na^{+}	38.2
Br^{-}	0.0	NH^{4+}	0.0
NO^{3-}	9.9	K^{+}	9.0
PO_4^{3-}	2.2	Mg^{2+}	2.9
SO_4^{2-}	21.3	Ca^{2+}	11.9

3. High performance liquid chromatography (HPLC) for the analyses of estrogens at high concentrations

The analysis of the estrogens in the samples corresponding to the experiment at high initial concentration in buffer solution (Experiment 1) was carried out through HPLC in an HP-1090 system equipped with a diode array detector at 210 nm. Chromatographic separation was performed on a Lichrocart 250-4 column packed with Lichrosphere 100 RP-18 5 μm (Merck) and 100 μL of injection volume was used. The mobile phase consisted of a binary mixture of solvents A (acetonitrile) and B (50 mM phosphate buffer, pH 4.5) at a flow rate of 0.8 mL/min. The gradient method and running times were programmed as follows: initial conditions were 40% A for 1 min, then linearly programmed up to 80% until 20 min and held for 4 min, followed by a linear program at 40% for 5 min and held for 1 min.

4. Gas chromatography mass spectrometry (GC-MS) for analyses of estrogens at low concentrations

Estrogens analysis corresponding to the experiments at low initial concentration in buffer solution or real wastewater (Experiments 2-3) were conducted by GC-MS using a Saturn 2100T (Varian) system with a CP Sil column (CP Sil 8 CB-MS low bleed (30 m x 0.25 mm x 0.25 µm). The analytical conditions are detailed in Table S2.

Table S2. Equipment and conditions for the determination of estrogens by GC-MS.

Chromatographic parameters	
Split-splitless injector	
Splitless time	2 min
Injection temperature	280ºC
Gas flow (He)	1 mL/min
Injector (volume)	1 µL
Solvent	Ethyl acetate
Temperature program	
Initial temperature	70ºC
Initial time	2 min
1st ramp	25ºC/min
Temperature 1	150ºC
2nd ramp	3ºC/min
Temperature 2	200ºC
3rd ramp	8ºC/min
Temperature 3	280ºC
Time 3	5 min
Mass Spectrometry	
Ionization mode	Electronic impact
Filament current	10 µA
Ion ramp temperature	220ºC
Transference line	280ºC
Voltage	1700 – 1750 V
Scan velocity	1s/scan
Mass spectrum	50-550 m/z

Limits of quantification (LOQ) of the system were 10 μg/L in the case of E1 and E2 and 40 μg/L for EE2. Anyhow, the concentration of the samples allowed the quantification of the compounds at concentrations of 1.5 and 6 μg/L.

5. Liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS-MS) for analyses of estrogens at environmental concentrations

The quantification of E1, E2 and EE2 present in the secondary effluent used as well as in the samples withdrawn during the experiment with non-spiked wastewater (Experiment 4) were performed by LC-APCI-MS-MS.

The LC system used (Alginet 1100, Alginet Technologies) was equipped with Synergi 4u MAX-RP 80A column (4.60 mm × 250 mm, 4 μm, Phenomenex) for the analytes separation. The injection volume was 10 μL and the mobile phase consisted of a binary mixture of 0.1% formic acid (v/v) in water (A) and 100% methanol (B) at a flow rate of 0.7 mL/min. The gradient was as follows: initial conditions were 70% B, increased linearly up to 88% until 2 min, followed by a linear program to 94% until 15 min.

Mass spectrometry was performed using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems) and MS/MS was carried out in the multiple reaction mode (MRM) using APCI in positive mode. The values of the main parameters of the source are shown in Table S3.

Table S3. Source-dependent parameters.

Parameter	
Collision gas (psig)	6
Curtain gas (psig)	14
Ion source gas nebulizer (psig)	15
Nebulizer current (μA)	3
Temperature (ºC)	450
Entrance potential (V)	10

Retention times were as follows: 9.2 min (EE2), 9.6 min (E2) and 9.7 min (E1). For each compound, two characteristic fragmentations were optimized, the most abundant one being used for quantitation, while the second one with lower intensity was used as a qualifier, as shown in Table S4.

Table S4. MRM conditions used for the analysis of the estrogens.

Compound	MRM transition → product ion	
E1	271.1 → 133.2,	271.1 → 159.2
E2	255.0 → 159.1,	255.0 → 133.1
EE2	279.2 → 133.1,	279.2 → 159.2

LOQs were 0.5 μg/L; however, the concentration of the samples (described in Materials and methods section) provided a reduction of the limits of quantification to 0.02 ng/L for the secondary effluent samples and 0.01 ng/L for the samples withdrawn from the EMR effluent during the Experiment 4.

In order to corroborate the removal results of E1, E2 and EE2, E2-d_4 was used as internal standard throughout the analytical procedure. The deuterated compound was added to the samples before the SPE (5 ng, which meant concentrations prior to SPE of 2.5 and 1 ng/L in the EMR influent and effluent samples, and 66.66 μg/L after concentration of the samples for subsequent LC/MS/MS analysis). The quantification of the estrogenic compounds was carried out using calibration curves built by plotting the ratio analyte peak area/E2-d_4 peak area versus the analyte concentration; calibration standards contained increasing amounts of the analytes in the range 0.5-70 μg/L, and a fixed concentration of the surrogate of 66.66 μg/L. The surrogate presented the following transitions: 259.2 → 161.1 and 259.2 → 135.0, and retention time 9.5 min.

6. Results obtained after the performance of the EMR for the treatment of synthetic water (buffered solution) at high and low estrogens initial concentrations

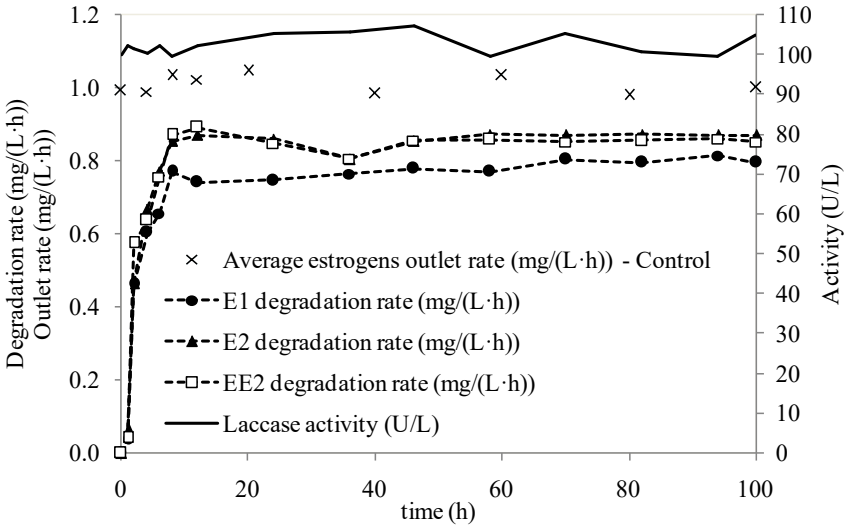

Figure S2. Time course of E1, E2 and EE2 degradation rates and laccase activity during the operation of the EMR for the treatment of synthetic waster containing 4 mg/L of each estrogen (feed addition rate 1 mg/(L·h)) by 100 U/L of initial laccase activity (Experiment 1), and average of the estrogens outlet rate during the corresponding control assay lacking laccase.

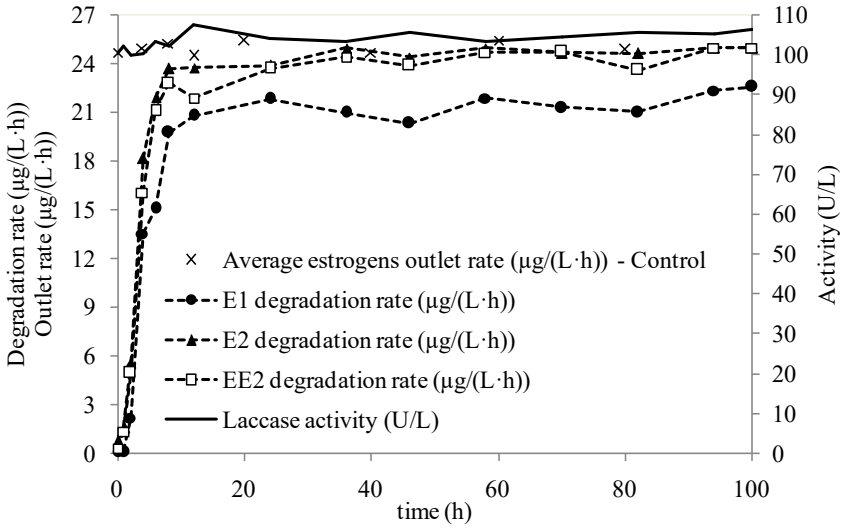

Figure S3. Time course of E1, E2 and EE2 degradation rates and laccase activity during the operation of the EMR for the treatment of synthetic water containing 100 µg/L of each estrogen (feed addition rate 25 µg/(L·h)) by 100 U/L of initial laccase activity (Experiment 2), and average of the estrogens outlet rate during the corresponding control assay lacking laccase.

7. Total ion chromatograms (TIC) and mass spectra resulted from the study of identification of laccase-catalyzed transformation products of E1, E2 and EE2 by LC-APCI analysis

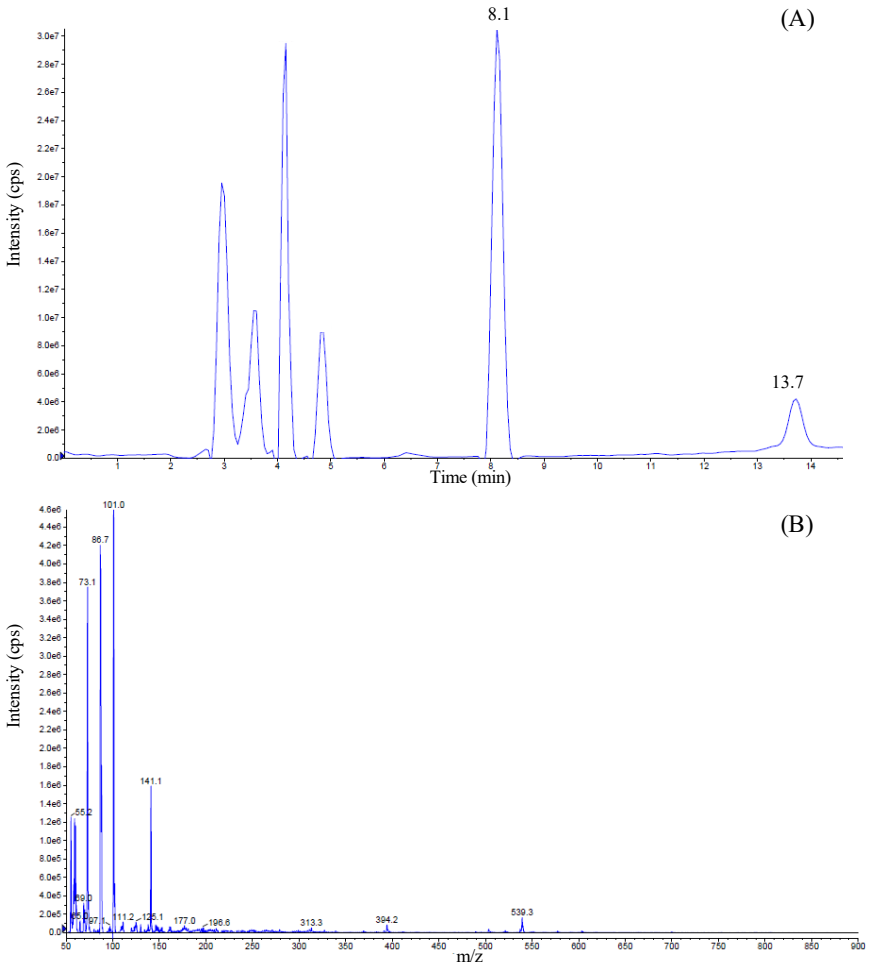

Figure S4. TIC of the sample after 24 h of laccase-catalyzed transformation of E1 (A) and mass spectrum of E1 dimer (B) obtained by LC-APCI positive in full scan mode.

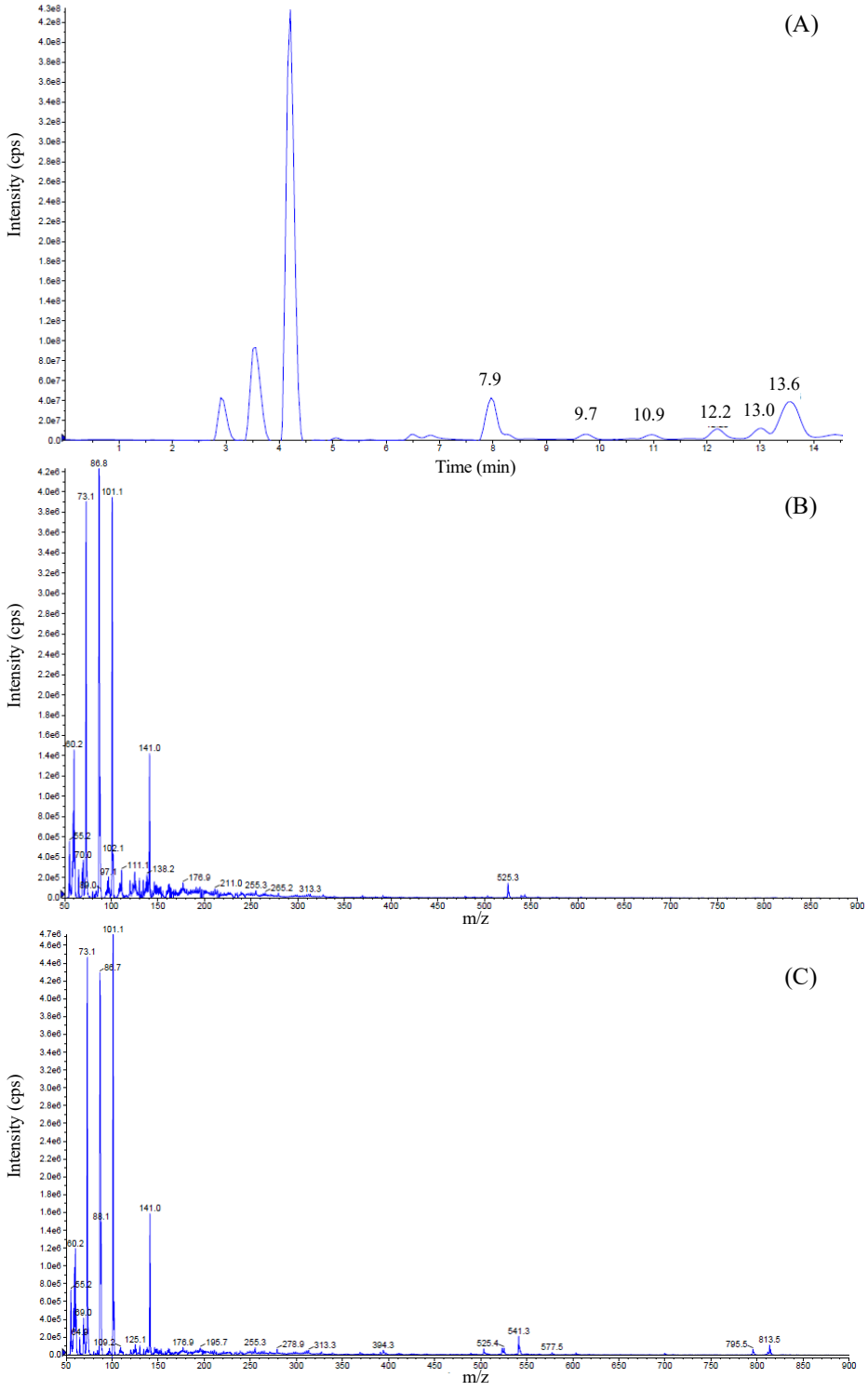

Figure S5. TIC of the sample after 24 h of laccase-catalyzed transformation of E2 (A) and mass spectra of E2 dimer I (B) and E2 trimer I (C) obtained by LC-APCI positive in full scan mode.

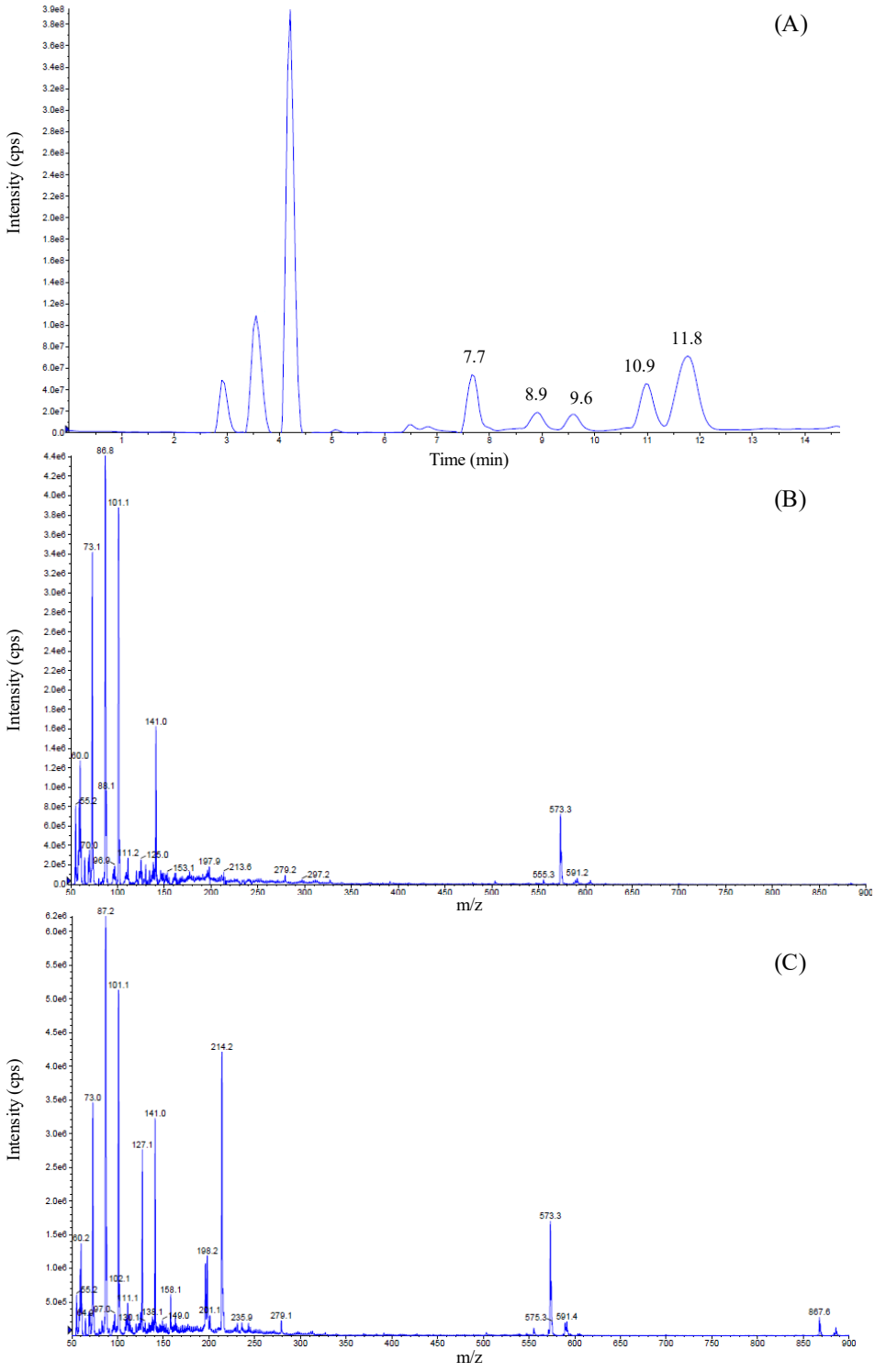

Figure S6. TIC of the sample after 24 h of laccase-catalyzed transformation of EE2 (A) and mass spectra of EE2 dimer I (B) and EE2 trimer I (C) obtained by LC-APCI positive in full scan mode.

8. Determination of accurate masses of biotransformation products by liquid chromatography electrospray time-of-flight mass spectrometry (LC-ESI-TOF)

Samples were analyzed by LC-ESI-TOF to obtain information concerning accurate masses. For this purpose, a LC system (Alginet 1100) equipped with a Zorbax Eclipse XDB (C18 3 mm x 250 mm, 5 µm) (Alginet) analytical column was used. A binary solvent comprised acetonitrile (ACN) was used as the mobile phase. Flow rate was set at 0.6 mL/min and the gradient was programmed as following: 0 minutes, 40% ACN; 10 minutes, 60% ACN; 40 minutes, 80% ACN; 43 minutes, 100% ACN; 44 minutes, 100% ACN; and the injection volume was 15 µL. This LC system was connected to a Microtof Bruker Daltonics mass spectrometer with an ESI source operated at negative mode and under the following conditions: capillary, 4.5 kV; drying gas, 8 L/min; gas temperature, 200ºC. The results obtained after the analyses of the samples from the batch experiments are shown in Table S5.

Table S5. Accurate masses of the transformation products (deprotonated compounds) detected by LC-ESI-TOF in the samples from the batch assays.

Parent compound	Suggested product	Elemental composition	Theoretical mass	Experimental mass	mDa	error (ppm)
E1	Dimer	$C_{36}H_{41}O_4$	537.3010	537.3037	-2.6	-5.0
				537.3038	-2.7	-5.2
E2	Dimer	$C_{36}H_{45}O_4$	541.3323	541.3320	0.3	0.6
				541.3315	0.8	1.5
				541.3334	-1.1	-2.0
				541.3325	0.9	-0.4
	Trimer	$C_{54}H_{67}O_6$	811.4943	811.4954	-1.1	-1.4
				811.4942	0.1	0.1
				811.4943	0.0	0.0
	E1	$C_{18}H_{21}O_2$	269.1547	269.1536	-1.6	4.1
EE2	Dimer	$C_{40}H_{45}O_4$	589.3323	589.3312	1.2	1.9
				589.3328	-0.4	-0.8
				589.3330	-0.6	-1.2
				589.3323	0.0	0.0
	Trimer	$C_{60}H_{67}O_6$	883.4943	883.4949	-0.5	-0.7
				883.4963	-2.2	-2.3
				883.4959	-1.6	-1.8
				883.4954	-1.1	-1.3