Chemical and enzymatic transformations of progesterone in simulation water treatment processes at laboratory scale

The removal of endocrine disrupting compounds (EDCs) present in waters and effluents has been proposed both by physical, chemical, and biological treatment methods and by the use of enzymes produced by living beings. While some methods prove efficient, it is known that steroid transformation can generate by-products with similar or increased disruptive capacity over the parent compounds. This study aimed to evaluate the removal of the progesterone molecule in oxidizing and enzymatic media and. to verify the formation of by-products. The samples were evaluated under different conditions when submitted to oxidizing media (pH, time, temperature, salinity) and enzyme medium (pH, time, enzymatic concentration). From the analyses, it was verified that the samples submitted to the oxidizing media presented lower average removal (18.7%) than the samples submitted to enzyme laccase (36.7%). Among the samples submitted to the oxidizing media, the positive influence of hydrogen peroxide on the average removal of progesterone (20.8%) was observed. While for the samples submitted to the enzymatic medium progesterone removal favored (43.9%) occurred in buffer solution pH 5 and the most extended incubation period (300 min). The generation of degradation byproducts was observed in the samples submitted to oxidizing media (12 by-products) and in the samples submitted to the enzymatic medium (a by-product). Thus, it is concluded that enzymatic media are more effective than oxidizing means in the removal of progesterone and that the generation of byproducts from progesterone occurs along with the exposure to both media.

Keywords: Endocrine Disrupting Compounds; Hormones; Byproducts.

Transformações químicas e enzimáticas da progesterona em processos de simulação de tratamento de água em escala laboratorial

A remoção de compostos desreguladores endócrinos (EDCs) presentes em águas e efluentes foi proposta tanto por métodos de tratamento físico, químico e biológico quanto pelo uso de enzimas produzidas por seres vivos. Embora alguns métodos se mostrem eficientes, é sabido que a transformação de esteróides pode gerar subprodutos com capacidade disruptiva semelhante ou aumentada sobre os compostos originais. Este estudo teve como objetivo avaliar a remoção da molécula de progesterona em meios oxidantes e enzimáticos e. para verificar a formação de subprodutos. As amostras foram avaliadas em diferentes condições quando submetidas a meios oxidantes (pH, tempo, temperatura, salinidade) e meio enzimático (pH, tempo, concentração enzimática). A partir das análises, verificou-se que as amostras submetidas ao meio oxidante apresentaram menor remoção média (18,7%) do que as amostras submetidas à lacase enzimática (36,7%). Entre as amostras submetidas ao meio oxidante, observou-se influência positiva do peróxido de hidrogênio na remoção média de progesterona (20,8%). Enquanto para as amostras submetidas ao meio enzimático favorecido a remoção da progesterona (43,9%) ocorreu na solução tampão pH 5 e no período de incubação mais prolongado (300 min). A geração de subprodutos de degradação foi observada nas amostras submetidas ao meio oxidante (12 subprodutos) e nas amostras submetidas ao meio enzimático (subproduto). Assim, conclui-se que os meios enzimáticos são mais eficazes que os meios oxidantes na remoção da progesterona e que a geração de subprodutos da progesterona ocorre juntamente com a exposição a ambos os meios.

Palavras-chave: Compostos desreguladores endócrinos; Hormônios; Subprodutos.

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INTRODUCTION

The contamination of environmental matrices by the presence of organic micropollutants from anthropic sources has become one of the most urgent issues to be solved during the last decades (GARCIA-MORALES et al., 2015). Some of these micropollutants are called endocrine disrupting compounds (EDCs) and have attracted attention. When present in the environment even at deficient concentrations ($\mu g\ L^{-1}$ to $ng\ L^{-1}$), they may interfere with the functioning of the endocrine system of humans, as well as other animals (FANG et al., 2016), blocking or mimicking the effect of hormones, affecting their synthesis or metabolism and altering the levels of hormone receptors (BARREIROS et al., 2016).

Among the endocrine disrupting compounds with the most significant capacity to negatively influence the endocrine system are natural and synthetic steroid hormones, such as estrogen (estrone, estradiol, estriol, 17-α-ethynylestradiol), androgenic (testosterone) and progestogenic (progesterone, levonorgestrel). These compounds are commonly excreted in the urine and feces of humans and animals, and their insertion into the environment is mainly attributed to the discharge of untreated sewage or ineffective treatment (CHANG et al., 2011; LEUSCH et al., 2017), which contributes to its presence in surface water and groundwater (FANG et al., 2016; FAYAD et al., 2013; GOTARDO et al., 2014; LEECH et al., 2009; STRECK, 2009).

The investigation of methods capable of reducing or inactivating such chemical compounds in water and effluent treatment plants is increasing. With technical proposals aimed at both physical/chemical treatment and the use of advanced oxidation processes (AOPs), ultralight exposure violet (UV), ozonation and adsorption; conventional biological treatment and making use of enzymes produced by microorganisms such as fungi, algae, and bacteria (ARCHER et al., 2017; VILELA et al., 2018).

Among the enzymes that stand out in the removal of EDC are the laccases produced by fungi. Its potential is related to its low specificity, being able to oxidize, in the presence and absence of mediators, a large variety of xenobiotic compounds, some of these recalcitrant, with high redox potential, including chlorinated and non-chlorinated phenolic compounds, pesticides and polycyclic aromatic compounds (GASSER et al., 2014; RAO et al., 2014).

Although some methods of removing EDCs at effluent treatment plants demonstrate, depending on the method and the compound, removal efficiency of 80 to 100% (CHANG et al., 2011), it is known that the transformation of steroids can generate byproducts with similar or increased estrogenicity concerning the generating compounds, offering environmental and public health risk. Thus, the disappearance of the parent compound does not necessarily imply that the treatment was effective (ALVES et al., 2017; GARCIA-MORALES et al., 2015).

In contrast to this approach of only evaluating the presence/absence of a particular compound, at the end of the use of a given technique, recent studies seek to identify how the mechanisms used in water and effluent treatment interact with EDCs by carefully analyzing which conditions can transform each one of the compounds and clarify the generation of by-products (CHEN et al., 2017; HE et al., 2016; IFELEBUEGU et

al., 2016; NOUTSOPOULOS et al., 2015; OJOGHORO et al., 2017; ZDARTA et al., 2018). Thus, considering the exposure of the progesterone molecule to different reaction media, as oxidizing and enzymatic means, it is sought to understand how they act and, whether or not they favor the transformation of the progesterone molecule and the generation of by-products.

MATERIALS AND METHODS

Materials

The standard Progesterone used was purchased from Sigma-Aldrich, (> 97%), and the enzyme Laccase Nova Nordisk BioChem (NS29033). The enzyme was in an aqueous solution, which was kept under refrigeration. All other materials were all purchased above 99% and proven by J. T. Baker. No other material was tested after further purification. Table 1 shows the physicochemical characteristics of Progesterone.

Table 1: Physical-chemical characteristics of the hormone progesterone.

Fórmula Empírica	MM (g mol^{-1})	Ws (mg L^{-1} a 25°C)	Log Kd	pKa	t$_{1/2}$ (h)	Número CAS
C$_{21}$H$_{30}$O$_2$	314,46	8,81	4,63	18,47	55,84	57-83-0

Source: Peng et al. (2014), MarvinSketch (2017), Sigma-Aldrich (2017).
MM: Molecular Weight; Ws: Solubility in water; Kd: Distribution constant; pKa: log de Ka (Acidity Constant); t$_{1/2}$: Half-life time; Number CAS: Register number at Chemical Abstracts Service

Methods

Chemical Transformation of Progesterone

Physical-chemical transformation tests consisted of the evaluation of the influence of different physical and chemical agents on the transformation of progesterone. To determine the experimental design, the Response Surface Methodology (MSR) was applied, in which the influence of 5 factors with two levels each was evaluated, namely: pH of the buffer solution (5 and 9); presence of reducing agent, Sodium Hypochlorite (NaClO) or oxidizing agent, Hydrogen Peroxide (H$_2$O$_2$); salinity (0 and 10 g L^{-1}); temperature (20 and 40 °C) and incubation time (30 and 120 min). Also, the central point of each factor was determined in order to estimate the experimental error and also to test if there are significant non-linear effects.

For the batch tests (in duplicate) were used Erlenmeyer of 200 mL, with the total volume of 50 mL; 5 mg solid progesterone, 38 ml acetate buffer (CH3COONa) 2 mol L^{-1} (pH 5.0) or boric acid buffer (H3BO3) / sodium hydroxide (NaOH) 0.1 mol L^{-1} (pH 9.0) and 12 ml of sodium hypochlorite (NaClO-) or; 46 mL acetate buffer (CH 3 COONa) 2 mol L^{-1} (pH 5.0) or boric acid buffer (H 3 BO 3) / sodium hydroxide (NaOH) 0.1 mol L^{-1} (pH 9.0) and 4 mL of hydrogen peroxide (H2O2). When the presence of the salinity factor was evaluated, 0.5 g sodium chloride (NaCl) was added to each Erlenmeyer, corresponding to 10 g L^{-1}.

The incubation of the samples was performed in the incubator chamber at the desired temperature (20 and 40 °C), under the agitation of 150 rpm. For the center point samples, 5 mg of solid progesterone, 50 ml of 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) and 0.25 g of sodium chloride (NaCl) were added to each Erlenmeyer flask. It was incubated at 30 °C for 75 min. For each incubation temperature control samples were prepared, whose Erlenmeyers contained only 50 mL of distilled water and 5 mg of solid progesterone.

After incubation, samples were transferred and concentrated in solid-phase extraction (SPE) cartridges, which were preconditioned three times with 5 mL of methanol and three times with 5 mL of water. All compounds of interest were extracted from the SPE with 5 mL of P.A Methanol and collected in a 10 mL test tube. The extracted volume was transferred to 1.5 ml amber flasks, which were kept at a temperature of less than 0 °C until the chromatographic analyses were carried out.

Enzymatic Transformations

The enzymatic activity of laccase was determined according to the methodology of Wolfendon and Wilson (1982), where the oxidation of 2,2-azinobis (3-ethylbenzthiazoline) -6-sulphonate (ABTS) was conducted in a reaction mixture of 2.1 mL, containing 1 mL of 0.1 mol L^{-1} phosphate buffer (pH 6.5), 1 mL of ABTS 1 mmol L-1 and 0.1 mL of enzyme solution at a temperature of 35 °C.

The oxidation of the substrate was carried out under greenhouse conditions at 35 °C and determined after 10 min of UV / visible spectrophotometer reaction at 420 nm (ε at 420 nm: 36000 mol L^{-1} cm^{-1}). The control samples were characterized by the absence of an enzymatic solution, which was replaced by 0.1 mL of distilled water.

One unit of laccase activity (one-unit enzyme) was defined as the variation in absorbance of the sample at 420 nm in 10 min. The change in absorbance at 10 min was equivalent to μ mol L^{-1} of oxidized ABTS in 10 min. This result was then converted to u mol L^{-1} of oxidized ABTS per min to calculate the enzymatic activity (IU). One unit of the enzyme (U.min^{-1}) was defined as the amount of enzyme capable of oxidizing 1 μmol of substrate per minute. For the enzymatic calculation, we used Equation 1 (SCMITT et al., 2009):

$$\frac{U}{mL} = \frac{\Delta Abs.60.V_{total}100000}{\epsilon_{ABTS}.V_{sample}.t}$$
Eq. (1)

Where: ΔAbs = Increased absorbance at wavelength 420 nm; ε = Absorbance coefficient for ABTS (ε420) (= 36,000 L mol^{-1} cm^{-1}); Vtotal = total volume (mL) of the mixing reaction in the cuvette; Vsample = volume (mL) of the enzymatic solution used in the mixing reaction; t = reaction time (min)

The experimental design of Enzyme Transformation Assays was also performed through Response Surface Methodology (MSR), where the influence of two concentrations of laccase was evaluated in two pH values and two incubation periods, aiming at determining which factors which favor the degradation of progesterone.

In order to perform the experiment, three factors were used: enzymatic concentration (250 and 750 U), buffer pH (5 and 6.5), and incubation time (60 and 300 min). The tests were performed in batch and duplicate. Central spot samples with 500 U enzymatic concentration and pH 5.75 buffer were also prepared. For these samples, the volume of 1.61 mL of the enzyme, 47.39 mL of 0.1 mol L^{-1} phosphate buffer solution, and 5 mg of progesterone solubilized in 1 mL of acetonitrile was used.

Samples were placed together in the incubator chamber at a temperature of 35øC and shaking at 150 rpm and, as the incubation time was complete (60, 180, and 300 min) were withdrawn for extraction. Control samples consisted of flasks containing only 50 mL of distilled water and 5 mg of progesterone solubilized in 1 mL of acetonitrile.

The samples were extracted as described for the physical-chemical tests. Each sample was then diluted by transferring 2 mL of the volume to a 250 mL volumetric flask and filling with distilled water. The diluted samples were transferred to two amber flasks of 1.5 ml each and kept in a freezer at a temperature below 0 °C until the chromatographic analyses were carried out.

A total of 200 mL Erlenmeyer was used, corresponding to 5 mg of progesterone solubilized in 1 mL of acetonitrile, 48.19 mL of 0.1 mol L^{-1} phosphate buffer solution (pH 5 or 6, 5), supplemented with 0.81 ml of enzyme to the enzymatic concentration of 250 U or; 46.58 ml of 0.1 mol L^{-1} phosphate buffer solution (pH 5 or 6.5) and complete with 2.42 ml of the enzyme for the enzymatic concentration of 750 U.

Progesterone determination

The determination of progesterone concentrations was performed according to Almeida et al. (2006), using a Dionex Ultimate 3000 HPLC with DAD detection. The mobile phase consisted of an aqueous solution of 10% (v / v) acetonitrile and acetonitrile PA, gradient from 0 to 100% acetonitrile over 60 min, and a flow of 1 ml min^{-1}. The column used was C18 (150 mm x 4 mm, five μm), and the detector used the 200 nm range for progesterone. The column temperature was 25 °C, and the injection volume was 200 μL. As the samples were concentrated in the SPE, the final concentration of progesterone was determined by calculations.

For the degradation by-products analysis, a liquid chromatography system (Agilent model LC 1260 Infinity) was used, with a 600 bar quaternary pump, automatic sampler model G1329B, equipped with an Eclipse Plus C18 column 50 mm long by 2.1 mm diameter and 1.8 μm porosity and 5 microns Eclipse Plus C18 2.1x 12.5 mm guard column coupled to the mass spectrometer (model 6420 Triple Quad LC / MS, Agilent), with electron ionization mode -spray (ESI). Progesterone molecular ions (m / z 315, 123, and 109) were monitored, and analysis was performed in Full-Scan mode for the identification of degradation byproducts.

Treatment and data analysis

Statistical analyses were performed using Statistica® 7.0 software, in which descriptive statistical analyses were performed, such as standard deviation, mean, statistical significance, confidence interval. The data obtained had their means compared by Analysis of Variance (ANOVA) according to a single criterion, with a level of significance (α) of 0.05. The Tukey test was applied when verified by ANOVA that the means of the treatments presented a significant difference in order to investigate which treatments differed from one another.

RESULTS AND DISCUSSION

Progesterone Analyzes

Analytical results, as well as linearity studies and limiting detection and quantification calculations, are presented in the supplemental material. The calibration curve had a linear adjustment according to the

presented model. In this way, the methodology was validated from the linearity studies, which were conclusive regarding the results and limits. Ultraviolet spectra obtained from the progesterone standard at 1.5 mg L-1 were used as standard. There was a mean correspondence of all ultraviolet spectra for the quantized signals of 97.46%. All quantified progesterone chromatographic peaks did not show a change in retention time greater than 0.3 minutes. In this way, and associated with the fine adjustments of linearity, we could trust the results presented in the study.

Table 1: Calibration curve data and Limites of detection and quantification (LOD and LOQ, respectively).

Compound	Retention Time (min)	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Progesterone	10,83	0,9990	0,14	0,43

Physico-chemical degradation of Progesterone

The final concentration of progesterone in the samples submitted to the physicochemical treatments varied from 55.69 to 95.67 µg L^{-1}, and the percentage of removal observed ranged from 0 to 41.3%. The final mean progesterone concentration of all samples submitted to chemical treatments was 77.05 µg L^{-1}, and the mean observed removal was 18.6%.

The lowest final concentrations of progesterone (55.69, 59.40, and 67.98 µg L^{-1}) and consequently the highest percentages of removal after treatments (41.3, 37.3 and 28.3%) were observed in samples subjected to the action of the hydrogen peroxide agent (H_2O_2).

Is was used the median concentrations of all the samples and observed against the effect of reducing agents hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaClO) in the removal of progesterone. It was observed that the lowest final concentration occurred in samples submitted to the action of hydrogen peroxide (75.07 µg L^{-1}), corresponding to the mean removal of 20.8%.
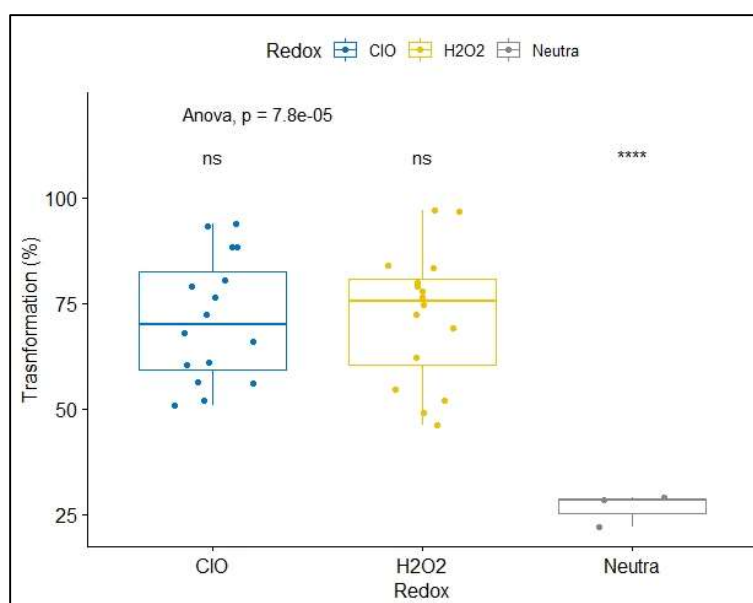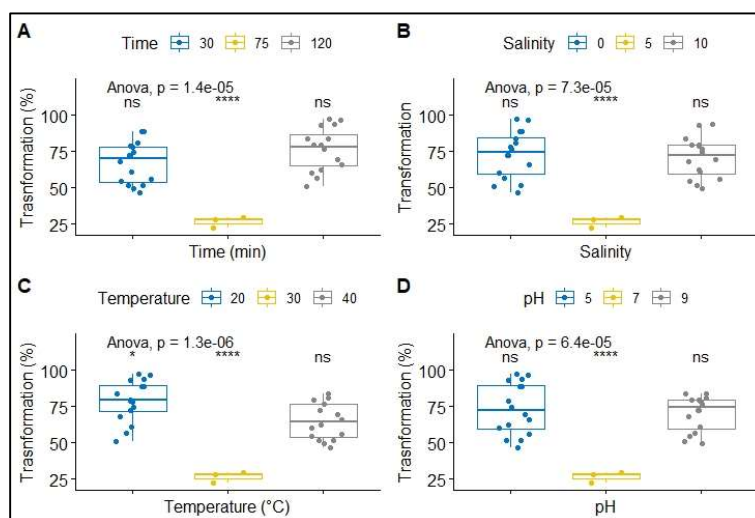

Figure 1: Final median progesterone concentration in the samples subjected to the action of Hydrogen Peroxide (H_2O_2), Sodium Hypochlorite (NaClO), and no reducing agent (central point).

The evaluation of the isolated use of hydrogen peroxide (H_2O_2) in the removal of EDCs in effluents is not a common practice since other techniques are usually used together to favor the formation of hydroxyl

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radicals, such as ultraviolet (UV), iron ion (II) (Fe^{2+}) or ozone (O_3). Thus, the percentages of removal observed were lower than those observed in studies that evaluated the removal of other EDCs by combining H_2O_2 with different chemical agents. Frontistis et al. (2015) evaluated the removal of EE2 using the UV / H_2O_2 combination and observed 100% removal after 15 min of treatment. Cédat et al. (2016) investigated the degradation of a mixture of estrogen hormones estrone, β-estradiol, and 17-α-ethynylestradiol) by UV / H_2O_2 photolysis in effluent and 80% of the initial estrogenic compounds could be removed.

When evaluating the action of hydrogen peroxide (H_2O_2) in the presence of other factors (pH value, experimental time, temperature and salinity), it was observed that the final concentration of progesterone was lower in samples submitted to pH 5 (55.69 and 59.40 µg L^{-1}), and the presence of salinity at the concentration of 10 g L^{-1} (51.45 and 56.27 µg L^{-1}), indicating that these conditions may favor its action.

Figure 2: Final medium progesterone concentration of samples subjected experiment time (A), Salinity (B), temperature (C) and salinity (D).

When performing the statistical analysis, it was observed that the agents that most influenced the decrease in concentration were the redox agent and salinity interaction. The presence of salinity and the longer experiment time would be favoring the removal of the progesterone molecule or, possibly, only causing a lower solubility (and in this case availability) of progesterone in the reaction medium. The salinity effect was the only one that present statistical difference when the median data was performed through ANOVA analysis (p< 0,001; α= 0,05).

Yang et al. (2016) found significant negative correlations between the concentrations of salinity and dissolved EDCs. This phenomenon has been widely referred to as salting out and is suggested as the cause of the decrease in the aqueous solubility of most EDCs due to the presence of soluble salts, resulting in the compound being more attracted to the solid phase. This condition may be indicative that of the amount of progesterone removed, a portion may be only insoluble, rather than totally degraded.

The response surface can show us a complete relationship between the chosen variables. In Figure 3, it can be seen that there is almost a linear relationship between the increase in salinity and the concentration of progesterone in the medium. That is, the less salt, the more degradation of it is favored.

Something similar, but with less intensity, can be observed in the variable oxidizing agent, where the lower values presented are related to the presence of H_2O_2.

Another condition that may have made possible the removal of progesterone was the interaction of the lower pH and the oxidizing agent hydrogen peroxide (H_2O_2), with higher percentages of progesterone removal in the samples submitted to pH 5.

Zhang et al. (2014) evaluated the effects of H_2O_2 dosage and pH on the removal of six endocrine disrupting compounds (estrone, 17β-estradiol, 17α-ethinylestradiol, estriol, bisphenol-A, and 4-nonylphenols) from TSE sludge with treatment by activated sludge and observed that the low pH value is favorable for the removal of EDCs in the different dosages of H2O2 tested (0.05-2 mol L^{-1}), being that in the dosage with pH three and H_2O_2 of 2 mol L^{-1}, the highest removal efficiencies of Estrone (E1), 17-α-ethinylestradiol (EE2), Estriol (E3), Bisphenol-A (BPA) and 4-nonylphenols (NP) were observed 73, 58, 58, 54 and 46%, respectively.

What may explain the favoring of the action of H_2O_2 in the removal of the EDCs at lower pH is that, in contrast, the increase in pH can cause the formation of an inactive hydroperoxide anion (HO_2^-) due to the dissociation of H_2O_2. This HO_2^- anion can act as an efficient eliminator of hydroxyl radicals, which leads to the inhibition of degradation (BOUASLA et al., 2010). Thus, H_2O_2 becomes unstable and decomposed to molecular oxygen at alkaline pH, losing its oxidation capacity (ZHANG et al., 2014).
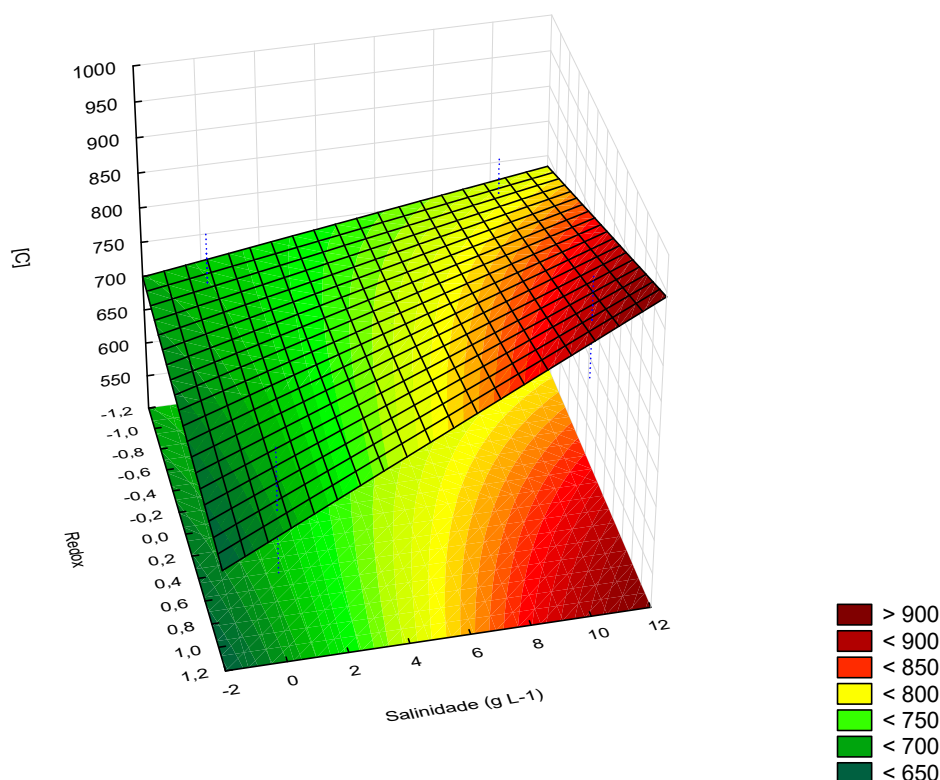
Figure 3: Response Surface for the relation between observed concentration, redox agent, and salinity. Redox value: H_2O_2 (-1); Neutral (0) and ClO^- (+1).

Hypochlorite (NaClO$^-$) samples showed concentrations at or above the central point (79.09 µg L^{-1}), which samples did not have a reducing agent. In the same way, the different conditions of pH, time of the experiment, temperature, and salinity did not stand out favoring the removal of progesterone by NaClO$^-$.

The variation in the pH value in the samples submitted to the action of sodium hypochlorite did not show any significant influence on the removal of progesterone. This condition is different from that observed in studies that studied the degradation of estradiol (E2) by hypochlorite (HE et al., 2016; LI et al., 2017), where increasing the pH value favored degradation.

Likewise, the two temperatures applied did not show a significant difference in progesterone removal performance, which differed from Ifelebuegu et al. (2016) who observed an increase in the rate of progesterone degradation with increasing temperature, a fact that attributed the improvement of the generation of hydroxyl radicals at high temperatures, increasing the rate of degradation and thus accelerating the degradation of the molecule.

Enzymatic Effect

The result of the enzymatic activity assay of laccase was 309.89 U mL^{-1} (standard deviation of 120.65 and coefficient of variation of 38.9%). From the result of the enzymatic activity, it was possible to determine the volume of the enzymatic solution used for each of the enzymatic activity tested.

The concentration of progesterone in samples submitted to laccase enzyme activity ranged from 72.35 to 107.55 µg L^{-1}. Removal values ranged from 44.8 to 17.9% and were below what Spina et al. (2015) observed when analyzing the application of fungal laccases in the removal of steroid hormones Estrone, 17-α-ethynylestradiol and 17-β -estradiol, which was 98.8; 100 and 100%, respectively.

Table 2: ANOVA Values for the enzymatic interactions with the removal of Progesterone.

| Factor	SS	df	MS	F	p
pH (Q)	153833,0	1	153833,0	828,2142	0,000009
Tempo (h)(L)	161,9	1	161,9	0,8715	0,403395
[C] Enzyme(L)	7,7	1	7,7	0,0416	0,848366
Error	743,0	4	185,7		
Total SS	154752,4	8			

Statistical analysis indicated that progesterone removal was favored in the samples, which were carried out in buffer solution at pH five and for a more extended period of 300 minutes (44 and 43.0%),

A similar result was obtained in the use of laccase enzyme for the removal of Bisphenol A (BPA). Zdarta et al. (2018) tested the removal of BPA in the pH range from 4 to 8 and recorded that the biodegradation efficiency for BPA increased to the maximum at pH 5 and decreased as the pH became higher. The authors related directly to the catalytic properties of laccase, which would have properties of higher catalytic activity and stability at pH 5 (ZDARTA et al., 2018).

Freitas et al. (2017) studied the ability of BPA removal by laccase enzymes of two fungi, concerning pH, both laccases were able to remove BPA at pH values ranging from 4 to 7, and the best results of degradation were obtained at pH 5.

The comparison of the removal with the enzymatic concentration showed no linear profile, and in identical enzymatic concentrations, significant removal differences were observed. This phenomenon may be due to the limited enzymatic affinity for the substrate, as well as diffusion problems of the substrate, being the concentration and bioavailability of the substrate a crucial and limiting factor (SPINA et al., 2015), availability that may have been affected by the others conditions, such as pH.

The pH was the only variable that presented significance ($p = 0.0001$; $\alpha = 0.05$), which was visualized in figure 4. The response surface presented a model of horse cell, and in it, it was found that the central point of the design was the one that less degraded the progesterone. This can be explained by a possible enzymatic inhibition in this pH range, making biological degradation impossible.
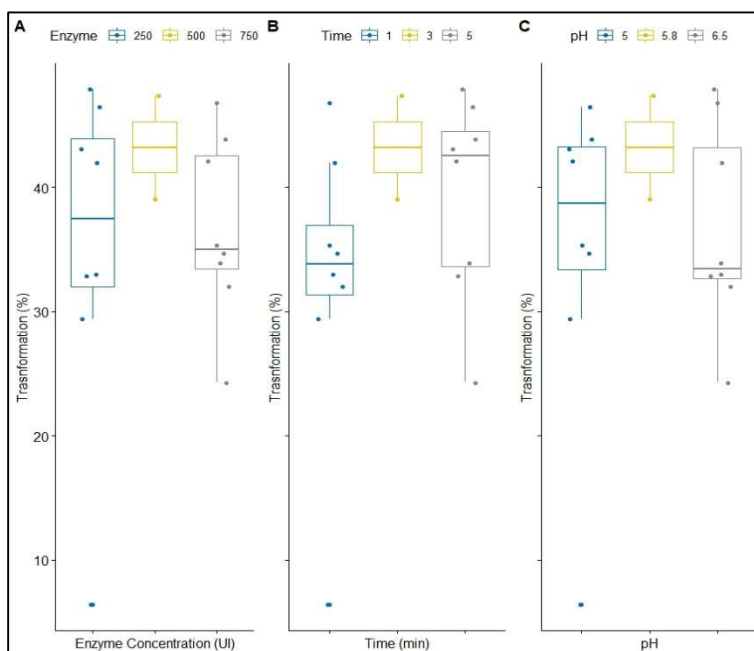
Figure 4: Enzymatic concentration on degradation of progesterone subjected by Enzyme concentration (A), Time (B) and pH (C).
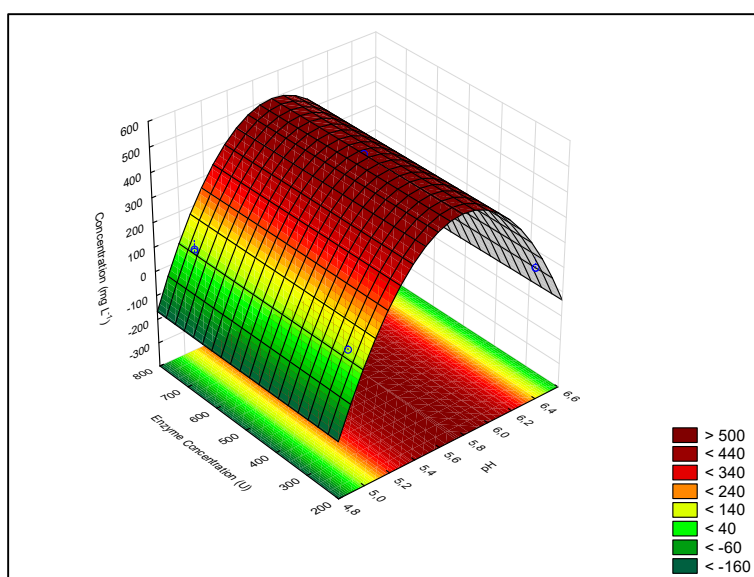
Figure 5: Response Surface for the relation between observed concentration, pH, and Enzymatic concentration os the media.

Degradation Byproducts

In the chemical transformation essay, was observed the generation of 12 degradation by-products, having the following mass / charge ratios (m/z): 395, 355, 393, 367, 355, 333, 315, 311, 270, 232, 149, 123. Progesterone must have a molecular weight of 314.47 g mol^{-1}. This indicates that in the chemical transformation, some products formed contain some or all of the progesterone molecule and some or all of the oxidant.

The by-product of m/z 395 was observed only in the samples subjected to the action of H_2O_2, whereas the by-product 355 only in the samples submitted to the action of $NaClO^-$. The by-products of m/z 393, 367, 333, 311, 270, 232, 149 were observed exclusively under the action of H_2O_2, under the condition of 40 ° C temperature and 30 min of the assay.

Ojoghoro et al. (2017) identified the generation of progesterone degradation byproducts in surface waters through laboratory tests simulating treatment plant tanks. The results showed the formation of six alleged transformation products of the nine monitored target products. The presence of two of the detected products was confirmed by the precise determination of their m / z ratios (MM = 312.2 and 286.2 g mol^{-1}), and a third product was detected in the HRMS, but with a lower degree of confidence and without the molecular formula (MM = 316.2 g mol^{-1}). The authors attributed the progesterone transformation predominantly to dehydrogenation reactions.

Ifelebuegu et al. (2016) observed changes in progesterone concentration during the Photo-Fenton degradation process. Among the degradation by-products identified, 17α-hydroxyl (MM = 331 g mol^{-1}), testosterone (MM = 288 g mol^{-1}) and dihydrotestosterone (MM = 290 g mol^{-1}) were detected after 10 min of progesterone (Mg = 104 g mol^{-1}), acetaldehyde (MM = 44 g mol-1), oxalic acid (MM = 90 g mol^{-1}), methanoic acid = 46 g mol^{-1}). The double bonds in the progesterone molecule between C4 and C5, the hydroxyl group in C12, and the double bond oxygen in C3 are the probable points of hydroxyl radical attack. The researchers concluded that there was a degradation of progesterone in several stages, involving several common byproducts of transformation that underwent further degradation and rupture of the ring to form simpler organic molecules such as carbon dioxide and water.

No mass/charge (m/z) ratios of the by-products identified by the progesterone transformation in the present study were equal to the molecular masses of the by-products identified in the two papers cited (IFELEBUEGU et al., 2016; OJOGHORO et al., 2017) only the by-product mass/charge ratio (m/z) 333 approached the 17α-hydroxyl mass (MM = 331 g mol^{-1}).

For the determination of which byproducts are generated, additional studies are necessary for equipment such as a high-resolution mass spectrometer. About the enzyme transformation assay samples, only the mass/charge (m/z) ratios of samples 9 and 18 correspondings to the center point were determined for this work.

When injecting sample 18, submitted to the enzymatic assay, two distinct signals can be observed at a retention time of 10.1 and 14.9 min. By filtering the chromatograms by the masses, it can be seen that the

first one is a progesterone (m / z 315), while the second may be some transformation product, but with a mass of 360.5. Through the mass breaks of this second signal, it is possible to observe breaks of m / z 315 (progesterone), m / z 243; m / z 149 and m / z 123.
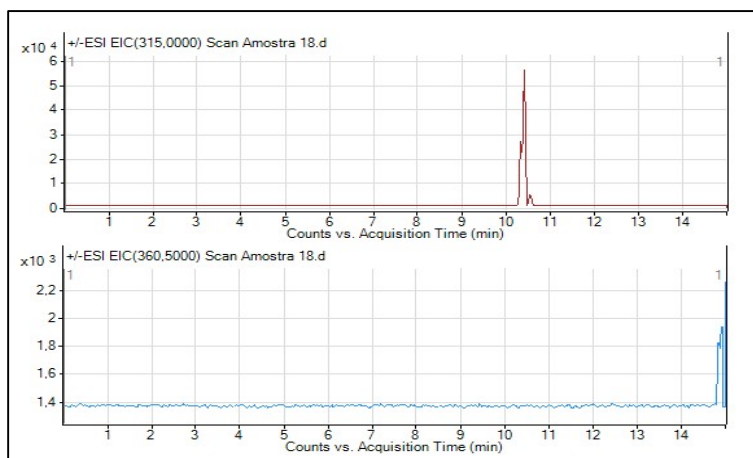
Figure 6: Chromatograms of sample 18, submitted to enzymatic medium, with progesterone signals (m / z 315) and some degradation product (m / z 360.5).

From the mass breaks, one can try to elucidate what the product of transformation would be. As losses of 14.4 ($CH2^+$) and 31.1 (=OH^+) are observed, and possibly, depending on the molecular structure, nucleophilic attacks would occur on progesterone instauration. A possible route of degradation of this compound is plausible with the molecular structure of progesterone, as observed in Figure 8. However, further studies are necessary to prove the existence of this molecule and may come from the ionization of the analytical method, or other conformation to be confirmed with subsequent molecular purifications and elucidation.
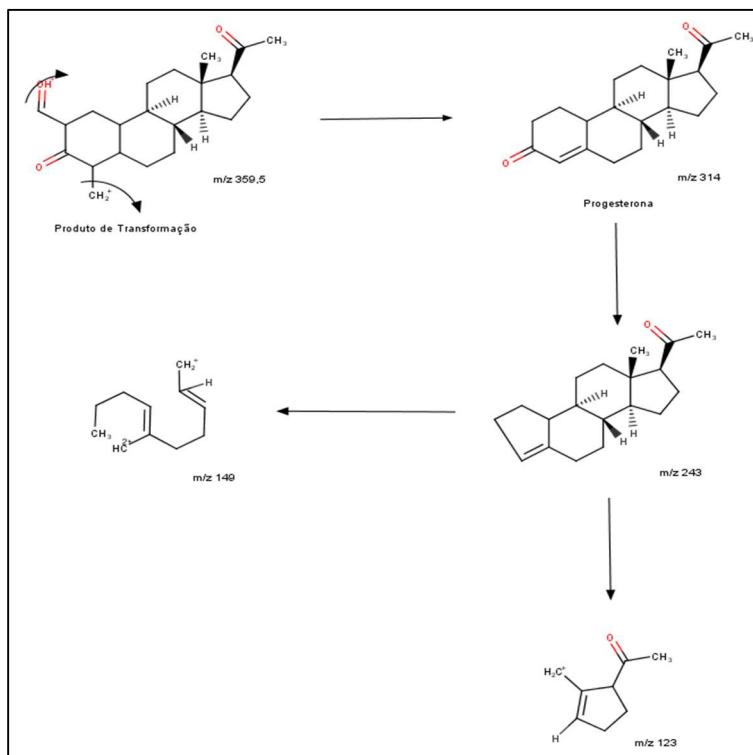
Figure 7: Schematic diagram suggesting possible route of transformation product observed in sample submitted to enzymatic medium.

CONCLUSIONS

The results obtained showed that the samples submitted to the oxidizing media had a lower average removal (18.6%) than those sampled under enzyme laccase (36.7%); Among the samples submitted to the oxidizing media, the ones submitted to hydrogen peroxide showed a higher average removal (20.8%). There were 12 by-products of degradation in the samples submitted to the oxidizing media, of which seven by-products were generated exclusively under the action of H_2O_2, in the condition of 40 ° C temperature and 30 min of the test.

In the center point samples of the enzymatic transformation assay, the presence of a by-product of the mass of 360.5 g mol^{-1} was observed. Through the mass breaks of this second signal, it was possible to observe m / z 315 (progesterone) breaks, m / z 243; m / z 149, and m / z 123. Thus, it is concluded that enzymatic media are more effective than oxidizing means in the removal of progesterone and that the generation of byproducts from progesterone occurs throughout the exposure to both oxidizing and enzymatic media.

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