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MONITORING OF CONTAMINATION OF URBAN SURFACE WATERS IN THE CITY OF CAMPO GRANDE/MS BY HORMONES 17β-ESTRADIOL AND 17α-ETHINYLESTRADIOL USING DISPERSIVE LIQUID-LIQUID MICROEXTRACTION AND HPLC-UV

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Abstract

Studies have shown that hormones have endocrine disrupting properties, which characterize them as potentially toxic to the aquatic environment. This study aims to monitor the water along Prosa Stream/Anhanduí River monthly, to evaluate its contamination by the hormones 17β-estradiol (E2) and 17α-ethinylestradiol (EE2). Dispersive liquid-liquid microextraction (DLLME) was used for analyte extraction, with acetone as a disperser solvent and carbon tetrachloride as the extraction solvent, followed by high-performance liquid chromatography with ultraviolet detector (HPLC-UV) as the analytical tool. The results of this application in natural samples indicated the presence of the natural hormone E2 in approximately 72% of the points evaluated, with its concentration values between 48 μg L^{-1} and 175 μg L^{-1}. In contrast, it was not possible to quantify the concentrations of synthetic hormone EE2, as these values were below the detection limit of the analytical method applied. Even though there is no national environmental legislation that limits amounts of hormones in surface water, these contaminations are significant, due to their already known toxicological potential. It was evident that along the river from its origin to the exit of the city there was the appearance and increase of the contamination of the waters by the hormone E2, thus it is clear that the urbanization around the rivers has become an environmental and health problem for providing the contamination of the aquatic environment.

Keywords: 17β-estradiol, 17α-ethinylestradiol, DLLME, endocrine disruptor, surface water.

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Introduction

Contamination by personal care and hygiene products, drugs, household cleaning chemicals and even nanoparticles (USEPA, 2008) in rivers and streams are currently one of the main issues affecting quality of life in large cities. It happens because the infrastructure for maintaining water quality and sewage sanitation has not grown significantly to follow the population and urbanization growth, especially in developing countries (Ferreira *et al.*, 2020).

Although developing countries may be experiencing economic growth and development, which causes a demographic expansion, the amount of public investment in wastewater treatment infrastructure rarely keeps pace with this demand. Consequently, most urban river systems in emergent nations are subject to water quality deterioration (Capps *et al*., 2016).

Because urbanization is largely unplanned, the land in these regions is used for multiple different purposes, including urban housing, industrial zones, small-scale urban agriculture, and informal settlements. Mixed land use, combined with a lack of treatment facilities, leads to a wide range of chemical and biological contaminants being introduced into rivers from point sources (Pongmala *et al.,* 2015) (Duvert *et al.,* 2019).

Modern society has been demanding an ever-increasing supply of a variety of products. This has increasingly caused contamination of river and groundwater by various chemical substances (Sorensen *et al.*, 2015). These contaminants are known as emerging contaminants (EC) and because they are not standardized in environmental legislation, they require studies on their toxicological intensities and impacting effects on the environment (Gaffney *et al.,* 2014).

ECs are defined as global organic contaminants with bioaccumulative, toxic and persistent characteristics. Substances such as alkylphenols and derivatives, artificial sweeteners, hormones, pesticides, illicit drugs, and by-products of water disinfection processes, are listed as ECs (Richardson and Kimura, 2016). These substances are transported into the rivers by direct flow or by indirect flow pathways through the subsurface. Variations in precipitation, short term and seasonal, exert direct control over the magnitude and time of entry of these contaminants into aquatic systems (Mouri *et al.,* 2011). This is significant in tropical countries such as Brazil, which face the challenge of intense rainy seasons that often result in high rates of rainwater overflow to urban rivers. High precipitation events can increase the risk of contaminants being transported by rainwater, as well as cause the dilution of sewage and wastewater (Duvert *et al.,* 2019).

Certainly, water is one of the most abundant chemical substances on Planet Earth, pointed out as a natural resource of vital importance for the support and progress of living beings. However, this source is finite, its greatest demands are required for human consumption and, in addition, it has a crucial importance in the industrial, agricultural, livestock and electrical sectors. With the

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population increase, accentuated year by year, these sectors have their frenetic amplification resulting in an eventual future scarcity of this natural resource, and in this way, making the use and consumption of water a worldwide complication. As a result, water quality stands out as an issue of great magnitude in the field of Environmental Chemistry (Chong *et al.,* 2010) (Gorga *et al.,* 2015) (Huang *et al.,* 2015).

There is no normative framework or legislation to control these contaminants in the environment. As such, routine environmental monitoring programs do not yet include some of these classes of chemical contaminants (Marcoux *et al.,* 2013).

These contaminants contribute to complications in the endocrine functions of aquatic communities. Substances that are related to the effect on the endocrine system are known as endocrine disruptors and are shown to be increasingly inserted in these ecosystems, being a potential risk to the life of aquatic organisms and even to humans (Gavrilescu *et al.,* 2015) (Benotti *et al*., 2009).

Studies indicate that some estrogen hormones enter the aquatic environment through domestic effluents, even after conventional treatment, which is insufficient for the elimination of these substances. Estrogenic hormones are extremely active compounds with a wide action potential in the endocrine system (Hamid *et al.,* 2012) (Chang *et al.,* 2011).

Among the various substances with endocrine disrupting properties, the hormones estrogen 17β-estradiol (E2), which is produced naturally by female mammals, and the synthetic 17α-ethinylestradiol (EE2), used in contraceptive tablets, have a considerable biological effect, and stand out for being identified in surface water bodies (Racz and Goel, 2010).

Dispersive Liquid-Liquid Microextraction (DLLME) is a very efficient technique used for the extraction of hormones in surface waters. This technique is based on the extraction of the analyte by partition between two liquid phases immiscible with each other, an aqueous phase (sample) and an organic phase (mixture of solvents). The solvents mixture consists of an associated disperser and extraction agents, where the disperser solvent, which is soluble in the aqueous sample and the extraction solvent, assists in the process by providing an increase in the contact surface between the extraction solvent (water-insoluble) and the sample by the generation of droplets in the same. This dispersion is formed from the rapid injection of the mixture over the sample (Martins *et al.,* 2012).

Campo Grande, capital of the state of Mato Grosso do Sul, has in its urban area eleven watersheds. Among them, the Anhanduí and Prosa watersheds stand out. One of the main water courses in the city of Campo Grande is the Anhanduí River (SEMAGRO, 2016). The Anhanduí River starts at the confluence of the Prosa and Segredo streams. Because it flows through densely populated regions,

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it may receive domestic sewage and/or effluents from commercial establishments along its route, also contributions from other microbasins. One of them is the microbasin of the Prosa Stream, which is in the central and eastern part of the urban area of Campo Grande. It goes through downtown, where part is channeled into the Segredo Stream, forming, from that part on, the Anhanduí River. Occasional releases may be due to the urbanization of the region, along the entire length of the Prosa Stream, and, in collaboration with its other affluents, downgrade the quality of these waters (SEMAGRO, 2016). Much of the extension of this river flows through the central region of the city, serving as a main route for rainwater.

In this context, the object was to optimize an analytical method to detect and measure the hormones E2 and EE2 as contaminants, by monitoring the surface waters of the Prosa Stream and the Anhanduí River monthly, to promote an essential tool for monitoring and evaluating hormones in these aquatic environments.

Experimental

Materials, reagents and chromatographic conditions

To carry out this study, the following reagents were used: Ultrapure water (18.2 MΩ cm); Acetonitrile (HPLC grade), J. T. Baker (Madrid, Spain); Carbon tetrachloride (99.5% PA), Dinâmica (Indaiatuba, Brazil); Hydrochloric Acid (37% PA), Vetec (Duque de Caxias, Brazil); Sodium Chloride (99.00% purity), Dinâmica (Indaiatuba, Brazil); Acetone (UV-HPLC-Spectroscopic grade), Vetec-Fine Chemistry (Duque de Caxias, Brazil); Sodium Hydroxide (97% purity), Vetec, (Duque de Caxias, Brazil); Standards 17β-estradiol (≥ 98%), Sigma Aldrich (Saint Louis, USA) and 17α-ethinylestradiol (≥ 98%), Sigma Aldrich (Saint Louis, USA). All solutions were filtered through a syringe filter (0.45 µm PVDF) and blanks were performed regularly to check the analyte in the reagents used.

The materials used to collect the samples were: a portable pH meter with automatic temperature compensation, Kasvi K39-0014PA (São José do Pinhais, Brazil); a polyvinyl chloride (PVC) container, approximately 1.5 L; a 500 mL stainless steel bucket with an adapted rod; 25 mL amber glass bottles; a thermal box; artificial ice.

The equipment and materials used for the extraction process were: a Vortex Shaker, Fisatom 772, with a fixed speed of 2800 rpm (São Paulo, Brazil); a centrifuge Sigma 4K15 (Osterode am Harz, Germany); PVDF syringe filters with 0.45 µm porosity and a 25 mm diameter, Filtrilo (Colombo, Brazil); a bench pH meter Hanna HI221 (Barueri, Brazil); a water purifier, Gehaka OS 50LX TQ (São Paulo, Brazil); 15 ml Falcon conical tubes, Cralplast (Cotia, Brazil); an analytical balance, Precisa XT-220A (Contagem, Brazil); micropipettes (10-100; 100-1000; 500-5000 µL), Labmate Pro, Satorius (Gottingen, Germany); a 50 µL glass syringe, Hamilton (Darmstadt, Germany).

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Chromatographic analyzes were performed in ambient temperature with a Varian Prostar liquid chromatograph with UV detector, model 320 – 20 µL manual injector; mobile phase acetonitrile/water, using the isocratic method 50/50 (v:v), with a total analysis time of 10 minutes at a flow rate of 1.0 mL min^{-1} and a wavelength adjusted to 281 nm; stationary phase column C18; ID 4.6 mm x 150 mm length; 5 µm particle size from Zorbax brand (Santa Clara, USA), guard-column with cartridge SB-C18, ID 4.6 x 12.5 mm length, 5 µm particle size from Zorbax brand (Santa Clara, USA).

Analytical methodology – HPLC-UV

Recovery studies of the analytes of interest were carried out using a mix of the hormones E2 and EE2 with a concentration of 5 mg L^{-1}. Injections were performed using the full loop technique (volumes of 25 µL). As the proportion of the mobile phase becomes less polar, increasing the amount of acetonitrile, the intensity of the chromatographic peaks improved significantly, however, it was noticed that the approximation of the retention times of the analytes impaired the chromatographic separation. A mixture of acetonitrile/water 50/50 (v/v) was the proportion of the mobile phase that showed the best separation between hormones. The flow was maintained at 1.0 mL min^{-1}. Equipment and sample blanks were evaluated to determine the need to use an internal standard during the analyses.

DLLME procedure

The collected samples were submitted to a previous treatment, aiming at the extraction of the analytes of interest. The DLLME technique was chosen for the treatment of raw samples because of its low cost, as well as speed and high efficiency in the extraction of target analytes (Martins *et al.,*2012). After verifying in the literature that most closely approximated the objective of the present study, the work of Hadjmohammadi and Ghoreishi (2011) was selected as a basis.

The solvents dichloromethane, chloroform and carbon tetrachloride as extraction solvent, and methanol, acetonitrile and acetone as disperser solvent were tested. The combinations used initially were 1 mL of disperser solvent, 50 µL of extraction solvent and 5 mL of sample. The solvents pair that formed the dispersion properly was acetone/carbon tetrachloride. Both were filtered through a hydrophobic PVDF syringe filter. A mixture of those two solvents was injected directly into the raw samples collected from the surface waters. After, this mixture (raw sample plus disperser-extraction solvent pair) was subjected to mechanical stirring in a vortex and subsequent centrifugation.

After centrifugation, 25 µL of the resulting sedimented phase was collected with a glass microsyringe and this volume was injected into the chromatographic system. The entire extraction procedure was adapted according to the work of Hadjmohammadi and Ghoreishi (2011) and a schematic of the process is shown in Figure 1.

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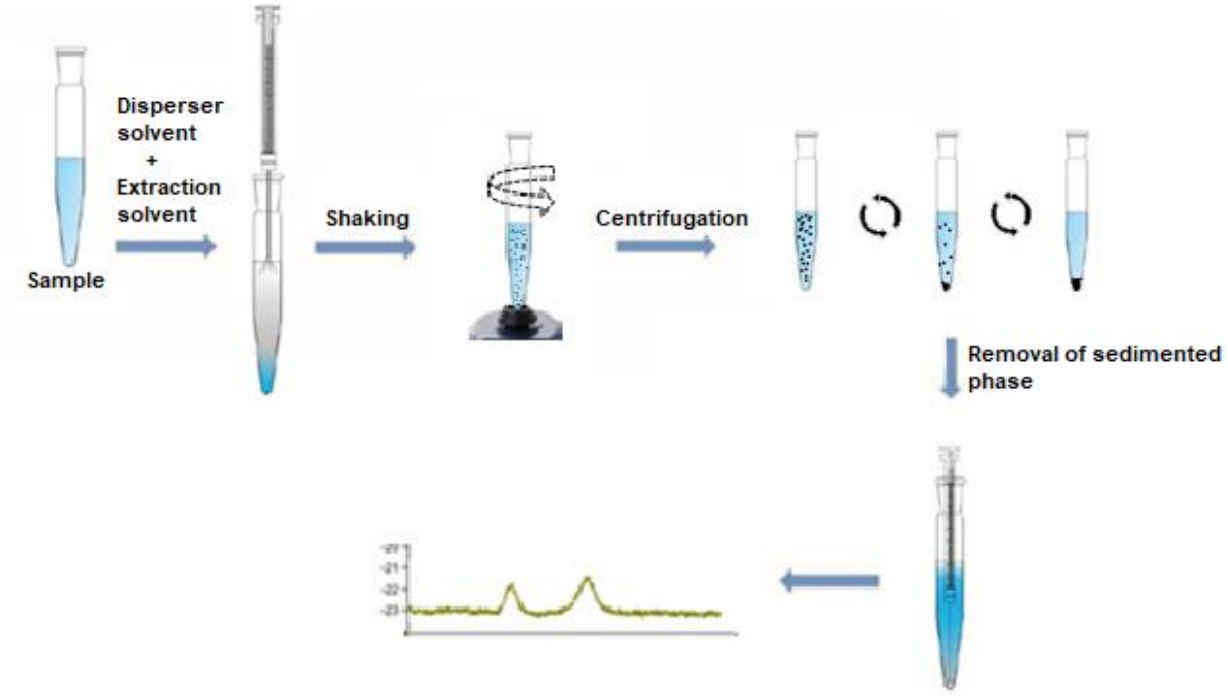

Figure 1. Dispersive Liquid-Liquid Microextraction (DLLME) procedure (adapted from Hadjmohammadi and Ghoreishi, 2011).

The DLLME technique was optimized to satisfy the best extraction conditions, considering the reality of the laboratory and available equipment. For this, a surface water matrix was used, free of the target analytes and spiked with a mix of hormones with a concentration of 80 µg L^{-1}. This allowed the evaluation of the following parameters: volume of the disperser solvent, volume of the extraction solvent, time of mechanical agitation in the vortex, ionic strength (% w/w of NaCl), pH of the samples, time and speed for centrifugation.

Sampling points

To monitor the waters of the Prosa Stream and the Anhanduí River, seven sampling points were established along the urban route of their water bodies. The monitoring points were selected considering the proximity of the source of the streams/rivers, mixing environments with other contributors, and indicative scenario of discharges that could interfere in the water quality. The collections were carried out in monthly campaigns from January to June 2019. Figure 2 shows the location of the collection points along the Prosa Stream and Anhanduí River.

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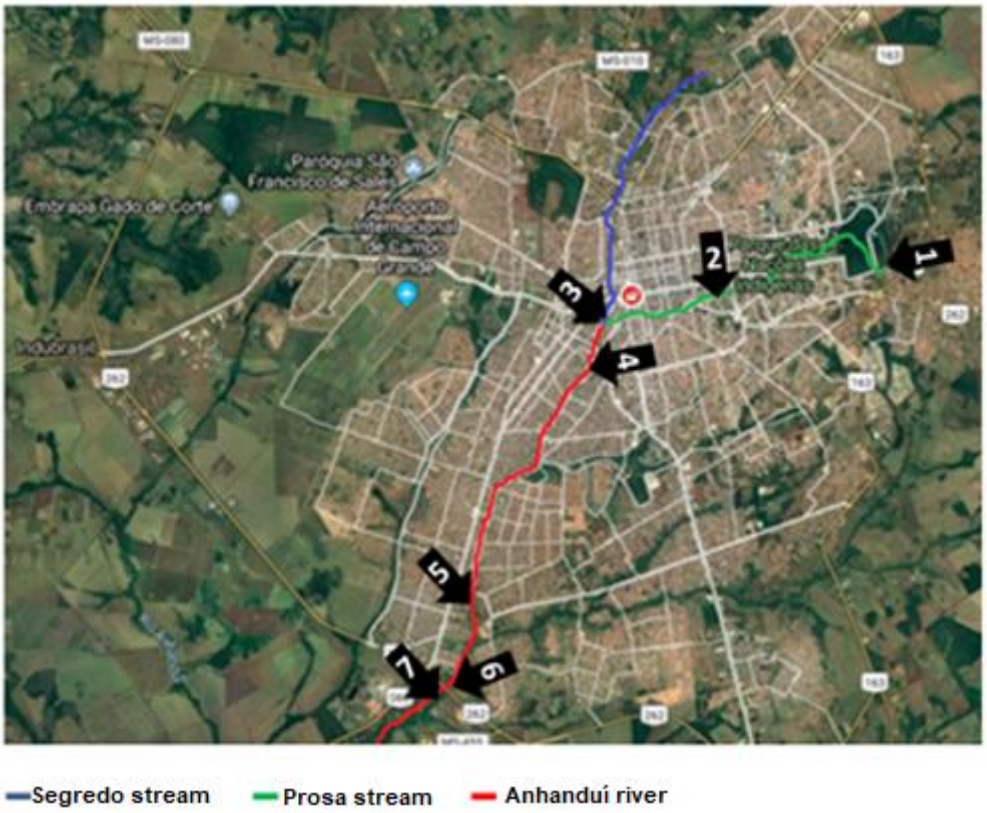

Figure 2. Location of the collection points along the Prosa Stream and Anhanduí River (Google Earth 2019).

Collections and field analyzes

The collections were carried out as described in "Guia Nacional de Coletas e Preservação de Amostras da Agência Nacional de Águas" (National Sample Collection and Preservation Guide of the National Water Agency) (CETESB, 2011) and the equipment used for sampling was made by the laboratory team itself. At each collection point the equipment was cleaned with deionized water and rinsed with water from the sampling point intended for collection.

Throughout the samplings carried out, field data was collected: the temperature of the samples (varying between 20°C and 29°C); the ambient temperature (varying between 21°C and 30°C); and the pH of the samples. The samples, after being collected, were placed in amber glass bottles and coded, then kept in a cooled box containing artificial ice for cooling. After this step, they were sent to the laboratory and stored in a refrigerator (between 4°-6°C). After 24h the extraction procedure was performed.

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6 de agosto de 2023

Results and discussion

Optimization of DLLME extraction parameters

For this purpose, a surface water matrix free of the target analytes was spiked with a mix of hormones at a concentration of 80 µg L^{-1}.

Volume of the extraction solvent

It is ideal that the extraction solvent has low water solubility, ensuring adequate separation of the organic phase after the extraction process, and a relatively high boiling temperature to avoid losses during the extraction process. In addition to those characteristics, it is important that the extraction solvent has good extraction efficiency for the analytes of interest (Liu *et al*, 2010).

The study of the effects of the variation of the extraction solvent volume was performed by fixing the volume of 1mL to the disperser solvent (acetone) and adding several volumes of the extraction solvent (carbon tetrachloride, CCl_4), constituting a mixture of disperser-extraction solvents. Volumes from 80 µL to 110 µL of the extraction solvent – CCl_4 were used. The results are shown in Figure 3.

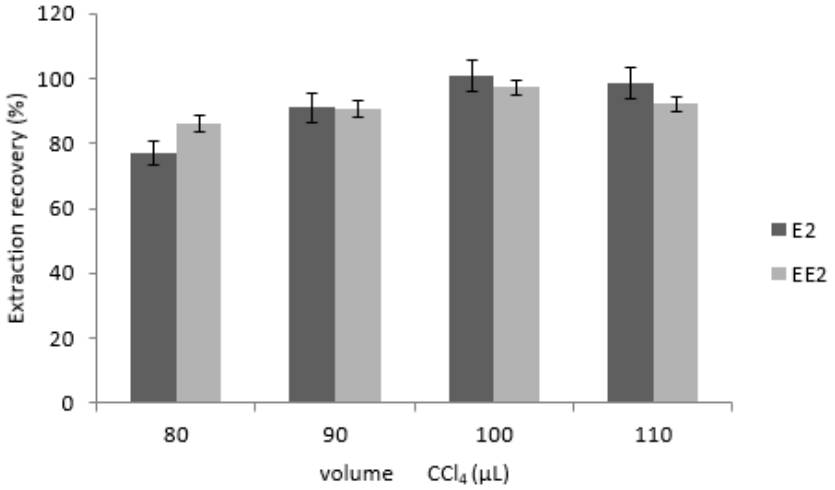

Figure 3. Effect of the variation of the volume of the extraction solvent (carbon tetrachloride – CCl_4 – µL) on the recovery of the hormones E2 (17β-estradiol) and EE2(17α-ethinylestradiol), n=3.

It is natural that the increase in the percentage of recovery of the analytes is favored with greater volumes of the extraction solvent. It was observed that the increase in CCl_4 resulted in greater recovery of the analytes and that from 100 µL of the extraction solvent the recovery of the analytes apparently kept it in a constant state, mostly for the hormone E2. With the intent of using smaller amounts of the extraction solvent, it seemed convenient to use a volume of 100 µL.

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Vol. 16, No.2, 656-677
6 de agosto de 2023

In Figure 3, the standard deviation bar, when analyzing the extraction using 90 µL or 100 µL, did not allow a conclusion of which volume of the extraction solvent resulted in better recovery behaviors. Using a Student *t* test, the null hypothesis was tested between the average of the groups referring to the volumes of 90 µL and 100 µL of the extraction solvent, in other words, null hypothesis (H0) – the averages of the two groups were equal, versus alternative hypothesis (Ha) – in which these averages were, in fact, different. Establishing a 95% confidence level, the $t_{calculated}$ value was 2.997 for the set of hormone E2 and 6.286 for the set of hormone EE2, against a t_{table} value equal to 2.776. Therefore, in both sets $t_{calculated}$ was greater than t_{table}, so the H0 hypothesis was rejected, and it was concluded that there is a significant difference between the averages of the two groups tested. Thus, the volume of 100 µL of the CCl_4 was established as an extraction solvent for the sequence of this work.

Volume of the disperser solvent

Volumes of acetone solvent used as disperser solvent, in portions of 0.25 mL to 2 mL, were evaluated. For this, the fixed volume of 100 µL to the CCl_4 was maintained. Figure 4 shows the effect resulting from the variation of the volume of the disperser solvent in the recovery of the hormones E2 and EE2.

It was observed that there was a gradual increase in recovery up to the volume of 1.0 mL of acetone where the greatest recovery was obtained. This happened because volumes below 1.0 mL of the disperser solvent were not enough to completely disperse the extraction solvent over the sample, resulting in low recoveries of the analytes (Krylov *et al*, 2011).

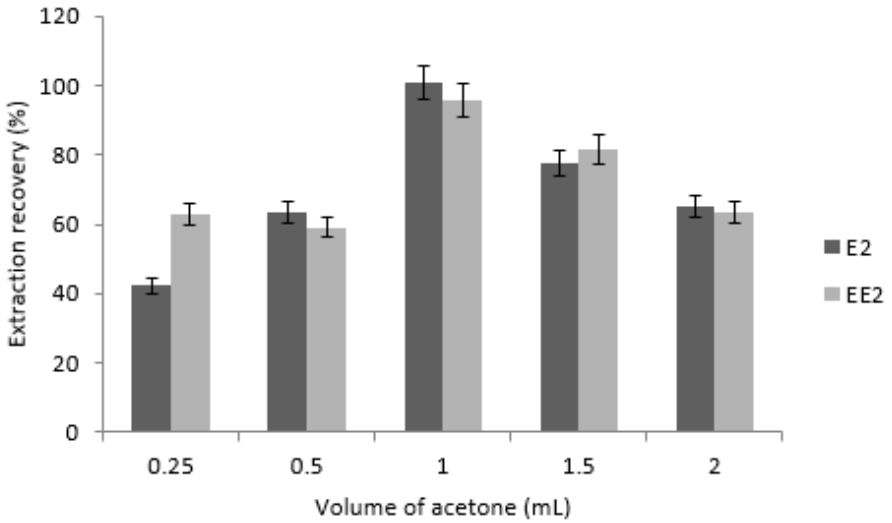

Figure 4. Effect of the variation of the volume of the disperser solvent (acetone – mL) on the recovery of the hormones E2 (17β-estradiol) and EE2(17α-ethinylestradiol), n=3.

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Vol. 16, No.2, 656-677
6 de agosto de 2023

At the volume of 1.5 mL of acetone and above the recoveries of the analytes decreased. This drop can be attributed to the increase in the volume of the disperser solvent that contributed to increase the solubilization of the hormones E2 and EE2 in the aqueous phase, resulting in smaller recoveries (Krylov *et al*, 2011).

This behavior was clearly seen in Figure 4, when 1.0 mL of the disperser solvent was used, the best result of hormone recovery was obtained. Thereby, this volume of acetone was established as a disperser solvent for the sequence of the studies.

Ionic strength

Figure 5 indicates successive increases in hormone recovery due to the increase in NaCl concentration (% w/w). The volumes of 1 mL of acetone (disperser) and 100 µL of the CCl_4 (extraction solvent) were used for these analyses. This increase is more evident in the recovery of the hormone E2 until it reaches the concentration of 2% (w/w) of NaCl in the aqueous phase, after that point a slight response decay happens. This decay can be attributed to the greater interaction of the salt ions and the analytes, reducing the ability of the analytes to move to the extraction solvent dispersed in the aqueous sample and, therefore, reducing their recovery. However, when considering the standard deviations, two groups stand out, those with NaCl concentrations of 1% and 2% (w/w).

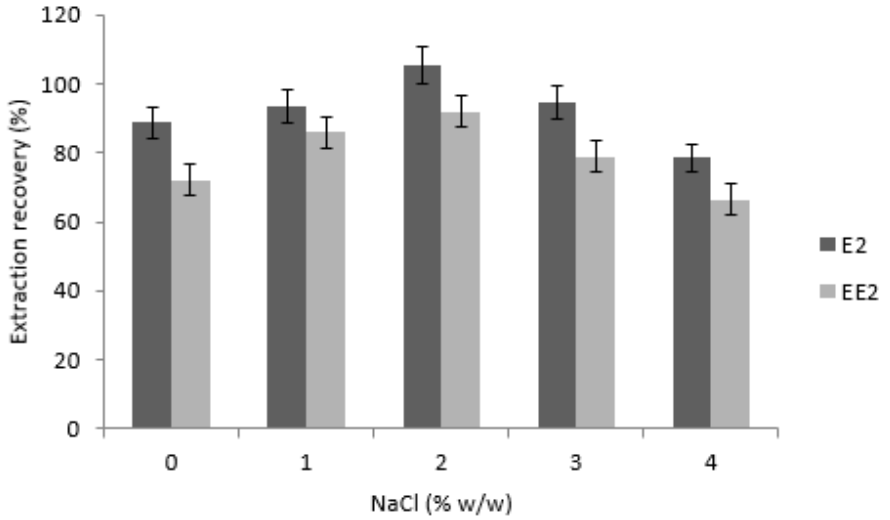

Figure 5. Effect of variation in NaCl concentration (% w/w) on the recovery of hormones E2 (17β-estradiol) and EE2(17α-ethinylestradiol), n=3.

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6 de agosto de 2023

Two hypotheses were tested to verify if there was a significant difference between the averages of those two groups, with a concentration of 1% and 2% (w/w) of NaCl. A Student *t* test with a 95% confidence level was applied, with the null hypothesis implying that the averages of the two groups studied were equal – H0, and the alternative hypothesis implying that the averages between the two groups studied were different – Ha. The studied groups, with NaCl concentration (% w/w) of 1% and 2%, presented $t_{calculated}$ values equal to 4.739 and 6.265, respectively for the hormones E2 and EE2. As both values were higher than the t_{table} value, which was equal to 2.776, hypothesis H0 was rejected, and hypothesis Ha was accepted. There is a significant difference between the average of the groups whose NaCl concentration (% w/w) is 1% and 2%. Thus, for the following analyzes, 2% NaCl (% w/w) was added to the aqueous sample.

Mechanical stirring time (vortex)

The extraction time in the DLLME is defined as the distance between the injection of the disperser/extraction solvent mixture in the aqueous phase and the end of the centrifugation. The stirring time is an important step, as it allows the transfer of the analytes that are in the aqueous phase to the extraction solvent dispersed in it, since the disperser solvent is soluble in the aqueous phase. For this, a vortex shaker was used, varying the stirring time in the time range from 0.5 to 2.0 minutes, and all the optimized conditions evaluated before were applied.

The best recovery obtained was in the agitation time of 1.0 minute. After this time, the decrease in the percentage of recovery of the analytes was noted. This behavior can be attributed to the solubilization of the disperser solvent in the aqueous phase. Thus, 1.0 minute was established as the ideal mechanical agitation time conditioned for this study.

Centrifugation time and rotational speed

For the evaluation of the resulting effect from the centrifugation time variation in the recovery of the hormones E2 and EE2 (n=3), the times of 1.0 to 7.0 minutes of centrifugation were considered, keeping all other previously optimized conditions. The percentage of recovery for the hormones did not suffer significant influence of centrifugation times of 1.0 and 3.0 minutes. On the other hand, with 5.0 minutes of centrifugation better results of recovery of the analytes were observed. After, the recovery response decays. Therefore, the time of 5.0 minutes was established for the sequence of studies.

The evaluated speeds were 1,000; 3,000 and 5,000 revolutions per minute (rpm). The percentages of recovery for the hormones showed a small variation, having an almost constant behavior for rotations of 3,000 and 5,000 rpm, conditioned to their standard deviations. A Student *t* test was applied with a 95% confidence level. H0 hypotheses were tested – in which the averages of the

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groups whose centrifuge rotation is 3,000 rpm are equal to the averages of the groups whose centrifuge rotation is 5,000 rpm – against the alternative hypothesis, Ha, where these averages were different.

The $t_{calculated}$ value was 3.251 for the set of hormone E2, and 5.498 for the set of hormone EE2. As both values were greater than the t_{table} value, which was equal to 2.776, the null hypothesis (H0) was rejected. The averages of the groups whose centrifuge rotation is 3,000 rpm was significantly different from the averages of the groups whose centrifuge rotation was 5,000 rpm. The alternative hypothesis (Ha) was accepted. Thus, the rotational speed of the centrifuge at 5,000 rpm was established as ideal, according to the tools adopted in this work, for further studies.

Sample pH

The increase in pH results in better recovery values from the extraction of analytes. This behavior is not observed in a basic environment. The best recovery values were obtained between pH 6.0 and pH 7.0 (maintaining all other optimized conditions for the DLLME). These two groups were submitted to a Student *t* test, assuming a 95% confidence level. Two hypotheses were evaluated: null hypothesis, H0, in which the average of the two groups were equal, and the alternative hypothesis, Ha, in which the averages of the two groups under study were significantly different.

$t_{calculated}$ values equal to 1.200 and 1.667 were obtained for the set of hormone E2 and EE2, respectively. The $t_{calculated}$ values were lower than the t_{table} value, which was equal to 2.776. Therefore, the H0 hypothesis was accepted, meaning that of the pH 6.0 and pH 7.0 groups were equal, with no significant difference between these groups, both for the hormone E2 and for EE2.

The results of this test made it possible to work with the raw samples, directly collected in the field, according to the nature of each water body studied and shown in Figure 6. Thus, it was not necessary to add reagents to correct the pH in the collected samples, considering that surface waters normally have pH values varying within the range of pH 6.0 to pH 7.0.

The pKas values for the natural hormone E2 and EE2 are 10.2 and 10.05, respectively, which means that the analytes are very weak acids, the non-dissociated forms prevails in the samples in neutral conditions, which facilitate the interaction with the extraction solvent (Aquino *et al*, 2013). Theses hormones, in high pH are converted in their ionized forms by loosing protons to the hydroxide ions, and because of the polar character that they assumed become more soluble in water than in the organic solvents (Ben Fredj *et al.,*2015) being unsuitable to the microextraction.

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Vol. 16, No.2, 656-677
6 de agosto de 2023

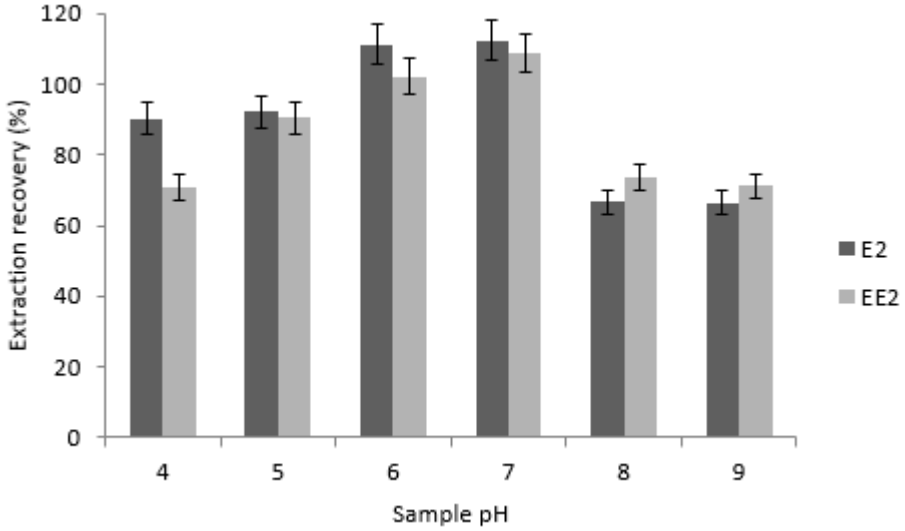

Figure 6. Effect resulting from the variation of the pH of the samples in the recovery of the hormones E2 (17β-estradiol) and EE2(17α-ethinylestradiol), n=3.

Optimized conditions for DLLME

For this study, the choice of optimal conditions considered for DLLME were based on the evaluation of the percentage of hormone recovery. The measured variable within each evaluated set that presented recovery percentages visibly higher in relation to the other variables evaluated in the set was assumed as a parameter considered ideal for work.

Table 1. Conditions established to carry out the study on the samples.

Stage	Parameter	Rated range	Optimized value
HPLC-UV	ACN/ H_2O mobile phase ratio (% V/V)	40/60 – 80/20	50/50 (%V/V)
	Injected volume	-	25µL
	Wavelength (λ)	-	281 nm
DLLME	Sample volume	-	5 mL
	Volume of the disperser solvent (acetone)	0.25 – 2 mL	1 mL
	Volume of the extraction solvent (CCl_4)	80 – 110 µL	100 µL
	Mechanical stirring time (vortex)	0.5 – 2 minutes	1 minute
	Ionic strength	0 – 4%	2%
	Sample pH	4,0 – 9,0	6,0 – 7,0
	Centrifugation time	1 – 7 minutes	5 minutes
	Rotational speed of the centrifuge	1000 – 5000 rpm	3000 rpm

HPLC-UV: High Perfomance Liquid Chromatography with Ultraviolet Detection, DLLME: Dispersive Liquid-Liquid Microextraction, ACN: Acetonitrile, CCl4: Carbon tetrachloride, %V/V: percentage in volume/volume.

Revista AIDIS de Ingeniería y Ciencias Ambientales:
Investigación, desarrollo y práctica. ISSN 0718-378X
http://dx.doi.org/10.22201/iingen.0718378xe.2023.16.2.83426
Vol. 16, No.2, 656-677
6 de agosto de 2023

Table 1 presents a summary of the suitable conditions, which were used for the extraction, detection and quantification of hormones in surface water matrices, conditioned to the reality of the laboratory used for this study. These conditions were maintained and used for further studies on the detection and quantification of hormones in samples collected from the Prosa Stream/ Anhanduí River, using DLLME-HPLC-UV.

Validation of extraction methods

Analytical response of the HPLC-UV system

Using a sample of surface water free of the target analytes, a linear regression curve was constructed by spiking the sample with a mix of hormones. The final concentrations were: 20; 40; 80; 100; 150; 200 µg L^{-1}. The samples were extracted by DLLME technique, and injection in the HPLC-UV system, according to conditions already informed. The linear regression, limits of detection (LOD) and of quantification (LOQ), calculated based on the equations of the linear regression curves (Ribani et al, 2004) (ANVISA, 2017) obtained, are shown in Table 2.

Table 2. Optimization parameters of the chromatographic system obtained with the fortified samples.

Analyte	Linear range (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	Linear Equation	R
E2	20 – 200	11	35	y = 213.48x + 737.11	0.9968
EE2	20 – 200	5	21	y = 222.14x + 370.36	0.9985

E2: 17β-estradiol, EE2: 17α-ethinylestradiol, LOD: Limit of detection, LOQ: Limit of quantitation, R: correlation coefficient.

Precision

Based on the analytical signals from the injections performed daily and during different days, of the spiked samples, the concentrations of 20 µg L^{-1}, 80 µg L^{-1} and 150 µg L^{-1} were used to constitute a control chart 29 (n=35). This tool provided an overview of the analytical responses with respect to intra-day and inter-day precision within statistically established limits.

Through the analysis of the control charts, it was observed that the studied points were reproducible throughout the tests. These studies were conducted in a universe of 35 analyzes, with part of these analyzes carried out on different days. The variations of the analytical signals followed the acceptance limits established in this method. In this way, it can be concluded that the analytical signals showed good repeatability and reproducibility, since they presented responses within the limits of acceptance according to the conditions used, both for intra-day and inter-day assessments. It is also possible to state that the analytes have good stability.

Revista AIDIS de Ingeniería y Ciencias Ambientales:
Investigación, desarrollo y práctica. ISSN 0718-378X
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Vol. 16, No.2, 656-677
6 de agosto de 2023

Accuracy

To verify the accuracy of the procedure, the percentages of recovery and relative standard deviation (RSD) (ANVISA, 2017) were evaluated from the analytical signals represented by control charts. Table 3 shows these datas.

Table 3. Recovery datas and standard deviation of control chart points.

Analyte	Fortification (µg L^{-1})	Recovery (%)	RSD (%) (n=35)
E2	20	83.0	2.85
	80	92.1	1.20
	150	95.8	1.52
EE2	20	89.8	2.32
	80	102.3	1.29
	150	101.8	1.35

RSD: relative standard deviation, E2: 17β-estradiol, EE2: 17α-ethinylestradiol.

The results of the percentage of recovery of the selected analytes varied between 83.0% to 102.3% with RSD below 3%. Therefore, the results were considered satisfactory, as they follow RDC 166 – ANVISA (2017), which provides guidelines for the validation of analytical methods.

The extraction procedure was adapted according from the work of Hadjmohammadi and Ghoreishi (2011), where some important parameters were reassessed, such as type and volume of extraction and dispersing solvents, extraction time, ionic strength and pH of the sample, after these step the optimized method proved to be totally efficient obtaining values of linear range of work, precision and accuracy consistent with the original method.

Daniel and Lima (2014), used solid phase extraction (SPE) cartridge, with octadecyl silica adsorbent (C18), this is one of the main methods for determining hormones in water, the method obtained recovery values of the order of 86.6% to 78.4% with quantification limits 5.41 and 3.75 µg L^{-1} for 17 α-ethinylestradiol and 17 β-estradiol, respectively. The recovery values are below the established in this work, which shows the efficiency of DLLME, with LOQ in the same order of magnitude.

When comparing the LOQs with the of Sorensen *et al.* (2015) work, which used a GC-MS, LOD and LOQ were also obtained in the same order of magnitude (µg L^{-1}), and in present work used an HPLC-UV. Thus, the method, in addition to complying with RDC 166 - ANVISA, is also in accordance with works reported in the literature. Proving to be a faster and cheaper technique to be implemented in a laboratory, presenting low solvent consumption, fast extraction time and high extraction power with the use of a small sample volume, as well as ease of operation associated with the simplicity of the process.

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Investigación, desarrollo y práctica. ISSN 0718-378X
http://dx.doi.org/10.22201/iingen.0718378xe.2023.16.2.83426
Vol. 16, No.2, 656-677
6 de agosto de 2023

Application of the optimized method

Figure 7 shows the chromatograms of the raw sample and of the same sample spiked. The sample used was collected in sample point 6, because it had a release of material from an effluent treatment station located in the region. The final concentration of hormones in the spiked sample was 80 µg L^{-1}. The sample from point 6, not spiked, showed an analytical signal for the hormone E2 in the retention time of approximately 5 minutes. According to the procedure adopted in this study, the same was not observed for EE2.

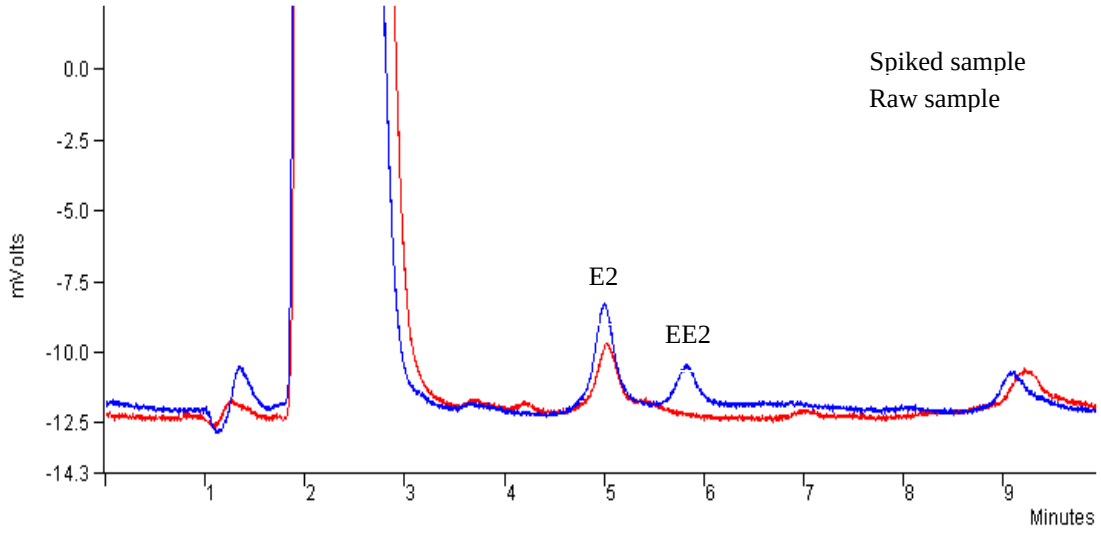

Figure 7. Chromatograms of the spiked sample and raw sample collected in point 6. Sample spiked with final concentration equal to 80 µg L^{-1} of the hormones E2 (17β-estradiol) and EE2 (17α-ethinylestradiol). Chromatographic conditions: v_{inj}=25µL, flow=1mL min^{-1}, λ=281 nm, mobile phase Acetonitrile/ H_2O 50% (v/v).

The chromatographic profile was evaluated using the spiked sample, as previously mentioned, with the mix solution of hormones in a final concentration of 80 µg L^{-1}, a blank sample from the equipment used for collection (blank equip) and a blank sample from the extraction procedure (blank extract). This study showed that there was no contribution from the concentration of analytes of interest due to cross contamination, as showing in Figure 8, and it was also possible to verify the lack of need to use an internal standard, as no interference with the retention time of the analytes selected for the study is observed.

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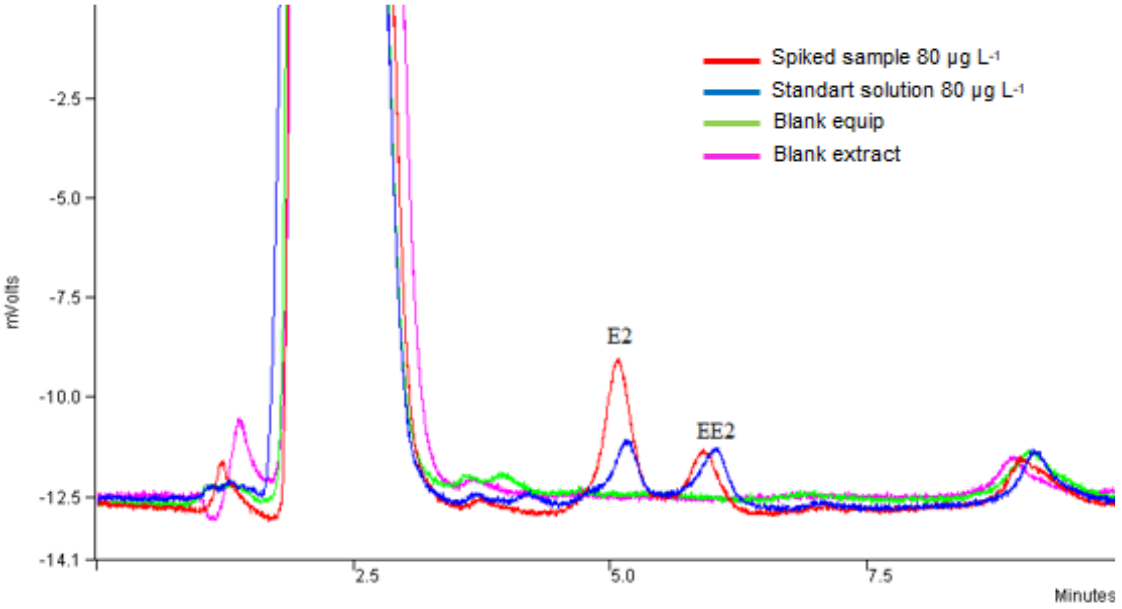

Figure 8. Chromatograms of the spiked sample and mixed solution of the hormones E2 (17β-estradiol) and EE2 (17α-ethinylestradiol), final concentration equal to 80 µg L^{-1}, blank equip and blank extract. Chromatographic conditions: v_{inj}=25µL, flow=1mL min^{-1}, λ=281 nm, mobile phase Acetonitrile/ H_2O 50% (v/v).

The quantification of the target analytes in the surface water samples was carried out through the difference of the average area of the analytical signals of the spiked samples (n=3) and the average area of the analytical signals of the mixed solution of the hormones (n=3). Then, this difference was attributed to the linear regression curve equation obtained for each of the studied hormones.

The results of the quantitative analyzes performed on the samples collected along the Prosa Stream/Anhanduí River during the campaigns are shown in Table 4.

In general, the sampling points selected in this work showed contamination by the natural hormone E2, except in an area of environmental preservation. Figure 9 presents an overview of the collection campaigns.

The monitoring of the surface waters selected in this work showed the presence of the environmental contaminant E2 along the studied water courses. The graph in Figure 9 clearly shows the influence of effluent discharge on surface waters. The greatest contamination of the hormone E2 was identified in points 6 and 7, which are located downstream of the effluent discharge from a local treatment plant.

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Vol. 16, No.2, 656-677
6 de agosto de 2023

Table 4. Results of quantitative analyzes on surface water samples.

Point identification	Campaign	E2 µg L^{-1} (n=3)	EE2 µg L^{-1} (n=3)
Point 1. Prosa stream – Parque das Nações Indígenas. S 20°27'10.8684'' W 54°33'55.9656''	January/19	< LOD	< LOD
	February/19	< LOD	< LOD
	March/19	< LOD	< LOD
	April/19	< LOD	< LOD
	May/19	< LOD	< LOD
	June/19	< LOD	< LOD
Point 2. Prosa stream - Fernando Corrêa da Costa Avenue with Sebastião Lima Street. S 20°28'6.7656'' W 54°36'29.7360''	January/19	< LOD	< LOD
	February/19	< LOD	< LOD
	March/19	< LOD	< LOD
	April/19	48	< LOD
	May/19	< LOD	< LOD
	June/19	< LOD	< LOD
Point 3. Prosa stream – Upstream of the Segredo stream S 20°28'16.9572'' W 54°37'29.1000''	January/19	75	< LOD
	February/19	66	< LOD
	March/19	49	< LOD
	April/19	< LOD	< LOD
	May/19	< LOD	< LOD
	June/19	< LOD	< LOD
Point 4. Anhanduí river- Ernesto Geisel Avenue with Brilhante Street. S 20°28' 27.6960'' W 54°37'34.5936''	January/19	65	< LOD
	February/19	71	< LOD
	March/19	66	< LOD
	April/19	< LOD	< LOD
	May/19	63	< LOD
	June/19	< LOD	< LOD
Point 5. Anhanduí river – 600 meters upstream of the effluent discharge. S 20°32'3.4764'' W 54°39'26.4852''	January/19	68	< LOD
	February/19	54	< LOD
	March/19	79	< LOD
	April/19	69	< LOD
	May/19	50	< LOD
	June/19	53	< LOD
Point 6. Anhanduí river – downstream of the effluent discharge. S 20°33'21.0312'' W 54°39'45.5976''	January/19	136	< LOD
	February/19	136	< LOD
	March/19	175	< LOD
	April/19	117	< LOD
	May/19	99	< LOD
	June/19	74	< LOD
Point 7. Anhanduí river – 110 meters downstream of the effluent discharge, after a bridge on Br 060. S 20°33'25.2036'' W 54°39'50.9652''	January/19	115	< LOD
	February/19	125	< LOD
	March/19	162	< LOD
	April/19	91	< LOD
	May/19	72	< LOD
	June/19	75	< LOD

LOD: Limit of detection (E2: 11 µg L^{-1}, EE2: 5 µg L^{-1}), E2: 17β-estradiol, EE2: 17α-ethinylestradiol.

Revista AIDIS de Ingeniería y Ciencias Ambientales:
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Vol. 16, No.2, 656-677
6 de agosto de 2023

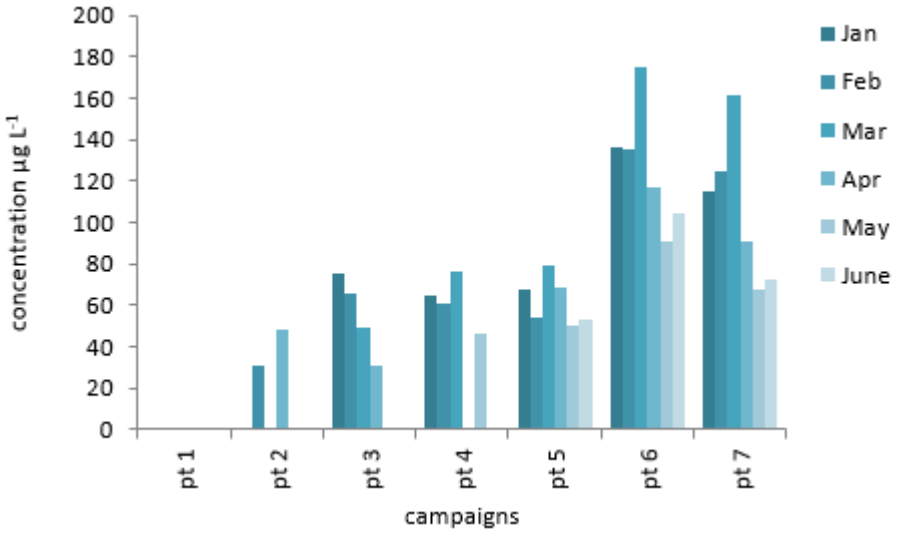

Figure 9. Overview of the analytical results of the collection campaigns (pt = point).

It was also observed that, along the collection points, contamination by this hormone has a gradual increase as the water body advances through the urban areas of the city, indicating a possible occasional effluent spill along the route.

This contamination can lead to damaging situations in the ecosystem, affecting microorganisms, small fish, etc., in other words, the producers, the base of the food chain. This can have effects, even indirectly, for the entire food chain and can cause irreversible effects to aquatic fauna and flora.

Conclusions

The optimization steps performed in the hormone extraction procedure in the waters of the Prosa Stream/ Anhanduí River proved to be efficient for the proposed purposes. It presented low solvent consumption, fast extraction time and high extraction power with the use of a small sample volume, as well an easy operation associated with the simplicity of the process.

The analytical method applied to detect and quantify the hormones E2 and EE2 was suitable, with a linear correlation coefficient (R) of 0.9968 and 0.9985 respectively, with a linear range also suitable for the study and conditioned to the employed working conditions.

The monitoring of these environmental contaminants in the sampled waters identified contamination along the surface water course evaluated in this work by the natural hormone E2.

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Vol. 16, No.2, 656-677
6 de agosto de 2023

Even if there is no national environmental legislation that limits the amount of hormones in surface waters, these contaminations can be significant.

The toxicological potential of substances with endocrine disrupting characteristics, such as natural estrogen E2, for living aquatic organisms and even humans is already known. This concern for the ecosystems signals the increasingly evident importance of establishing guiding limits for such substances in the environmental control standards.

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