Identification of new ozonation disinfection byproducts of 17β-estradiol and estrone in water

Renata de Oliveira Pereira [a] [c] [1], Miren Lopez de Alda[a], Jesús Joglar[b], Luiz Antonio Daniel[c], Damià Barceló[a,d]

[a] Institute of Environmental Assessment and Water Research, IDAEA-CSIC, C/ Jordi Girona 18-26, 08034, Barcelona, Spain.

[b] Department of Biological Chemistry and Molecular Modelling, Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), C/ Jordi Girona 18-26, 08034, Barcelona, Spain

[c] São Carlos Engineering School, São Paulo University, Av. Trabalhador São-carlense, 400, 13566-590, São Carlos, SP, Brazil

[d] Catalan Institute for Water Research (ICRA), C/ Emili Grahit 101, Edifici H_2O, Parc Científic i Tecnologíc de la Universitat de Girona, 17003, Girona, Spain.

[1] Corresponding author, permanent address - Department of Hydraulics and Sanitation of the São Carlos Engineering School - São Paulo University, Campus de São Carlos, Av. Trabalhador São-carlense, 400, Arnold Schimidt, São Carlos - São Paulo - Brazil - 13566-590 tel: 055-32-32323808, *e-mail: p*ereira.or@gmail.com.

Abstract

Estrogens are a class of micro-pollutants found in water at low concentrations (in the ng L^{-1} range), but often sufficient to exert estrogenic effects due to their high estrogenic potency. Disinfection of waters containing estrogens through oxidative processes has been shown to lead to the formation of disinfection byproducts, which may also be estrogenic. The present work investigates the formation of disinfection byproducts of 17β-estradiol (E2) and estrone (E1) in the treatment of water with ozone. Experiments have been carried out at two different concentrations of the estrogens in ground water (100 ng L^{-1} and 100 μg L^{-1}) and at varying ozone dosages (0-30 mg L^{-1}). Detection of the estrogens and their disinfection byproducts in the water samples has been performed by means of ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with a triple quadrupole (QqQ) and a quadrupole-time of flight (QqTOF) instrument. Both E2 and E1 have been found to form two main byproducts, with molecular mass (MM) 288 and 278 in the case of E2, and 286 and 276 in the case of E1, following presumably the same reaction pathways. The E2 byproduct with MM 288 has been identified as 10epsilon-17beta-dihydroxy-1,4-estradieno-3-one (DEO), in agreement with previously published results. The molecular structures and the formation pathways of the other three newly identified byproducts have been suggested. These byproducts have been found to be formed at both high and low concentrations of the estrogens and to be persistent even after application of high ozone dosages.

Keywords: estrogen; drinking water; oxidation process; disinfection byproduct.

1. Introduction

Ozone can be used in the drinking water process, since it is a powerful oxidant and disinfectant. However, it can form disinfection byproducts through oxidation with inorganic and organic compounds (Richardson, 2003).

The presence of some ozonation disinfection byproducts (DBPs), like bromate, trihalomethanes (THMs) and haloacetic acids (HAAs), is regulated in drinking water to control and minimize human exposure to these hazardous compounds (USEPA; European-Communities, 1998). However, there are potentially many more ozonation DBPs, and until 2003 only less than 50% of the assimilable organic carbon (AOC) formed during ozonation of water has been characterized (Richardson, 2003).

Compounds such as 17β-estradiol (E2) and estrone (E1), which have endocrine disrupting properties, are often found in surface water (Kuster et al., 2008; Yoon et al., 2010). If present in the water used as source for abstraction of drinking water these micro-pollutants can react with ozone forming disinfection byproducts that can pose a potential risk to the population served with this water.

Sewage treatment effluents are one of the main sources of estrogens in the aquatic environment. The extent of their degradation during sewage treatment and their subsequent load into the receiving natural waters is variable, depending on the type of treatment applied, the hydraulic residence time, the sludge retention time, and other operational factors (Auriol, 2006; Cargouet et al., 2004; Esperanza, 2007). Furthermore, since estrogens are female sex hormones naturally produced by vertebrates, non-point sources, especially in rural areas with intense farming activities, may also be relevant (Ying et al., 2009).

Of the various natural and synthetic free estrogens E1 and E2 are the most frequently detected. Their mean/median effluent concentrations in wastewater are

situated in the range of 20–55 ng L^{-1} for E1 and 9–20 ng L^{-1} for E2 (D'Ascenzo et al., 2003). Meanwhile, in surface water the concentrations reported for these two estrogens in the last years have ranged between 0.7 and 143 ng L^{-1} for E1 (Rodriquez-Mozaz et al., 2004a, b; Ying et al., 2009) and between 1.4 and 34 ng L^{-1} for E2 (Chen et al., 2007; Ying et al., 2009).

After entering into the drinking water treatment process E1 and E2, alike other estrogens and endocrine disrupting compounds (EDCs), are usually gradually and efficiently, although not necessarily completely, removed (Chang, 2009; Chen et al., 2007). Thus, E2 and E1 have been detected, for instance, in finished drinking water from Brazil at concentrations up to 6.8 ng L^{-1} and 0.1 ng L^{-1}, respectively (Gerolin, 2008, Lopes et al., 2010; Sodre et al., 2010). However, in general, the treatments applied in drinking water treatment plants (DWTPs) are successful at removing estrogens, i.e., at eliminating the parent compounds (although this does not necessarily imply full elimination by e.g. mineralization), and oxidation by ozone and chlorine are among the most effective and extensively used processes (Benotti et al., 2009b). Other oxidants, apart from ozone and chlorine (Alum et al., 2004; Bila et al., 2007; Broséus et al., 2009; Deborde et al., 2005; Hashimoto et al., 2006; Kim et al., 2004; Lin et al., 2009; Maniero et al., 2008; Westerhoff et al., 2005), that have been studied for the removal of estrogens (parent compound) from water have been chlorine dioxide (Huber et al., 2005), photolysis (Mazellier et al., 2008), photo-Fenton (Yaping and Jiangyong, 2008; Zhao et al., 2008), titanium dioxide photocatalysis (TiO_2/UV) (Benotti et al., 2009a; Ohko et al., 2002; Zhang et al., 2007), ferrate (Lin et al., 2009), electrochemical degradation (Murugananthan et al., 2007) and sonolysis (Suri et al., 2007). In comparison with most of these oxidants, ozone presents the advantage of having high rate constants (Huber et al., 2005) and being a powerful disinfectant.

Typical doses of ozone (< 2 mg L^{-1}) applied during treatment of water containing E1 and E2 have reached 96 to 99% removal of the parent compound (Alum et al., 2004; Bila et al., 2007; Broséus et al., 2009; Kim et al., 2004; Maniero et al., 2008;Westerhoff et al., 2005). However, some works have reported the formation of byproducts after the removal of E1 and E2 (Pereira et al., 2011). In this context, the objective of this work was to investigate the formation of E1 and E2 byproducts during ozonation, to try to identify them, and to estimate the dose of ozone necessary for the complete removal of these byproducts.

2. Materials and Methods

2.1. Reagents and solutions

HPLC-grade acetone, methanol and water were supplied by Merck (Darmstadt, Germany). Pure standards of 17β-estradiol (E2), estrone (E1), 6α-hydroxyestradiol, 4-hydroxyestradiol, and 2-hydroxyestradiol were purchased from Sigma Aldrich (Steinheim, Germany). Individual stock solutions of the analytes were prepared at 100 mg L^{-1} by dissolving 10 mg of each compound in 100 mL of methanol. Estrogens in methanol solutions stored in the dark at 4°C are stable for at least 6 months. The solutions at 100 μg L^{-1} and 100 ng L^{-1} were freshly prepared before every batch of experiments by appropriate dilution of the individual stock solutions in ground water.

Ground water from the University of São Paulo (São Carlos Campus, Brazil) collected before chlorination was used to carry out the assays. After collection it was stored at 4 ºC until use. This water source was chosen because of the similarity of its characteristics with those of drinking water, with the required quality in terms of average turbidity (0.4 ± 0.2 NTU) and average color (1.0 ± 1.5 Pt-Co units).

2.2. Ozonation experiments

The ozonation experiments were carried out in a pilot scale experimental unit (Supplementary Information - 1). The ozonation reactor had a total height of 3.5 m, a working height of 3.0 m, an external diameter of 110 mm and a volume of 28.5 L. The ozone generator (EAGLESAT®, Brazil) was a corona discharge type and had an oxygen generation unit (PSA method - Pressure Swing Adsorption) and a compressor to generate ozone. The production of ozone in the gas phase was measured with an ozone analyzer (IN USA, ASX-Mod H1) coupled to a rotameter. The ozone was applied to the base of the reactor using a porous diffuser and the off-gas was captured in a flask containing potassium iodide solution at 2% (w/w). The total ozone consumed was calculated according to equation 1.

$$CO = AO - OF - RO \quad (1)$$

Where, CO is the consumed ozone (mg L^{-1}), AO is the applied ozone (mg L^{-1}), OF is the ozone in the off-gas (mg L^{-1}), and RO is the residual ozone in the liquid phase (mg L^{-1}). Therefore, the concentration of ozone expressed in the present work is the ozone consumed during the reaction time.

Experiments were performed at two different initial concentrations of E2 and E1 (100 µg L^{-1} and 100 ng L^{-1}), in separated batches for each estrogen and one treatment was done for each dose of ozone (batch experiments). The experiments at 100 µg L^{-1} were conducted first. The objective in this case was to identify the main byproducts formed during ozonation. Then the experiments at 100 ng L^{-1} were performed in order to track and confirm the presence of the previously identified byproducts but at concentrations closer to those found in real samples (surface water). In all cases the dose of ozone applied ranged from 0.4 to 28 mg L^{-1}, the temperature was set at 19 ± 2ºC and the natural pH of the ground water at the beginning of the treatments was 7.0 ± 0.3.

After the ozone application, aliquots of 200 mL were withdrawn from the reactor and the residual ozone in the water samples was measured by indigo colorimetric method 4500-O_3 B (APHA, 1998) immediately after the assays.

2.3. Analysis

2.3.1 Sample preparation

Samples (200 mL) were preconcentrated by solid phase extraction (SPE) with C18 cartridges (Accubond, 500 mg) from Agilent Technologies (Santa Clara, CA, USA) following a procedure adapted from Ternes et al. (1999). After sample loading the cartridges were rinsed with 10 mL of HPLC water and eluted with 4 mL of acetone. Main differences with respect to the method described by Ternes et al. (1999) refer to the sample volume (200 mL *vs* 1 L) and the performance of a washing step of the cartridge prior to elution. The extracts obtained were then blown down to dryness under nitrogen, reconstituted with methanol to a final volume of 0.5 mL, and stored in the dark at 4°C for subsequent LC–MS/MS analysis. Under these conditions both E1 and E2 are stable for at least 60 days (recovery percentage ± relative standard deviation equal to 96 ± 1% and 102 ± 5% for E1 and E2, respectively).

2.3.2. Identification of byproducts by UPLC-QqToF-MS/MS

Identification of E1 and E2 ozonation byproducts in the water samples extracts coming from the experiments performed at 100 µg L^{-1} (see section 2.2) was carried out by UPLC-QqTOF-MS/MS on a Waters Acquity UPLC system (Waters Corp., Mildford, MA) equipped with a binary solvent delivery system and an autosampler coupled to a Waters QqToF-Micro MS/MS detector. Chromatographic separation was performed on a Hibar HR Purospher STAR RP-18 column (30 x 2.1 mm, 2 µm particle size) from Merck (Darmstadt, Germany). The column temperature was set to 35 ºC and the samples compartment temperature was kept to 10 ºC. The injection volume was 5 µL

and the column flow rate 0.4 mL min^{-1}. The mobile phase consisted of A (acetonitrile) and B (water). The gradient started with 10% A, composition that was kept constant for 2 min, then linearly increased to 25% A over 2 minutes, kept constant for 1 min, linearly increased to 50% A over 1 min, kept constant for 2 min, raised to 100% A in 2 min, maintain constant for 1 min, and decreased to 10% A within 1 minute. Total run time, including reequilibration of the column to the initial conditions, was 12 minutes.

Detection was performed with an electrospray (ESI) interface in the negative ionization (NI) and with the capillary voltage set to 2800 V and cone voltages varying between 10 and 50 V. The nebulization gas (nitrogen) was set to 500 L h^{-1} at a temperature of 450 ^{o}C; the cone gas (nitrogen) was set to 30 L h^{-1}, and the source temperature to 150 ^{o}C. For MS experiments, the instrument was operated in a wide pass quadrupole mode with the TOF data being collected between *m*/*z* 50 and 600 and low collision energy (CE) of 4 eV. The product ion MS/MS experiments were performed at variable CE (10–50 eV). All analyses were performed using an independent reference spray (lockSpray) to ensure accuracy and reproducibility. Val-Tyr-Val was used as lock mass (*m*/*z* 380.2029) for internal mass calibration.

Instrument control, data acquisition, and evaluation were done by means of MassLynx V4.1 software (Waters Corp.). This software (Waters Corp.) calculates all possible elemental compositions for a given accurate mass and is thus a potent tool for hypothesizing possible structures of unknown compounds. Final identification can then be performed based on the accurate mass measurements of the parent ions and fragments obtained in MS/MS experiments.

2.3.3. Analysis of byproducts by UPLC-QqQ-MS/MS

Analysis of the previously identified byproducts in the water extracts coming from the experiments performed at low initial concentration of estrogens (100 ng L^{-1}) was

carried out by UPLC-QqQ-MS/MS on a Waters Acquity UPLC system coupled to a Waters TQD MS/MS detector. Chromatographic conditions were the same as described above for UPLC-QqToF-MS/MS analysis except for the injection volume which was set to 8 µL to increase sensitivity. MS/MS detection was performed in the selected reaction monitoring (SRM) mode using ESI in the NI mode and basically the same MS/MS conditions described above: capillary voltage, 2800 V; nebulization gas flow rate, 650 L h^{-1}; desolvation temperature, 450 ºC; cone gas flow rate, 30 L h^{-1}; source temperature, 150 ºC. SRM transitions and the cone and collision voltages selected for the monitoring of the previously identified byproducts is showed in Supplementary Information - 2. Optimization of these conditions was performed with the samples coming from the high concentration experiments conducted before. Instrument control and data acquisition and evaluation were done with the software MassLynx V4.1.

3. Results and Discussion

Figure 1 shows the disappearance of E1 and E2 during ozonation in the experiments conducted with initial concentration of 100 µg L^{-1} of the estrogens in water. As it can be seen, both estrogens vanish rapidly, although the reaction of ozone with E1 is slightly faster than with E2. Parallel to the disappearance of the estrogens, a number of other new peaks appear in the UPLC-QqTOF-MS chromatograms obtained throughout the experiments. The *m/z* ratios and the corresponding retention times of the major peaks identified in the degradation of E1 and E2 are depicted in Figure 2. The main byproducts of E1 (with nominal *m/z* ratios 285, 275, 301, and 305) are similar to those of E2 (with nominal *m/z* ratios 287, 277, 303, and 307) with a difference of two mass units between them, which coincides with the difference of two mass units also in the molecular mass (MM) of the parent compounds (270.36608 g/mol for E1 and 272.38196 g/mol for E2). Since this difference is due to the presence of a ketone group

224 in position 17 of the molecular structure of E1 and a hydroxyl group in the same
225 position of the E2 molecule, it is clear that ozone attacks the aromatic moiety, where the
226 high electronic densities on the carbons located in the ortho and para positions favor the
227 process (Figure 1).

228 **(Figure 1)**

229 In both cases the most abundant and persistent byproducts are the ones with lower
230 *m/z* ratios: 285 and 275 for E1, and 287 and 277 for E2. The other byproducts, with *m/z*
231 301 and 305 in the case of E1, and *m/z* 303 and 307 in the case of E2, are formed in
232 much less quantity and are therefore not considered further for discussion.

233 **(Figure 2)**

234 Figure 2A shows that the E1 byproduct with *m/z* 285 is fairly stable until an ozone
235 dosage of 12 mg O_3 L^{-1}, decreasing thereafter but not disappearing. Meanwhile the
236 compound with *m/z* 275 and retention time 4.27 min rises until the ozone dosage
237 reaches 16.7 mg O_3 L^{-1} and its concentration keeps fairly constant afterwards. As it is
238 shown in Figure 2B, the E2 byproducts show a rather similar behavior, except for the
239 fact that the byproduct with *m/z* 287 starts to decrease at a lower ozone dosage, 4.0 mg
240 O_3 L^{-1}, than the E1 byproduct with *m/z* 285.

241 The elemental compositions proposed by the Masslynx software for the
242 experimental accurate masses measured by the QqTOF-MS/MS detector, after applying
243 basic organic chemical rules to eliminate nonfeasible formulae and possible degradation
244 scenarios for the parent compounds, were $C_{18}H_{23}O_3$ for *m/z* 287 and $C_{16}H_{21}O_4$ for *m/z*
245 277. However, considering the structure of E2 and that the most probable attack occurs
246 at the aromatic ring (high electronic densities) there are more than one possible structure
247 for *m/z* 287 or, in other words, with mass 288. The structures proposed so far in the
248 scientific literature for E2 disinfection byproducts with molecular mass 288 have been:

2-hydroxyestradiol (2OH-E2), identified by both Bila et al. (2007) and Maniero et al. (2008), monohydroxylated E2 (Irmak et al., 2005), 10e-17b-dihydroxy-1,4-estradieno-3-one (DEO) (Bila et al., 2007) and testosterone (Bila et al., 2007; Maniero et al., 2008) in ozonation experiments. In experiments performed with other types of oxidation processes such as, photo-Fenton, photolysis, or TiO_2 photocatalysis the compounds previously identified were: 6-hydroxyestradiol (6OH-E2) (Mazellier et al., 2008; Zhao et al., 2008), again 2OH-E2 , testosterone (Ohko et al., 2002; Zhao et al., 2008) and DEO (Mai et al., 2008; Zhao et al., 2008). The E2 byproduct with mass 278 (*m/z* 277) has never been reported in the literature.

Trying to elucidate the structure of the byproduct with mass 288 the standards that were available (2OH-E2, 4OH-E2, and 6OH-E2) were acquired and analyzed. However, as it can be seen in Figure 3 the retention times of the standards 2OH-E2 (5.69 min) and 4OH-E2 (5.79 min) were very different from the retention time of the E2 byproducts with MM 288 (4.05 and 4.36 min) detected in our ozonation experiments (16.7 mg O_3 L^{-1}). On the contrary the retention time of the standard 6OH-E2 (3.99 min) was quite similar, but when the molecules were fragmented their fragments were totally different from each other, indicating that 6OH-E2 is not the targeted byproduct. The standard of estriol (E3), which also has a MM of 288, was tested as well, but the LC-MS data did not fit either. Apart from these there are other structure possibilities for MM 288, but these structures do not have their corresponding counterparts for MM 286 when the same degradation mechanism is applied to the E1 molecule. The compound testosterone was also disregarded because its formulae ($C_{19}H_{28}O_2$) contains only two oxygens (instead of three). Thus the byproduct with MM 288 is expected to be the compound named DEO. DEO was suggested by Ohko et al. in 2002 as byproduct in the mechanism of degradation of E2 by TiO_2 photocatalysis (Ohko et al., 2002) and further confirmed

by Mai et al. in 2008 in similar oxidative processes by LC-MS/MS analysis (Mai et al., 2008). Furthermore, the fragments observed by Mai et al. (2008) were similar to those found in the present study.

(Figure 3)

The fragmentation of the E2 and E1 byproducts with *m/z* 287 and *m/z* 285, respectively, is shown in Figure 4. The part marked in the square is virtually identical, i.e., presents the same fragment ions, in both compounds, whereas the circled part shows a similar fragmentation pattern but with a difference of two mass units. Based on these spectra and on previously published works (Bila et al., 2007; Mai et al., 2008; Ohko et al., 2002; Zhao et al., 2008) the E2 byproduct with *m/z* 287 is believed to be DEO, and the main E1 byproduct with *m/z* 285 is believed to correspond to a similar compound but with a ketone group instead of a hydroxyl group in position 17, i.e. to 10-hydroxy-1,4-estradieno-3,17-dione (HEDO).

(Figure 4)

The formation pathway of DEO from E2 has already been suggested by Mai et al. (2008) and according to it the formation pathway of the above suggested E1 byproduct would be the same.

It may be worth mentioning that the pH of the samples (measured at the beginning and at the end of the experiments) decreased as the formation of the byproducts increased (following the loss of H^+ and the addition of OH in the molecule) and vice versa, as it is shown in Supplementary Information - 3 for the byproduct with *m/z* 285.

For the E2 and E1 byproducts with *m/z* 277 and *m/z* 275, respectively, the pathway of degradation based on the mechanism of phenol ozonolysis (Komissarov et al., 2006) is suggested. This pathway is illustrated in Figure 5 for E2. The pathway for E1 would be similar but maintaining the ketone group that is present in position 17 in the original

E1 structure. Figure 6 shows the product ion mass spectra and the purported fragmentation of the byproducts with *m/z* 277 and *m/z* 275 that support the proposed structures.

The finding of these byproducts, not reported previously by other authors, could be due to the use of different equipments and/or experimental conditions. In the works performed by Bila et al. (2007) and Maniero et al. (2008) the ozone doses and the initial concentration of E2 were comparable to those used in the present study but the identification of byproducts was carried out by GC/MS. In the work conducted by Irmak et al. (2005) the detection of byproducts was performed by LC-MS/MS but the conditions of the experiment were very different from ours with higher initial estrogen concentrations and unclear doses of ozone consumed. Another possible difference could be the reactive form triggering the process in each work, which in our study, conducted at pH around 7, would be basically molecular ozone and in other studies OH radicals.

(Figure 5)

(Figure 6)

The ozonation experiments carried out at lower initial concentrations of the estrogens (100 ng L^{-1}) were conducted with the double objective of, first, confirming the formation of the previously identified main byproducts at concentrations in water closer to those found in the environment and, secondly, determining the ozone dosage that would have to be applied to remove them. Figure 7 shows the evolution of the targeted byproducts during the ozonation experiments. As it can be seen, some byproducts were formed and reached their maximum concentration at an ozone dosage of approximately 0.5 mg O_3 L^{-1}, decreasing afterwards. Comparatively, the E2 byproducts, that persisted until an ozone dosage of 16 mg O_3 L^{-1}, were more recalcitrant than the E1 byproducts, that disappeared at a lower ozone dosage of 10 mg O_3 L^{-1}.

The E2 and E1 byproducts with *m/z* 287 and *m/z* 285, respectively, had the same behavior, being both completely removed (below equipment detection limits) after an ozone dosage of 4 mg O_3 L^{-1} (Figure 7). The E1 byproduct with *m/z* 275 and the E2 byproduct with *m/z* 277 remained longer in the solution, until the ozone dosage reached 10 and 16 mg O_3 L^{-1}, respectively.

(Figure 7)

Several authors that have investigated the estrogenicity of disinfected waters have observed estrogenic activity after water ozonation (Alum et al., 2004; Huber et al., 2004; Kim et al., 2004; Maniero et al., 2008), indicating the occurrence of byproducts and that these byproducts are also biologically active even after application of high ozone dosages. An increase in the concentration of ozone from 0.5 to 10 mg L^{-1} has been shown to reduce the estrogenicity of the original water by a factor of 200 to 1000 (Huber et al., 2004). This residual estrogenicity, although low, may still be relevant if we consider that water is just one of many possible routes of exposure to estrogenic compounds.

4. Conclusions

E2 and E1 have shown to be efficiently removed from water through ozonation, but to form each of them two major byproducts. These byproducts with MM 288 and 278, and 286 and 276, respectively, have been tentatively identified (the last three for the first time) by means of UPLC-QqTOF-MS/MS. E2 and E1 are believed to follow the same degradation pathway since their corresponding byproducts, alike them, differ in two mass units. The total elimination of these byproducts occurred only with high ozone dosages (10 mg and 16 mg O_3 L^{-1} for the E1 and E2 byproducts, respectively).

The ozonation byproducts can pose a risk not only to drinking water consumers but also to aquatic organisms that can become exposed to them through reclaimed water

having been subjected to ozonation or when ozone treated wastewater is released into the environment. Thus, for a proper evaluation of the risk associated to the use of ozonized water, the ecotoxicity and estrogenicity of the byproducts should be evaluated.

5. Acknowledgments

This work has been supported by the Spanish Ministry of Science and Innovation through the projects SCARCE (Consolider-Ingenio 2010 CSD2009-00065) and CEMAGUA (CGL2007-64551/HID). Renata Pereira thanks the CNPQ (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico) for the doctorate scholarship (process 200100/2009-2) and gratefully acknowledges FAPESP for its support (process 2008/070439) in the development of the project in Brazil. Merck (Darmstadt, Germany) is also acknowledged for the gift of LC columns.

6. References

Alum, A., Yoon, Y., Westerhoff, P., Abbaszadegan, M., 2004. Oxidation of bisphenol A, 17β-estradiol, and 17α-ethynyl estradiol and byproduct estrogenicity. Environ.Toxicol. 19, 257-264.

American Public Health Association/American Water Works Association/Water Environment Federation, 1998. Standard Methods for the Examination of Water and Wastewater. 20th ed., Washington DC, USA.

Auriol, M., Filali-Meknassi, Y., Tyagi, R.D., Adams, C.D., Surampalli, R.Y., 2006. Endocrine disrupting compounds removal from wastewater, a new challenge. Process Biochem. 41, 525-539.

Benotti, M.J., Stanford, B.D., Wert, E.C., Snyder, S.A., 2009a. Evaluation of a photocatalytic reactor membrane pilot system for the removal of pharmaceuticals and endocrine disrupting compounds from water. Water Res. 43, 1513-1522.

373 Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder,
374 S.A., 2009b. Pharmaceuticals and endocrine disrupting compouds in U.S. drinking
375 water. Environ. Sci. Technol. 43, 597-603.
376 Bila, D., Montalva, A.F., Azevedo, D.A., Dezotti, M., 2007. Estrogenic activity removal
377 of 17β-estradiol by ozonation and identification of by-products. Chemosphere 69, 736-
378 746.
379 Broséus, R., Vincent, S., Aboulfadl, K., Daneshvar, A., Sauvé, S., Barbeau, B., Prévost,
380 M., 2009. Ozone oxidation of pharmaceuticals, endocrine disruptors and pesticide
381 during drinking water treatment. Water Res. 43, 4707-4717.
382 Cargouet, M., Perdiz, D., Mouatassim-Souali, A., Tamisier-Karolak, S., Levi, Y., 2004.
383 Assessment of river contamination by estrogenic compounds in Paris area (France). Sci.
384 Total Environ. 324, 55-66.
385 Chang, H.S., 2009. The methods of identification, analysis, and removal of endocrine
386 disrupting compounds (EDCs) in water. J. hazard. mater. 172, 1-12.
387 Chen, C.-Y., Wen, T.-Y., Wang, G.-S., Cheng, H.-W., Lin, Y.-H., Lien, G.-W., 2007.
388 Determining estrogenic steroids in Taipei waters and removal in drinking water
389 treatment using highflow solid-phase extraction and liquid chromatography. Sci. Total
390 Environ. 378, 352-365.
391 D'Ascenzo, G., Di Corcia, A., Gentili, A., Mancini, R., Mastropasqua, R., Nazzari, M.,
392 Samperi, R., 2003. Fate of natural estrogen conjugates in municipal sewage transport
393 and treatment facilities. Sci.Total Environ. 302, 199-209.
394 Deborde, M., Rabouan, S., Duguet, J.P., Legube, B., 2005. Kinetics of aqueous ozone-
395 induced oxidation of some endocrine disrupters. Environ. Sci. Technol. 39, 6086-6092.
396 Esperanza, M., 2007. Fate of sex hormones in two pilot-scale municipal wastewater
397 treatment plants: conventional treatment. Chemosphere 66, 1535-1544.

European-Communities, 1998. Council directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal of the European Communities.

Gerolin, E.R.R., 2008. Occurrence and removal of endocrine disrupters in public supply water from Campinas and Sumaré - São Paulo. PhD thesis in Civil Engineering. Campinas State University, Campinas. Link to access: <http://cutter.unicamp.br/document/?code=000436059.

Hashimoto, T., Takahashi, K., Murakami, T., 2006. Characteristics of estrogen decomposition by ozonation. Water Sci. Technol. 54, 87-93.

Huber, M.M., Korhonen, S., Ternes, T.A., von Gunten, U., 2005. Oxidation of pharmaceuticals during water treatment with chlorine dioxide. Water Res. 39, 3607-3617.

Huber, M.M., Ternes, T.A., von Guten, U., 2004. Removal of estrogenic activity and formation of oxidation products during ozonation of 17α-ethynylestradiol. Environ. Sci. Technol. 38, 5177-5186.

Irmak, S., Erbatur, O., Akgerman, A., 2005. Degradation of 17β-estradiol and bisphenol A in aqueous medium by using ozone and ozone/UV techniques. J. Hazard. Mater. B 126, 54-62.

Kim, S.E., Yamada, H., Tsuno, H., 2004. Evaluation of estrogenicity for 17β-estradiol decomposition during ozonation. Ozone-Sci. Eng. 26, 563-571.

Komissarov, V., Zimin, Y., Khursan, S., 2006. On the mechanism of phenol ozonolysis. Kinet. Catal+ 47, 850-854.

Kuster, M., López de Alda, M.J., Hernando, M.D., Petrovic, M., Martin-Alonso, J., Barcelo, D., 2008. Analysis and occurrence of pharmaceuticals, estrogens, progestogens

422 and polar pesticides in sewage treatment plant effluents, river water and drinking water
423 in the Llobregat river basin (Barcelona, Spain). J. Hydrol. 358, 112-123.
424 Lin, Y., Peng, Z., Zhang, X., 2009. Ozonation of estrone, estradiol, diethylstilbestrol in
425 waters. Desalination 249, 235-240.
426 Lopes, L.G., Marchi, M.R.R., Souza, J.B.G., Moura, J.A., Lorenzon, C.S., Cruz, C.,
427 Amaral, L.A., 2010. Estrogens in natural and treated waters in the region of Jaboticabal
428 - São Paul, Brazil. Quim. Nova 33, 639-643.
429 Mai, J., Sun, W., Xiong, L., Liu, Y., Ni, J., 2008. Titanium dioxide mediated
430 photocatalytic degradation of 17β-estradiol in aqueous solution. Chemosphere 73, 600-
431 606.
432 Maniero, M.G., Bila, D.M., Dezotti, M., 2008. Degradation and estrogenic activity
433 removal of 17β-estradiol and 17α-ethinylestradiol by ozonation and O_3/H_2O_2. Sci. Total
434 Environ. 407, 105-115.
435 Mazellier, P., Méité, L., Laat, J.D., 2008. Photodegradation of the steroid hormones
436 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in dilute aqueous solution.
437 Chemosphere 73, 1216-1223.
438 Murugananthan, M., Yoshihara, S., Rakuma, T., Uehara, N., Shirakashi, T., 2007.
439 Electrochemical degradation of 17β-estradiol (E2) at boron-doped diamond (Si/BDD)
440 thin film electrode. Electrochim. Acta 52, 3242-3249.
441 Nakamura, H., Kuruto-Niwa, R., Uchida, M., Terao, Y., 2007. Formation of chlorinated
442 estrones via hypochlorous disinfection of wastewater effluent containing estrone.
443 Chemosphere 66, 1441-1448.
444 Ohko, Y., Iuchi, K., Niwa, C., Tatsum A, T., Nakashima, T., Iguchi, T., Kubota, Y.,
445 Fujishima, A., 2002. 17ß-Estradiol degradation by TiO_2 photocatalysis as a means of
446 reducing estrogenic activity. Environ. Sci. Technol. 36, 4175-4181.

447 Pereira, R.O., Postigo, C., de Alda, M.L, Daniel, L.A., Barceló, D., 2011. Removal of
448 estrogens through water disinfection processes and formation of by-products.
449 Chemosphere 82, 789-799.
450 Richardson, S., 2003. Disinfection by-products and other emerging contaminants in
451 drinking water. TRAC-Trends Anal. Chem. 22, 666-684.
452 Rodriquez-Mozaz, S., de Alda, M.J.L., Barceló, D., 2004a. Monitoring of estrogens,
453 pesticides and bisphenol A in natural waters and drinking water treatment plants by
454 solid-phase extraction-liquid chromatography-mass spectrometry. J. Chromatogr. A
455 1045, 85-92.
456 Rodriquez-Mozaz, S., de Alda, M.J.L., Barceló, D., 2004b. Picogram per liter level
457 determination of estrogens in natural water and waterworks by a fully automated on-line
458 solid-phase extraction-liquid chromatography-electrospray tandem mass Spectrometry
459 method. Anal. Chem. 76, 6998-7006.
460 Sodre, F.F., Locatelli, M.A.F., Jardim, W.F., 2010. Occurrence of emerging
461 contaminants in brazilian drinking waters: a sewage-to-tap issue. Water Air Soil Poll.
462 206, 57-67.
463 Suri, R.P.S., Nayak, M., Devaiah, U., Helmig, E., 2007. Ultrasound assisted destruction
464 of estrogen hormones in aqueous solution: effect of power density, power intensity and
465 reactor configuration. J. Hazard. Mater. 146, 472-478.
466 Ternes, T.A., Stumpf, U.M., Mueller, J., Haberer, K., Wilken, R.-D., Servos, M., 1999
467 Behavior and occurrence of estrogens in municipal sewage treatment plants - I.
468 Investigations in Germany, Canada and Brazil. Sci. Total Environ., 225, 81-90.
469 USEPA, 2009. National Primary Drinking Water Regulations.

Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. Environ. Sci. Technol. 39, 6649-6663.

Yaping, Z., Jiangyong, H., 2008. Photo-Fenton degradation of 17β-estradiol in presence of α-FeOOHR and H_2O_2. Appl. Catal. B-Environ. 78, 250-258.

Ying, G.-G., Kookana, R.S., Kumar, A., Mortimer, M., 2009. Occurrence and implications of estrogens and xenoestrogens in sewage effluents and receiving waters from South East Queensland. Sci. Total Environ. 407, 5147-5155.

Yoon, Y., Ryu, J., Oh, J., Choi, B.-G., Snyder, S.A., 2010. Occurrence of endocrine disrupting compounds, pharmaceuticals, and personal care products in the Han River (Seoul, South Korea). Sci. Total Environ. 408, 636-643.

Zhang, Y., Zhou, J.L., Ning, B., 2007. Photodegradation of estrone and 17β-estradiol in water. Water Res. 41, 19-26.

Zhao, Y., Hu, J., Jin, W., 2008. Transformation of oxidation products and reduction of estrogenic activity of 17β-estradiol by a heterogeneous photo-Fenton reaction. Environ. Sci. Technol. 42, 5277-5284.

***Highlights**

17β-Estradiol and estrona in wastewater treatment → Surface water or reuse of water → drinking water→ oxidative processes: ozone → identification of some byproducts and its persistency

Identification of new ozonation disinfection byproducts of 17β-estradiol and estrone in water

Renata de Oliveira Pereira [a c 1], Miren Lopez de Alda[a], Jesús Joglar[b], Luiz Antonio Daniel[c], Damià Barceló[a,d]

[a] Institute of Environmental Assessment and Water Research, IDAEA-CSIC, C/ Jordi Girona 18-26, 08034, Barcelona, Spain.

[b] Department of Biological Chemistry and Molecular Modelling, Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), C/ Jordi Girona 18-26, 08034, Barcelona, Spain

[c] São Carlos Engineering School, São Paulo University, Av. Trabalhador São-carlense, 400, 13566-590, São Carlos, SP, Brazil

[d] Catalan Institute for Water Research (ICRA), C/ Emili Grahit 101, Edifici H_2O, Parc Científic i Tecnologíc de la Universitat de Girona, 17003, Girona, Spain.

[1] Corresponding author, permanent address - Department of Hydraulics and Sanitation of the São Carlos Engineering School - São Paulo University, Campus de São Carlos, Av. Trabalhador São-carlense, 400, Arnold Schimidt, São Carlos - São Paulo - Brazil - 13566-590 tel: 055-32-32323808, *e-mail: p*ereira.or@gmail.com.

Abstract

Estrogens are a class of micro-pollutants found in water at low concentrations (in the ng L^{-1} range), but often sufficient to exert estrogenic effects due to their high estrogenic potency. Disinfection of waters containing estrogens through oxidative processes has been shown to lead to the formation of disinfection byproducts, which may also be estrogenic. The present work investigates the formation of disinfection byproducts of 17β-estradiol (E2) and estrone (E1) in the treatment of water with ozone. Experiments have been carried out at two different concentrations of the estrogens in ground water (100 ng L^{-1} and 100 μg L^{-1}) and at varying ozone dosages (0-30 mg L^{-1}). Detection of the estrogens and their disinfection byproducts in the water samples has been performed by means of ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with a triple quadrupole (QqQ) and a quadrupole-time of flight (QqTOF) instrument. Both E2 and E1 have been found to form two main byproducts, with molecular mass (MM) 288 and 278 in the case of E2, and 286 and 276 in the case of E1, following presumably the same reaction pathways. The E2 byproduct with MM 288 has been identified as 10epsilon-17beta-dihydroxy-1,4-estradieno-3-one (DEO), in agreement with previously published results. The molecular structures and the formation pathways of the other three newly identified byproducts have been suggested. These byproducts have been found to be formed at both high and low concentrations of the estrogens and to be persistent even after application of high ozone dosages.

Keywords: estrogen; drinking water; oxidation process; disinfection byproduct.

1. Introduction

Ozone can be used in the drinking water process, since it is a powerful oxidant and disinfectant. However, it can form disinfection byproducts through oxidation with inorganic and organic compounds (Richardson, 2003).

The presence of some ozonation disinfection byproducts (DBPs), like bromate, trihalomethanes (THMs) and haloacetic acids (HAAs), is regulated in drinking water to control and minimize human exposure to these hazardous compounds (USEPA; European-Communities, 1998). However, there are potentially many more ozonation DBPs, and until 2003 only less than 50% of the assimilable organic carbon (AOC) formed during ozonation of water has been characterized (Richardson, 2003).

Compounds such as 17β-estradiol (E2) and estrone (E1), which have endocrine disrupting properties, are often found in surface water (Kuster et al., 2008; Yoon et al., 2010). If present in the water used as source for abstraction of drinking water these micro-pollutants can react with ozone forming disinfection byproducts that can pose a potential risk to the population served with this water.

Sewage treatment effluents are one of the main sources of estrogens in the aquatic environment. The extent of their degradation during sewage treatment and their subsequent load into the receiving natural waters is variable, depending on the type of treatment applied, the hydraulic residence time, the sludge retention time, and other operational factors (Auriol, 2006; Cargouet et al., 2004; Esperanza, 2007). Furthermore, since estrogens are female sex hormones naturally produced by vertebrates, non-point sources, especially in rural areas with intense farming activities, may also be relevant (Ying et al., 2009).

Of the various natural and synthetic free estrogens E1 and E2 are the most frequently detected. Their mean/median effluent concentrations in wastewater are

situated in the range of 20–55 ng L^{-1} for E1 and 9–20 ng L^{-1} for E2 (D'Ascenzo et al., 2003). Meanwhile, in surface water the concentrations reported for these two estrogens in the last years have ranged between 0.7 and 143 ng L^{-1} for E1 (Rodriquez-Mozaz et al., 2004a, b; Ying et al., 2009) and between 1.4 and 34 ng L^{-1} for E2 (Chen et al., 2007; Ying et al., 2009).

After entering into the drinking water treatment process E1 and E2, alike other estrogens and endocrine disrupting compounds (EDCs), are usually gradually and efficiently, although not necessarily completely, removed (Chang, 2009; Chen et al., 2007). Thus, E2 and E1 have been detected, for instance, in finished drinking water from Brazil at concentrations up to 6.8 ng L^{-1} and 0.1 ng L^{-1}, respectively (Gerolin, 2008, Lopes et al., 2010; Sodre et al., 2010). However, in general, the treatments applied in drinking water treatment plants (DWTPs) are successful at removing estrogens, i.e., at eliminating the parent compounds (although this does not necessarily imply full elimination by e.g. mineralization), and oxidation by ozone and chlorine are among the most effective and extensively used processes (Benotti et al., 2009b). Other oxidants, apart from ozone and chlorine (Alum et al., 2004; Bila et al., 2007; Broséus et al., 2009; Deborde et al., 2005; Hashimoto et al., 2006; Kim et al., 2004; Lin et al., 2009; Maniero et al., 2008; Westerhoff et al., 2005), that have been studied for the removal of estrogens (parent compound) from water have been chlorine dioxide (Huber et al., 2005), photolysis (Mazellier et al., 2008), photo-Fenton (Yaping and Jiangyong, 2008; Zhao et al., 2008), titanium dioxide photocatalysis (TiO_2/UV) (Benotti et al., 2009a; Ohko et al., 2002; Zhang et al., 2007), ferrate (Lin et al., 2009), electrochemical degradation (Murugananthan et al., 2007) and sonolysis (Suri et al., 2007). In comparison with most of these oxidants, ozone presents the advantage of having high rate constants (Huber et al., 2005) and being a powerful disinfectant.

Typical doses of ozone (< 2 mg L^{-1}) applied during treatment of water containing E1 and E2 have reached 96 to 99% removal of the parent compound (Alum et al., 2004; Bila et al., 2007; Broséus et al., 2009; Kim et al., 2004; Maniero et al., 2008;Westerhoff et al., 2005). However, some works have reported the formation of byproducts after the removal of E1 and E2 (Pereira et al., 2011). In this context, the objective of this work was to investigate the formation of E1 and E2 byproducts during ozonation, to try to identify them, and to estimate the dose of ozone necessary for the complete removal of these byproducts.

2. Materials and Methods

2.1. Reagents and solutions

HPLC-grade acetone, methanol and water were supplied by Merck (Darmstadt, Germany). Pure standards of 17β-estradiol (E2), estrone (E1), 6α-hydroxyestradiol, 4-hydroxyestradiol, and 2-hydroxyestradiol were purchased from Sigma Aldrich (Steinheim, Germany). Individual stock solutions of the analytes were prepared at 100 mg L^{-1} by dissolving 10 mg of each compound in 100 mL of methanol. Estrogens in methanol solutions stored in the dark at 4°C are stable for at least 6 months. The solutions at 100 μg L^{-1} and 100 ng L^{-1} were freshly prepared before every batch of experiments by appropriate dilution of the individual stock solutions in ground water.

Ground water from the University of São Paulo (São Carlos Campus, Brazil) collected before chlorination was used to carry out the assays. After collection it was stored at 4 ºC until use. This water source was chosen because of the similarity of its characteristics with those of drinking water, with the required quality in terms of average turbidity (0.4 ± 0.2 NTU) and average color (1.0 ± 1.5 Pt-Co units).

2.2. Ozonation experiments

The ozonation experiments were carried out in a pilot scale experimental unit (Supplementary Information - 1). The ozonation reactor had a total height of 3.5 m, a working height of 3.0 m, an external diameter of 110 mm and a volume of 28.5 L. The ozone generator (EAGLESAT®, Brazil) was a corona discharge type and had an oxygen generation unit (PSA method - Pressure Swing Adsorption) and a compressor to generate ozone. The production of ozone in the gas phase was measured with an ozone analyzer (IN USA, ASX-Mod H1) coupled to a rotameter. The ozone was applied to the base of the reactor using a porous diffuser and the off-gas was captured in a flask containing potassium iodide solution at 2% (w/w). The total ozone consumed was calculated according to equation 1.

$$CO = AO - OF - RO \quad (1)$$

Where, CO is the consumed ozone (mg L^{-1}), AO is the applied ozone (mg L^{-1}), OF is the ozone in the off-gas (mg L^{-1}), and RO is the residual ozone in the liquid phase (mg L^{-1}). Therefore, the concentration of ozone expressed in the present work is the ozone consumed during the reaction time.

Experiments were performed at two different initial concentrations of E2 and E1 (100 µg L^{-1} and 100 ng L^{-1}), in separated batches for each estrogen and one treatment was done for each dose of ozone (batch experiments). The experiments at 100 µg L^{-1} were conducted first. The objective in this case was to identify the main byproducts formed during ozonation. Then the experiments at 100 ng L^{-1} were performed in order to track and confirm the presence of the previously identified byproducts but at concentrations closer to those found in real samples (surface water). In all cases the dose of ozone applied ranged from 0.4 to 28 mg L^{-1}, the temperature was set at 19 ± 2ºC and the natural pH of the ground water at the beginning of the treatments was 7.0 ± 0.3.

After the ozone application, aliquots of 200 mL were withdrawn from the reactor and the residual ozone in the water samples was measured by indigo colorimetric method 4500-O_3 B (APHA, 1998) immediately after the assays.

2.3. Analysis

2.3.1 Sample preparation

Samples (200 mL) were preconcentrated by solid phase extraction (SPE) with C18 cartridges (Accubond, 500 mg) from Agilent Technologies (Santa Clara, CA, USA) following a procedure adapted from Ternes et al. (1999). After sample loading the cartridges were rinsed with 10 mL of HPLC water and eluted with 4 mL of acetone. Main differences with respect to the method described by Ternes et al. (1999) refer to the sample volume (200 mL *vs* 1 L) and the performance of a washing step of the cartridge prior to elution. The extracts obtained were then blown down to dryness under nitrogen, reconstituted with methanol to a final volume of 0.5 mL, and stored in the dark at 4°C for subsequent LC–MS/MS analysis. Under these conditions both E1 and E2 are stable for at least 60 days (recovery percentage ± relative standard deviation equal to 96 ± 1% and 102 ± 5% for E1 and E2, respectively).

2.3.2. Identification of byproducts by UPLC-QqToF-MS/MS

Identification of E1 and E2 ozonation byproducts in the water samples extracts coming from the experiments performed at 100 μg L^{-1} (see section 2.2) was carried out by UPLC-QqTOF-MS/MS on a Waters Acquity UPLC system (Waters Corp., Mildford, MA) equipped with a binary solvent delivery system and an autosampler coupled to a Waters QqToF-Micro MS/MS detector. Chromatographic separation was performed on a Hibar HR Purospher STAR RP-18 column (30 x 2.1 mm, 2 μm particle size) from Merck (Darmstadt, Germany). The column temperature was set to 35 ºC and the samples compartment temperature was kept to 10 ºC. The injection volume was 5 μL

and the column flow rate 0.4 mL min^{-1}. The mobile phase consisted of A (acetonitrile) and B (water). The gradient started with 10% A, composition that was kept constant for 2 min, then linearly increased to 25% A over 2 minutes, kept constant for 1 min, linearly increased to 50% A over 1 min, kept constant for 2 min, raised to 100% A in 2 min, maintain constant for 1 min, and decreased to 10% A within 1 minute. Total run time, including reequilibration of the column to the initial conditions, was 12 minutes.

Detection was performed with an electrospray (ESI) interface in the negative ionization (NI) and with the capillary voltage set to 2800 V and cone voltages varying between 10 and 50 V. The nebulization gas (nitrogen) was set to 500 L h^{-1} at a temperature of 450 ^{o}C; the cone gas (nitrogen) was set to 30 L h^{-1}, and the source temperature to 150 ^{o}C. For MS experiments, the instrument was operated in a wide pass quadrupole mode with the TOF data being collected between *m*/*z* 50 and 600 and low collision energy (CE) of 4 eV. The product ion MS/MS experiments were performed at variable CE (10–50 eV). All analyses were performed using an independent reference spray (lockSpray) to ensure accuracy and reproducibility. Val-Tyr-Val was used as lock mass (*m*/*z* 380.2029) for internal mass calibration.

Instrument control, data acquisition, and evaluation were done by means of MassLynx V4.1 software (Waters Corp.). This software (Waters Corp.) calculates all possible elemental compositions for a given accurate mass and is thus a potent tool for hypothesizing possible structures of unknown compounds. Final identification can then be performed based on the accurate mass measurements of the parent ions and fragments obtained in MS/MS experiments.

2.3.3. Analysis of byproducts by UPLC-QqQ-MS/MS

Analysis of the previously identified byproducts in the water extracts coming from the experiments performed at low initial concentration of estrogens (100 ng L^{-1}) was

carried out by UPLC-QqQ-MS/MS on a Waters Acquity UPLC system coupled to a Waters TQD MS/MS detector. Chromatographic conditions were the same as described above for UPLC-QqToF-MS/MS analysis except for the injection volume which was set to 8 μL to increase sensitivity. MS/MS detection was performed in the selected reaction monitoring (SRM) mode using ESI in the NI mode and basically the same MS/MS conditions described above: capillary voltage, 2800 V; nebulization gas flow rate, 650 L h^{-1}; desolvation temperature, 450 ºC; cone gas flow rate, 30 L h^{-1}; source temperature, 150 ºC. SRM transitions and the cone and collision voltages selected for the monitoring of the previously identified byproducts is showed in Supplementary Information - 2. Optimization of these conditions was performed with the samples coming from the high concentration experiments conducted before. Instrument control and data acquisition and evaluation were done with the software MassLynx V4.1.

3. Results and Discussion

Figure 1 shows the disappearance of E1 and E2 during ozonation in the experiments conducted with initial concentration of 100 μg L^{-1} of the estrogens in water. As it can be seen, both estrogens vanish rapidly, although the reaction of ozone with E1 is slightly faster than with E2. Parallel to the disappearance of the estrogens, a number of other new peaks appear in the UPLC-QqTOF-MS chromatograms obtained throughout the experiments. The *m/z* ratios and the corresponding retention times of the major peaks identified in the degradation of E1 and E2 are depicted in Figure 2. The main byproducts of E1 (with nominal *m/z* ratios 285, 275, 301, and 305) are similar to those of E2 (with nominal *m/z* ratios 287, 277, 303, and 307) with a difference of two mass units between them, which coincides with the difference of two mass units also in the molecular mass (MM) of the parent compounds (270.36608 g/mol for E1 and 272.38196 g/mol for E2). Since this difference is due to the presence of a ketone group

224 in position 17 of the molecular structure of E1 and a hydroxyl group in the same
225 position of the E2 molecule, it is clear that ozone attacks the aromatic moiety, where the
226 high electronic densities on the carbons located in the ortho and para positions favor the
227 process (Figure 1).

228 **(Figure 1)**

229 In both cases the most abundant and persistent byproducts are the ones with lower
230 *m/z* ratios: 285 and 275 for E1, and 287 and 277 for E2. The other byproducts, with *m/z*
231 301 and 305 in the case of E1, and *m/z* 303 and 307 in the case of E2, are formed in
232 much less quantity and are therefore not considered further for discussion.

233 **(Figure 2)**

234 Figure 2A shows that the E1 byproduct with *m/z* 285 is fairly stable until an ozone
235 dosage of 12 mg O_3 L^{-1}, decreasing thereafter but not disappearing. Meanwhile the
236 compound with *m/z* 275 and retention time 4.27 min rises until the ozone dosage
237 reaches 16.7 mg O_3 L^{-1} and its concentration keeps fairly constant afterwards. As it is
238 shown in Figure 2B, the E2 byproducts show a rather similar behavior, except for the
239 fact that the byproduct with *m/z* 287 starts to decrease at a lower ozone dosage, 4.0 mg
240 O_3 L^{-1}, than the E1 byproduct with *m/z* 285.

241 The elemental compositions proposed by the Masslynx software for the
242 experimental accurate masses measured by the QqTOF-MS/MS detector, after applying
243 basic organic chemical rules to eliminate nonfeasible formulae and possible degradation
244 scenarios for the parent compounds, were $C_{18}H_{23}O_3$ for *m/z* 287 and $C_{16}H_{21}O_4$ for *m/z*
245 277. However, considering the structure of E2 and that the most probable attack occurs
246 at the aromatic ring (high electronic densities) there are more than one possible structure
247 for *m/z* 287 or, in other words, with mass 288. The structures proposed so far in the
248 scientific literature for E2 disinfection byproducts with molecular mass 288 have been:

2-hydroxyestradiol (2OH-E2), identified by both Bila et al. (2007) and Maniero et al. (2008), monohydroxylated E2 (Irmak et al., 2005), 10e-17b-dihydroxy-1,4-estradieno-3-one (DEO) (Bila et al., 2007) and testosterone (Bila et al., 2007; Maniero et al., 2008) in ozonation experiments. In experiments performed with other types of oxidation processes such as, photo-Fenton, photolysis, or TiO_2 photocatalysis the compounds previously identified were: 6-hydroxyestradiol (6OH-E2) (Mazellier et al., 2008; Zhao et al., 2008), again 2OH-E2 , testosterone (Ohko et al., 2002; Zhao et al., 2008) and DEO (Mai et al., 2008; Zhao et al., 2008). The E2 byproduct with mass 278 (*m/z* 277) has never been reported in the literature.

Trying to elucidate the structure of the byproduct with mass 288 the standards that were available (2OH-E2, 4OH-E2, and 6OH-E2) were acquired and analyzed. However, as it can be seen in Figure 3 the retention times of the standards 2OH-E2 (5.69 min) and 4OH-E2 (5.79 min) were very different from the retention time of the E2 byproducts with MM 288 (4.05 and 4.36 min) detected in our ozonation experiments (16.7 mg O_3 L^{-1}). On the contrary the retention time of the standard 6OH-E2 (3.99 min) was quite similar, but when the molecules were fragmented their fragments were totally different from each other, indicating that 6OH-E2 is not the targeted byproduct. The standard of estriol (E3), which also has a MM of 288, was tested as well, but the LC-MS data did not fit either. Apart from these there are other structure possibilities for MM 288, but these structures do not have their corresponding counterparts for MM 286 when the same degradation mechanism is applied to the E1 molecule. The compound testosterone was also disregarded because its formulae ($C_{19}H_{28}O_2$) contains only two oxygens (instead of three). Thus the byproduct with MM 288 is expected to be the compound named DEO. DEO was suggested by Ohko et al. in 2002 as byproduct in the mechanism of degradation of E2 by TiO_2 photocatalysis (Ohko et al., 2002) and further confirmed

by Mai et al. in 2008 in similar oxidative processes by LC-MS/MS analysis (Mai et al., 2008). Furthermore, the fragments observed by Mai et al. (2008) were similar to those found in the present study.

(Figure 3)

The fragmentation of the E2 and E1 byproducts with *m/z* 287 and *m/z* 285, respectively, is shown in Figure 4. The part marked in the square is virtually identical, i.e., presents the same fragment ions, in both compounds, whereas the circled part shows a similar fragmentation pattern but with a difference of two mass units. Based on these spectra and on previously published works (Bila et al., 2007; Mai et al., 2008; Ohko et al., 2002; Zhao et al., 2008) the E2 byproduct with *m/z* 287 is believed to be DEO, and the main E1 byproduct with *m/z* 285 is believed to correspond to a similar compound but with a ketone group instead of a hydroxyl group in position 17, i.e. to 10-hydroxy-1,4-estradieno-3,17-dione (HEDO).

(Figure 4)

The formation pathway of DEO from E2 has already been suggested by Mai et al. (2008) and according to it the formation pathway of the above suggested E1 byproduct would be the same.

It may be worth mentioning that the pH of the samples (measured at the beginning and at the end of the experiments) decreased as the formation of the byproducts increased (following the loss of H^+ and the addition of OH in the molecule) and vice versa, as it is shown in Supplementary Information - 3 for the byproduct with *m/z* 285.

For the E2 and E1 byproducts with *m/z* 277 and *m/z* 275, respectively, the pathway of degradation based on the mechanism of phenol ozonolysis (Komissarov et al., 2006) is suggested. This pathway is illustrated in Figure 5 for E2. The pathway for E1 would be similar but maintaining the ketone group that is present in position 17 in the original

E1 structure. Figure 6 shows the product ion mass spectra and the purported fragmentation of the byproducts with *m/z* 277 and *m/z* 275 that support the proposed structures.

The finding of these byproducts, not reported previously by other authors, could be due to the use of different equipments and/or experimental conditions. In the works performed by Bila et al. (2007) and Maniero et al. (2008) the ozone doses and the initial concentration of E2 were comparable to those used in the present study but the identification of byproducts was carried out by GC/MS. In the work conducted by Irmak et al. (2005) the detection of byproducts was performed by LC-MS/MS but the conditions of the experiment were very different from ours with higher initial estrogen concentrations and unclear doses of ozone consumed. Another possible difference could be the reactive form triggering the process in each work, which in our study, conducted at pH around 7, would be basically molecular ozone and in other studies OH radicals.

(Figure 5)

(Figure 6)

The ozonation experiments carried out at lower initial concentrations of the estrogens (100 ng L^{-1}) were conducted with the double objective of, first, confirming the formation of the previously identified main byproducts at concentrations in water closer to those found in the environment and, secondly, determining the ozone dosage that would have to be applied to remove them. Figure 7 shows the evolution of the targeted byproducts during the ozonation experiments. As it can be seen, some byproducts were formed and reached their maximum concentration at an ozone dosage of approximately 0.5 mg O_3 L^{-1}, decreasing afterwards. Comparatively, the E2 byproducts, that persisted until an ozone dosage of 16 mg O_3 L^{-1}, were more recalcitrant than the E1 byproducts, that disappeared at a lower ozone dosage of 10 mg O_3 L^{-1}.

The E2 and E1 byproducts with *m/z* 287 and *m/z* 285, respectively, had the same behavior, being both completely removed (below equipment detection limits) after an ozone dosage of 4 mg O_3 L^{-1} (Figure 7). The E1 byproduct with *m/z* 275 and the E2 byproduct with *m/z* 277 remained longer in the solution, until the ozone dosage reached 10 and 16 mg O_3 L^{-1}, respectively.

(Figure 7)

Several authors that have investigated the estrogenicity of disinfected waters have observed estrogenic activity after water ozonation (Alum et al., 2004; Huber et al., 2004; Kim et al., 2004; Maniero et al., 2008), indicating the occurrence of byproducts and that these byproducts are also biologically active even after application of high ozone dosages. An increase in the concentration of ozone from 0.5 to 10 mg L^{-1} has been shown to reduce the estrogenicity of the original water by a factor of 200 to 1000 (Huber et al., 2004). This residual estrogenicity, although low, may still be relevant if we consider that water is just one of many possible routes of exposure to estrogenic compounds.

4. Conclusions

E2 and E1 have shown to be efficiently removed from water through ozonation, but to form each of them two major byproducts. These byproducts with MM 288 and 278, and 286 and 276, respectively, have been tentatively identified (the last three for the first time) by means of UPLC-QqTOF-MS/MS. E2 and E1 are believed to follow the same degradation pathway since their corresponding byproducts, alike them, differ in two mass units. The total elimination of these byproducts occurred only with high ozone dosages (10 mg and 16 mg O_3 L^{-1} for the E1 and E2 byproducts, respectively).

The ozonation byproducts can pose a risk not only to drinking water consumers but also to aquatic organisms that can become exposed to them through reclaimed water

having been subjected to ozonation or when ozone treated wastewater is released into the environment. Thus, for a proper evaluation of the risk associated to the use of ozonized water, the ecotoxicity and estrogenicity of the byproducts should be evaluated.

5. Acknowledgments

This work has been supported by the Spanish Ministry of Science and Innovation through the projects SCARCE (Consolider-Ingenio 2010 CSD2009-00065) and CEMAGUA (CGL2007-64551/HID). Renata Pereira thanks the CNPQ (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico) for the doctorate scholarship (process 200100/2009-2) and gratefully acknowledges FAPESP for its support (process 2008/070439) in the development of the project in Brazil. Merck (Darmstadt, Germany) is also acknowledged for the gift of LC columns.

6. References

Alum, A., Yoon, Y., Westerhoff, P., Abbaszadegan, M., 2004. Oxidation of bisphenol A, 17β-estradiol, and 17α-ethynyl estradiol and byproduct estrogenicity. Environ.Toxicol. 19, 257-264.

American Public Health Association/American Water Works Association/Water Environment Federation, 1998. Standard Methods for the Examination of Water and Wastewater. 20th ed., Washington DC, USA.

Auriol, M., Filali-Meknassi, Y., Tyagi, R.D., Adams, C.D., Surampalli, R.Y., 2006. Endocrine disrupting compounds removal from wastewater, a new challenge. Process Biochem. 41, 525-539.

Benotti, M.J., Stanford, B.D., Wert, E.C., Snyder, S.A., 2009a. Evaluation of a photocatalytic reactor membrane pilot system for the removal of pharmaceuticals and endocrine disrupting compounds from water. Water Res. 43, 1513-1522.

373 Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder,
374 S.A., 2009b. Pharmaceuticals and endocrine disrupting compouds in U.S. drinking
375 water. Environ. Sci. Technol. 43, 597-603.
376 Bila, D., Montalva, A.F., Azevedo, D.A., Dezotti, M., 2007. Estrogenic activity removal
377 of 17β-estradiol by ozonation and identification of by-products. Chemosphere 69, 736-
378 746.
379 Broséus, R., Vincent, S., Aboulfadl, K., Daneshvar, A., Sauvé, S., Barbeau, B., Prévost,
380 M., 2009. Ozone oxidation of pharmaceuticals, endocrine disruptors and pesticide
381 during drinking water treatment. Water Res. 43, 4707-4717.
382 Cargouet, M., Perdiz, D., Mouatassim-Souali, A., Tamisier-Karolak, S., Levi, Y., 2004.
383 Assessment of river contamination by estrogenic compounds in Paris area (France). Sci.
384 Total Environ. 324, 55-66.
385 Chang, H.S., 2009. The methods of identification, analysis, and removal of endocrine
386 disrupting compounds (EDCs) in water. J. hazard. mater. 172, 1-12.
387 Chen, C.-Y., Wen, T.-Y., Wang, G.-S., Cheng, H.-W., Lin, Y.-H., Lien, G.-W., 2007.
388 Determining estrogenic steroids in Taipei waters and removal in drinking water
389 treatment using highflow solid-phase extraction and liquid chromatography. Sci. Total
390 Environ. 378, 352-365.
391 D'Ascenzo, G., Di Corcia, A., Gentili, A., Mancini, R., Mastropasqua, R., Nazzari, M.,
392 Samperi, R., 2003. Fate of natural estrogen conjugates in municipal sewage transport
393 and treatment facilities. Sci.Total Environ. 302, 199-209.
394 Deborde, M., Rabouan, S., Duguet, J.P., Legube, B., 2005. Kinetics of aqueous ozone-
395 induced oxidation of some endocrine disrupters. Environ. Sci. Technol. 39, 6086-6092.
396 Esperanza, M., 2007. Fate of sex hormones in two pilot-scale municipal wastewater
397 treatment plants: conventional treatment. Chemosphere 66, 1535-1544.

398 European-Communities, 1998. Council directive 98/83/EC of 3 November 1998 on the
399 quality of water intended for human consumption. Official Journal of the European
400 Communities.
401 Gerolin, E.R.R., 2008. Occurrence and removal of endocrine disrupters in public supply
402 water from Campinas and Sumaré - São Paulo. PhD thesis in Civil Engineering.
403 Campinas State University, Campinas. Link to access:
404 <http://cutter.unicamp.br/document/?code=000436059.
405 Hashimoto, T., Takahashi, K., Murakami, T., 2006. Characteristics of estrogen
406 decomposition by ozonation. Water Sci. Technol. 54, 87-93.
407 Huber, M.M., Korhonen, S., Ternes, T.A., von Gunten, U., 2005. Oxidation of
408 pharmaceuticals during water treatment with chlorine dioxide. Water Res. 39, 3607-
409 3617.
410 Huber, M.M., Ternes, T.A., von Guten, U., 2004. Removal of estrogenic activity and
411 formation of oxidation products during ozonation of 17α-ethynylestradiol. Environ. Sci.
412 Technol. 38, 5177-5186.
413 Irmak, S., Erbatur, O., Akgerman, A., 2005. Degradation of 17β-estradiol and bisphenol
414 A in aqueous medium by using ozone and ozone/UV techniques. J. Hazard. Mater. B
415 126, 54-62.
416 Kim, S.E., Yamada, H., Tsuno, H., 2004. Evaluation of estrogenicity for 17β-estradiol
417 decomposition during ozonation. Ozone-Sci. Eng. 26, 563-571.
418 Komissarov, V., Zimin, Y., Khursan, S., 2006. On the mechanism of phenol ozonolysis.
419 Kinet. Catal+ 47, 850-854.
420 Kuster, M., López de Alda, M.J., Hernando, M.D., Petrovic, M., Martin-Alonso, J.,
421 Barcelo, D., 2008. Analysis and occurrence of pharmaceuticals, estrogens, progestogens

422 and polar pesticides in sewage treatment plant effluents, river water and drinking water
423 in the Llobregat river basin (Barcelona, Spain). J. Hydrol. 358, 112-123.
424 Lin, Y., Peng, Z., Zhang, X., 2009. Ozonation of estrone, estradiol, diethylstilbestrol in
425 waters. Desalination 249, 235-240.
426 Lopes, L.G., Marchi, M.R.R., Souza, J.B.G., Moura, J.A., Lorenzon, C.S., Cruz, C.,
427 Amaral, L.A., 2010. Estrogens in natural and treated waters in the region of Jaboticabal
428 - São Paul, Brazil. Quim. Nova 33, 639-643.
429 Mai, J., Sun, W., Xiong, L., Liu, Y., Ni, J., 2008. Titanium dioxide mediated
430 photocatalytic degradation of 17β-estradiol in aqueous solution. Chemosphere 73, 600-
431 606.
432 Maniero, M.G., Bila, D.M., Dezotti, M., 2008. Degradation and estrogenic activity
433 removal of 17β-estradiol and 17α-ethinylestradiol by ozonation and O_3/H_2O_2. Sci. Total
434 Environ. 407, 105-115.
435 Mazellier, P., Méité, L., Laat, J.D., 2008. Photodegradation of the steroid hormones
436 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in dilute aqueous solution.
437 Chemosphere 73, 1216-1223.
438 Murugananthan, M., Yoshihara, S., Rakuma, T., Uehara, N., Shirakashi, T., 2007.
439 Electrochemical degradation of 17β-estradiol (E2) at boron-doped diamond (Si/BDD)
440 thin film electrode. Electrochim. Acta 52, 3242-3249.
441 Nakamura, H., Kuruto-Niwa, R., Uchida, M., Terao, Y., 2007. Formation of chlorinated
442 estrones via hypochlorous disinfection of wastewater effluent containing estrone.
443 Chemosphere 66, 1441-1448.
444 Ohko, Y., Iuchi, K., Niwa, C., Tatsum A, T., Nakashima, T., Iguchi, T., Kubota, Y.,
445 Fujishima, A., 2002. 17ß-Estradiol degradation by TiO_2 photocatalysis as a means of
446 reducing estrogenic activity. Environ. Sci. Technol. 36, 4175-4181.

447 Pereira, R.O., Postigo, C., de Alda, M.L, Daniel, L.A., Barceló, D., 2011. Removal of
448 estrogens through water disinfection processes and formation of by-products.
449 Chemosphere 82, 789-799.
450 Richardson, S., 2003. Disinfection by-products and other emerging contaminants in
451 drinking water. TRAC-Trends Anal. Chem. 22, 666-684.
452 Rodriquez-Mozaz, S., de Alda, M.J.L., Barceló, D., 2004a. Monitoring of estrogens,
453 pesticides and bisphenol A in natural waters and drinking water treatment plants by
454 solid-phase extraction-liquid chromatography-mass spectrometry. J. Chromatogr. A
455 1045, 85-92.
456 Rodriquez-Mozaz, S., de Alda, M.J.L., Barceló, D., 2004b. Picogram per liter level
457 determination of estrogens in natural water and waterworks by a fully automated on-line
458 solid-phase extraction-liquid chromatography-electrospray tandem mass Spectrometry
459 method. Anal. Chem. 76, 6998-7006.
460 Sodre, F.F., Locatelli, M.A.F., Jardim, W.F., 2010. Occurrence of emerging
461 contaminants in brazilian drinking waters: a sewage-to-tap issue. Water Air Soil Poll.
462 206, 57-67.
463 Suri, R.P.S., Nayak, M., Devaiah, U., Helmig, E., 2007. Ultrasound assisted destruction
464 of estrogen hormones in aqueous solution: effect of power density, power intensity and
465 reactor configuration. J. Hazard. Mater. 146, 472-478.
466 Ternes, T.A., Stumpf, U.M., Mueller, J., Haberer, K., Wilken, R.-D., Servos, M., 1999
467 Behavior and occurrence of estrogens in municipal sewage treatment plants - I.
468 Investigations in Germany, Canada and Brazil. Sci. Total Environ., 225, 81-90.
469 USEPA, 2009. National Primary Drinking Water Regulations.

Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. Environ. Sci. Technol. 39, 6649-6663.

Yaping, Z., Jiangyong, H., 2008. Photo-Fenton degradation of 17β-estradiol in presence of α-FeOOHR and H_2O_2. Appl. Catal. B-Environ. 78, 250-258.

Ying, G.-G., Kookana, R.S., Kumar, A., Mortimer, M., 2009. Occurrence and implications of estrogens and xenoestrogens in sewage effluents and receiving waters from South East Queensland. Sci. Total Environ. 407, 5147-5155.

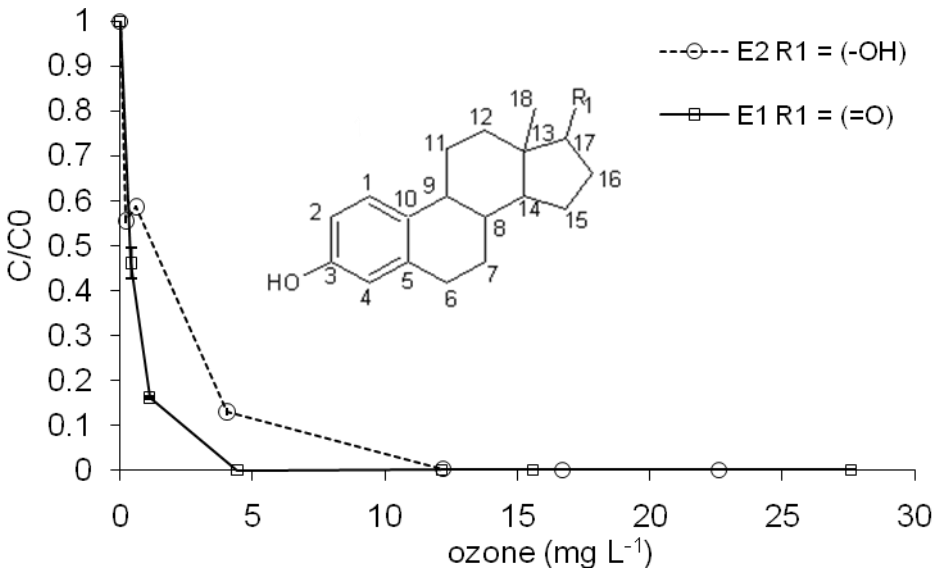
Yoon, Y., Ryu, J., Oh, J., Choi, B.-G., Snyder, S.A., 2010. Occurrence of endocrine disrupting compounds, pharmaceuticals, and personal care products in the Han River (Seoul, South Korea). Sci. Total Environ. 408, 636-643.

Zhang, Y., Zhou, J.L., Ning, B., 2007. Photodegradation of estrone and 17β-estradiol in water. Water Res. 41, 19-26.

Zhao, Y., Hu, J., Jin, W., 2008. Transformation of oxidation products and reduction of estrogenic activity of 17β-estradiol by a heterogeneous photo-Fenton reaction. Environ. Sci. Technol. 42, 5277-5284.

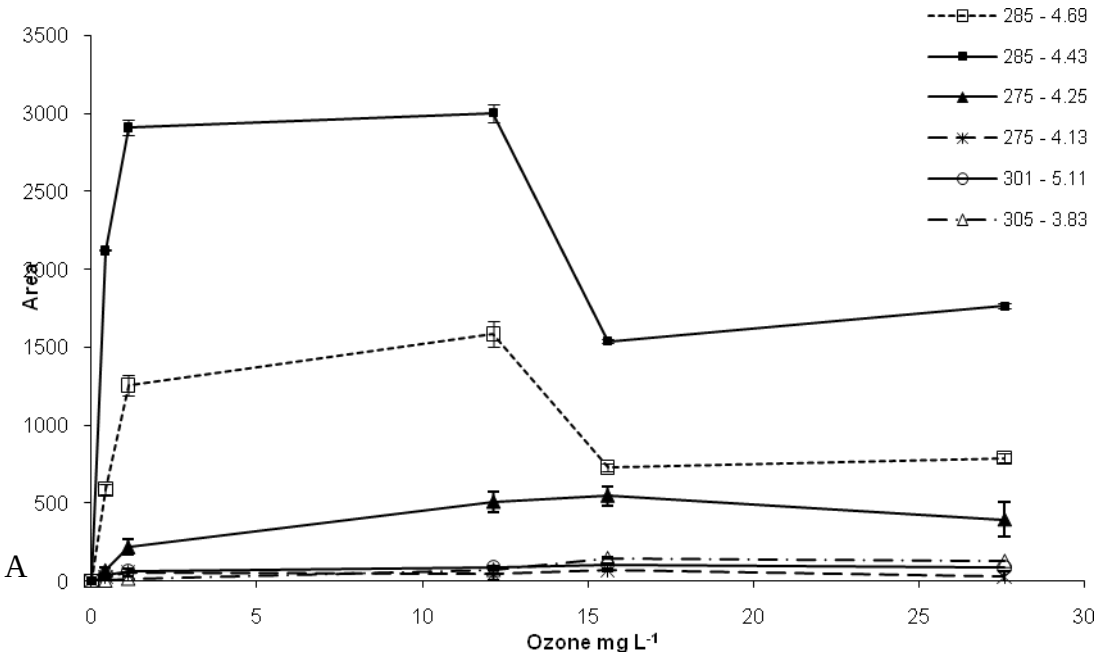
Figure

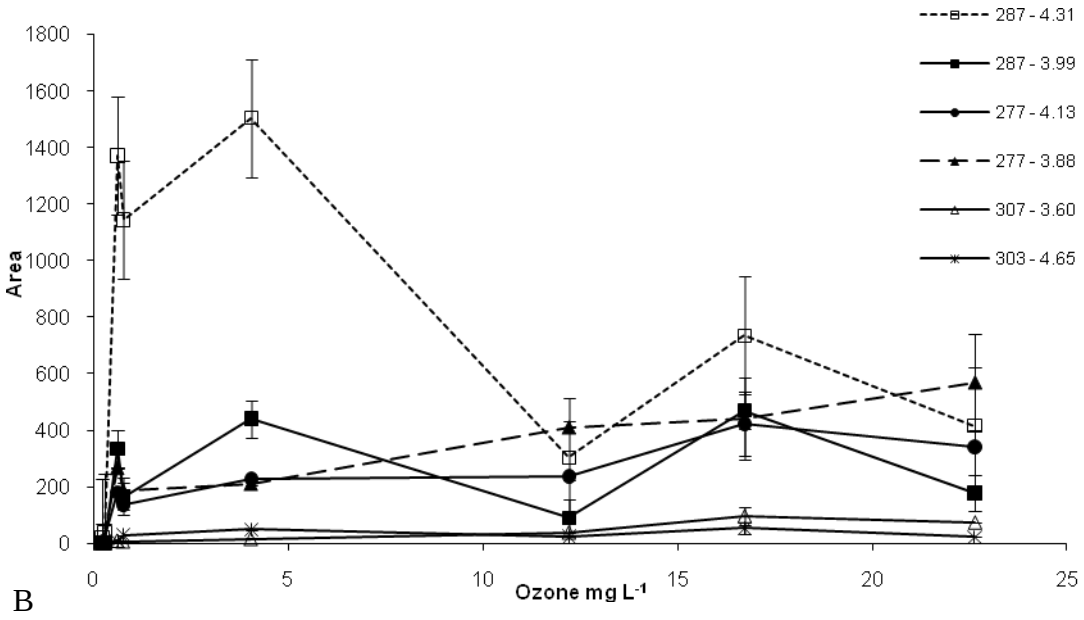
Figure 1



Figure

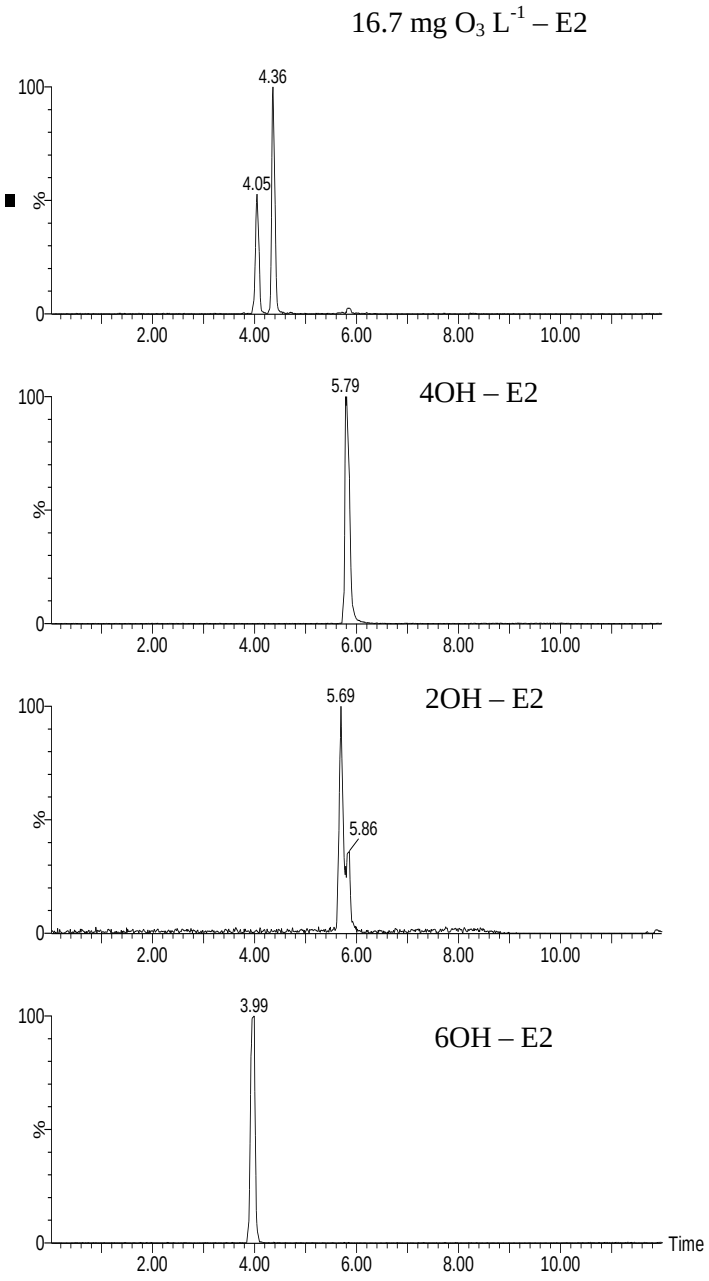
Figure 2





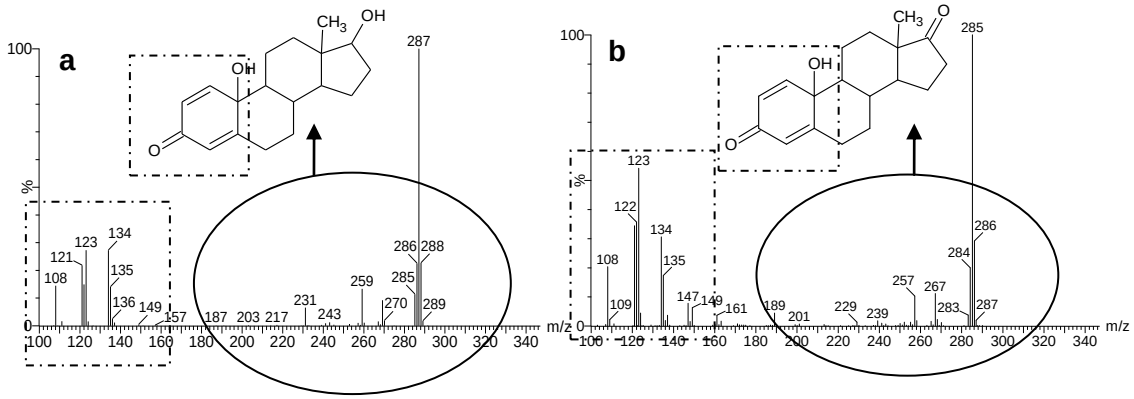
Figure

Figure 3



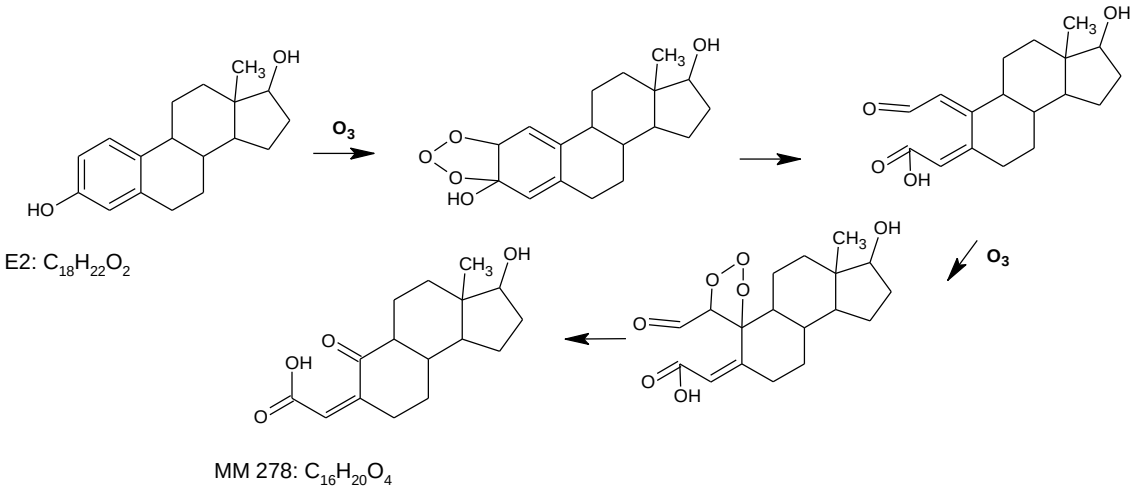
Figure

Figure 4



Figure

Figure 5



E2: $C_{18}H_{22}O_2$

MM 278: $C_{16}H_{20}O_4$

Figure

Figure 6

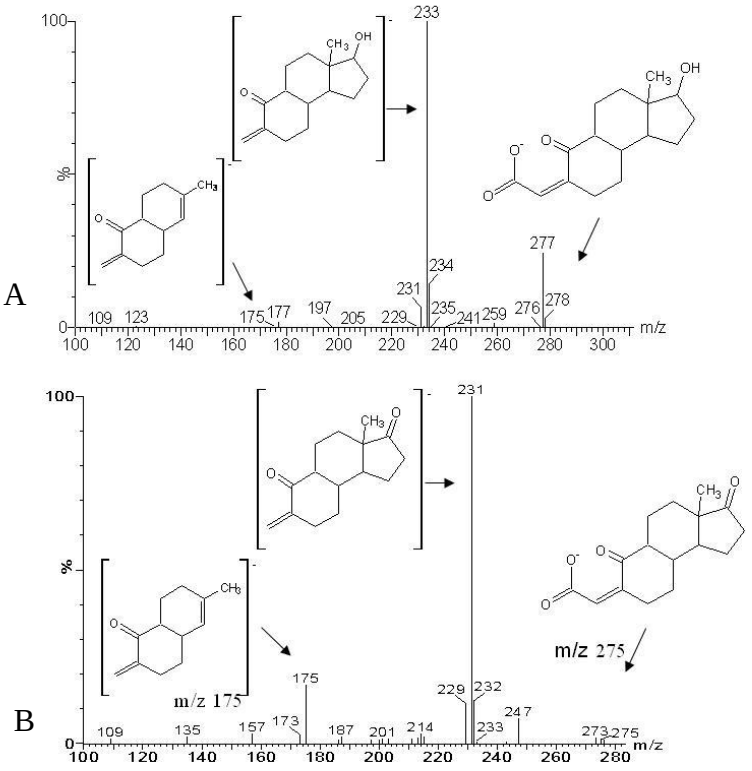


Figure 7

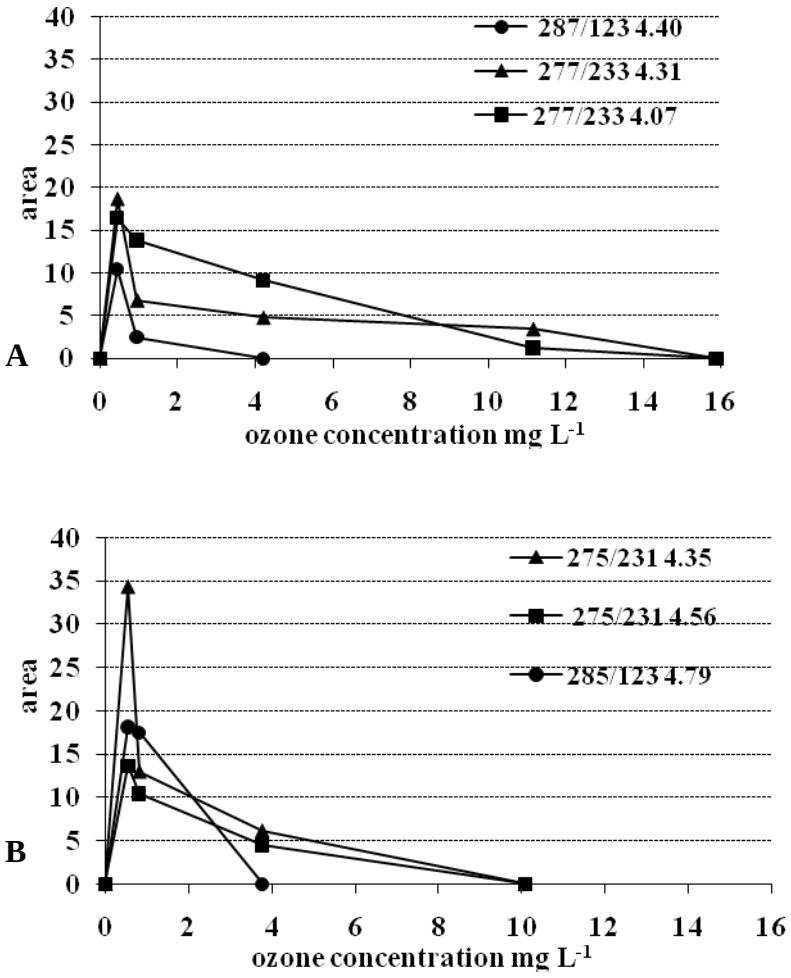

A
area
ozone concentration mg L-1
287/123 4.40
277/233 4.31
277/233 4.07
B
area
ozone concentration mg L-1
275/231 4.35
275/231 4.56
285/123 4.79

Figure captions

Figure 1. Degradation of E1 and E2 during ozonation in the experiments carried out with an initial concentration of the estrogens in water of 100 μg L^{-1}.

Figure 2. Mass-to-charge (*m/z*) ratio and retention time of the main byproducts identified with different doses of ozone in the degradation of: (A) E1 and (B) E2 with an initial concentration of the estrogens in water of 100 μg L^{-1}.

Figure 3. Extracted ion (*m/z* 287) UPLC-QqTOF-MS chromatograms obtained from the analysis of E2 treated with 16.7 mg O_3 L^{-1} and from the analysis of standards of 4OH-E2, 2OH-E2 and 6OH-E2.

Figure 4. Product ion mass spectra obtained from the UPLC-QqTOF-MS/MS analysis of (a) an E2 experiment sample (parent ion 287) and (b) an E1 experiment sample (parent ion 285), and suggested corresponding structures. Cone voltage, 30 V; CE, 20 eV.

Figure 5. Proposed pathway for the formation of the E2 byproduct with *m/z* 277 observed in the ozonation experiments.

Figure 6. Product ion mass spectra and purported fragmentation of the byproducts with m/z 277 (A) and m/z 275 (B) formed during ozonation of E2 and E1, respectively.

Figure 7. Formation and degradation of the main E2 (A) and E1 (B) byproducts during ozonation. Initial concentration of estrogens in water 100 ng L^{-1}.

Electronic Supplementary Material (online publication only)

Click here to download Electronic Supplementary Material (online publication only): Supplementary Information 1.doc

Electronic Supplementary Material (online publication only)

Click here to download Electronic Supplementary Material (online publication only): Supplementary Information 2.doc

Electronic Supplementary Material (online publication only)

Click here to download Electronic Supplementary Material (online publication only): Supplementary Information 3.doc