Mass Spectrometry investigation of 17α-Ethinylestradiol and Drospirenone complete removal from synthetic wastewater using Ozonation

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ABSTRACT

17α-ethinylestradiol (EE2) and drospirenone (DRO) are steroidal birth control compounds of widespread use and the presence of these two compounds in effluents and drink water have been related with a variety of diseases such as cancer and metabolic changes in humans. This paper describes the use of FT-ICR-MS and HPLC-MS/MS to investigate the removal of EE2 and DRO from synthetic wastewater simulating an industrial pilot plant residue, using an ozone treatment pilot plant. Ab initio calculations have been also performed suggesting that the lower energy pathway for drug degradation involves the action of singlet oxygen to these steroidal species. Total elimination of EE2 and DRO were achieved after 3 and 28 min, respectively. Estrogens degradations were screened via FT-ICR MS analyses in both positive and negative ion modes, and no residual organic molecules could be detected showing extensive oxidation and near total conversion to small volatiles or inorganic material.

INTRODUCTION

Impact of pharmaceutical industrial residues, which cause the long term toxicity are major concerns for the pharmaceutical industries. The search for environmental friendly molecules must be taking in account. Nowadays, environmental changes related to modern life are becoming a matter of great concern (Corvalan *et al.,* 2005). Many studies (Caliman and Gavrilescu, 2009; Bergman *et al*., 2012; Blode *et al*., 2012; diCalo *et al.,* 2011) have indicated that steroidal organic compounds when present in substantial amounts in the environment, mostly in drinking water, may interfere with the regular function of the endocrine system of humans and wildlife. This interference on metabolism might be towards: (i) mimicking

or antagonizing the effect of exogenous hormones; (ii) antagonizing the effect of endogenous hormones; (iii) altering the pattern of synthesis and metabolism of hormones, or (iv) modifying hormone receptor levels. Drospirenone (DRO) and 17α-ethinylestradiol (EE2) (Fig. 1) are two examples of "endocrine disrupting compounds" (EDC), which are commonly used together in contraceptive formulations or used in hormone replacement therapy. Water contamination by synthetic hormones such as DRO and EE2 are critical at municipal sewage treatment plants (STPs) that receives effluents containing hospital wastewater, and wastewater from private STPs of direct discharging chemical or pharmaceutical manufacturing plants (Nasuhoglu *et al*., 2012). During the last decades, extensive research has been carried out to find efficient degradation processes for these hormones in sewage treatment plants (STP) to avoid the potential risks caused by estrogens in aquatic environments. The kinetics and mechanisms for the ozonation of EE2 have been widely reported (Koumeir *et al.,* 2015), but we failed to find any report on the behavior of its

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degradation with DRO concomitant formulation or even DRO alone. There are a variety of methodologies based on conventional or advanced treatments that have been investigated to eliminate pharmaceuticals from water using physical (sorption, membrane filtration) (Silva *et al.,* 2012) or biological (bacteria, microalgae and ligninolytic enzymes from fungi) methods (Larcher and Yargeau, 2013; Cajthaml *et al.,* 2009). Advanced oxidation processes (AOP) have been recently investigated as complementary or alternative methods (Larcher *et al.,* 2012; Homlok *et al.,* 2013) whereas processes such photolysis, heterogeneous photocatalysis, strong oxidizers, combination of UV and strong oxidizers or ozonolysis are considered as AOP and have been reported as highly effective to degrade hormones in water (Silva *et al.,* 2012; Chen *et al.,* 2012; Zuo *et al.,* 2006; Hwang *et al.,* 2008). Recently, ozone (O_3) has gained popularity as a strong oxidizer for hormones (steroids) due to its high effectiveness on oxidation of their rigid structures. Ozone has also been demonstrated capable of removing hormones of emerging concern such as EE2 (Larcher *et al.,* 2012), but we found no reports on the degradation of drospirenone. A study on the degradation of EE2 by ozone reported 53.9% of mineralization (Zhang *et al.,* 2006), but total mineralization should also be considered the ideal scenario since it eliminates the risk of formation of dangerous or eventually even more dangerous by-products than the precursor molecules. The strategy investigated here is under development on a pharmaceutical unit in Brazil in which an ozone treatment pilot plant is being installed right after the EE2 and DRO production pilot plant. The plan is to use substantially higher concentrations of ozone, then for instance that was used before leading to partial mineralization (Zhang *et al.,* 2006), and then to force total mineralization of all intermediates, mostly organic acids, as previously observed. Ozonation of organic compounds is believed to be initiated by the attack of hydroxyl radicals (Zhang *et al.,* 2006), therefore *ab initio* calculations were also performed to verify whether the energies of the HOMO or LUMO of the studied reactive species are compatible with those of the neutral steroids to allow for proper reactivity (Fukui *et al.,* 1952). To evaluate the efficiency of these wastewater treatments, selective and sensitive techniques might be used. Sensitivity is particularly important for hormones analyses because of their very low concentrations, often as low as ppt, in wastewater combined with the high potential risk to public health. Hormone degradation have been usually monitored by high performance liquid chromatographic (HPLC) equipped with diode array or fluorescence detectors after selective extraction and concentration steps (Zhang *et al.,* 2012; Patrolecco *et al.,* 2013). Although HPLC with DAD or fluorescence detectors are often used for many degradation studies, it lack the capability of speciation and identification of by-products, and sometimes it lacks proper selectivity to comprehensively evaluate the whole process and to properly characterize unknown intermediates. HPLC separation followed by mass spectrometric characterization is becoming therefore the state of the art technique in the analysis of drug degradation products (Amaral *et al.,* 2011; Athanasiadou

et al., 2013; Fernandes *et al.,* 2011) organic analysis due to the unmatched specificity, selectivity, and sensitivity. In addition, the ultrahigh resolution provided by MS analysis performed via Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) offers a powerful tool in this area since it is able to provide unequivocal structural characterization of the degradation products (Skotnicka-Pitak *et al.,* 2008). Proper characterization of degradation products or total mineralization is highly important in remediation processes for drugs since the mere disappearance of the original drug as revealed by the HPLC-DAD and HPLC-Fluorescence chromatogram does not necessarily imply that the treatment was efficient, since sometimes degradation products may have lost the chromophores, responsible for UV detection. Comprehensive monitoring of undesirable by-products is essential to compose the big picture for the process. In this study, we aim to employ FT-ICR-MS and HPLC-MS/MS methods to evaluate the ozone oxidation process of EE2 and DRO in water aiming to either intercept un-mineralized intermediates or to probe that indeed total mineralization has occurred. The conditions used tried to mimic those of an industrial ozone treatment pilot plant being installed in a pharmaceutical company in Brazil. The wastewater used as model was the highly concentrated wastewater generated from the oral contraceptive production facility of a production pilot plant (~15.0 ng mL^{-1}) from Medley Pharmaceutical, Brasília-Brazil. A similar synthetically reproduced solution with high concentrated excipients that mimics the actual effluent was used in most studies. The ozone treatment pilot plant evaluated was designed to employ a high ozone flux (470 μmol min^{-1}) aiming to achieve total mineralization.

MATERIAL AND METHODS

Chemicals

EE2 with 100% purity and DRO with 99.6% purity (Figure 1) were obtained from USP (USA). Methanol and acetonitrile (ACN) were used of the HPLC grade from Honeywell (Morristown, NJ, USA). All the chemicals were used without further purification. Water (H_2O) was purified on a Milli-Q system from Millipore (Medford, MA, USA).
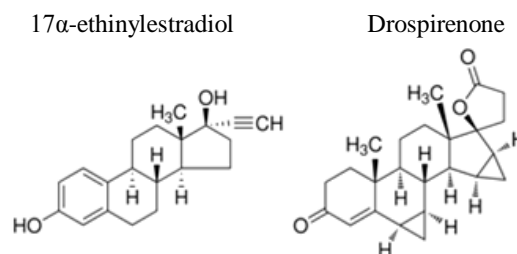

Fig. 1: Molecular structure of 17α-ethinylestradiol and drospirenone.

Solutions

Stock solutions of 1.0 μg mL^{-1} of EE2 and DRO were prepared in ACN:H2O (1:1) and stored at 4°C. Theses stock solutions were diluted with ACN:H2O (1:1) to prepare solutions of the analytes at varying concentrations: 10.5; 12.0; 15.0; 18.0; 19.5

ng mL^{-1}, and were used to obtain the calibration curve. A solution of 1.0 µg.mL^{-1} EE2 and DRO plus the placebo was also prepared in ACN: H$_2$O (1:1) simulating the residual solution from the for validation of the method. A solution of 15.0 ng mL^{-1} of both steroids was used or quality control. In addition, 50 mL of the placebo solution was also prepared using 50 mg of the excipients (cellulose, silicon dioxide, magnesium stearate, starch glycolate, talc and water) used in the formulations.

Degradation Methodology

         Ozone degradation was carried out in an ozone treatment pilot plant installed at the Medley production pilot plant located in Brasilia-Brazil. The ozonation pilot plant is constituted of an ozone generator (home-made) that was set up to produce 470 µmol O$_3$ min^{-1} (45 mg L^{-1}) using a mixture of pure oxygen and pure nitrogen as feed gases at a flow rate of 0.5 L min^{-1}. Both gases were purchased from White Martins (Danbury, CT, USA). Synthetic wastewater simulating the wastewater produced at the pharmaceutical processing pilot plant was submitted to ozonation experiments (25 ^{o}C, 1 atm) and in a batch reactor at different exposure time. Samples were filtered through a polyvinylidene difluoride (PVDF) membrane filter of a 0.45 µm pore size before analysis.

Calculations

         Ab initio calculations were based on density functional theory as implemented in the OpenMX code, that is based on ab initio linear-combination-of-pseudo-atomic-orbitals formalism (Ozaki, 2003). Pseudo atomic orbital were generated using the Troullier–Martins norm-conserving pseudopotentials (Troullier and Martins, 1993). For all molecules we used a polarized double-zeta basis set. The Perdew–Burke–Ernzerhof version of the generalized gradient approximation (GGA) was applied to the exchange-correlation functional (Perdew *et al.,* 1996). A vacuum separation of 20 Å was used to avoid the interactions between the images in the neighboring cells. The energy levels of all molecules were aligned with respect to the vacuum level.

MASS SPECTROMETRY

FT-ICR MS

         Previously to the MS analysis, additives such as an aqueous solution of formic acid 0.1 v/v % for the positive ion mode or ammonium hydroxide 10.0 mmol.L^{-1} for the negative ion mode were added to the analyte solutions. The samples were injected into the LTQ FT Ultra (thermoScientific, Bremen, Germany) with a syringe pump (Havad). The electrospray ionization (ESI) conditions were: capillary voltage (3.10 KV), tube lens (148 V) in positive mode and -100 V in negative mode, the flow rate was 5uL min^{-1} in both modes.

HPLC-MS/MS

         EE2 and DRO solutions were injected into the LC 1200 series HPLC from Agilent (Santa Clara CA, USA) equipped with a

Waters SunFire$^{®}$ C18 column (150 x 2.1 mm, 3.5 µm) for separation. HPLC operating conditions were as follow: mobile phase of 50% ACN and 50% ultrapure water with 1% acetic acid; flow rate of 0.6 mL min^{-1}; injection volume of 10 µL and oven temperature of 40 °C. Quantitation was performed in a Qtrap 5500 System® from AB SCIEX (Framingham, MA, USA). The positive ion mode was used to monitor DRO as its protonated molecule [DRO + H^{+}]$^{+}$ and the negative ion mode was used to monitor EE2 as its deprotonated molecule [EE2 - H]$^{-}$. The following optimized source parameters were used: ion spray voltage of 1500 V, curting gas (Nitrogen 4.0) at 20 psi, ion source gas at 1.15 psi, ion source gas at 2.25 psi and dessolvation temperature of 450 °C. The fragmentation patterns were studied by ESI-MS/MS at optimal collision energies (CE). Table 1 displays the MS parameters optimized for each hormone. Data for quantitation and confirmation, respectively, were acquired in MRM (multiple reaction monitoring) mode using the transitions channels 295>145 and 295>143 for DRO and 367>97 and 367>91 for EE2. Data acquisition and processing were controlled using Analyst® software (AB Sciex., version 1.4).

Table 1: Mass spectrometry parameters and retention time for EE2 and DRO.

Analyte	Transitions	DP (V)	EP (V)	CE (eV)	CXP (V)	RT (min)
EE2	295 > 145 295 > 143	135	10	50 72	15 15	2.90
DRO	367 > 97 367 > 91	1	10	29 73	14 12	2.22

Validation

         The proposed method was fully validated, including the following parameters: stability, specificity, selectivity, accuracy, limit of detection (LOD), limit of quantification (LOQ), linearity, repeatability and robustness. For robustness, we have evaluated mobile phase flow, column and temperature. A calibration curve was constructed for each compound by plotting peak area versus concentrations and the target compounds from wastewater samples were measured using the external standard method.
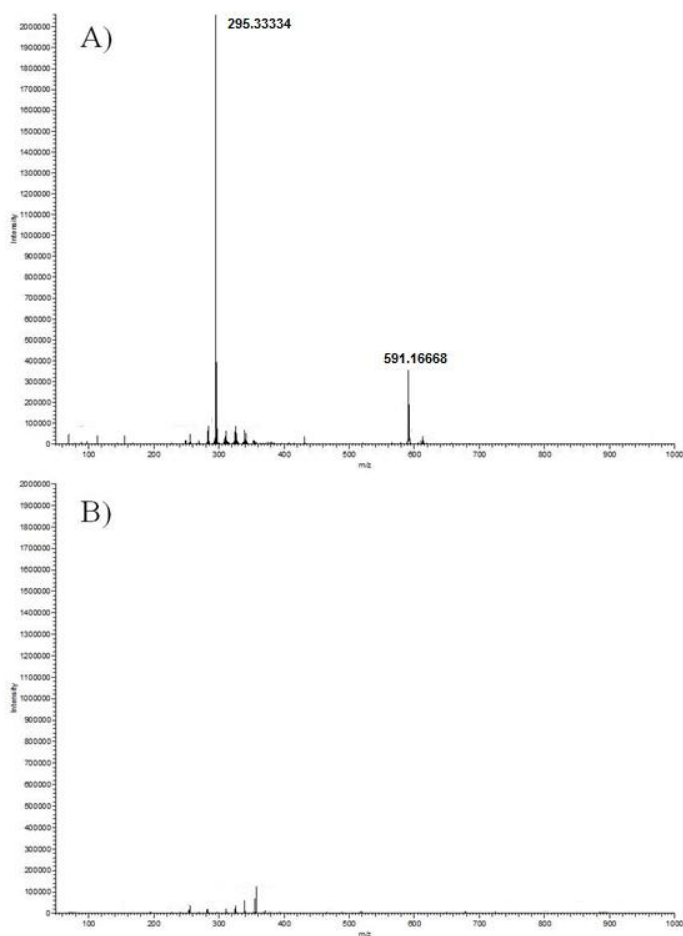
RESULT AND DISCUSSION

FT-ICR MS

         The first experiment was the evaluation of the possible residual products, which are already mentioned, may be eventually more active or more dangerous than the precursors. Samples prior and after ozonation during 28 min were then analyzed by FT-ICR MS using either ESI(+) or ESI(-) as ionization technique. Fig. 2 and 3 display typical mass spectra for these solutions with started at high concentrated solutions (15.0 ng mL^{-1}) of EE2 and DRO plus excipients, here called synthetic wastewater samples, which mimic the actual solutions processed by the pharmaceutical processing pilot plant wastewater, before and after ozonation.

         Ozone is commonly used in steroidal hormone degradation and the interception and characterization of by-products has been sometimes reported (Larcher *et al.,* 2012; Zhang *et al.,* 2006). Fig. 2 and 3 indicate however that the ozonation

process in the condition used herein was able to promote extensive, perhaps complete mineralization producing solutions in which none of the precursor product or any organic byproduct or intermediate could be detected by ESI-MS. The failure of ESI to detect any of such molecules becomes even more important when considering that ozonation usually produces more polar by-products (Hwang *et al.,* 2008) than the precursor molecules, in our case the synthetic hormones due to the oxygen organic functions that are formed upon ozonation. It would therefore be expected that such molecules would respond with even more efficiency to the highly sensitive ESI technique.

generator to produce high amounts of ozone, that is 470 μmol min^{-1}, which is much higher than those used in previous works (Zhang *et al.,* 2006; Maniero *et al.,* 2008; Zhang *et al.,* 2012). The intermediates observed by Zang were likely therefore further oxidised by ozone thus producing either volatile organic compounds or CO_2 plus H_2O, or both.
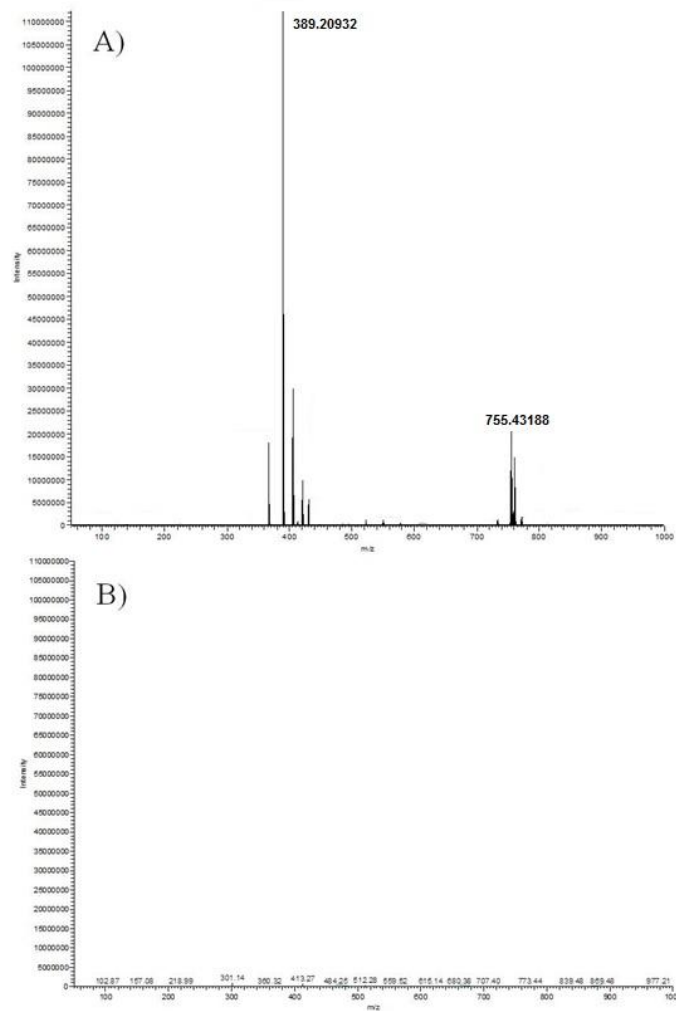
Fig. 3: ESI(+)FT-ICR MS for aqueous synthetic wastewater samples before (A) and after (B) ozonation.

Fig. 2: ESI(-) FT-ICR MS for the aqueous synthetic wastewater samples before (A) and after (B) ozonation.

CALCULATIONS

The mass spectra of Figs. 2 and 3 are shown as a function of absolute abundances to better highlight the likely full removal of both hormones, and the low abundant ions detected after ozonolysis mostly likely correspond to background and solvent ions. The ESI-MS data indicate therefore that ozonation was able to promote extensive mineralization of the hormones to CO_2 and H_2O, assuming the GC-MS monitoring as discussed as well as the report by Zhang[15] that partial ozonation produce essentially polar compounds. A feature that likely have favored extensive or even total mineralization in our stydy was the setting of the ozone

Ab initio calculations clearly indicate that the HOMO orbitals of DRO and EE2 are much closer in energy to the LUMO orbitals of the neutral steroids, as expected. Fig. 4 summarizes therefore the energies of the HOMO orbitals of both DRO and EE2 as well as the LUMO energies of the likely reactive species during ozonation, with include hydroxyl radicals or O_2 or O_3 in their triplet or singlet states.

The most interesting finding is that the HOMO energy of the singlet oxygen is the closest level to the LUMO of the hormones. It might indicate that the singlet oxygen is the most reactive specie on the process (Fig. 4). From the plot of the wave

function of the HOMO orbitals of the molecules in Fig. 4, we note that the attack should occur in the aromatic part of the steroidal species.
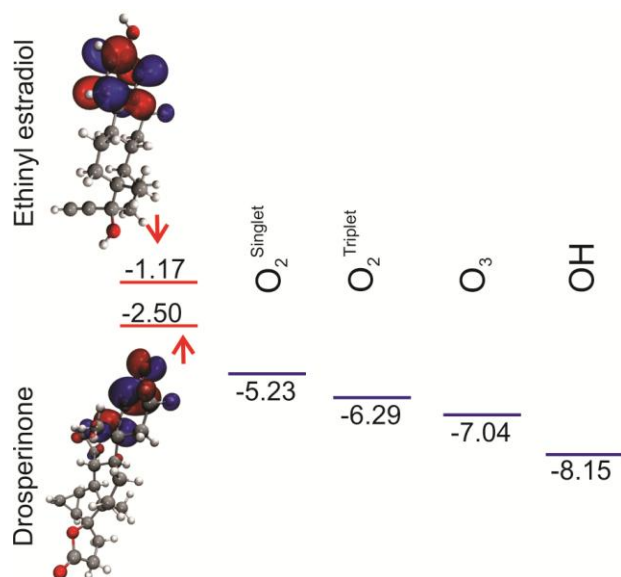

Fig. 4: HOMO energies of the steroids DRO and EE2 and LUMO energies of the possible reactive species from ozone.

HPLC-MS/MS method developed and validation
Optimization of MS/MS detection parameters

The ESI-MS/MS responses for both synthetic hormones were also examined testing both the positive and negative ion modes using ACN:H2O as the mobile phase. MS/MS data were acquired in order to establish the best ion transitions to be used in the MRM analysis. The settings were adjusted to maximize the response of each precursor-production combination. Chromatographic separation of both compounds were achieved on a reverse-phase column in isocratic mode with a total run time of 5 min. Fig. 5 illustrates typical chromatograms for standard solution, in which the [EE2 − H]$^-$ ion was monitored via the *m/z* transitions 295>145 and 295>143. The [DRO + H$^+$]$^+$ ion was monitored via the *m/z* transitions 367>97 and 367>91.
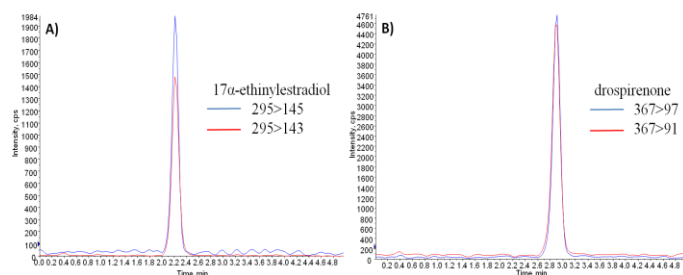

Fig. 5: HPLC-MS/MS data using the MRM mode for EE2 (A) and DRO (B) standards (25 ng mL^{-1}) in the positive ion mode in (A) and the negative ion mode in (B). The *m/z* transitions were as follow: 295>145 and 295>143 for EE2 (A) and 367>97 and 367>91 for DRO (B).

Fragmentations of multicyclic steroidal molecules as proposed for EE2 (Fig. 6) and DRO (Fig. 7) are not favorable

hence it occurs with no highly preferential channels leading to many fragment ions with relatively low abundances.
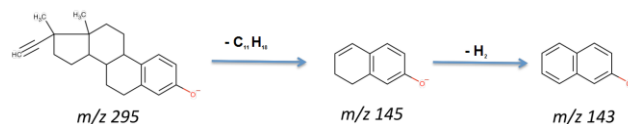

Fig. 6: Proposed fragmentation route for the [EE2 − H]$^-$ anion of *m/z* 295.
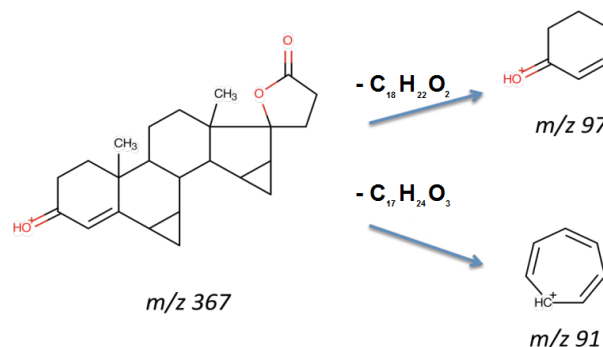

Fig. 7: Proposed fragmentation for the [DRO − H]$^-$ anion of *m/z* 367.

Linearity

Linearity for each compound was established by analysis of EE2 and DRO standard solutions ranging from 10.0 to 20.0 ng.mL^{-1}. Such range of concentrations was chosen considering the high concentration observed in the pilot plant effluent. The analytical curves were found to be linear with determination coefficients > 0.99 (Fig. 8).
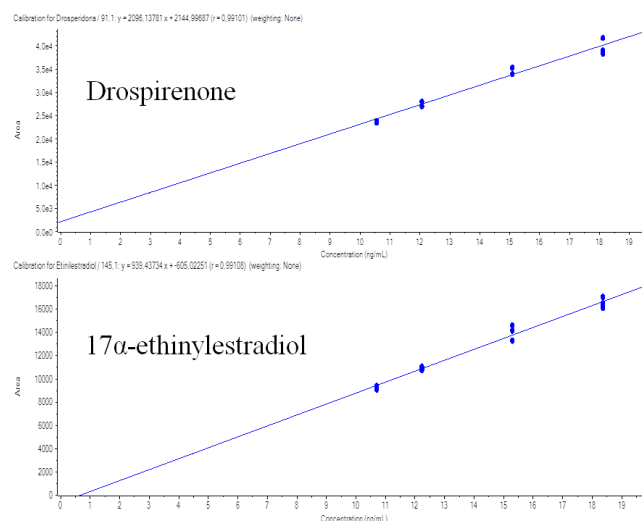

Fig. 8: Calibration curves for EE2 and DRO standards.

Detection and quantification limits (sensitivity)

The limits of detection (LOD) and limits of quantification (LOQ) for both hormones were determined based on

the equations LOD=3.3 s/S and LOQ=10 s/S, where s is the standard deviation of the blank response (n = 10) and the S is the slope of the calibration curve. The LOQ were 0.22 ng mL^{-1} for EE2 and 0.49 ng mL^{-1} for DRO. The LOD were 0.07 ng mL^{-1} for EE2 and 0.16 ng mL-1 for DRO.

According to the world health organization (WHO), liquid waste effluents should be handled in a manner that avoids environmental contamination risk, but certainly more research is needed to obtain a fuller picture of the health and environment impacts of endocrine disruptors such as synthetic hormones. The legislation about the maximum levels allowed in waste effluent has also not been accorded. Considering the low LOQ obtained in this work when compared to other methods for the same application (Zhang *et al.,* 2006), the proposed method seems suitable to monitor the residual concentration of these hormones in wastewater from a pharmaceutical processing plant. Specificity was also examined by analyzing standards plus placebo solution. There were no significant interferences at the retention time of the standard or even in the response.

Precision and accuracy

The precision and accuracy of the HPLC-MS/MS method was determined by analysis of medium level for both standards and also for standard plus placebo solutions. Intra-day precision was assessed by running the samples with six replicates, and the inter-day precision was determined by running the samples with six replicates in another day. Values in the range between 85% and 115% were considered satisfactory. Table 2 summarizes the intra-assay and inter-assay coefficients of variation (CV) at concentration of 15 ng mL^{-1}.

Table 2. CVs measurements on standard and standard plus placebo solutions

Hormone		Intra-assay		Inter-assay	
		Standard	Standard plus placebo	Standard	Standard plus Placebo
17α-ethinylestradiol	Mean*	107.30	106.35	104.70	99.30
	SD	4.78	4.97	5.97	8.46
	CV (%)	4.46	4.67	5.70	1.42
Drospirenone	Mean*	100.55	105.68	91.93	102.82
	SD	9.99	3.38	3.55	2.97
	CV (%)	9.93	3.20	3.86	2.89

Recovery

The analytes recoveries and the matrix effect were investigated by spiking placebo with three concentrations (10.5, 15.0, and 19.5 ng mL^{-1}) of standard solutions. The matrix effect was investigated by comparing the peak areas of the standard solutions prepared in methanol with placebo spiked at the same nominal concentrations. As a result, the ion signal suppression was found to be negligible for the analyte detection and the average recoveries obtained for both compounds in all tested concentrations were higher than 85 %, considering the minimum value accepted. These results show that the developed method

exhibits excellent sample recoveries, as Table 3 summarizes, for both compounds and the matrix effect was not concentration-dependent.

**Table 3.** Recoveries obtained by spiking different concentrations in placebo

Hormone (ng mL^{-1})	10.5	15.0	19.5
17α-ethinylestradiol (%)	93.03 ± 0.89	99.46 ± 7.64	93.50 ± 5.61
Drospirenone (%)	90.70 ± 0.77	100.33 ± 7.05	92.70 ± 2.65

Stability and Robustness

A control quality solution containing 15 ng mL^{-1} was repeatedly analyzed after each developed method stage. EE2 and DRO were stable during the whole method validation period with relative standard deviation (RSD) less than 10.0 %. It can be therefore concluded that the stability of target molecules is suitable for chromatographic analysis. Experiments relative to mobile phase flow, column and temperature demonstrated the robustness of the developed method with RSDs less than 10 % for both compounds.

Degradation kinetic

The optimized method was therefore applied to determine the decay of EE2 and DRO in wastewater samples obtained during the ozonation process. The HPLC-MS/MS chromatograms (Fig. 9) show indeed a quite fast decay of both hormones (EE2 and DRO) as a function of ozonation time, more so for EE2, and then degradation percentages were calculated. Fig. 10 indicates that degradation of EE2 and DRO is achieved by ozone treatment to a level below the LOD after 3 and 28 min. of ozone treatment, respectively.
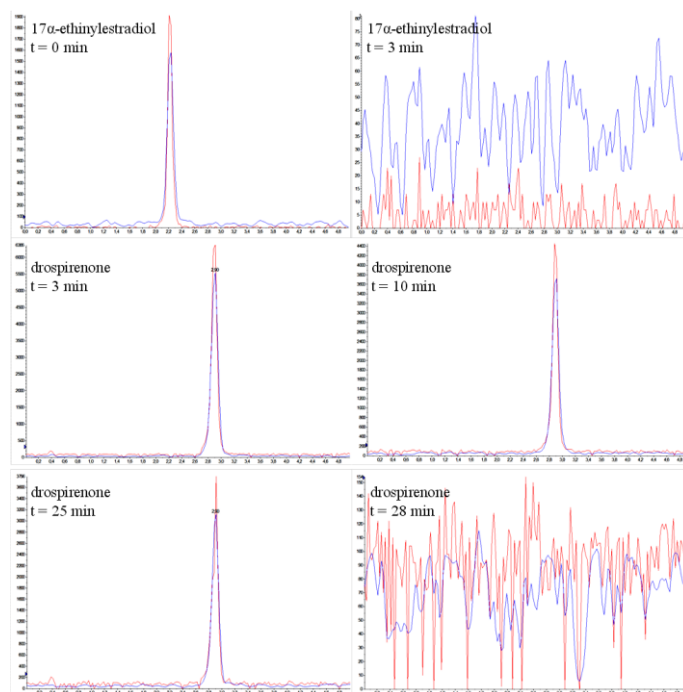
Fig. 9: HPLC-MS/MS chromatograms for EE2 and DRO after different ozonation time.
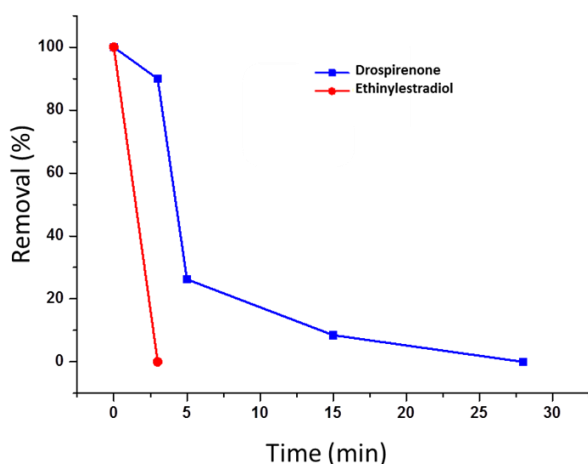
Fig. 10: Degradation efficiency of 17α-ethinylestradiol and drospirenone by ozonation as monitored by HPLC-MS/MS.

The ozone degradation process used herein appears therefore to be more quite advantageous to to lead to extensive if not total mineralization. It seems also to be quite more efficient than other methods previusly report, as for instance, the one in which the biodegradation by R.rhodochrouns showed 100 % of EE2 removal but after 48 h of treatement (Koumeir *et al.,* 2015). Catalytic oxidative degradation by FeIII-TAML, an iron tetra-amido macrocyclic system plus H_2O_2 consumed about 60 min to achieve total EE2 removal (Larcher *et al.,* 2012). The ozonation conditions used herein was shown to achieve 100% removal for both EE2 and DRO present in residual wastewater from a pharmaceutical processing pilot plant in less than 30 min. but with an crucail additional feature: total mineralization.

CONCLUSION

FT-ICR MS and HPLC-MS/MS methods have been used or developed to monitor ozone degradation of EE2 and DRO in synthetic wastewater matrix. FT-ICR MS data demonstrated that the final solution is nearly or completely free of organic molecules detectable by ESI suggesting extensive or full mineralization of EE2 and DRO after ca. 30 min.

ACKNOWLEDGEMENTS

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