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Development and Validation of a Spectrophotometric Method in the UV-Vis Region to Evaluate the Interactions of Estrogens with Humic Substances in an Aqueous Environment

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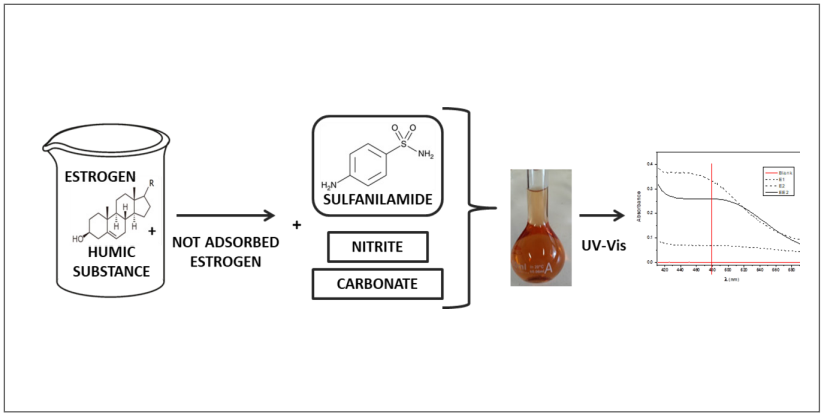
Natural and synthetic sex hormones are potent endocrine disruptors (EDs) that have been detected in aquatic environments on all continents. Studies show that humic substances (HS) are the main compounds capable of interacting with hormones, interfering in the bioavailability processes. In the present work, a methodology based on the colorimetric properties of the azo-compounds was developed and validated using diazotized sulfanilamide as a reagent to study the interactions with HS of estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2). As an alternative method to quantify hormones during interactions, ultraviolet-visible molecular absorption spectroscopy was employed. An analytical curve was constructed for concentrations ranging from 10.0 to 28.0 μg mL^{-1}. The results were compatible with chromatography, also applied in this work. For the study of the interaction, samples of the hormone standard and HS solution were used under constant agitation. The reduction in the concentration of estrogens has been detected since the beginning of the process, with EE2 being the best result. In the aliquot removed from the system after 48 hours of agitation, 51.1% EE2 was removed in the presence of 10.0 μg mL^{-1} of the HS and 75.8% at 20 μg mL^{-1}. Simulations can predict the behavior of hormones in natural aquatic environments rich in HS.

Graphical abstract



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1. Introduction

The transformation of watershed areas as sanitary sewage receivers, treated or not, has gained notoriety in recent decades due to the potential capacity to contaminate surface water in urban areas [1-3]. Among the substances that impact aquatic bodies, the contaminants of emerging concern, deserve special attention, because even present in small concentrations, these substances are capable of triggering effects on the systems in which they are introduced [4].

Compounds called endocrine disruptors (EDs) are important pollutants of emerging concern. Recognized for their ability to cause adverse effects on living organisms and its descendants, due to changes in functions of the endocrine system [5,6]. Defined as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural body hormones that are responsible for maintaining homeostasis, reproduction, development and behavior" [7, 8].

Natural or synthetic, sex hormones, are potent EDs, which have been detected in different environmental matrices, on all continents. It has been reported [9-14] that the presence of estrogens: estrone (E1), 17 β-estradiol (E2) e 17 α-ethinylestradiol (EE2) in various aquatic compartments (water, sediments and biota) and Wastewater Treatment Plant (WWTP) effluents, in concentrations ranging from ng L^{-1} to μg L^{-1}. In this context, the occurrence of estrogenic compounds in the aquatic environment can be considered a worldwide public health problem [2, 8, 15-17], which is mainly related to the inefficiency of the effluent and sewage treatment processes [9, 17]. Moreover, contamination of estrogenic compounds has an important environmental impact on aquatic bodies. Studies has shown that these hormones can affect the reproductive system of aquatic biota, especially fishes, even when the concentrations are low [3, 16].

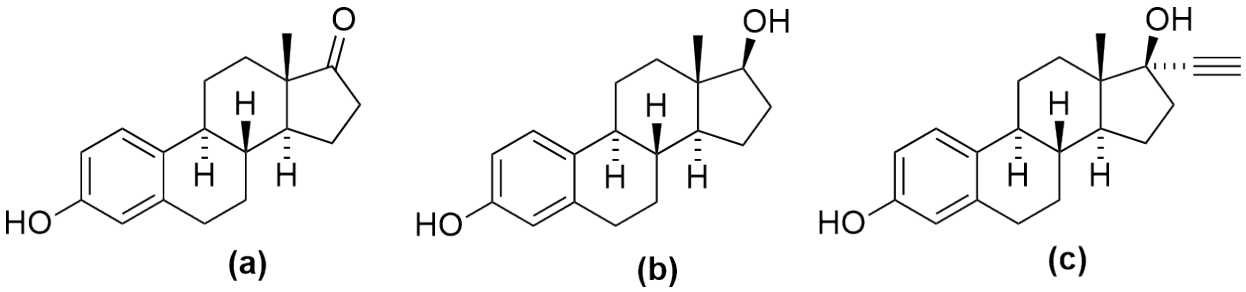

Fig. 1. Chemical structure of endocrine disrupters: (a) estrone (E1); (b) 17 β-estradiol (E2); (c) 17 α- Ethinylestradiol (EE2).

Natural and synthetic estrogens are excreted by urine in biologically active forms in different concentrations, depending on age, diet, health status, pregnancy, among other conditions [18]. Although they have a relatively short half-life when compared to other organic compounds, such as pesticides, however, estrogens are continuously introduced into the environment, which gives them a persistent character [19-21].

There are several routes of degradation and hormonal complexation in the aquatic environment that arouse great interest, especially the interactions between hormones and HS, which are potential compounds to reduce the bioavailability of DEs in the environment. The estrogen occurrence in aquatic environmental systems and its possible interactions with humic substances, are topics of great relevance. However, studies depend on analytical techniques for sample preparation and analysis, considering the complexity of the matrix and the level of analyte concentration. Therefore, in this study, the first stage and of great relevance importance was the validation of two methodological approaches. Then, from the validation data, the objective was to assess the behavior of estrogens against HS in bench studies. *This study can serve as a basis for others investigating removal alternatives.*

2. Material and Methods

The present study was based on two different Analytical Methodologies to assess the estrogens presence and behavior in surface waters, also identifying the feasibility of different methodological approaches. The first analytical method was Molecular Absorption Spectroscopy [22] and the second, High Performance Liquid Chromatography (HPLC) with UV-Vis detector [23]. These methodologies were developed based on the literature on the standardization of the colorimetric method and HPLC for quantification of estrogens in water, and its removal with adsorbent material [24].

The most common methodology for studying hormones in aqueous media is High Performance Liquid Chromatography (HPLC), with detection by diode array (DAD) or UV-Vis, with good results. However, the high cost for its development and the restricted access to this equipment can make the study unfeasible, or at least, limit the approach. On the other hand, the availability of pre-concentration techniques using devices such as cartridges for solid phase extraction, has brought the prospect of using cheaper and widely available methodologies, such as those based on molecular absorption. The study of both methodologies, the traditional and the alternative, carried out in a comparative way, observing the obtained results, is the proposal that we make in the present work.

To prepare the solutions, rinse the electrodes, and glassware, purified water was used, using Milli-Q system (Millipore) with 18.2 MΩ resistivity to produce high purity water. All reagents used for the experiments were analytical grade. Estrogens and humic acid (HA) from Sigma Aldrich®. Absolute ethanol, sodium nitrite p.a. and sulfanilamide from Synth®. Calcium chloride p.a., 37% fuming hydrochloric acid and anhydrous sodium carbonate p.a., Merck®. For

chromatographic analyzes, HPLC grade methanol from Merck® was used.

2.1 UV/VIS Absorption Spectrophotometry Analysis

2.1.1 Colorimetric reaction

Initially, a stock solution (1000 µg mL^{-1}) of each of the estrogens was prepared in absolute ethyl alcohol [22]. In ice bath, 0.1% (w/v) of sulfanilamide solution dissolved in 0.1 mol L^{-1} hydrochloric acid solution was added with 0.5% (w/v) sodium nitrite and stored for 2 min for stabilization. Then, 10 mL of the standard solution of estrogens was added at concentrations of 10.0; 12.0; 14.0; 16.0; 18.0; 20.0; 22.0; 24.0; 26.0 and 28.0 µg mL^{-1} excepted to the blank. The solution was stirred for 1 min and a 10% (w/v) sodium carbonate solution was added and completed to 50 ml with ultrapure water [22].

2.1.2 Analysis and Construction of an analytical curve for E1, E2 and EE2

The absorbance was measured in triplicates for all concentrations of the three estrogens, aiming at the construction of the analytical curve, at 480 nm wavelength. The measurements were performed on a VARIAN CARY® 50 spectrophotometer, using quartz cells with 3 mL capacity and an optical path of 5 mm. The analytical performance of the method was evaluated using the following parameters of merit: linearity, accuracy, precision, limit of detection (LD) and limit of quantification (LQ) [25].

2.2 High performance liquid chromatography (HPLC) analysis

Was evaluated in triplicates of estrogens solutions with 10.0; 15.0; 20.0; 25.0 and 30.0 µg mL^{-1}, maintaining similarity with the concentration range used for the spectroscopic method. A chromatograph model YL9100 and a C18 column brand Luna Kinetex (4.6x250mmx5µm) with UV/Vis detector were used. The analytes separation were achieved by gradient elution using ultrapure water (A) and methanol HPLC grade (B) as mobile phase: A:B (41:59) by 9 min and then to 49% A: 51% B was held until the end of the run. The chromatographic conditions used are shown in Table 1.

Table 1. Chromatographic conditions.

Run time	30 min
Volume injection	20 µL
Column temperature	40°C
Flow rate	1.2 mL min^{-1}
wavelength	230 nm

2.3 Humic Acid (HA) analysis

To evaluate the functional groups in the infrared spectrum of the HA was performed in the range of 400 to 4000 cm^{-1}. The equipment used was a SHIMADZU® spectrophotometer, model IR PRESTIGE 21. The pastilles were prepared with 100 mg of KBr spectroscopic grade (dried at 110°C and sprayed), and 1 mg of standard HA.

2.4 Estrogen-SH interactions

The interaction of hormones E1, E2 and EE2 with HA was performed at room temperature (~ 25°C) and pH ~ 7.0. The ionic strength was kept constant with calcium chloride 0.01 mol L^{-1} [24]. Experiments varyied the HA concentration to 5.0; 10.0 and 20.0 µg mL^{-1} against the hormone solution at a constant initial concentration of 20.0 µg mL^{-1}. The interaction was performed under constant agitation at 80 rpm on a Scientec® shaker and accompanied by periodic collections of solution for periods of maximum 48 h, with quantification using the methods validated in the present research.

3. Results and Discussion

3.1 Validation Process of UV/VIS Absorption Spectrophotometry

Initially, the spectral behavior for the UV-Vis spectroscopy method was verified. The absorption spectra of the products obtained from the coupling reaction of the studied hormones at a concentration of 20.0 mg / mL, and the diazotized sulfanilamide showed absorption curves with a maximum absorption at 480 nm (Figure 2).

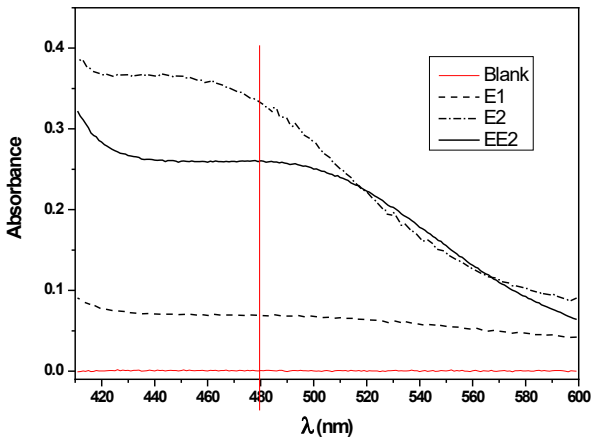

Fig. 2. Absorption spectrum of the product formed in the coupling reaction of diazotized sulfanilamide and E1, E2 and EE2 (20.0 µg / mL).

In the reaction, sodium nitrite is used, which reacts with sulfanilamide in an acidic medium forming a compound diazo, followed by coupling with the hormone [22]. The compound formed has low stability and immediate spectroscopic analysis after the colorimetric reaction is recommended.

This colorimetric reaction is noticeable in the UV-Vis range and corresponds to the absorption spectrum of the hormones E1, E2 and EE2 at 480nm. Reading was performed at 480 nm, checking, as expected, the variation in absorption according to the concentration.

Linearity was determined by analyzing in triplicate at the same day, 10 different concentrations (from 10.0 to 28.0 µg mL^{-1}), obtaining an average correlation coefficient (R^2) of three analytical curves of 0.994 for E1, 0.994 for E2 and 0.981 for EE2.

After calculating the average of the triplicates (experimental values) for each point, the analytical curves were constructed and are shown in Figure 3.

The average correlation coefficients obtained from the three curves, close to 1, meet the required criteria. Which demonstrates the data variation obtained, and the linear relationship between the concentration of the studied estrogens and the absorbance in the concentration range 10.0 - 28.0 µg mL^{-1} [26]. For the calculation of accuracy, a

linear range was established, from 3 concentrations: low (10.0 μg mL^{-1}), medium (20.0 μg mL^{-1}) and high (28.0 μg mL^{-1}), all analyzed in triplicates (Table 2).

The method accuracy was established through the relationship between the average concentration determined experimentally and the corresponding theoretical concentration. All obtained values are within the Brazilian legislation, which establishes Accuracy between 80 and 120% of the analyte content in research [27]. Method precision was assessed in terms of relative standard deviation (RSD) or the coefficient of variation (CV%), with no values higher than 15% allowed. Once the Standard Deviation (SD) was obtained, RSD was calculated which shows the accuracy of the method. This value is the ratio of the standard deviation (SD) to the determined mean concentration (DMC) expressed as a percentage. Considering the absorbance for the estrogen concentration range in the analytical curve, at 480 nm, the following obtained results are presented in Table 3.

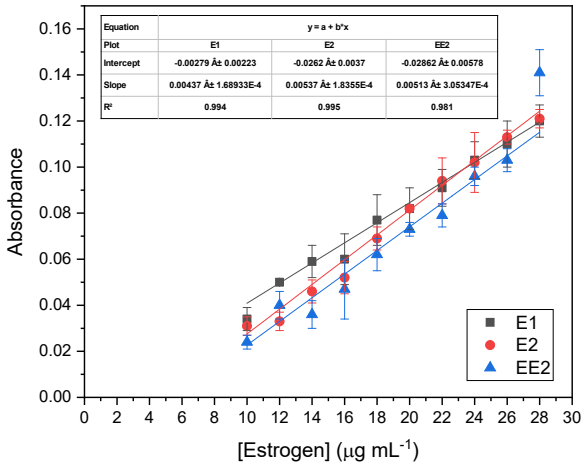

Fig. 3. Analytical Curves for Estrogens by UV/Vis method in 480 nm.

Table 2. Analytical test for assessing the accuracy of the method.

Theoretical Concentration (μg mL^{-1})	E1		E2		EE2	
	Experimental average concentration (μg mL^{-1})	Accuracy (%)	Experimental average concentration (μg mL^{-1})	Accuracy (%)	Experimental average concentration (μg mL^{-1})	Accuracy (%)
10.0	9.8	98.5	10.7	107.1	11.3	113.0
20.0	19.7	98.5	19.7	98.5	20.8	104.0
28.0	28.4	101.4	27.9	99.6	27.7	98.9

Table 3. Relative standard deviation for the concentration ranges from 10 to 28 μg mL^{-1} of E1, E2 and EE2.

	E1			E2			EE2		
C (μg mL^{-1})	Average absorbance	σ	RSD (%)	Average absorbance	σ	RSD (%)	Average absorbance	σ	RSD (%)
10	0.034	0.005	14.7	0.031	0.004	12.9	0.024	0.003	15.0
12	0.050	0.001	2.0	0.033	0.004	12.1	0.040	0.006	15.0
14	0.059	0.007	11.8	0.046	0.005	10.8	0.036	0.006	8.1
16	0.060	0.011	15.0	0.052	0.007	13.4	0.047	0.013	11.2
18	0.077	0.011	14.2	0.069	0.005	7.2	0.062	0.007	11.8
20	0.082	0.009	10.9	0.082	0.001	1.2	0.073	0.003	3.9
22	0.091	0.008	8.8	0.094	0.010	10.6	0.079	0.005	6.0
24	0.103	0.008	7.7	0.102	0.013	12.7	0.096	0.004	4.6
26	0.110	0.010	9.0	0.113	0.003	2.6	0.103	0.005	4.5
28	0.120	0.007	5.8	0.121	0.004	3.3	0.141	0.010	8.1

Therefore, the RSD, for the implemented methodology according to the experimental conditions described above, the average values of 9.4% for E1, 8.3% for E2 and 8.1% for EE2, corresponding to the methods accuracy within the relative standard deviation limit below 15%. In the present study, the limits were determined based on the relationship between the standard deviation of the response and the slope of the analytical curve [28] (Table 4).

Table 4. Limit of detection and limit of quantification calculated for E1, E2 and EE2.

Estrogen	σ	Slope	LD calculated (μg mL^{-1})	LQ calculated (μg mL^{-1})
E1	0.005	0.00458	3.27	10.91
E2	0.004	0.00524	2.29	7.63
EE2	0.003	0.00501	1.79	5.98

After the limits were determined, they were tested with independent samples, to verify if the trend and precision achieved were satisfactory. For trace level analysis, LQ can be adopted as the lowest concentration of the analytical curve [28]. After validation of the colorimetric method with quantification by UV/Vis spectroscopy, the methodology was then validated using HPLC with UV/Vis detector, comparing the obtained results in order to confirm the adequacy of the spectroscopic method for the purposes of the present work.

3.2 Chromatographic method

After sample injection, running times were identified in approximately 24.5 minutes for E2, 26 minutes for E1 and 29 minutes for EE2. The peaks for E1 and E2 have close retention times under the presented chromatographic conditions and thus, the peaks related to the hormones E1 and E2 overlap when analyzed simultaneously, in the chromatogram resulting in only one broad peak. For this

reason, E2 was injected separately. The chromatogram is shown in Figure 4.

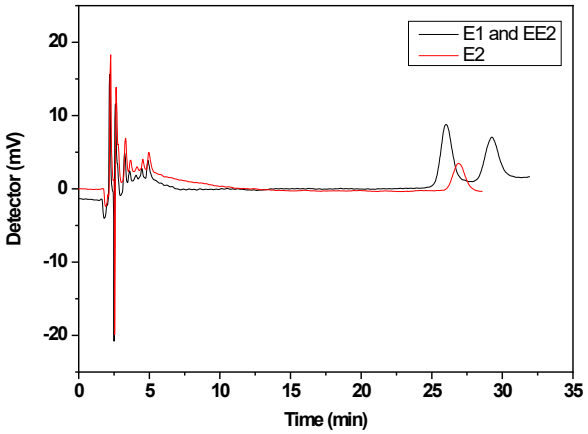

Fig. 4. Chromatogram for E1, E2 and EE2.

The chromatographic peaks observed between 1 and 6 minutes can be attributed to the solvent used to prepare the standard solutions (ethanol) and to possible impurities.

3.2.1 Validation Process of chromatographic method

As the aim of the present work is to make a comparison between the chromatographic method and the spectroscopic method, the same figures of merit were used for validation.

Linearity was determined [26, 27] by the triplicate analysis of 5 different concentrations (10.0 to 30.0 μg mL^{-1}), obtaining a determination coefficient (R^2) of three analytical curves from 0.990 to E1; 0.987 for E2 and 0.995 for EE2. The experimental values obtained for the construction of the analytical curve through the average of the triplicate analysis of each of the 5 chosen points are shown in Table 5.

Table 5. Average values of the area of the chromatographic peaks ($mV.s^{-1}$) of estrogens E1, E2 and EE2 and standard deviation.

Concentration (μg mL^{-1})	E1		E2		EE2	
	Average (area)	σ	Average (area)	σ	Average (area)	σ
10.0	111.009	8.7	139.234	7.9	91.062	6.3
15.0	242.207	13.4	280.446	6.2	194.014	12.4
20.0	300.006	6.6	358.195	5.8	266.145	5.2
25.0	391.649	9.9	480.244	8.3	324.271	6.4
30.0	455.975	3.2	511.883	5.3	408.125	5.1

After calculating the average of the triplicates (experimental values) for each point, the analytical curves were constructed from the relationship between the different concentrations and the mean area values (mV s^{-1}) produced by the chromatographic peaks and are shown in Figure 5.

The average correlation coefficients obtained for the three curves, close to 1, meet the required criteria. They demonstrated the variation of the data obtained, and a linear relationship between the concentration of the studied estrogens and the area of the chromatographic peaks range 10.0 to 30.0 μg mL^{-1} [28, 30]. For the calculation of accuracy, a linear range was established, from 3 concentrations: low (10.0 μg mL^{-1}), medium (20.0 μg mL^{-1}) and high (28.0 μg mL^{-1}), all analyzed in triplicates (Table 6).

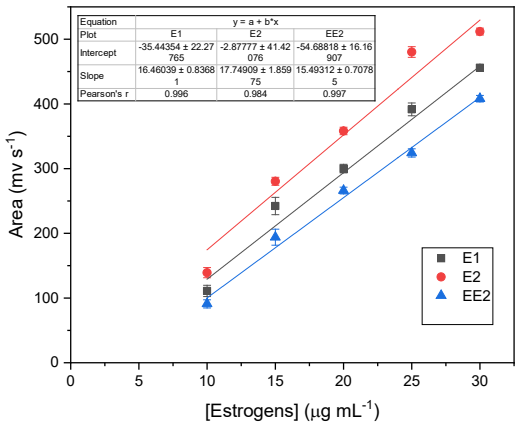

Fig. 5. Analytical Curves for estrogens for chromatographic method.

Table 6. Analytical test for assessing the accuracy of the chromatographic method.

Theoretical Concentration (μg mL^{-1})	E1		E2		EE2	
	Experimental average concentration (μg mL^{-1})	Accuracy (%)	Experimental average concentration (μg mL^{-1})	Accuracy (%)	Experimental average concentration (μg mL^{-1})	Accuracy (%)
10.0	10.37	103.7	9.28	92.8	10.59	105.9
20.0	19.62	98.1	20.34	101.7	20.01	100.05
28.0	28.71	102.5	28.06	100.2	27.92	99.7

The accuracy of the method was established through the relationship between the average concentration determined experimentally and the corresponding theoretical concentration. All the values obtained are within the legislation, which establishes accuracy between 80 and 120% of the analyte content in research [26]. The relative standard deviation to establish the precision of the chromatographic method was calculated based on the area of the chromatographic peaks for the concentration range of estrogens E1, E2 and EE2 on the analytical curve (Table 7).

Concerning precision, that is, the evaluation of the proximity of the results obtained in a series of measures of multiple sampling, the method showed precision within the limit of RSD or the CV%, which should not be higher than 15% [28]. Thus, the RSD, for the methodology implemented according to the experimental conditions described above,

has an average value of 3.7% for E1, 2.4% for E2 and 3.6% for EE2. The limits were calculated based on the Analytical Methods Committee (Royal Society of Chemistry). The LD calculated values for the chromatographic method are 1.56 μg mL^{-1} for E1; 1.28 μg mL^{-1} for E2 and 1.24 μg mL^{-1} for EE2. The LQ calculated values for the chromatographic method are 5.22 μg mL^{-1} for E1; 4.27 μg mL^{-1} for E2 and 4.13 μg mL^{-1} for EE2. The LD and LQ values for the chromatographic method are lower than those obtained by UV-Vis Spectroscopy, which substantiates data from the literature that points to chromatography as a more sensitive method [29].

Table 7. Relative standard deviation for the concentration ranges from 10 to 28 μg mL^{-1} of E1, E2 and EE2.

C (μg mL^{-1})	E1			E2			EE2		
	Average (area)	σ	RSD (%)	Average (area)	σ	RSD (%)	Average (area)	σ	RSD (%)
10.0	111.009	8.7	7.8	139.234	7.9	5.6	91.062	6.3	6.9
15.0	242.207	13.4	5.5	280.446	6.2	2.2	194.014	12.4	6.3
20.0	300.006	6.6	2.1	358.195	5.8	1.6	266.145	5.2	1.9
25.0	391.649	9.9	2.5	480.244	8.3	1.7	324.271	6.4	1.9
30.0	455.975	3.2	0.7	511.883	5.3	1.0	408.125	5.1	1.2

3.3 UV/Vis spectroscopy *vs* chromatography

After the validation of the method of quantification of estrogens E1, E2 and EE2 by UV/Vis molecular absorption spectroscopy and by HPLC, a simultaneous analysis was carried out using the two methods for solutions specifically prepared for this test, obtaining the results that follow according to Table 8.

Table 8. Results obtained in the simultaneous analysis of E1, E2 and EE2 solutions by UV/Vis and HPLC.

Theoretical concentration (μg mL^{-1})	E1 (μg mL^{-1})		E2 (μg mL^{-1})		EE2 (μg mL^{-1})	
	HPLC	UV/Vis	HPLC	UV/Vis	HPLC	UV/Vis
10.0	10.4	9.7	9.3	10.4	10.6	10.8
20.0	19.6	19.7	20.3	19.7	20.0	20.5
28.0	28.7	28.7	28.0	27.6	27.9	27.5

Notwithstanding of the chromatographic method being the most recommended for the quantification of estrogens, the spectroscopic method applied in this work showed satisfactory results, close to those obtained by chromatography, recalling that standard solutions prepared in the laboratory, with known chemical composition, with no interferers were used. The comparison of the UV/Vis method with HPLC adds value and consistency to the results of the spectroscopic method, what possibilities to continue this study to the interaction evaluation between the hormones E1, E2 and EE2 with HS.

3.4 Spectroscopic analysis of HA employing infrared

The spectrum obtained (Figure 6) for the analysis of HA used in the tests of interactions with estrogens, revealed bands characteristic of phenolic and carboxylic groups, with the presence of aromatic compounds.

However, the intense and large band in the region of 3550 - 3200 cm^{-1} can be attributed to the ν(O-H) stretch, which corresponds to several groups containing phenolic OH [32] which can be observed in 3400 cm^{-1}. Bands that indicate the presence of aromatic compounds appear in the region of 1600 - 1585 cm^{-1} and 1500 - 1400 cm^{-1} and are attributed to skeletal vibration involving the axial deformation of the C-C bonds of the aromatic ring [30]. Further, there are also intense bands located in the region of 1750 - 1500 cm^{-1}, where the ν(C=O) stretches of carboxylic groups and asymmetric ν_{as}(C=O) stretches of the carboxylates appeared, indicating a high concentration of carboxylic groups. Following, the presence of bands in this region also receives a contribution from the δ (N-H) deformation [31]. The bands that appear in the spectrum that correspond to carboxylic acids in the regions of 1320 - 1210 cm^{-1} and 1440 - 1395 cm^{-1} derive from the axial strain of C-O and the angular strain of O-H, respectively. The band located in the region of 3000 - 2800 cm^{-1} is attributed to the stretch ν(C-H). Moreover, there is also a band in the region between 1120 - 1050 cm^{-1} that may be attributed to the ν(C-O) stretch of alcohols. Subsequently, at 1095 cm^{-1} a peak is observed for the Si-O groups present, indicating the presence of silica in the sample [30-32]. The sample of HS analyzed that corresponds to the material used in the study of this work has, therefore, structural characteristics that can be indicative of its reactivity and its tendency to participate in reactions such as complexation or adsorption. Altogether, the presence of phenolic functional groups, quinones and semiquinones in their structure, is an indicator of redox activity in natural systems [33, 34].

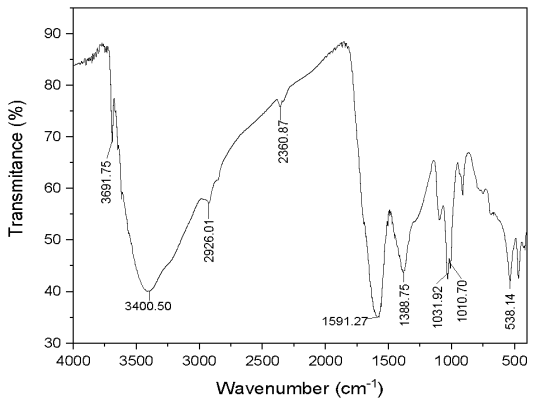

Fig. 6. Graphic of HA transmitance by IR analysis.

3.5 Interaction between estrogens and HS

To study the interaction between hormones and SH, HA was used in low concentrations (5 to 20 μg mL^{-1}), to eliminate interferences during measurement by UV-Vis spectroscopy, since the solution of HA is colored. The interaction of the hormones E1, E2 and EE2 in an initial concentration of 20.0 μg mL^{-1} with HA in concentrations of 5.0; 10.0 and 20.0 μg mL^{-1} were availed in a maximum period of 48 hours. The results obtained are shown in Figure 7.

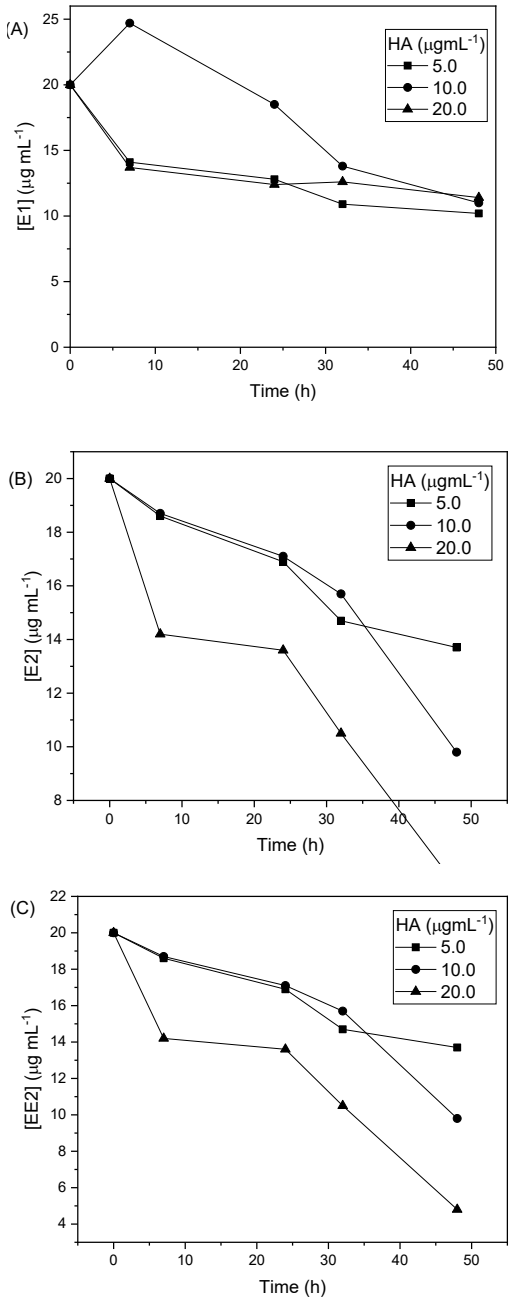

Fig. 7. Hormones interaction with HA for 48 h (a) E1, (b) E2 and (c) EE2.

Although the reduction in the concentration of estrogens in the study medium, when in the presence of HA was detected since the beginning of the process. In all cases, it was noticed an increase in hormone removal with an increase of the contact time. Typically, for the concentration of 5 μg mL^{-1} of HA, at the end of 48 h, the concentration of E1 was reduced from 20.0 μg mL^{-1} to 10.2 μg mL^{-1}, that is, a 49% removal. When the concentration of HA was increased to 10 and 20 μg mL^{-1}, there was again a reduction in the concentration of the studied estrogens, over time (Figure 7a). However, for E1 the percentage removed was practically the same, and it can be affirmed that there is a minimal difference in the removal capacity when compared with the HA solution 5.0 μg mL^{-1}. In the presence of 10.0 μgmL^{-1} of HA, 44.85% (9.0 μg mL^{-1}) of E1 was removed, while in 20.0 μg mL^{-1} of HA, 43.0% was removed (8.6 μg mL^{-1}) of E1 estrogen.

The interaction of 5.0 μg mL^{-1} of HA with E2 and EE2, promoted a reduction in the concentration of hormones of approximately 35%, with concentrations of 12.0 μg mL-1 of E2 and 13.7 μg mL^{-1} of EE2 at the end of the allotted time. Further, in the case of the hormone E2 (Figure 7 b), there was a reduction in the concentration of estrogen with an increase in contact time, in the different conditions studied, but the removal of this estrogen was less expressive with the increase in HA concentration, which was 44.40% (8.9 μg mL^{-1}) in the presence of 10.0 μg mL^{-1} of HA and 54.45% (10.9 μg mL^{-1}) in the presence of 20.0 μ gmL^{-1} of HA.

The hormone EE2 showed the best results of complexing with HA in the studied conditions. When increasing the concentration of HA, there was a significant increase in the removal of estrogen (Figure 7 c). Considering the aliquot removed from the system, after 48 h of agitation, 51.1% (10.2 μg mL^{-1}) was removed in the presence of 10.0 μg mL^{-1} of HA and 75.8% (15.2 μg mL^{-1}) in 20.0 μg mL^{-1}.

As may be seen, solutions containing the highest concentrations of HA, the contact time to reach the same values of percentage of removal was shorter than for solutions containing the lowest concentrations of HA. Based on these results, it can be assumed that, in an environment rich in HS, the order of complexation of estrogens concerning time will be EE2>E2>E1. Figure 8 represents the graph that shows the relationship between the percentage of hormone removal and the concentration of HA.

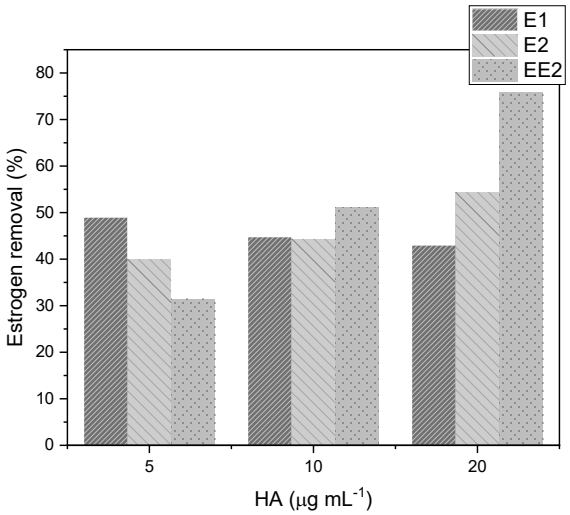

Fig. 8. Relationship between hormone removal percentage and HA concentration.

Scientific works that searched to quantify estrogen hormones in surface waters and sediments, observed the highest concentrations in the sediment, which indicates the adsorption of the hormones [35, 36]. Therefore, the presence of HS in the aquatic environment considerably reduces the availability of these estrogens and probably other EDs and, consequently, their interaction with the biota. These estrogens are hydrophobic organic compounds, moderately soluble in water and which preferentially adsorb to sediments and solid particles. Consequently, it is expected that adsorption in soil, or biological sludge or sludge is a significant factor in reducing the concentration of these estrogens in the aqueous phase [37, 38]. The amount difference of hormone complexed with HA may be associated with the structural peculiarities of each hormone. The EE2 has more non-polar characteristics, due to the presence of the ethinyl group in its structure, being able to alter its affinity for HA [24].

However, investigations on the adsorption of estrogens in sediments [39] revealed that synthetic estrogens are removed more easily from the aqueous phase than natural estrogens, besides, the sorption of estrogens to sediments was directly related to the levels of total organic carbon of the medium.

The different groups present in HS, such as carbonyls and phenolic hydroxyls, cause them to assume a polyelectrolytic behavior, acting as complexing agents for several metal ions; they are also able to adsorb various organic pollutants, such as hormones and pesticides, thus decreasing their needs in the environment [40]. The solubility and, therefore, the availability of organic pollutants in surface waters, is totally dependent on temperature, pH, dissolved salts, and on the existence of humic substances or suspended materials [17]. Adsorption is the main interaction phenomenon and occurs through humic substances dissolved in the aquatic environment, and also through suspended materials of any origin, but covered with humic substances. The details of this process, as we intend in the present study, can provide subsidies for new strategies for the removal or immobilization of dissolved hormones in different media.

Physic-chemical data indicates that the humic molecule has a flexible structure due to intra and intermolecular interactions and hydrogen bonds [41]. For the studied EDs, it can be assumed that the main existing interaction with HS, represented by HA, is the hydrogen bonds of the oxygenated groups and the (OH) groups present in these molecules. Additionaly, they have many hydrogens available for Van der Waals interactions in their structures, the studied estrogens can occupy the complexation sites present in humic micelles [41].

4. Conclusions

Considering that UV/Vis molecular absorption spectroscopy has the important advantage of being a simple, fast, low-cost technique that require less solvent compared to HPLC, the method, as described in this study, may be an alternative for the quantification of standard samples of estrogens. The results obtained demonstrated that this methodology has adequate analytical characteristics according to the current legislation and specialized scientific literature. The validation process of this methodology for estrogens E1, E2 and EE2 was successfully achieved, as well as for the methodology based on chromatographic analysis (HPLC). The spectroscopic analyzes showed results compatible with the chromatographic ones, within the concentration range worked, and considering the experimental bench conditions. The quantification of estrogens for the study of interactions with HS was made possible by both instrumental techniques.

According to that, the limitations of the validated techniques include the impossibility of simultaneous detection in mixtures of these hormones due to the lack of the selectivity of the spectroscopic method and the overlapping of the peaks for E2 in the chromatographic analysis. Moreover, regarding the analyzed concentrations, the study of real samples should include sample pre-concentration techniques, as well as more sensitive detectors and hyphenated techniques for chromatographic methods.

In the present work, we used UV-Vis spectroscopic analysis in order to study the behavior of hormones against humic substances, however the methodology may be more comprehensive. If the objective is to detect the hormones E1, E2 or EE2 in an aqueous sample, the methodology also applies, in this case associated with the sample pre-concentration procedure, using techniques such as solid phase extraction (SPE). Pre-concentration seeks to reach the level of detection for hormones with the applied methodology. Furthermore, if the determination of the concentration of hormones is carried out in real aqueous samples, with high concentrations of HS, the effect of the matrix can be minimized, using a sample blank to reset the equipment. White can correct possible color errors or turbidity in the sample before adding reagents. In this case too, one can use the pattern addition technique, and proceed with the UV-Vis methodology already discussed.

To conclude, the results demonstrate that the availability of estrogens may be related to the presence of HS in the aquatic environment, consequently, similar studies are important for a better understanding of the mobility, transport and reactivity of emerging contaminants in aquatic systems. Considering the presence of HS in several natural environments, it is believed that the study of the impact caused by the supply of estrogens in the environment and its consequences for living organisms, must include the interactions verified in this work. Studies involving emerging pollutants are increasingly important due to the risks they pose to the environment and health, as well as new perspectives for analysis and quantification.

Author Contributions

Sheisa Fin Dantas Sierpinski contributed with formal analysis, investigation, methodology, writing original draft and review & editing. Aline Jorge contributed with investigation and review & editing. Vitor da Silveira Freitas, Rômulo Domingues and Laíz M. França constributed with investigation. Adriano Gonçalves Viana and Giovana K. Wiecheteck contributed with supervision. Elizabeth Weinhardt O. Scheffer contributed with conceptualization, supervision and writing original draft.

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