INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

UNIVERSIDADE FUMEC/FEA FACULDADE DE ENGENHARIA E ARQUITETURA

MONITORING OF 17β-ESTRADIOL IN RAW AND TREATED SAMPLES OF WASTEWATER TREATMENT PLANTS

NATHALIA SILVA FOUREAUX

Thesis presented to Escola Superior Agrária de Bragança to the fulfilment of requirements to obtain the Master degree of Environmental Technology, in the scope of the double degree with Fundação Mineira de Educação e Cultura (FUMEC)

SUPERVISORS

Professor Ana Queiroz
Professor António Ribeiro

CO-SUPERVISOR

Professor Eduardo Carneiro

This Dissertation takes into account the changes and suggestions made by the Jury

BRAGANÇA
JULY 2021

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”
Marie Curie

ACKNOWLEDGEMENTS

I would like to thank IPB and FUMEC for allowing me an incredible and fulfilling professional experience, an opportunity that I will always treasure and carry close to my heart. To my supervisors, Ana Queiroz, António Ribeiro, Paulo Brito and Eduardo Carneiro, I have learnt so much with you throughout this process, thank you for your support and guidance. Dr. Paula Plasencia and Verónica Machado, working with you in the laboratory was a pleasure, thank you for all the help.

Above all, I thank my family, the most important people in my life, the foundation of everything. Aline and Alexandre, mom and dad, you have given me all the tools I needed to be here, you have shown me a world that allowed me to dream and encouraged me to pursue my dreams, I am forever grateful to be your daughter. Henrique (Paipadro), Raquel, Júlia, Pedro, Vó Emília, you have given me so much love, I feel so lucky to have you in my life, thank you for everything you do for me. My beloved uncle Guilherme, aunt Flavia, cousins Vitor and Mateus and my absent grandparents, even though you are no longer here with us, you will forever be a huge part of who I am and I share this moment with you, miss you.

Maria Fernanda, Walkíria and Thatiana, your friendship has carried me through the most difficult times and have also given me my happiest laughs, lucky me to have amazing, strong woman like you to share life with. Perpétua, words are not enough to express my gratitude for your care with me, my most sincere thank you.

To the most important person in my life, whom I do not imagine life without, my sister Alessandra. To be your sister has been one of the greatest joys of my life, thank you for your friendship, but most importantly, thank you for being my biggest supporter in life, I love you sister. My love, Mateus, this would not have been possible without your endless support and love. You have made the past year lighter and brighter by just being by my side.

ABSTRACT

Aquatic environment pollution is a keen subject in the most important discussions surrounding global issues. With the increase of industrialization, globalization and urbanization, as consequence, there is an increase in production of high-level pollutants. Emerging pollutants (EP's) are compounds which are, usually, not found in natural water sources. Data concerning the occurrence and concentrations of some pharmaceuticals in effluents from WWTPs and surface waters, shows that EP concentrations in effluents fluctuate widely, most probably due to inconsistent efficiency of wastewater treatment.

Endocrine disrupting compounds (EDCs), are an important group of EP's, considering they are often found in different aquatic matrices. Nowadays, monitoring the concentration levels of estrogens in treated wastewaters of wastewater treatment plants (WWTP) is an environmental mandatory task to minimize or eliminate water pollution.

The present work is divided in two main experimental stages. First, an SPE/HPLC-UV experimental methodology is optimized to detect and quantify 17β-Estradiol (E2) present in aqueous samples. The HPLC-UV operating conditions were selected by performing a screening between 10 different mobile phase compositions. A pure methanol composition was selected based in the lower retention time and the highest UV detector signal. The solid phase extraction optimization involves a three-level Box-Behnken experimental design with four factors (sample volume, sample pH, adsorbent drying time and solvent composition used for the washing step), combined with a response surface methodology.

The implementation of the optimized experimental methodology occurred by the monitoring of estradiol in a wastewater influent and effluent samples. E2 was detected and quantified in three different samples collected from three distinct point of a WWTP. Sample 1 was collected from the entrance point, sample 2 was collected from the activated sludge aeration tank and sample 3 was the completely treated effluent. The concentration of E2 found in three points was higher than what was anticipated, but coherent with other works.

Keywords: wastewater treatment plant, solid phase extraction, high performance liquid chromatography, estradiol.

RESUMO

A poluição do ambiente aquático é um assunto importante nas discussões mais importantes em torno de questões globais. Com o aumento da industrialização, globalização e urbanização, como consequência, há um aumento na produção de poluentes de alto nível. Poluentes emergentes (PE) são compostos que, geralmente, não são encontrados em fontes naturais de água. Dados relativos à ocorrência e concentração de alguns produtos farmacêuticos em efluentes de ETARs e águas superficiais, mostram que as concentrações de PE em efluentes variam amplamente, muito provavelmente devido à eficiência inconsistente do tratamento de águas residuais.

Compostos desreguladores endócrinos (CDEs), são um importante grupo de PE, considerando que são freqüentemente encontrados em diferentes matrizes aquáticas. Atualmente, monitorar os níveis de concentração de estrogênios em águas residuárias tratadas de estações de tratamento de efluentes (ETE) é uma tarefa ambiental de suma importância para minimizar ou eliminar a poluição da água.

O presente trabalho está dividido em duas etapas experimentais principais. Primeiro, uma metodologia experimental SPE/HPLC-UV é otimizada para detectar e quantificar o 17β-estradiol (E2) presente em amostras aquosas. As condições operacionais de HPLC-UV foram selecionadas realizando uma triagem entre 10 composições de fase móvel diferentes. Uma composição de metanol puro foi selecionada com base no menor tempo de retenção e no maior sinal do detector de UV. A otimização da extração em fase sólida envolve um projeto experimental Box-Behnken de três níveis com quatro fatores (volume da amostra, pH da amostra, tempo de secagem do adsorvente e composição do solvente usado para a etapa de lavagem), combinado com uma metodologia de superfície de resposta. A implementação da metodologia experimental otimizada ocorreu por meio do monitoramento do estradiol em uma amostra de efluente e afluente de água residuária.

O E2 foi detectado e quantificado em três diferentes amostras coletadas em três pontos distintos de uma ETAR. A amostra 1 foi coletada do ponto de entrada, a amostra 2 foi coletada do tanque de aeração de lodo ativado e a amostra 3 foi o efluente

completamente tratado. A concentração de E2 encontrada em três pontos foi maior do que o previsto, mas coerente com outros trabalhos.

Palavras-chave: estação de tratamento de efluentes, extração em fase sólida, cromatografia líquida de alta eficiência, estradiol.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

EP	Emerging Pollutants
EDC	Endocrine Disruptive Compound
ACN	Acetonitrile
MeOH	Methanol
E2	17b-Estradiol
EE2	17α-Ethinylestradiol
E1	Estrone
E3	Estriol
HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
CV	Coefficient of variation
SD	Standard Deviation
SPE	Solid Phase Extraction
UV	Ultraviolet
WWTP	Wastewater Treatment Plant

1 - INTRODUCTION

1.1 - MOTIVATION AND OBJECTIVES

Nowadays, there has been a growing concern regarding the possible consequences of exposure to estrogens through its direct or indirect consumption. The increasing utilization of estrogenic compounds, such as natural and synthetic estrogens, pharmaceuticals and pesticides has resulted in their continual occurrence in the aquatic environment.

The risk that endocrine disrupting compounds (EDCs) cause to human life and wildlife, is one of the reasons why studies concerning their detection and removal from diverse aquatic environment are so important. Even at low concentration levels, EDCs can induce unhealthy changes to human lives [1]. Moreover, prolonged exposure to these substances can possibly be a causal factor in diseases such as breast cancer and testicular germ cell cancer, as well as the decreasing sperm count [2]. The occurrence and, more importantly, the destination of these compounds are matters of utmost importance towards a better public health.

Estrogens are some of the most potent endocrine disrupting compounds [3]. Monitoring the levels of estrogens is, currently, highly necessary due to its frequent detection in treated wastewaters of Wastewater Treatment Plants (WWTP´s) [4]. Estrone (E1), 17β-estradiol (E2) and estriol (E3) are natural female sex hormones produced by humans, mammals and other vertebrates. Ethinylestradiol (EE2) is a synthetic estrogen that has therapeutic uses, such as oral contraceptive [5].

These conjugated estrogens are excreted through urine and feces making it usual to found them in WWTP´s. This fact combined with the facts that E2 and EE2 are components used in highly consumed pharmaceuticals and wastewater

treatment plants were not specifically designed to remove these compounds [6], are important reasons why estrogenic compounds are found in effluents from WWTP´s and ultimately, identified in the aquatic environment.

1.2 - OBJECTVES

1.2.1 - MAIN OBJECTIVE

The present study aims to contribute to researches concerning water quality, by developing and validating an experimental methodology to be used in monitoring the levels of an important EDC, that is E2, in treated effluents from wastewater treatment plants.

1.2.2 - SPECIFIC OBJECTIVES

The present work is divided in two main experimental stages. First, an SPE/HPLC-UV [4] experimental methodology is optimized to detect and quantify 17β-Estradiol (E2) present into aqueous samples. The HPLC-UV operating conditions were selected by performing a screening of the mobile phase composition (10 different compositions).

The solid phase extraction optimization involves a three-level Box-Behnken (BBD) experimental design [7] with four factors (sample volume, sample pH, adsorbent drying time and solvent composition in the washing step), combined with a response surface methodology. Secondly, the validation of the optimized experimental methodology is done by the monitoring of estradiol in wastewater influent and effluent samples from a Wastewater Treatment Plant.

1.2.3 - REPORT ORGANIZATION

This thesis is organized in five main chapters. The first one consists of an introduction to the context of the present study, the relevance of the proposed work and the objectives to be met. The second chapter gathers a thorough literature review, with published works in the field of detection and quantification of EDC's, specially estrogens. Third chapter presents the guidance through the experimental methodology developed in this work. In the fourth chapter, the main experimental results are presented and discussed. Fifth and final chapter gathers the main conclusions and suggestions for future works.

2 - LITERATURE REVIEW

2.1 - EMERGING POLLUTANTS

Aquatic environment pollution is a keen subject in the most important discussions surrounding global issues. With the increase of industrialization, globalization and urbanization, as consequence, there is an increase in production of high-level pollutants. Emerging pollutants (EP) can be defined as pollutants that are currently not included in routine monitoring programs at the European level and which may be candidates for future regulation, depending on research on their (eco)toxicity, potential health effects and public perception and on monitoring data regarding their occurrence in the various environmental compartments. These pollutants can be originated from natural occurring processes in human's body or anthropogenic contribution, such as industrialization, urbanization and uncontrolled disposal of wastewater [8].

The aqueous pollutants can be classified into two large groups, inorganic and organic. Within the organic group there are: residues from oil, food, pharmaceutical production and refinery industries. Personal care products, surfactants, hormones, steroids and antibiotics are also included in that group. These compounds of different origin and chemical nature, essentially organic, are considered emerging due to the fact their entry into the ecosystem and their occurrence limits are not yet regulated [9]. Even though, the presence of those chemicals in the environment can cause adverse ecological and human health effects. According to the NORMAN network, at least 700 substances categorized into 20 classes, have been identified in the European aquatic environment [10, 71].

Data concerning the occurrence and concentrations of some pharmaceuticals in effluents from WWTPs and surface waters, shows that EP concentrations in effluents fluctuate widely, most probably due to inconsistent efficiency of wastewater treatment. Nevertheless, information considering the nature, variability, transport and fate of these

compounds in wastewater and treatment facilities must be improved, because knowledge in this area is still limited [11]. Figure 1 presents a flowchart with the main routes of environment contamination by EP´s [11].

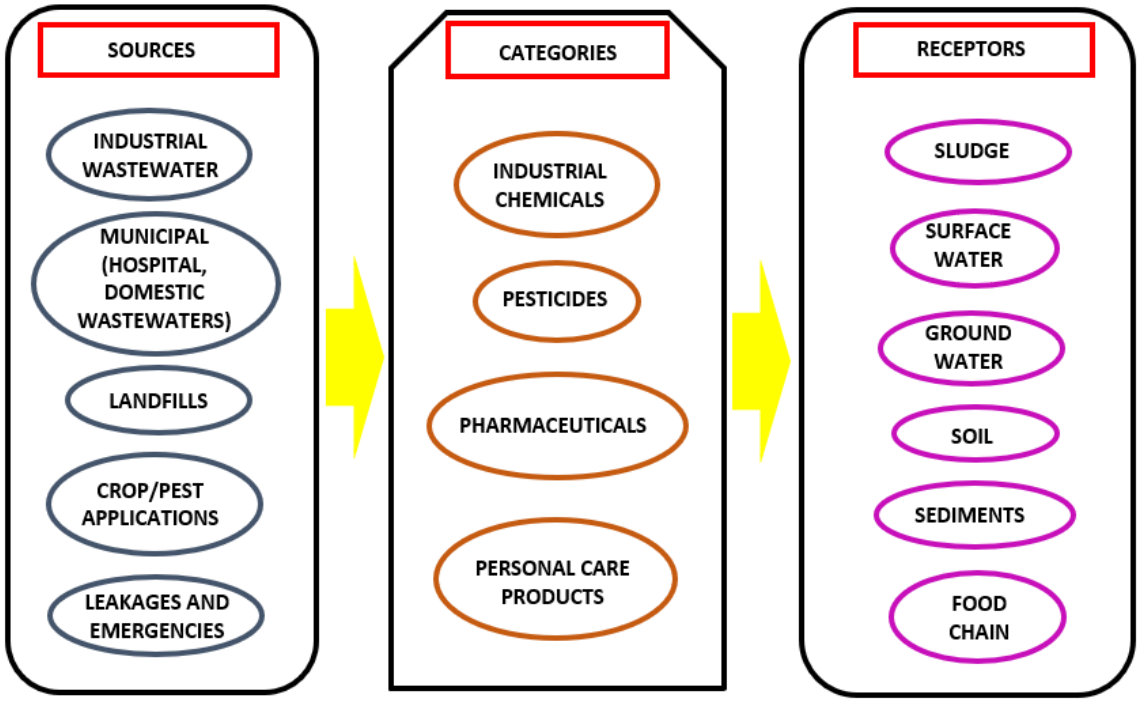

Figure 1. Environmental occurrence and fate of EP´s.

There are few studies devoted to monitoring and understanding the processes involved in conventional or innovative wastewater treatment in eliminating or reducing the concentrations of a large diversity of emerging pollutants at wastewater facilities [12].

Yet, it is unlikely that the conventional treatment of wastewater or drinking water will be able to remove in its totality estrogens, androgens or detergent components due to the chemical structural stability of these compounds, as well as their low bioavailability, which affects biodegradation [11]. In addition, municipal sewage sludge is also a repository system for these emerging pollutants and only recently has been an effort to assess their occurrence and biotreatment potential. Therefore, the analysis of the efficiency in the wastewater treatment plants regarding the removal of those EP´s is critical to a better

understanding of the current problem as a whole. Figure 2 presents a flowchart with pathways of some emerging pollutants from sources to receptors [11].

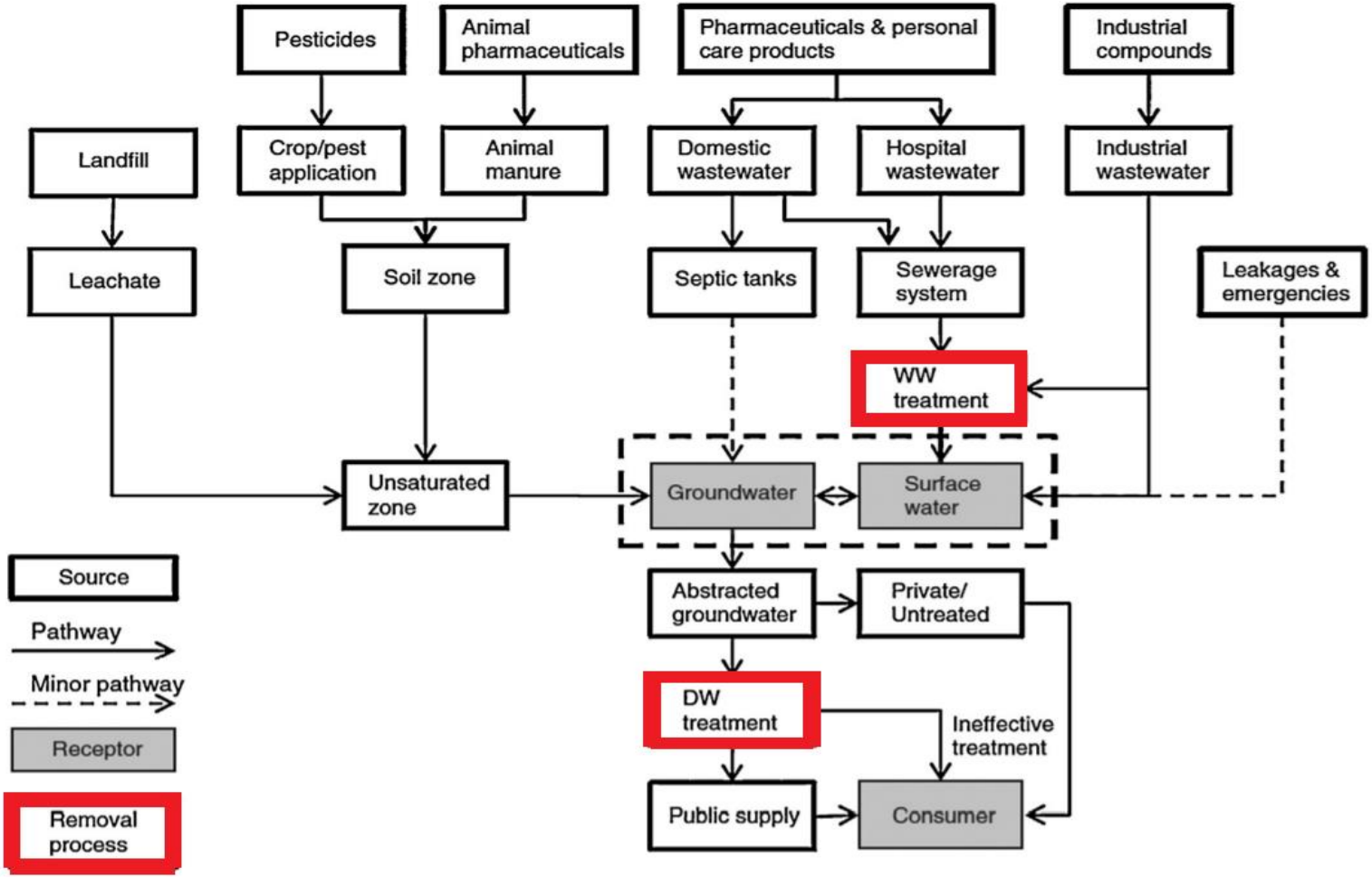

Figure 2. Adapted scheme of pathways of some emerging pollutants from sources to receptors.

2.2 - ESTROGENIC COMPOUNDS

Estrogenic compounds are those with similar properties to the hormone 17β-Estradiol, E2, (main natural estrogen produced by the ovaries), whose effects are induced by interactions with the estrogen receptor and cell systems [13]. There are several natural and synthetic compounds found in effluents that can bind to estrogen receptors, which means those compounds have some level of estrogenic potency. The estrogenic potency of a chemical is determined by its ability to bind to the estrogen receptor, thereby mimicking or blocking the activity of natural estrogens [3,14].

From all categories of EDCs, the sex hormones 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) represent the highest estrogenic potency (≥ 1), even at low concentrations [15]. On account of that elevated estrogenic potency, these compounds are part of a European Union “watch list” regarding emerging aquatic pollutants [16].

There are four estrogens most commonly found in wastewater, they are three natural steroids, 17β-estradiol (E2), estrone (E1) and estriol (E3); and one synthetic compound, 17α-ethinyl estradiol (EE2), E2 and EE2 are used in contraceptives and hormone replacement therapy [3]. Hereinafter, the figure 3 adapted [17], illustrates the structural representation of those estrogens.

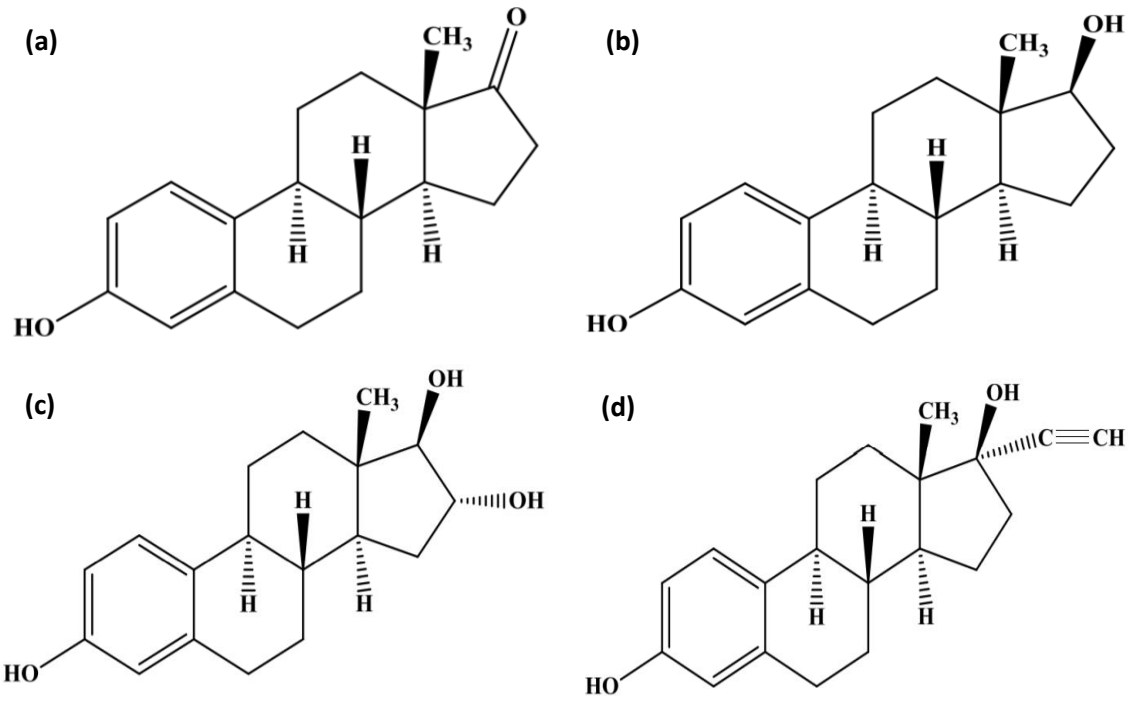

Figure 3. Molecular structural representation: (a) Estrone (E1), (b) 17β-estradiol (E2), (c) Estriol (E3), (d) 17α-ethinyl estradiol (EE2).

2.3 - ESTROGENIC COMPOUND IN STUDY

The E2 hormone, 17β-estradiol, object of this study, is a natural estrogen that is commonly found in diverse aquatic environment, but especially in wastewater. In addition to that, it presents a high estrogenic potency as previous stated. Table 1 presents some properties of 17β-estradiol (E2) [17].

Table 1. Structures and properties of 17β-estradiol (E2).

ESTROGENIC COMPOUND	CAS	MOLECULAR FORMULA	MOLAR MASS ($g.mol^{-1}$)	Log Kow	K sorption	WATER SOLUBILITY (mg/L, 20°C)	pKa
17β-estradiol (E2)	50-28-2	$C_{18}H_{24}O_2$	272,30	4,01	3300	3,6	10,4

17β-estradiol is a natural estrogen that stimulates proliferation and growth in the organs of the reproductive tract, activating the development of uterus endometrium [18]. The molecule of E2 (presented in the Figure 3) is constituted of 18 carbon atoms, a hydroxyl group connected to a five-carbon ring and one phenolic compound, which is the structural responsible for the high affinity to connection to the estrogen receptor. Thus, processes that can alter the phenolic compound, tend to suppress the high affinity to connection to the estrogen receptor [17]. Moreover, the hydroxyl group present in the 17-carbon atom can be in an equatorial or axial position, which will influence in its estrogenic potency. Considering that, the 17β-estradiol is 10 times more potent than 17α-estradiol [19].

2.4 - ESTROGENS IN WASTEWATER TREATMENT

Since effluents from wastewater treatment systems are discharged continuously into the environment, it is critical to understand the behavior of estrogens in wastewater, in order to gather more information concerning the most effective methods to identify and remove them before they accumulate into the environment.

A typical layout of a wastewater treatment plant involves primary, secondary, and tertiary treatment units. In every wastewater treatment unit, the secondary treatment plays the most important role in removing steroid estrogens. A very low rate of steroid estrogen removal is obtained in primary treatment [3, 20, 21].

In the secondary treatment is where it happens the organic matter removal, through a suspended growth system, such as an activated sludge, or through an attached growth system, as a trickling filter (TF) and a rotating biological contactor (RBC). Activated sludge treatment systems have microorganisms that breaks down organic material with aeration and agitation. In TF treatment systems, the biofilm grows when the wastewater trickles through a circular bed of plastic media or coarse

stones, while RBC treatment systems allows microorganisms to grow on the surface of closely spaced parallel discs mounted on a rotating shaft where biodegradation takes place [22].

Steroid estrogens, during the secondary treatment, are removed from aqueous phase by sorption into the micro-flocs and subsequently biodegraded by bacteria. Biodegradation is the primary removal means for estrogens in wastewater. This mechanism includes (1) deconjugation, (2) degradation as a carbon source for heterotrophic bacteria, (3) co-metabolism with nitrifying biomass, or (4) other co-metabolisms [3, 23].

Therefore, it is noticeable that the biodegradation mechanism plays a big role in steroid estrogen removal. Also, biodegradation is more rapid and complete under aerobic conditions through catabolic pathways. Yet, studies have shown that both sludge retention times (SRT) and hydraulic retention time (HRT) have appeared to be especially important parameters in removing estrogens from secondary treatment systems [22].

Regarding to tertiary treatment, also known as “advanced treatment”, it improves the secondary effluent quality by nitrogen removal, chlorination, and ultraviolet (UV) disinfection. From nitrogen removal perspectives, both nitrifying and denitrifying activities in tertiary treatment can degrade natural steroid estrogens, while synthetic estrogen can only be degraded in nitrifying conditions [24].

In Figure 4 is presented a flowchart with pathways of estrogens from sources to receptors, adapted [25].

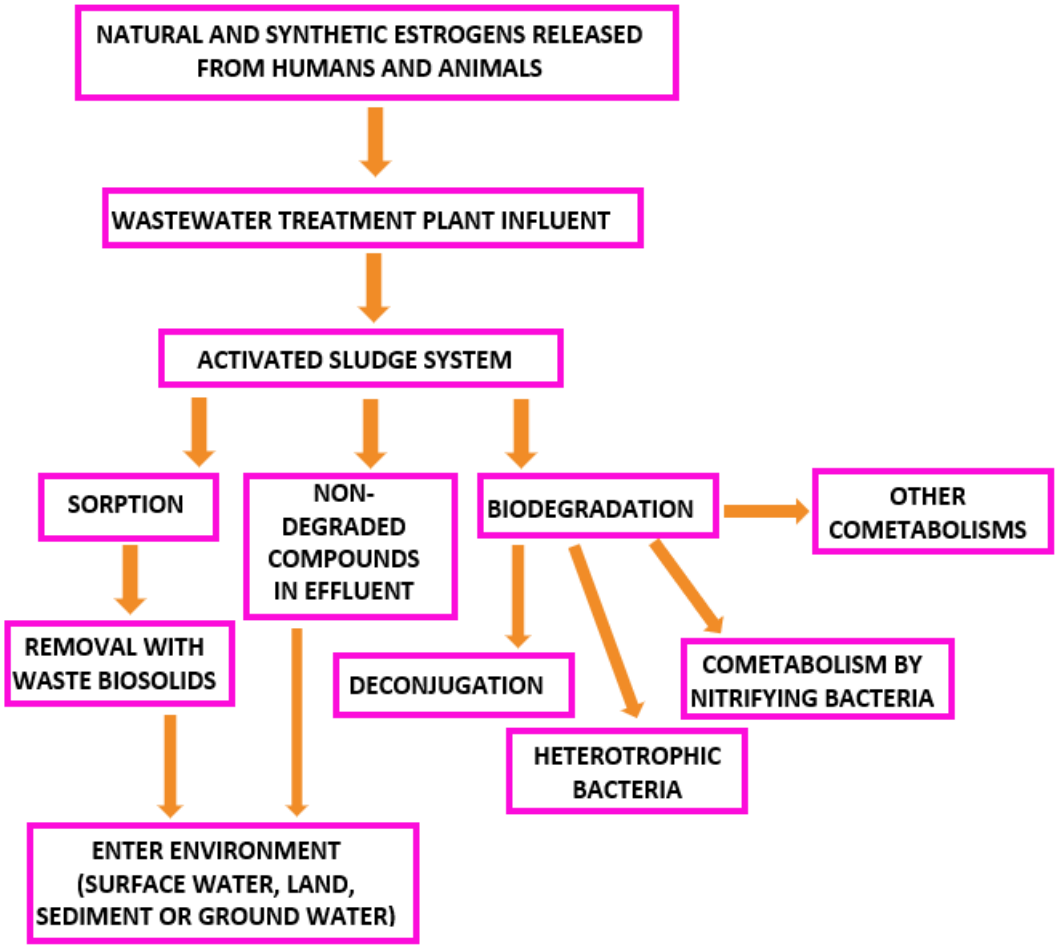

Figure 4. Fate of estrogens in a wastewater treatment plant and the environment.

However, there are a few variations in wastewater treatment processes and operational conditions, such as temperature changes throughout the year, that are associated to fluctuations in estrogen removal efficiencies making its removal not total, allowing it to enter into the environment through different aquatic matrices. For instance, an increase in temperature usually leads to better removal efficiency, as the metabolic rate of microorganisms in the biological treatment plant increases. Another fact to be taken into consideration is the charge that is being received into the WWTP, in particular, natural estrogens are poorly removed in highly loaded plants [25].

In Table 2, is presented the removal efficiency of E2 in different countries and different wastewater treatment systems [22].

Table 2. Removal efficiency of E2 in different countries.

Country	Type of WWTP	Removal efficiency of E2 (%)	Reference
Italy	CAS	76	[26]
China	CAS	73	[27]
UK	CAS	86	[28]
China	CAS	69,3	[29]
Iran	CAS	68,2	[30]
China	CAS	66,7	[31]
South Africa	AL	73,4	[32]
Brazil	AL	62	[33]
Canada	CASc	39,5	[24]
	CASuv	75,9	

CAS: Conventional activated sludge; AL: Aerated lagoon; CASc: CAS with chlorination; CASuv: CAS with ultraviolet.

An additional example is the fact that bacterial communities in municipal wastewater treatment sludge have a much greater capacity to biodegrade estrogens than industrial wastewater treatment sludge. Eventually, in some circumstances, that wastewater effluent is discharged into a body of water that at some point could be used as a water source [34, 35].

This scenario explains how estrogens can potentially be found in drinking water. In addition, estrogens can accumulate in wastewater sludge and can accumulate even more on soluble organic compounds found in soils [36].

2.5 - ANALYTICAL PROCEDURES

Detection of estrogens in different aquatic matrices, especially in wastewater influent and effluent, is a procedure that is undergoing continuous study and research, in which many efforts have been taken, aiming the development of new

techniques and improvement of those already used. In light of this, the present chapter aims to put together the most utilized analytical procedures when it comes to detect estrogens in wastewater, from different literatures from the recent years regarding this subject.

Tables 3, 4, 5, 6, 7 and 8 contemplates the title of the literature, the compound analyzed, the analytical methodology utilized and their respective references.

Table 3. Some literature references for estrogens analysis in wastewater samples.

TITLE	COMPOUND ANALYZED	ANALYTICAL METHODOLOGY	REFERENCE
Removal of seven endocrine disrupting chemicals (EDCs) from municipal wastewater effluents by a freshwater green alga	Estrone (E1) 17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE HPLC-DAD	[37]
Solid phase extraction using molecular imprinting polymers (MISPE) for the determination of estrogens in surface water by HPLC	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE HPLC-DAD	[38]
17α-Ethinylestradiol and 17β-estradiol removal from a secondary urban wastewater using an RBC treatment system	17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SBSE HPLC-DAD	[15]
Transformation and fate of natural estrogens and their conjugates in wastewater treatment plants: Influence of operational parameters and removal pathways	Estrone (E1) 17β-Estradiol (E2) Estriol (E3)	SPE UPLC	[40]

Table 4. Some literature references for estrogens analysis in wastewater samples.

TITLE	COMPOUND ANALYZED	ANALYTICAL METHODOLOGY	REFERENCE
Determination of Estrogens in Raw and Treated Wastewater by High Performance Liquid Chromatography-Ultraviolet Detection	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE HPLC-UV	[4]
Efficiency of selected wastewater treatment processes in removing estrogen compounds and reducing estrogenic activity using the T47D-KBLUC reporter gene assay	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE (LC-MS/MS)	[41]
Determinação de hormônios estrógenos em água potável usando CLAE-DAD	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE HPLC-DAD	[42]
Sensitive Estrogens Determination in Wastewater Samples by HPLC and Fluorescence Detection	17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE HPLC-FLD	[43]

Table 5. Some literature references for estrogens analysis in wastewater samples.

TITLE	COMPOUND ANALYZED	ANALYTICAL METHODOLOGY	REFERENCE
Estrogenic activity in Finnish municipal wastewater effluents	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE LC-MS/MS	[44]
Removal of estrogens by activated sludge under different conditions using batch experiments	Estrone (E1) 17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE HPLC-DAD	[45]
Optimization of Analytical Conditions to Determine Steroids and Pharmaceuticals Drugs in Water Samples Using Solid Phase-Extraction and HPLC	17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE HPLC-DAD	[46]
Quantification of selected steroid hormones (17 β-Estradiol and 17 α-Ethynylestradiol) in wastewater treatment plants in Klang Valley (Malaysia)	17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE ELISA	[47]
Comparison of different advanced treatment processes in removing endocrine disruption effects from municipal wastewater secondary effluent	Estrone (E1) 17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE GC/MS	[48]

Table 6. Some literature references for estrogens analysis in wastewater samples.

TITLE	COMPOUND ANALYZED	ANALYTICAL METHODOLOGY	REFERENCE
The use of peracetic acid for estrogen removal from urban wastewaters: E2 as a case study	17β-Estradiol (E2)	SBSE HPLC-DAD	[39]
Fate of selected estrogenic hormones in an urban sewage treatment plant in Tunisia (North Africa)	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE GC/MS	[49]
Fate and Analysis of Endocrine-Disrupting Compounds in a Wastewater Treatment Plant in Portugal	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE UPLC-ESI-MS/MS	[50]
Assessing the estrogenic potency in a Portuguese wastewater treatment plant using an integrated approach	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) 17α-Ethinylestradiol (EE2)	SPE LC-MS-MS	[51]

Table 7. Some literature references for estrogens analysis in wastewater samples.

TITLE	COMPOUND ANALYZED	ANALYTICAL METHODOLOGY	REFERENCE
Presence of estrogenic endocrine disruptors in three European estuaries in Northwest Iberian Peninsula (Portugal)	Estrone (E1) 17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE ELISA	[52]
Multiresidue Determination of Endocrine Disrupting Compounds in Sewage Treatment Plants (SPE-HPLC-DAD)	Estrone (E1) 17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE HPLC-DAD	[53]
Determinação dos desreguladores endócrinos bisfenol-A, β-estradiol, 17αetinilestradiol e estrona no Rio Paraíba do Sul	Bisfenol-A Estrone (E1) 17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE HPL-UV	[56]
A Microextraction Coupled with HPLC-UV for a Sensitive Detection of Estrogens in Water	17β-Estradiol (E2) 17α-Ethinylestradiol (EE2) diethylstilbestrol (DES)	Microextraction HPLC-UV	[59]

Table 8. Some literature references for estrogens analysis in wastewater samples.

TITLE	COMPOUND ANALYZED	ANALYTICAL METHODOLOGY	REFERENCE
Determination of estrogens in water by HPLC-UV using cloud point extraction	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) Progesterone (P)	Cloud point extraction (CPE) HPLC-UV	[60]
Determination of 17 b-estradiol in pharmaceutical preparation by UV spectrophotometry and high performance liquid chromatography methods	17β-Estradiol (E2)	HPLC-UV	[61]
HPLC determination of estradiol, its degradation product, and preservatives in new topical formulation Estrogel HBF	Estrone (E1) 17β-Estradiol (E2)	HPLC-UV	[62]
Determinação simultânea de estriol, 17β-estradiol, 17α-etinilestradiol e estrona empregando-se extração em fase sólida (SPE) e cromatografia líquida de alta eficiência (HPLC)	Estrone (E1) 17β-Estradiol (E2) 17α-Ethinylestradiol (EE2)	SPE HPLC-UV	[69]

2.6 - ANALITYCAL METHODOLOGY

Considering all the literature gathered concerning methods of quantification of emergent contaminants and more specifically, estrogens, in wastewater influent and effluent samples, the majority cites HPLC as the most utilized method to determinate estrogenic compounds with low limits of detection.

The one information that is unanimous between literatures is the necessity of a prior extraction. Solid phase extraction (SPE) is recognized as a very common sample pre-treatment methodology for concentrating the target analytes in biological and environmental samples [4]. In conclusion, SPE coupled with HPLC is an effective method for determination of trace organic compounds, presenting enough sensibility to detect and quantifying 17b-estradiol (E2) in aquatic matrices.

2.6.1 - SOLID PHASE EXTRACTION

Solid Phase Extraction (SPE), is a liquid-solid separation technique utilized to extract semi-volatile and non-volatile analytes from liquid samples. It was developed to supply the disadvantages of classic liquid-liquid extraction such as, elevated solvent consumption and as consequence, elevated generation of toxic waste and low percentages of analyte recovery. SPE has high recovery capacity of analytes and its manifolds and sorbents are available commercially [17, 54].

The separation mechanisms that occurs in the SPE are related to physical, chemical and mechanical processes, being the main mechanisms, adsorption, partition (normal phase and reverse phase), ion exchange and exclusion. In the case of reverse phase, the main chemical and physical forces that act between the analyte and sorbent molecules are those of Van der Waals, among the carbon-hydrogen bonds of the analyte with the functional groups of the silica surface. In normal phase,

the main interactions are between the polar groups of the solid phase and the analyte, for hydrogen bonding, π-π interactions, dipole-dipole, induced dipole-dipole and dipole interactions induced-induced dipole. In ion exchange separation, selective extractions of the analytes occur through ionic interactions [17, 55].

Generally, SPE procedures consist of 4 steps: 1) conditioning or activation of the sorbent present in the cartridge through the elution of a suitable solvent; 2) percolation of the sample, when occurs the retention of the analyte or, sometimes, the retention of some interferents; 3) cleaning the cartridge with an appropriate solution to remove interferents that are less retained than the analytes, a step called washing or clean-up and 4) elution and analyte collect [54]. The procedure is illustrated in Figure 5, adapted [72], and Figure 6 is the equipment used in this study.

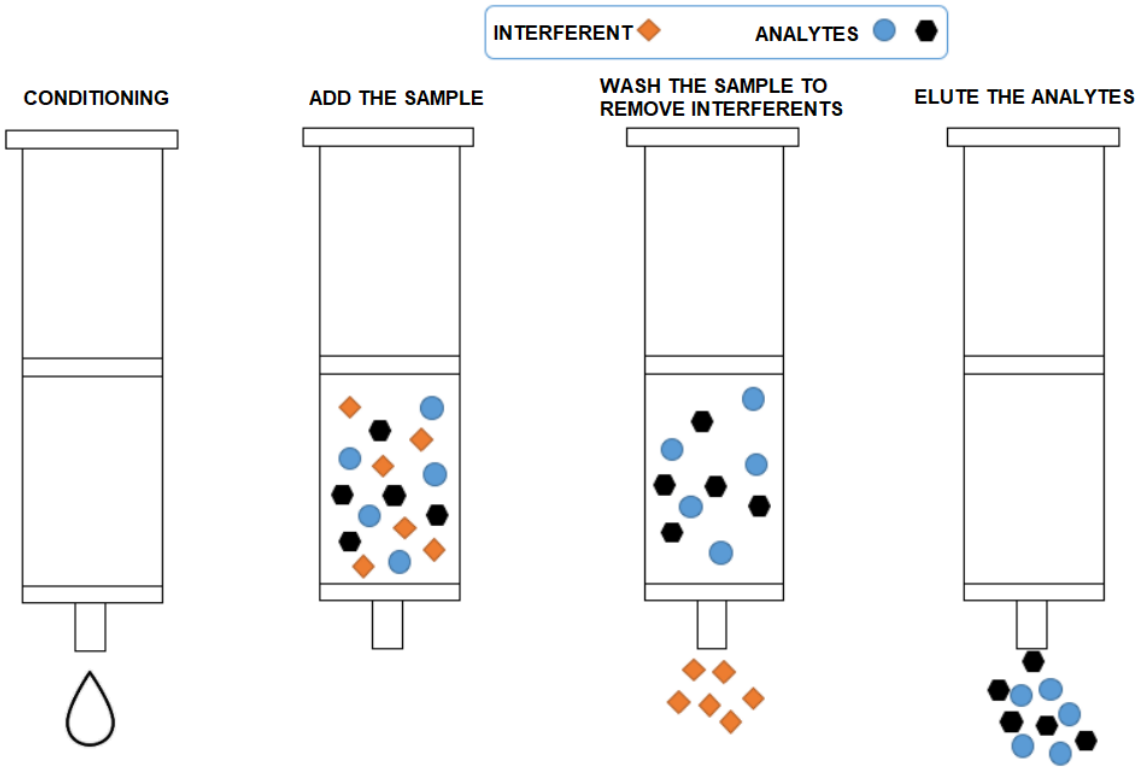

Figure 5. Four steps used in SPE basic procedure.

Figure 6. SPE equipment used in this work.

2.6.2 - HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatography is a widely used method that allows separation, identification and determination of very similar chemical compounds in complex mixtures. In this technique there are two phases, one of which remains stationary, immobilized in one column or on a flat surface, while the other moves through it. In all chromatographic separations the sample is transported by the mobile phase which is forced to pass through a stationary phase. The mobile phase can be a supercritical liquid, gas or fluid. During the elution of the mobile phase over the stationary phase the components of the mixture are distributed by them so that each of them is selectively retained by the stationary phase, which results in differential migrations velocities of these components [64].

Chromatographic methods are classified according to the physical environment that the stationary phase comes into contact, if this is in a tube is called chromatography in column, however if it is supported on a flat plate or on the surface of a paper it is called planar chromatography. Regarding the mobile phase, the chromatographic methods are divided into gas chromatography, liquid chromatography and chromatography with supercritical fluid.

High Performance Liquid Chromatography (HPLC) uses columns packed with specially prepared materials and a mobile phase eluted under high pressures. The equipment used in the HPLC system can be completely automated and it consists basically of a mobile phase reservoir, a pressure pump, an injector, a chromatographic column, a detector (one or more) and a data acquisition system. The most used detector class in the HPLC is the optic, which includes fluorescence, refractive index, light scattering and absorbance (fixed wavelength photometric, long spectrophotometric variable wave, spectrophotometric by diode array). Spectrophotometric detectors are based on the absorbance of light by the sample, by passing through it any electromagnetic radiation with a given wavelength [64, 65].

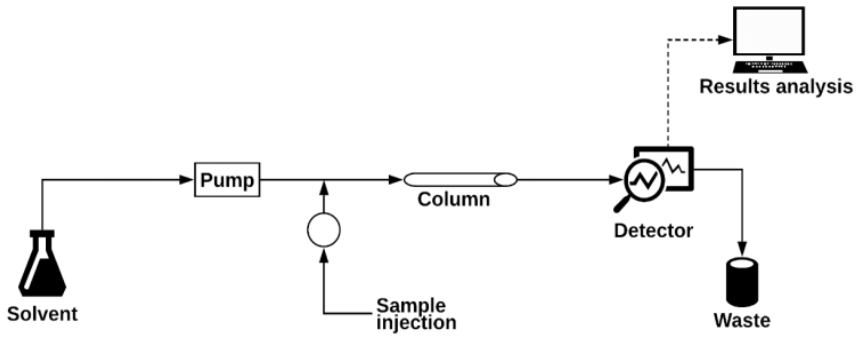

Figure 7. Standard HPLC system.

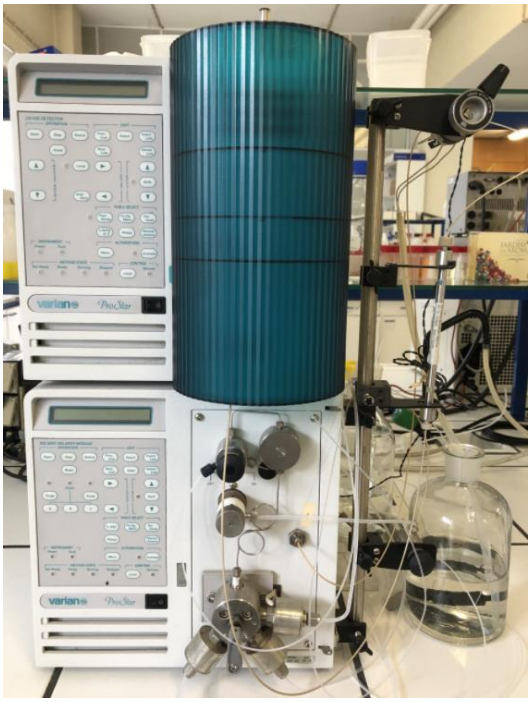

Figure 8. HPLC-UV equipment used in the present study.

2.7 - ANALYTICAL METHODOLOGY VALIDATION

In analytical chemistry one important step is the optimization of an experimental methodology. However, this process needs to be put through thorough evaluation in order to estimate its efficiency. This evaluation is known as analytical methodology validation.

It is really relevant to analyze the correlation between experimental results and the questions that the proposed method is created to respond. The evaluation will demonstrate that the method in itself is appropriated to answer those questions [57, 58].

The validation parameters applied to this study are presented in the next topics.

2.7.1 - LINEARITY

The relation between the measured signal (peak area) and the concentration of the analyte is expressed by a mathematical equation, called a calibration curve. To properly define the relationship between the concentration and the area, it is necessary to use between 5 to 8 different concentration levels, without including the zero point. The most used method is the linear model, using the least squares method, in which the independent variable (x) is the concentration and the dependent variable (y) the chromatographic response, the area [66, 67].

$$y = ax + b \quad (1)$$

Using the experimental areas, it is possible to calculate the correlation coefficient (r). Linearity is often tested using r. When r = 1, this parameter indicates that all points are exactly on a positive slope line, when r = 0, it indicates the lack of correlation between the dependent variable (y) and the independent variable (x). That means, if closer to 1, greater the degree of linearity and the relation between the variables.

2.7.2 - PRECISION

The precision of a method is a measure of dispersion that characterizes the analytical values considering their mean. Being defined as the degree of agreement between the values of analytical tests series repetition. It can be evaluated at three levels, namely: repeatability, intermediate precision and reproducibility.

Repeatability method is a measurement of the method under optimal conditions accuracy, in the same instrumental conditions on the same sample, over the course of a series of tests carried out in a short period of time. Precision is calculated by the coefficient of variation (CV) of the responses obtained.

$$CV(\%) = \frac{SD}{\bar{X}} \times 100 \quad (2)$$

The intermediate precision assesses the influence of variations within the same environment, on different days. This study is generally under a greater variability, which is why it is considered as a more representative measurement of the results to be observed [66,67].

2.7.3 - LIMIT OF DETECTION (LOD)

The detection limit is defined as the smallest amount of analyte in a given sample that can be detected, but not necessarily quantified. The LOD can be determined by visual assessment, signal/noise ratio and methods based on calibration curve parameters.

The visual assessment method consists of adding a known concentration of the analyte to a matrix and establishing the LOD as the smallest amount that can be detected. The signal/noise ratio is obtained by comparing the signals presented by samples with known low concentrations, with the signal presented by the matrix without the analyte. Although, the most usual method is the one applying the calibration curves parameters [66, 67, 68].

$$LOD = \frac{LOQ \times 3.3}{100} \quad (3)$$

2.7.4 - LIMIT OF QUANTIFICATION (LOQ)

The limit of quantification is defined as the lowest amount of analyte in a sample that can be quantified with acceptable precision. The LOQ is equivalent to the lowest concentration of the calibration curve, in a curve that uses at least 5 independent standards.

Once the LOQ is established, this value must be respected as an operational limit, that is, extrapolations below this value are not recommended because they are not accurate [66, 67, 68].

2.7.5 - ACCURACY

The accuracy of a method is defined as the ability of a given analytical method to produce results as close as possible to the true value. It is common to execute this assessment by adding a known quantity of reference of the substance to the matrix. Accuracy is given by the difference between the amount of analyte added, which is known, with the concentration obtained by the method used.

A usual process to evaluate accuracy is through a recovery measurement experiment. The recovery percentage is calculated through equation (3). Where C1 is the measured concentration in the eluted sample, C2 is the measured concentration in not-fortified sample and C3 is the concentration that was added [66, 67, 68].

$$R = \frac{C1 - C2}{C3} \times 100 \quad (4)$$

3 - MATERIALS AND METHODS

3.1 - MATERIALS

3.1.1 - SOLVENTS AND STANDARD

- Acetonitrile HPLC grade, Carlo Erba, +99.9%
- Ultrapure water (resistivity value below 18.2 MΩ.cm - Type I)
- Commercial ethanol, Aga, 96%
- Methanol HPLC grade, Carlo Erba, +99.9%
- Trifluoracetic acid, Sigma Aldrich, +99%
- 17b-estradiol, Sigma, ≥98%
- Three wastewater samples, 5000 mL each, collected from a wastewater treatment plant

3.1.2 - EQUIPMENT

- Chromatographic analytical column Nucleosil 100-5 C18 with a particle size diameter of 5 μm, 150 mm x 4.6 mm from Macherey-Nagel
- Analytical balance ADA 210/C, ±0.0002 g, Adam Equipment
- pH meter HI 2020-02 from Hanna
- Chromabond HLB SPE cartridges, 60 μm; 6 mL/500 mg from Macherey-Nagel
- Cytiva Glass Vacuum Filtration Device, with 0.2 μm pore size filters
- HPLC system Varian Prostar UV/VIS
- SPE vacuum manifold system

3.2 - EXPERIMENTAL METHODOLOGY

The present work is divided in two main experimental stages. First, an SPE/HPLC-UV [4] experimental methodology is optimized to detect and quantify 17β-Estradiol (E2) present into aqueous samples. Secondly, the validation of the optimized experimental methodology is done by the monitoring of estradiol in wastewater influent and effluent samples from a Wastewater Treatment Plant.

3.2.1 - STOCK AND STANDARD SOLUTIONS PREPARATION

During experimental work, all glassware was cleaned first with distillated water and next with methanol to remove any impurities.

Stock solution was prepared by measuring 10 mg of E2 into a 100 mL volumetric flask and completing the remaining volume with methanol, reaching a final concentration of 100 mg/L. The standard solutions were prepared in a dilution series from stock solution (Table 9). All prepared standard solutions were transferred to glass flasks, sealed with film and stored at -18ºC until analysis.

Table 9. Stock and standard solution preparation.

STANDARD SOLUTION	CONCENTRATION (mg/L)	DILUTED FROM
Stock solution C1	100	-
C2	80	C1
C3	40	C2
C4	20	C3
C5	10	C4
C6	5	C5
C7	1	C6
C8	0.5	C7
C9	0.25	C8

3.2.2 - WAVELENGTH SELECTION

The ultraviolet-visible equipment, Jasco V-730 spectrophotometer, was used in scanning mode between the wavelengths of 240 nm and 740 nm, using quartz cuvettes to select the most appropriate wavelength for the analysis of estradiol. Methanol was used as the reference solvent and a solution of estradiol in methanol with a concentration of 100 mg/L.

The maximum absorption value outside the solvent cut-off region (205 nm) is obtained at a wavelength of 281 nm. The obtained spectrum is shown in Figure 9.

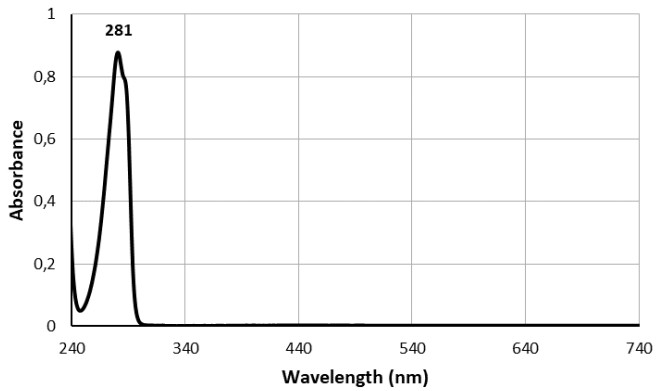

Figure 9. Analysis by wavelength scanning with ultraviolet-visible spectrometry using a 100 mg/L estradiol solution.

3.2.3 - OPTIMIZATION OF MOBILE PHASE COMPOSITION

To optimize selective and sensitive analytical methods is an important step to provide well founded data concerning E2 in WWTPs. So, the first variable to go through this process is the mobile phase composition. According to literature about detection and quantification of E2 in WWTPs effluents, the mobile phase is a combination of acetonitrile (ACN) and ultrapure water (W) or methanol (MeOH) and ultrapure water (W), in different proportions [37, 38, 4].

The first thing to be considered was the column to be used and the chosen one was, Nucleosil 100-5 C18, dp = 5 mm, 150 mm x 4.6 mm da Macherey-Nagel. This specific column works with a solvent pH value between 2 and 8, so firstly, it was measured the mobile phase combinations pH value, that are presented in Table 10.

Table 10. Mobile phase conditions optimized.

MOBILE PHASE	COMPOSITION	pH
1	50 ACN : 50 W	7.6
2	80 ACN : 20 W	5.9
3	100 ACN	5.2
4	50 ACN : 50 ÁGUA + 0.02 TFA	2.5
5	80 ACN : 20 ÁGUA + 0.02 TFA	2.1
6	100 ACN + 0.005 TFA	1.7
7	100 MET	5.8
8	80 MET : 20 ÁGUA	6.1
9	70 MET : 30 ÁGUA	6.4
10	100 MET + 0.005 TFA	2.0

Due to the different polarities of acetonitrile and water, the interactions between the analytes and the mobile phase changes for different compositions. The main objective in this step is to obtain a higher response for E2, with a lower retention time, in order to minimize the use of solvents. Considering that, in selected mobile phase composition, it was used Trifluoracetic acid (TFA) to verify if in lower pH values the response for E2 would be bigger and obtained using less solvent.

To be able to better verify the interference of only mobile phase composition, parameters as column, volume of injection, wavelength value and flowrate were constant for all analysis, as presented in Table 11.

Table 11. Constant mobile phase conditions.

PARAMETER	CONDITION
Column	Nucleosil 100-5 C18, dp = 5 mm, 150 mm x 4.6 mm da Macherey-Nagel
Volume of injection	20 μL

Wavelength	281 nm
Flowrate	1 mL/min

3.2.3.1 - LINEARITY OF THE HPLC-UV ANALYSIS

Linearity was verified by constructing a calibration curve to every mobile phase composition, then injecting stock solution and 8 standard solutions of E2 prepared in methanol with the respective concentrations: 100, 80, 40, 20, 10, 5, 1, 0.5 and 0.25 mg/L, each solution was injected three times. After construction of the calibration curve, the coefficients of correlation were calculated to attest the linearity.

To evaluate repeatability, nine different concentrations (between stock and standard solutions) were analyzed, all in triplicate, for all compositions of mobile phase. Then, to evaluate precision of the chosen mobile phase composition, nine different concentrations were analyzed, all in triplicate, in three distinct days.

3.2.3.2 - LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

LOQ was defined as the lowest concentration of a E2 solution that allowed a chromatography integration, for each mobile phase composition. LOD was defined by equation (3).

3.2.4 - OPTIMIZATION OF SPE CONDITIONS

To validate the Solid Phase Extraction (SPE) methodology, an optimization planning of experiments was created using the software *Design-Expert 11*. This planning consists of a three-level Box-Behnken (BBD) experimental design with four parameters to be changed, these being the pH value of ultrapure water (2, 5 and 8), the volume of the sample (500, 1000 and 1500 mL), the drying time of the cartridge

(10, 35 and 60 minutes) and the composition of the washing step, changing the percentage of methanol used (0, 5 and 10%), in order to obtain the highest response (chromatographic area) Table 12. The combinations of the levels and parameters to optimize resulted in 27 experiments, as presented in Table 13.

Table 12. Experimental planning using the three-level Box-Behnken experimental design.

PARAMETERS	LEVELS		
	-1	**0**	**+1**
Sample Volume (mL)	500	1000	1500
Sample pH	2	5	8
Adsorbent drying time (min)	10	35	60
Solvent composition in washing (%)	0	5	10

Table 13. Combination of SPE experiments generated by Design-Expert 11 software.

RUN	SAMPLE VOLUME (mL)	SAMPLE pH	ADSORBENT DRYING TIME (MIN)	SOLVENT COMPOSITION IN WASHING STEP (%)
1	0	+1	0	0
2	-1	-1	-1	-1
3	-1	0	0	0
4	0	0	-1	0
5	-1	+1	-1	+1
6	+1	-1	-1	-1
7	+1	+1	+1	+1
8	0	-1	0	0
9	-1	+1	+1	+1
10	0	0	0	-1
11	+1	+1	-1	+1
12	0	0	0	0
13	+1	+1	-1	-1
14	+1	-1	-1	+1
15	+1	-1	+1	+1
16	0	0	0	0
17	0	0	0	0
18	-1	+1	-1	-1
19	-1	+1	+1	-1
20	-1	-1	+1	+1
21	0	0	0	+1
22	0	0	+1	0

23	+1	0	0	0
24	-1	-1	-1	+1
25	-1	-1	+1	-1
26	+1	-1	+1	-1
27	+1	+1	+1	-1

These experiments follow the guidelines for performing SPE described in Table 14.

Table 14. SPE operating conditions.

	STEP	CONDITION APPLIED	MEASUREMENT	FLOWRATE (mL/min)
1	Conditioning A	Methanol	5 (mL)	1
2	Conditioning B	Acetonitrile	5 (mL)	1
3	Conditioning C	Ultrapure water, (pH 2, 5 or 8)	5 (mL)	1
4	Loading	E2 in ultrapure water with pH value of 2, 5 or 8	500, 1000 or 1500 (mL)	4
5	Washing	Ultrapure water with a pH value of 2, 5 or 8, with addition of methanol (0, 5 or 10%)	10 (mL)	1
6	Cartridge drying	Vacuum	10, 35 or 60 (min)	-
7	Elution	Acetonitrile	10 (mL)	1
8	Evaporation	Heating plate	70°C until dry	-
9	Reconstitution	Methanol	0.5 mL	-

All samples referred to the “loading” step, were prepared using the volume of 1 liter of ultrapure water, added of 100 μL of the standard solution of estradiol in methanol at a concentration of 10 mg/L. The final concentration of the loading sample is always 1 μg/L. The pH of the ultrapure water was adjusted daily as planned, using HCl to obtain a pH value of 2 and KOH to pH value of 8. Ultrapure water without the addition of any components already has a pH value of approximately 5.

The cartridges used were all from the same brand, Chomabond HLB (60 mm / 6 mL / 500 mg) - MN, and in order to maintain the integrity of the adsorbent present in the cartridges, they were used only once per experiment. After performing all the experiments of Box-Behnken planning, the samples were injected in the HPLC-UV

system, using the mobile phase selected in the process of optimizing HPLC-UV conditions. The experiments that presented a higher response, were selected, and between them, the one that allowed a higher recovery of E2, was chosen.

3.3 - APPLICATION OF THE DEVELOPED METHODOLOGY

The application of the developed methodology was done by collecting three samples from a Wastewater treatment plant. Three samples, five liters each, were collected from different phases of the wastewater treatment plant. Sample number 1 was in the entrance point, and consisted of raw wastewater, sample number 2 was retrieved from the activated sludge aeration tank and sample number 3 was at the final point, the treated effluent. Figure 10 illustrates the points where the samples were collected.

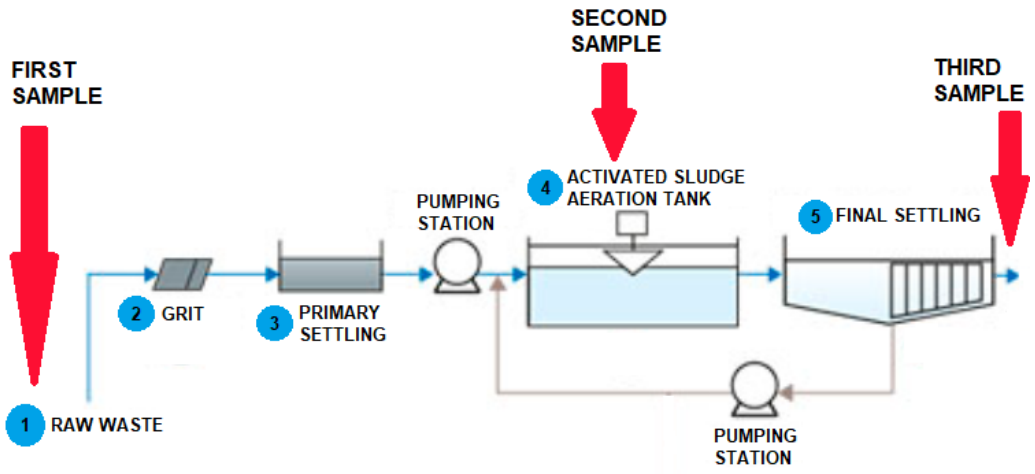

Figure 10. Illustration of a typical biological wastewater treatment plant, with the indication of the retrieval samples points.

In order to analyze the efficiency of removal of E2 in a biological wastewater treatment plant, the three points were selected in a way that allowed that analysis. After collecting the samples, they were stored in the refrigerator (4°C). Next, each sample went

through a process of filtration with a 0.2 μm filer (Figure 11) and again restored at 4°C until the moment of analyses.

Figure 11. Filtration equipment used in this work.

4 - RESULTS AND DISCUSSION

4.1 - ANALYTICAL METHODOLOGY DEVELOPMENT

The present chapter gathers the main results obtained from the analytical methodology developed in this study.

4.1.1 - MOBILE PHASE COMPOSITION FOR THE HPLC-UV SYSTEM

As preview stated, in total, there were 10 combinations of mobile phase analyzed in the HPLC-UV system and their respective results are presented next. The main objective of this analysis of mobile phase composition is to work with one that will decrease the amount of solvent needed, while also presenting the best conditions of quantification of E2.

4.1.1.1 - COMPOSITION OF ACN : W : TFA

4.1.1.1.1 - MOBILE PHASE 1

The HPLC system was conditioned with acetonitrile and ultrapure water, in a proportion of 50% each, and with a flow rate of 1 mL/min for 2 hours. After stabilizing the baseline, a total system pressure of approximately 93 atm was observed. Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 7.0 minutes, with estradiol's retention time of 5.13 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, 0.5 and 0.25 mg/L were analyzed in triplicates each. The analyzes stopped at a

concentration of 0.25 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5, 1 and 0.5 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 15. In Figures 12 and 13, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

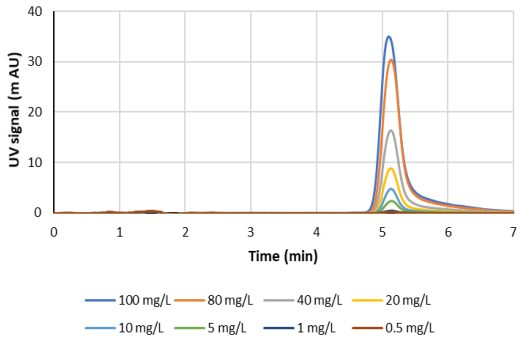

Figure 12. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 50ACN:50W.

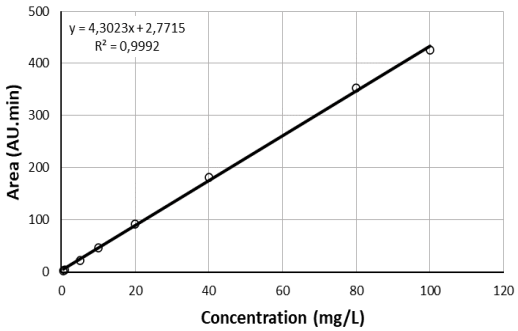

Figure 13. Estradiol calibration curve for the linearity range between 0.5 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 50% ACN and 50 % W.

Table 15. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 0.5 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
0.5	1961	1934	1924	1940	19.1	0.99
1	4159	4146	4130	4145	14.5	0.35
5	22381	22452	22659	22497	144.4	0.64
10	46829	46566	46681	46692	131.8	0.28
20	91014	91524	91746	91428	375.3	0.41
40	181315	180892	180181	180796	573.1	0.32
80	352846	352584	352080	352503	389.3	0.11
100	425509	425897	425740	425715	195.2	0.05

4.1.1.1.2 - MOBILE PHASE 2

The HPLC system was conditioned with acetonitrile and ultrapure water, in a proportion of 80% and 20% respectively, and at a flow rate of 1 mL/min for 1 hour. After stabilizing the baseline, a total system pressure of approximately 58 atm was observed. Then, successive analyzes of the standard estradiol solution with a concentration of 100 mg/L in methanol were performed until the chromatographic peak of estradiol in three successive analyzes was overlapping. The analysis time required with this mobile phase composition is approximately 4.00 min, with estradiol's retention time of 2.26 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, 0.5 mg/L were analyzed in triplicates. The analyzes stopped at the concentration of 0.5 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the experimental areas obtained, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5 and 1 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 16. In Figures 14 and 15, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

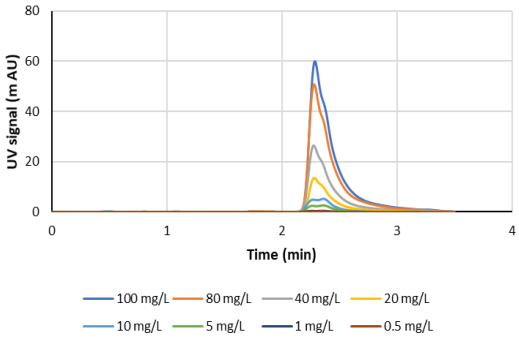

Figure 14. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 80ACN:20W.

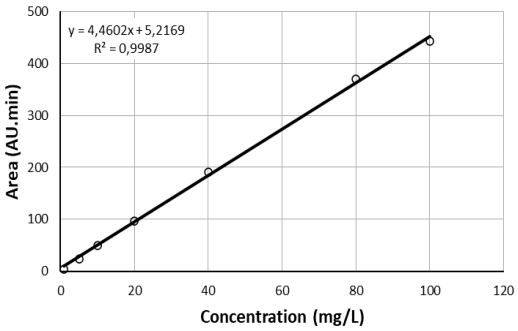

Figure 15. Estradiol calibration curve for the linearity range between 1 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 80% ACN and 20 % W.

Table 16. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 1 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
1	4275	4277	4249	4267	15.6	0.37
5	23957	24076	23978	24004	63.5	0.26
10	50547	50203	49898	50216	324.7	0.65
20	97729	97623	97143	97498	312.3	0.32
40	191237	191031	190759	191009	239.8	0.13
80	369473	370037	368267	369259	904.2	0.24
100	442248	442306	441682	442079	344.7	0.08

4.1.1.1.3 - MOBILE PHASE 3

The HPLC system was conditioned with acetonitrile, 100%, with a flow rate of 1 mL/min for 1 hour. After stabilizing the baseline, a total system pressure of approximately 55 atm was observed. Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 4.0 minutes, with estradiol's retention time of 2.45 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1 and 0.5 mg/L were analyzed in triplicates each. The analyzes stopped at a concentration of 0.5 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5 and 1 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 17. In Figures 16 and 17, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

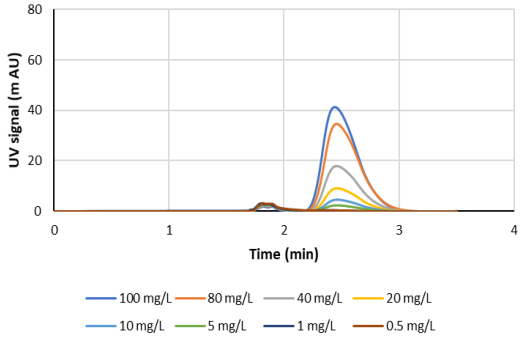

Figure 16. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 100ACN.

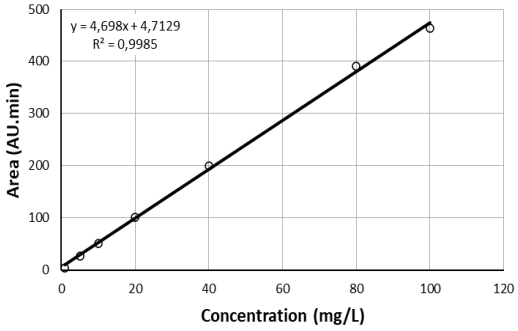

Figure 17. Estradiol calibration curve for the linearity range between 1 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 100% ACN.

Table 17. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 1 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
1	3755	3702	3743	3733	27.8	0.74
5	26234	26132	26301	26222	85.1	0.32
10	51507	51921	51077	51502	422.0	0.82
20	100004	100682	100624	100437	375.8	0.37
40	199981	200011	199736	199909	150.9	0.08
80	390801	390749	388608	390053	1251.4	0.32
100	463741	463810	463942	463831	102.1	0.02

In order to study the first peak identified in the chromatogram, with a retention time of 1.8 min, 20 µL of 100% Methanol (Figure 18) were injected in the HPL-UV system, confirming that the peak in question refers to the methanol used in the preparation of the estradiol stock and standard solutions.

When analyzing the chromatographic graphics for the estradiol standard with a concentration of 100 mg/L (Figure 19), using the three types of mobile phase mentioned above, as well as when comparing their respective areas (Table 18), it is possible to conclude that the mobile phase composed of 100% acetonitrile represents the lowest retention time and highest area values. Mobile phases that allow less retention time, imply in a lower analysis time and consequently, less use of solution, thus optimizing the process.

On the other hand, a composition of 50% acetonitrile and 50% water obtained a lower LOQ (0.5 mg/L).

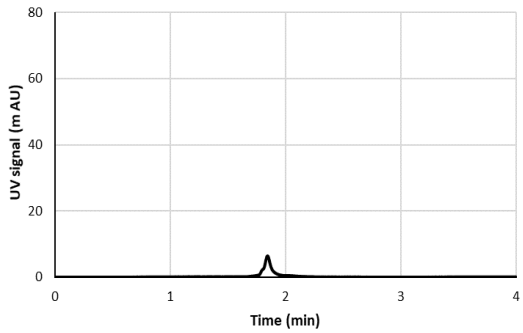

Figure 18. HPLC-UV analysis of a solution of 100% methanol in a mobile phase consisting of 100% acetonitrile.

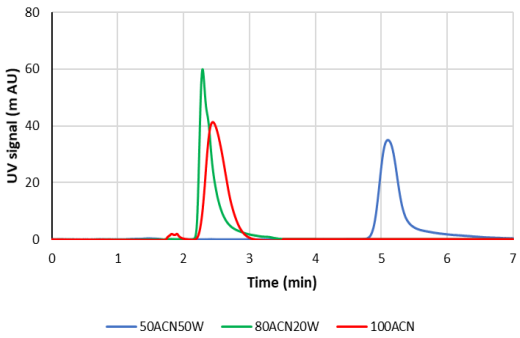

Figure 19. Chromatograms (overlapping) obtained by HPLC-UV relative to the estradiol standard with a concentration of 100 mg/L injected using the mobile phases 50ACN: 50W, 80 ACN: 20W and 100ACN.

Table 18. Average experimental areas and their respective retention times for the estradiol stock solution with concentration of 100 mg/L in mobile phases of 50ACN:50W, 80ACN:20W and 100ACN.

Mobile Phase	Average area (mAU.min)	Retention Time (min)	LOQ (mg/L)
50ACN:50W	425715	5.13	0.5
80ACN:20W	442079	2.26	1.0
100ACN	463831	2.45	1.0

Then, to verify the possibility of optimizing even more the previous mobile phase conditions mentioned, trifluoroacetic acid (TFA) was added to decrease the pH, in order to test whether the addition of TFA would significantly change the values of the areas obtained, LOQ and retention time.

4.1.1.1.4 - MOBILE PHASE 4

The HPLC system was conditioned with acetonitrile and ultrapure water, in a proportion of 50% each and 0.02% of TFA, with a flow rate of 1 mL/min for 1 hour. After stabilizing the baseline, a total system pressure of approximately 82 atm was observed.

Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 7.0 minutes, with estradiol's retention time of 5.52 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, 0.5 and 0.25 mg/L were analyzed in triplicates each. The analyzes stopped at a concentration of 0.25 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5, 1 and 0.5 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 19. In Figures 20 and 21, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

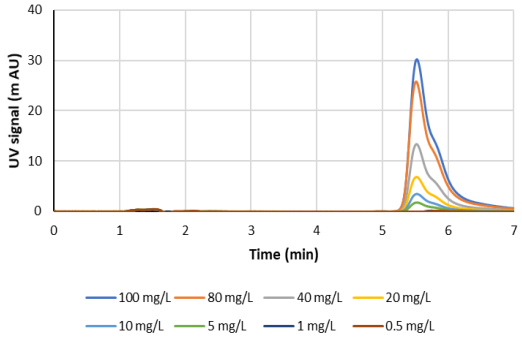

Figure 20. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 50ACN:50W:0.02TFA.

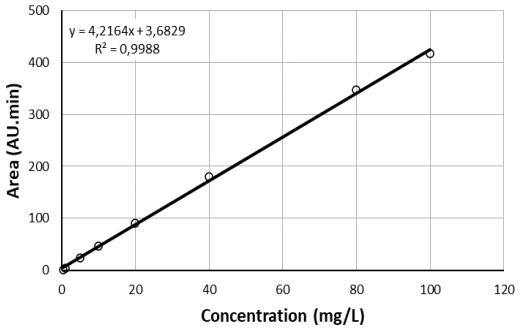

Figure 21. Estradiol calibration curve for the linearity range between 0.5 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 50% ACN, 50 % W and 0.02% TFA.

Table 19. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 0.5 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
0.5	1749	1794	1782	1775	23.3	1.31
1	4052	4102	4141	4098	44.6	1.03
5	23418	23065	23329	23271	183.6	0.79
10	46419	47024	46169	46537	439.6	0.94
20	91014	91426	91074	91171	222.6	0.24
40	180342	180302	180047	180230	160.0	0.09
80	347216	347214	347977	347469	439.9	0.13
100	417266	415826	416172	416421	751.7	0.18

4.1.1.1.5 - MOBILE PHASE 5

The HPLC system was conditioned with acetonitrile and ultrapure water, in a proportion of 80% acetonitrile, 20% ultrapure water and 0.02% of TFA, with a flow rate of 1 mL/min for 1 hour. After stabilizing the baseline, a total system pressure of approximately 82 atm was observed. Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 4.0 minutes, with estradiol's retention time of 2.27 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, 0.5 and 0.25 mg/L were analyzed in triplicates each. The analyzes stopped at a concentration of 0.25 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5, 1 and 0.5 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 20. In Figures 22 and 23, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

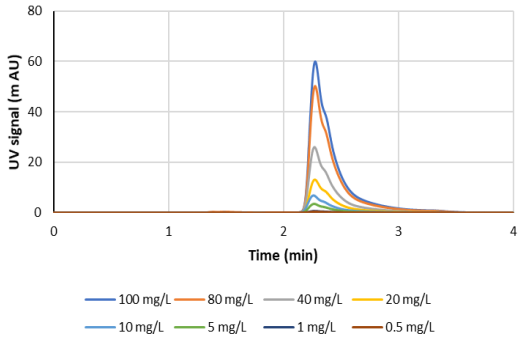

Figure 22. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 80ACN:20W:0.02TFA.

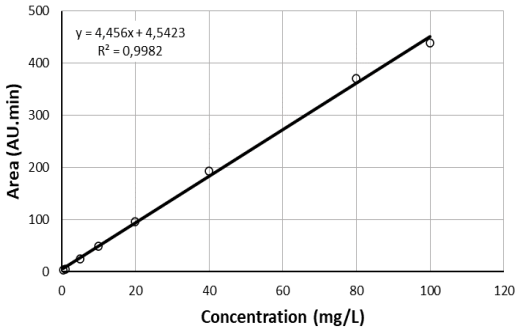

Figure 23. Estradiol calibration curve for the linearity range between 0.5 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 80% ACN, 20 % W and 0.02% TFA.

Table 20. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 0.5 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
0.5	2730	2792	2781	2768	33.1	1.20
1	4932	4941	4934	4936	4.7	0.10
5	24156	24427	24676	24420	260.1	1.07
10	49201	49283	49264	49249	42.9	0.09
20	96291	96496	96039	96275	228.9	0.24
40	193243	193156	193122	193174	62.4	0.03
80	370745	369503	370306	370185	629.8	0.17
100	437721	438994	438205	438307	642.6	0.15

4.1.1.1.6 - MOBILE PHASE 6

The HPLC system was conditioned with acetonitrile 100% and 0.005% of TFA, with a flow rate of 1 mL/min for 1 hour. After stabilizing the baseline, a total system pressure of approximately 67 atm was observed. Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 4.0 minutes, with estradiol's retention time of 2.50 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, and 0.5 mg/L were analyzed in triplicates each. The analyzes stopped at a concentration of 0.5 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5, and 1 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 21. In Figures 24 and 25, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

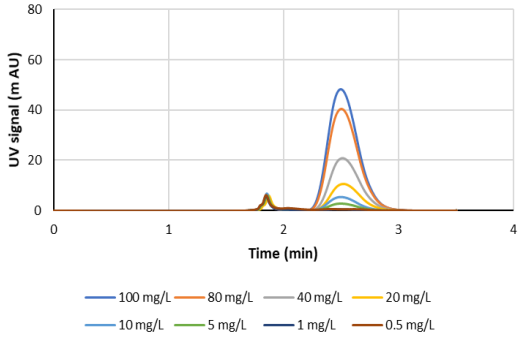

Figure 24. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 100ACN:0.005TFA.

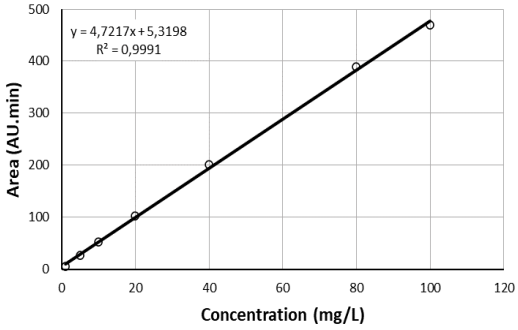

Figure 25. Estradiol calibration curve for the linearity range between 1 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 100% ACN and 0.005% TFA.

Table 21. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 1 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
1	4644	4621	4629	4631	11.7	0.25
5	27096	27152	27101	27116	31.0	0.11
10	52156	52570	52156	52294	239.0	0.46
20	102717	102595	102310	102541	208.9	0.20
40	200433	200533	200314	200427	109.6	0.05
80	389071	389922	389991	389661	512.4	0.13
100	469606	469202	469140	469316	253.1	0.05

4.1.1.2 - COMPOSITION OF MET : W : TFA

4.1.1.2.1 - MOBILE PHASE 7

The HPLC system was conditioned with methanol, 100%, with a flow rate of 1 mL/min for 1.50 hours. After stabilizing the baseline, a total system pressure of approximately 80 atm was observed. Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 3.0 minutes, with estradiol's retention time of 1.97 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, 0.5 and 0.25 mg/L were analyzed in triplicates each. The analyzes stopped at a concentration of 0.25 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5, 1 and 0.5 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 22. In Figures 26 and 27, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

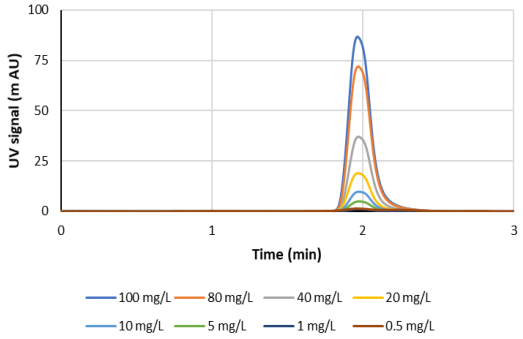

Figure 26. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 100MET.

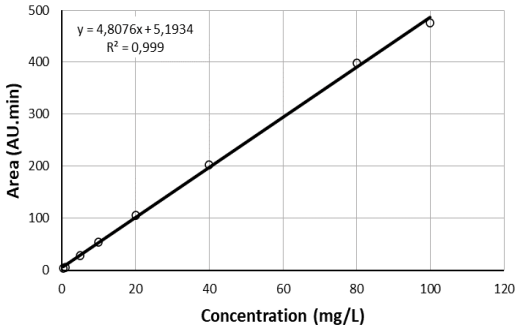

Figure 27. Estradiol calibration curve for the linearity range between 0.5 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 100% MET.

Table 22. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 0.5 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
0.5	4534	4215	4371	4373.3	159.5	3.65
1	5875	5841	5744	5820.0	68.0	1.17
5	28143	28116	28275	28178.0	85.1	0.30
10	54351	54081	54255	54229.0	136.9	0.25
20	104861	104668	104749	104759.3	96.9	0.09
40	202647	202212	202319	202392.7	226.7	0.11
80	399070	398845	398679	398864.7	196.2	0.05
100	476312	476038	475856	476068.7	229.5	0.05

4.1.1.2.2 - MOBILE PHASE 8

The HPLC system was conditioned with methanol and ultrapure water, 80% and 20% respectively, with a flow rate of 1 mL/min for 1 hour. After stabilizing the baseline, a total system pressure of approximately 144 atm was observed. Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 6.0 minutes, with estradiol's retention time of 3.40 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, 0.5 and 0.25 mg/L were analyzed in triplicates each. The analyzes stopped at a concentration of 0.25 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5, 1 and 0.5 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 23. In Figures 28 and 29, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

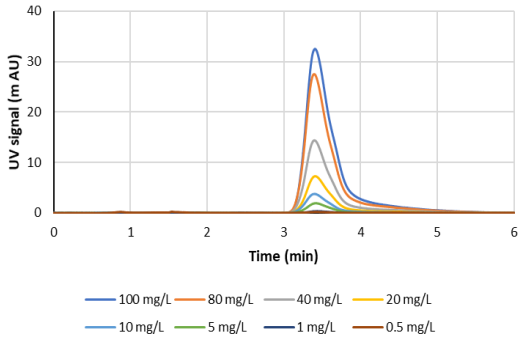

Figure 28. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 80MET:20W.

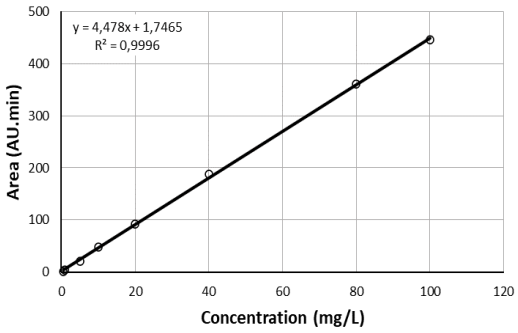

Figure 29. Estradiol calibration curve for the linearity range between 0.5 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 80% MET and 20% W.

Table 23. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 0.5 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
0.5	1791	1723	1755	1756.3	34.0	1.94
1	4139	4162	4125	4142.0	18.7	0.45
5	21676	21376	21619	21557.0	159.3	0.74
10	48058	48315	48290	48221.0	141.7	0.29
20	92884	92245	92393	92507.3	334.5	0.36
40	187653	187538	187246	187479.0	209.8	0.11
80	361560	361221	361313	361364.7	175.3	0.05
100	445521	445824	445296	445547.0	265.0	0.06

4.1.1.2.3 - MOBILE PHASE 9

The HPLC system was conditioned with methanol and ultrapure water, 70% and 30% respectively, with a flow rate of 1 mL/min for 1 hour. After stabilizing the baseline, a total system pressure of approximately 159 atm was observed. Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 8.0 minutes, with estradiol's retention time of 5.64 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, 0.5 and 0.25 mg/L were analyzed in triplicates each. The analyzes stopped at a concentration of 0.25 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5, 1 and 0.5 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 24. In Figures 30 and 31, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

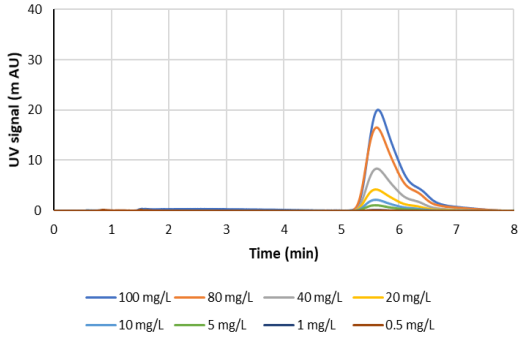

Figure 30. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 70MET:30W.

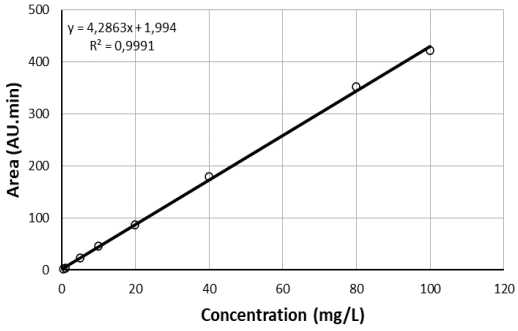

Figure 31. Estradiol calibration curve for the linearity range between 0.5 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 70% MET and 30% W.

Table 24. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 0.5 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
0.5	1535	1582	1504	1540.3	39.3	2.55
1	3523	3581	3524	3242.7	255.2	7.87
5	23256	23899	23523	23559.3	323.0	1.37
10	46012	46207	46185	46134.7	106.8	0.23
20	86387	86197	86078	86220.7	155.9	0.18
40	179921	179667	179421	179669.7	250.0	0.14
80	352447	352199	352289	352311.7	125.5	0.04
100	422309	422682	422242	422411.0	327.1	0.06

When analyzing the chromatographic curves for the stock solution of E2 with a concentration of 100 mg/L (Figure 32), injected using the three types of mobile phase mentioned above (100M, 80M:20W and 70M:30W), as well as when comparing their respective areas (Table 25), it is possible to conclude that the mobile phase composed of 100% methanol, is the mobile phase that has lower retention time and higher area values. Mobile phases that allow a lower retention time, imply less analysis time and consequently less use of solution, therefore optimizing the process.

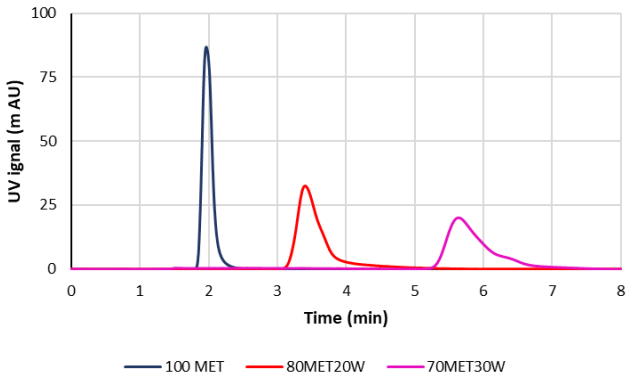

Figure 32. Chromatograms (overlapping) obtained by HPLC-UV relative to the estradiol stock solution with a concentration of 100 mg/L injected using mobile phases 100M, 80M:20W and 70M:30W.

Table 25. Average experimental areas and their respective retention times for the estradiol stock solution with concentration of 100 mg/L in mobile phases of 100MET, 80MET:20W and 70MET:30W.

Mobile Phase	Average area (mAU.min)	Retention Time (min)	LOQ (mg/L)
100MET	476068.7	1.97	0.5
80MET:20W	445547.0	3.40	0.5
70MET:30W	422411.0	5.64	0.5

To verify the possibility of optimizing the conditions for the mobile phase of 100% methanol, trifluoroacetic acid (TFA) was added to decrease the pH, in order to test whether the addition of TFA would significantly alter the area values obtained, LOQ and retention time.

4.1.1.2.4 - MOBILE PHASE 10

The HPLC system was conditioned with methanol 100% and 0.005% of TF, with a flow rate of 1 mL/min for 1 hour. After stabilizing the baseline, a total system pressure of approximately 60 atm was observed. Then, successive analyzes of the standard estradiol solution were performed with a concentration of 100 mg/L in methanol until the chromatographic peak of estradiol in three successive analyzes was overlapping. The time required for this analysis is approximately 3.0 minutes, with estradiol's retention time of 1.95 min.

After stabilization of the chromatographic column, concentrations of 100, 80, 40, 20, 10, 5, 1, 0.5 and 0.25 mg/L were analyzed in triplicates each. The analyzes stopped at a concentration of 0.25 mg/L, since with that concentration and using a signal/noise ratio of 2 (Y/N = 2), chromatographic integration is no longer possible. With the obtained experimental areas, a calibration curve was constructed using the standards with concentrations of 100, 80, 40, 20, 10, 5, 1 and 0.5 mg/L. The experimental data obtained, as well as their statistical treatment, are shown in Table 26. In Figures 33 and 34, is presented the chromatograms obtained and used in the construction of the calibration curve with the present mobile phase, and its respective calibration curve.

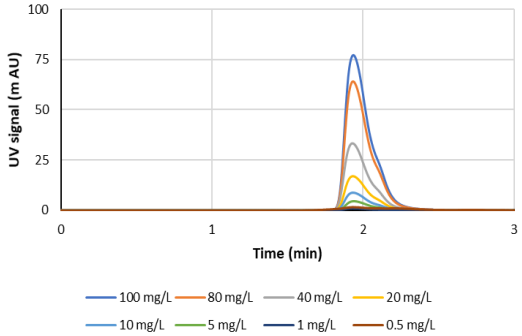

Figure 33. Chromatograms (overlapping) obtained by HPLC-UV considering the standards used for the construction of the calibration curve, in 100MET and 0.005TFA.

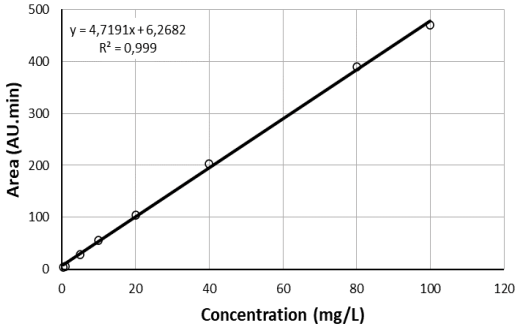

Figure 34. Estradiol calibration curve for the linearity range between 0.5 and 100 mg/L, obtained using an HPLC-UV system, with mobile phase of 100% MET and 0.005% TFA.

Table 26. Experimental results obtained and respective statistical treatment related to the estradiol calibration curve obtained by HPLC-UV for a concentration range between 0.5 and 100 mg/L.

Concentration (mg/L)	Area 1 (mAU.min)	Area 2 (mAU.min)	Area 3 (mAU.min)	Average (mAU.min)	Standard deviation (S, mAU.min)	Coefficient of Variation (CV, %)
0.5	4019	4272	4112	4134.3	128.0	3.10
1	6470	6287	6324	6360.3	96.8	1.52
5	28114	28153	28579	28282.0	257.9	0.91
10	55204	55384	55285	55291.0	90.1	0.16
20	104813	104035	104778	104542.0	439.4	0.42
40	202612	202834	202612	202722.7	111.0	0.05
80	389644	388425	389949	389672.7	263.2	0.07
100	469167	469766	469808	469580.3	358.6	0.08

4.1.1.3 - DISCUSSION

After analyzing the chromatographic curves for the estradiol stock solution with a concentration of 100 mg/L (Figure 35), injected using the two types of mobile phase, 100% methanol with and without TFA, as well as when comparing their respective areas (Table 27), it is possible to conclude that there is no significant variation in the retention time when adding TFA to the composition of the mobile phase 100% methanol, in addition to a decrease in the value referring to the average area. On that account, the addition of TFA does not prove to be advantageous. Considering all the mobile phase compositions used, the 100% methanol composition showed the best results (Figure 36).

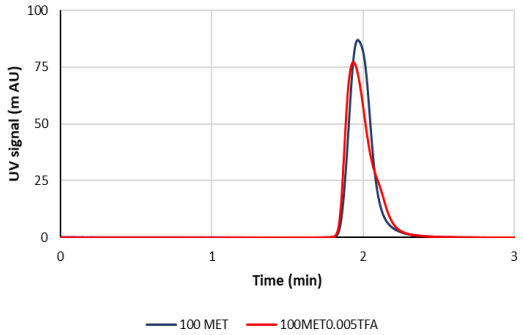

Figure 35. Chromatograms (overlapping) obtained by HPLC-UV relative to the estradiol stock solution with a concentration of 100 mg/L injected using mobile phases 100MET and 100MET:0.005TFA.

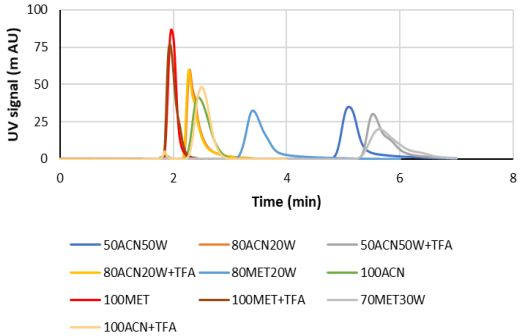

Figure 36. HPLC-UV chromatographic pulses of a E2 standard solution (100 mg/L) injected in 10 different mobile phase combinations.

Table 27. Average experimental areas and their respective retention times for the estradiol stock solution with concentration of 100 mg/L in mobile phases of 100MET and 100MET:0.005TFA.

Mobile Phase	Average area (m AU.min)	Retention Time (min)	LOQ (mg/L)
100MET	476068.7	1.97	0.5
100MET:0.005TFA	469580.3	1.95	0.5

4.1.1.4 - VALIDATION OF THE HPLC-UV METHODOLOGY OPTMIZED

To calculate the linearity of the parameters optimized, a linear equation for the stock and standard solution of E2 were made, considering at least 7 concentration levels injected, in triplicates, in the HPLC-UV system with mobile phase combinations stated prior. Linearity results are presented in Table 28.

All calibration curves for all mobile phase compositions proven to be linear, considering that all correlation coefficients (R^2) were higher than 0.99, proving that there is a strong correlation between the area of the chromatographic peak and the concentration of the standard solutions of E2 injected.

When compared with the literature gathered, the retention times obtained are significantly lower. Considering that one of the important objectives established in the beginning of the process of optimization, was the reduced amount of solvent necessary to perform the analysis, so, the results attended that specific objective.

The achieved limits of detection and limits of quantification, on the other hand, were higher from the ones observed in compared studies. The main reasons for that fact can be attributed to the pH value of the mobile phase, as shown previously, different pH values have influence in peak sizes, or different columns used in the studies.

With the analytical methodology optimized for the solid phase extraction, there is a pre concentration, the samples are concentrated 2000 times from their initial concentration. Then, the higher LOD achieved will not interfere with the results.

Table 28. Treatment of values obtained by HPLC-UV analysis.

MOBILE PHASE	N	Range (mg/L)	Linear Regression		pH	Retention Time (min)	LOD (mg/L)	LOQ (mg/L)	Calibration Curve	CV (%) mean
			$a \pm t.S_a$	$b \pm t.S_b$						
1-50ACN:50W	8	0.5 - 100	1697 ± 25256	4309 ± 82	7.55	5.13	0.165	0.5	Y=4.3023x+2.7715 R^2=0.9992	0.39
2-80ACN:20W	7	1 - 100	2585 ± 34924	4482 ± 111	5.85	2.26	0.33	1	Y=4.4602x+5.2169 R^2=0.9987	0.29
3-100ACN	7	1 - 100	2335 ± 38911	4717 ± 121	5.19	2.45	0.33	1	Y=4.698x+4.7129 R^2=0.9985	0.38
4-50ACN:50W:0.02TFA	8	0.5 - 100	2255 ± 31215	4226 ± 103	2.49	5.52	0.165	0.5	Y=4.2164x+3.6829 R^2=0.9988	0.60
5-80ACN:20W:0.02TFA	8	0.5 - 100	2782 ± 41400	4468 ± 132	2.05	2.27	0.165	0.5	Y=4.456x+4.5423 R^2=0.9982	0.38
6-100ACN:0.005TFA	7	1 - 100	2636 ± 32345	4744 ± 100	1.68	2.50	0.33	1	Y=4.7217x+5.3198 R^2=0.9991	0.18
7-100MET	8	0.5 - 100	3180 ± 35458	4821 ± 109	5.8	1.97	0.165	0.5	Y=4.8076x+5.1934 R^2=0.999	0.71
8-80M:20W	8	0.5 - 100	1069 ± 19023	4482 ± 61	6.1	3.40	0.165	0.5	Y=4.478x+1.7465 R^2=0.9996	0.50
9-70M:30W	8	0.5 - 100	1180 ± 27513	4292 ± 90	5.4	5.64	0.165	0.5	Y=4.2872x+1.9275 R^2=0.999	0.69
10-100M:0.005TFA	8	0.5 - 100	3838 ± 34922	4735 ± 108	2.0	1.95	0.165	0.5	Y=4.7191x+6.2682 R^2=0.999	0.84

As to the precision parameter, intermediate precision, regarding the mobile phase chosen of 100% methanol, Table 29 presents the coefficients of variation obtained. CV values obtained are < 1%.

Table 29. Intermediate precision (%) for mobile phase 100% methanol.

INTERMEDIATE PRECISION (%)		
CV 1	**CV 2**	**CV 3**
0.71	0.71	0.99

4.1.2 - SOLID PHASE EXTRACTION OPERATING CONDITIONS

The main objective of this step is to analyze between the 27 SPE experiments made, which one will present a higher surface response, consequently a higher peak area for an estradiol solution.

All SPE experiments were executed following the guidelines described in section 3.2.4. It is considered that the maximum chromatographic area is obtained with a 500 mL sample with a concentration of 1 μg/L, then, for the chromatographic areas obtained from samples with volumes of 1000 and 1500 mL, the value of these areas were divided by 2 and 3 respectively, so that it is possible to compare the area values obtained. The chromatographic curves also follow the same methodology and were divided by 2 and 3 when necessary, so that the comparison between graphics was possible.

Figures 37-63 presents the chromatographic curves obtained by injecting the final sample of 0.5 mL from each SPE experiment in the HPLC-UV system and Table 30 presents their respective peak areas.

Table 30. Description of the 27 SPE experiments generated by Design-Expert 11 software.

RUN	SAMPLE VOLUME (mL)	SAMPLE pH	ADSORBENT DRYING TIME (MIN)	SOLVENT COMPOSITION IN WASHING STEP (%)	SURFACE RESPONSE - CHROMATOGRAPHIC AREA (m AU.min)
1	1000	8	35	5	32331
2	500	2	10	0	68393
3	500	5	35	5	44964
4	1000	5	10	5	25822
5	500	8	10	10	70243
6	1500	2	10	0	76397
7	1500	8	60	10	17143
8	1000	2	35	5	273025
9	500	8	60	10	53560
10	1000	5	35	0	38220
11	1500	8	10	10	44295
12	1000	5	35	5	27113
13	1500	8	10	0	47199
14	1500	2	10	10	131986
15	1500	2	60	10	87068
16	1000	5	35	5	38670
17	1000	5	35	5	39134
18	500	8	10	0	70553
19	500	8	60	0	47715
20	500	2	60	10	343387
21	1000	5	35	10	19780
22	1000	5	60	5	28636
23	1500	5	35	5	17237
24	500	2	10	10	88150
25	500	2	60	0	291333
26	1500	2	60	0	76215
27	1500	8	60	0	26007

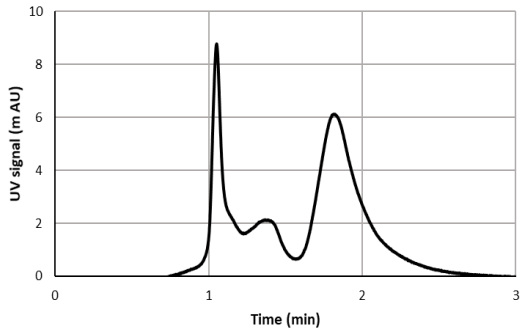

Figure 37. Chromatogram obtained by RUN 2.

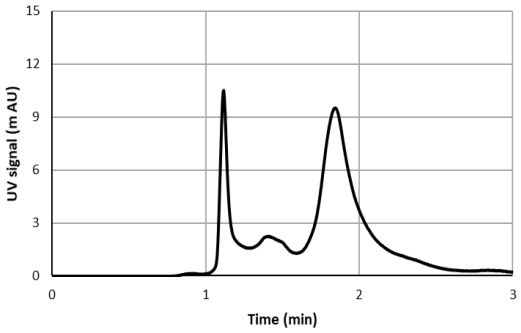

Figure 38. Chromatogram obtained by RUN 24.

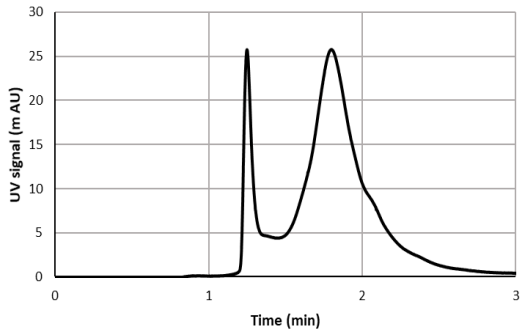

Figure 39. Chromatogram obtained by RUN 20.

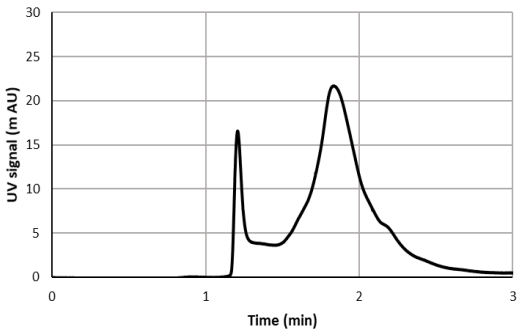

Figure 40. Chromatogram obtained by RUN 25.

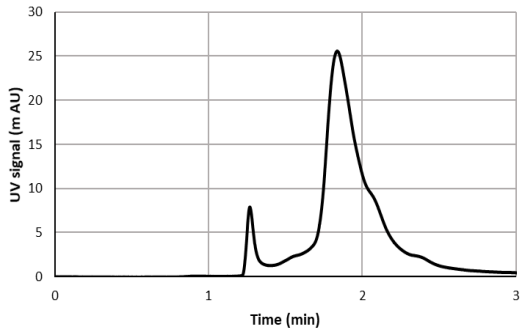

Figure 41. Chromatogram obtained by RUN 8.

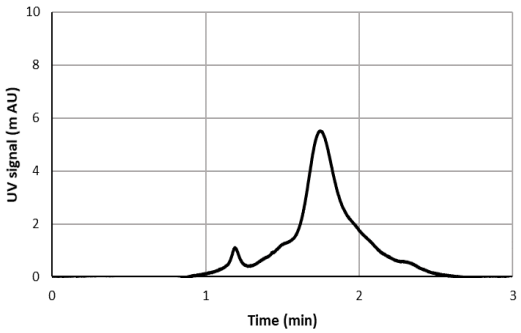

Figure 42. . Chromatogram obtained by RUN 5.

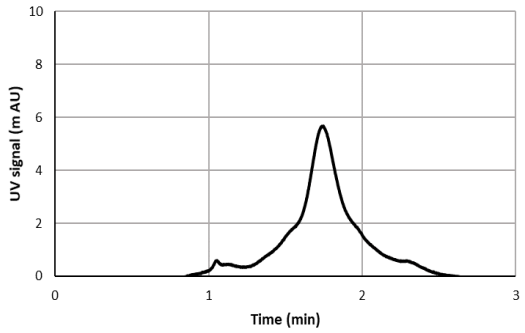

Figure 43. Chromatogram obtained by RUN 18.

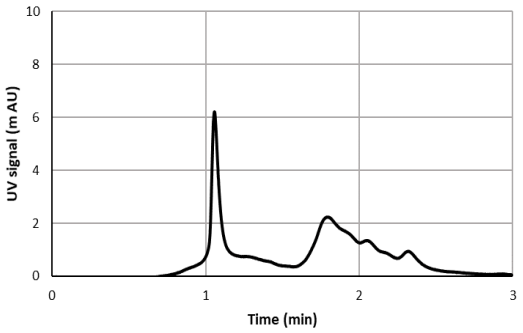

Figure 44. Chromatogram obtained by RUN 1.

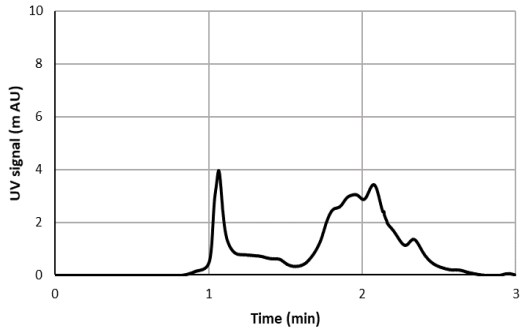

Figure 45. Chromatogram obtained by RUN 11.

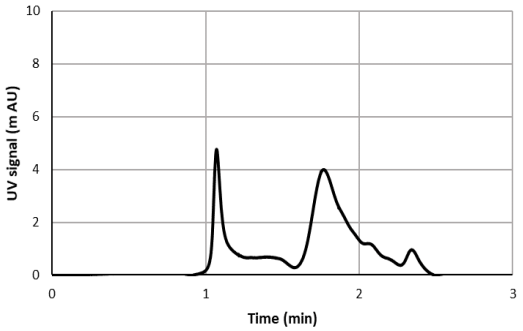

Figure 46. Chromatogram obtained by RUN 13.

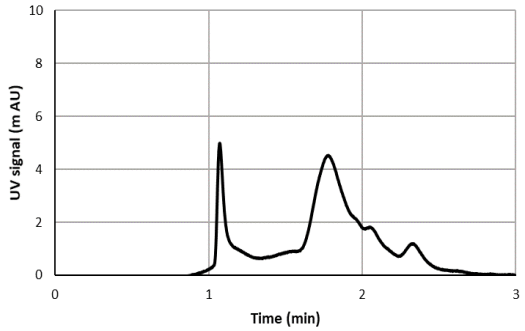

Figure 47. Chromatogram obtained by RUN 9.

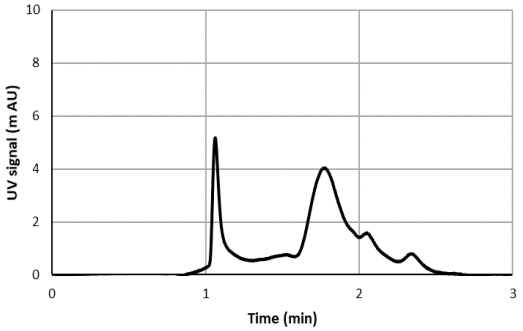

Figure 48. Chromatogram obtained by RUN 19.

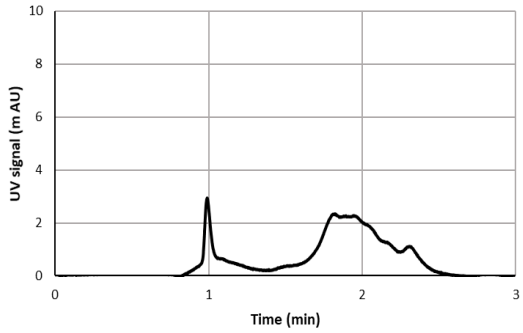

Figure 49. Chromatogram obtained by RUN 3.

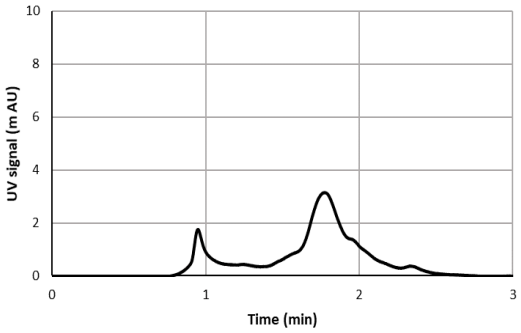

Figure 50. Chromatogram obtained by RUN 10.

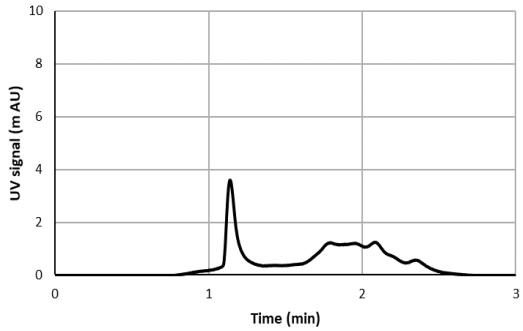

Figure 51. Chromatogram obtained by RUN 12.

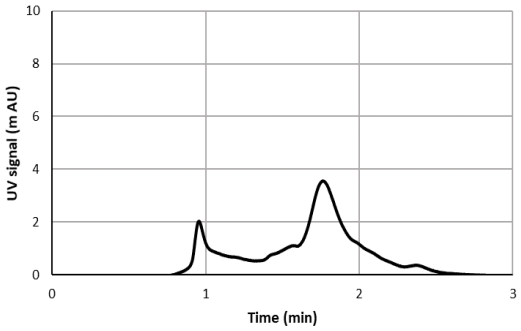

Figure 52. Chromatogram obtained by RUN 16.

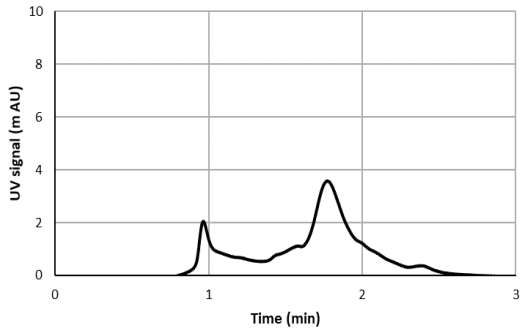

Figure 53. Chromatogram obtained by RUN 17.

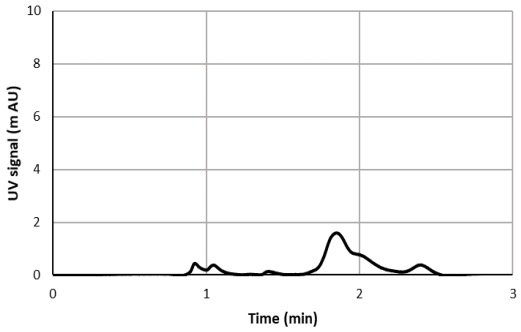

Figure 54. Chromatogram obtained by RUN 21.

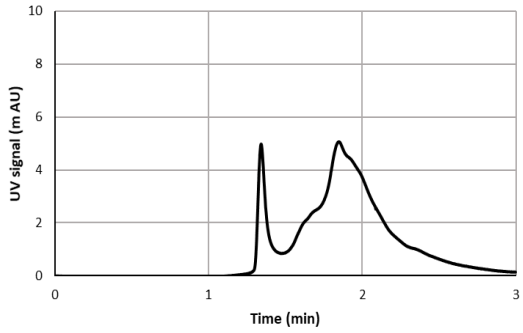

Figure 55. Chromatogram obtained by RUN 6.

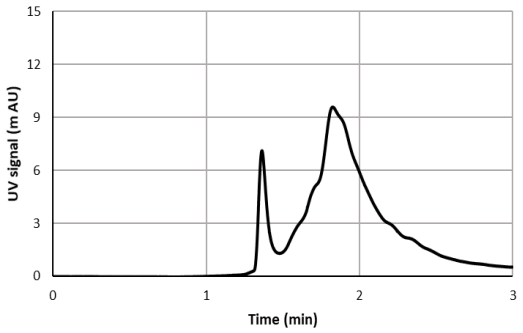

Figure 56. Chromatogram obtained by RUN 14.

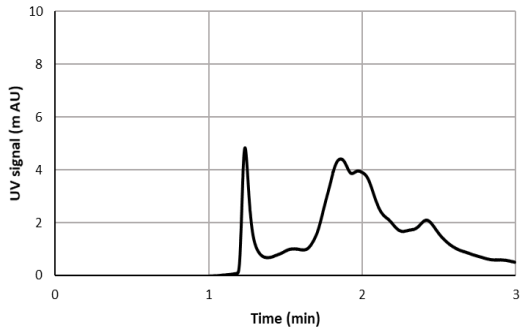

Figure 57. Chromatogram obtained by RUN 26.

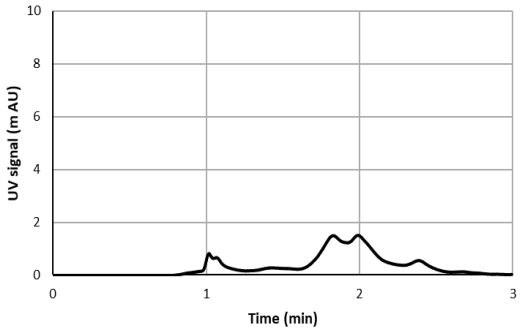

Figure 58. Chromatogram obtained by RUN 27.

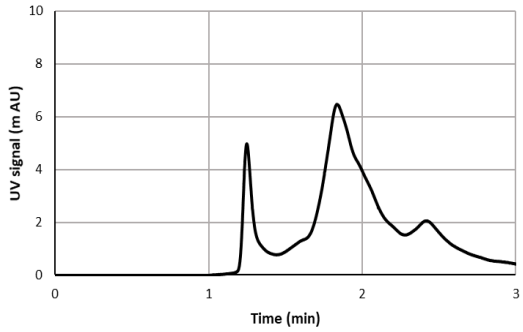

Figure 59. Chromatogram obtained by RUN 15.

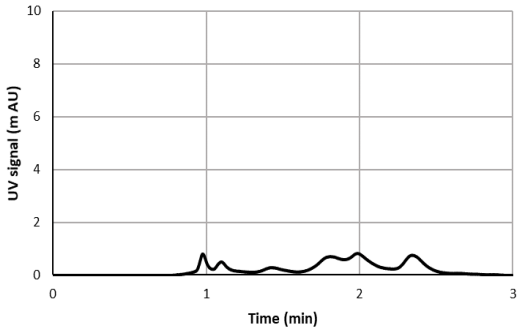

Figure 60. Chromatogram obtained by RUN 7.

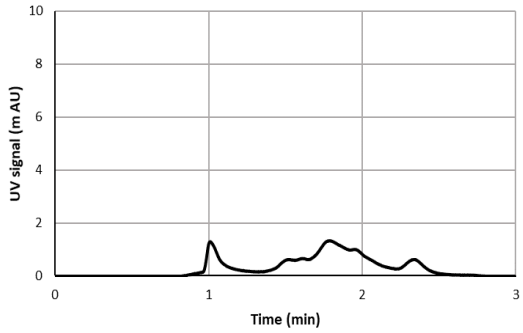

Figure 61. Chromatogram obtained by RUN 4.

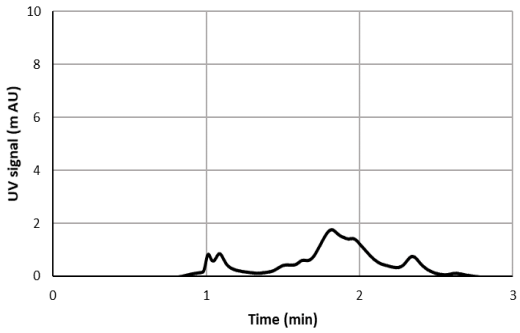

Figure 62. Chromatogram obtained by RUN 22.

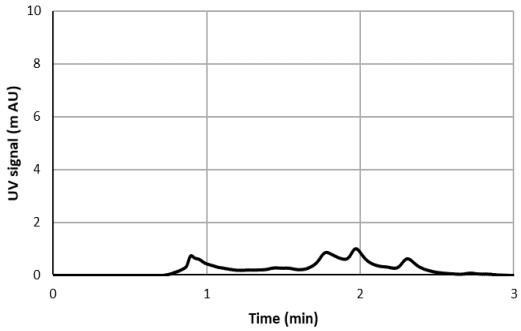

Figure 63. Chromatogram obtained by RUN23.

It was conducted, simultaneously, experiments to possibly identify the origin of the chromatographic peaks observed in retention times prior to 1.5 min. These experiments and its results are presented in Appendix A.

4.1.2.1 - DISCUSSION

After analyzing the statistical processing of data regarding the surface response for each of the 27 SPE experiments made, it was observed that four experiments presented a higher chromatographic area value (Run 8, 20, 25 and 14) and they were compared (Figure 64). One parameter in common between them is the pH value of 2.

Experiments with higher peak areas, are consequently the experiments that present a higher recovery of E2. By observing the perturbation graphic generated by the software

Design-Expert 11, presented in Figure 65, it is possible to state that pH value is the parameter that has bigger influence on the recovery of E2 and the pH value of 2 allows a bigger response. Figures 66-71 present the surface graphic relating all four parameters.

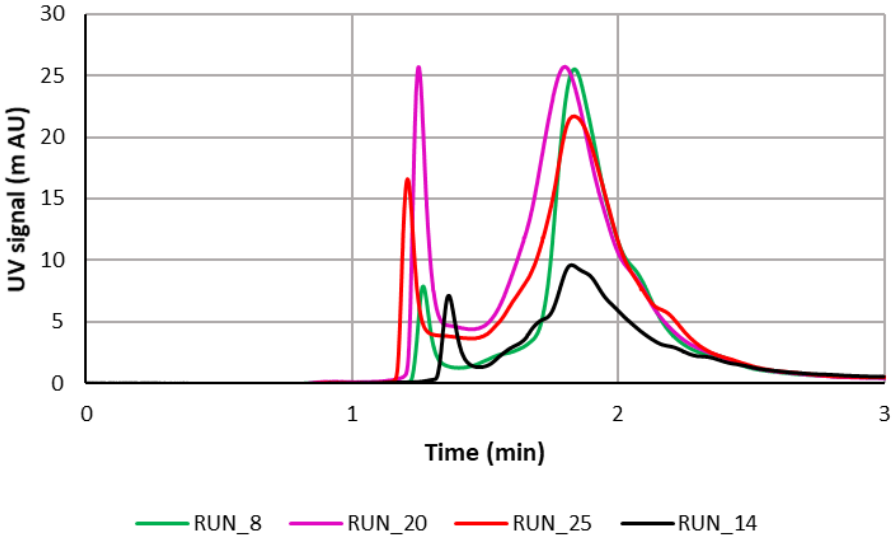

Figure 64. Chromatographic curves of experiments Run 8, 20, 25 and 14.

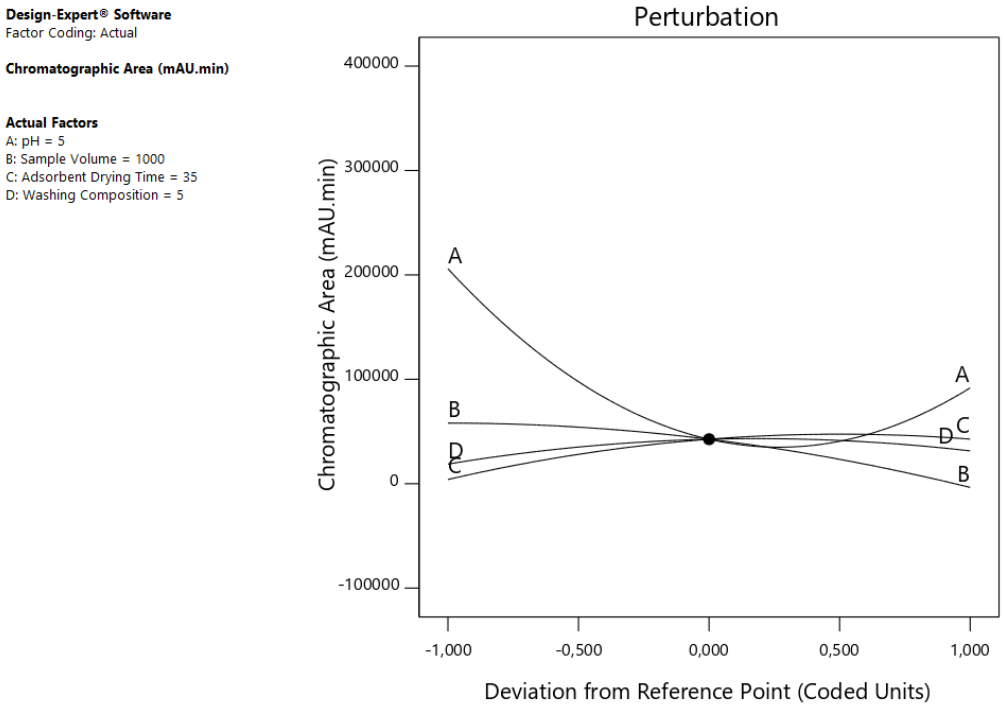

Figure 65. Perturbation graphic with the influence that all four parameters have in the surface response.

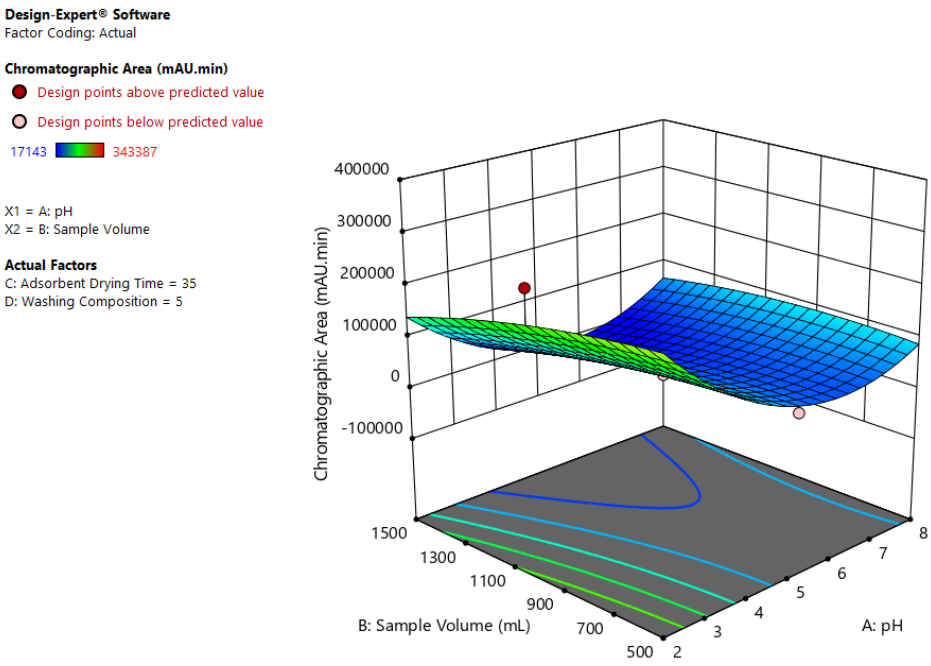

Figure 66. Surface response graphic relating parameters sample volume and pH value.

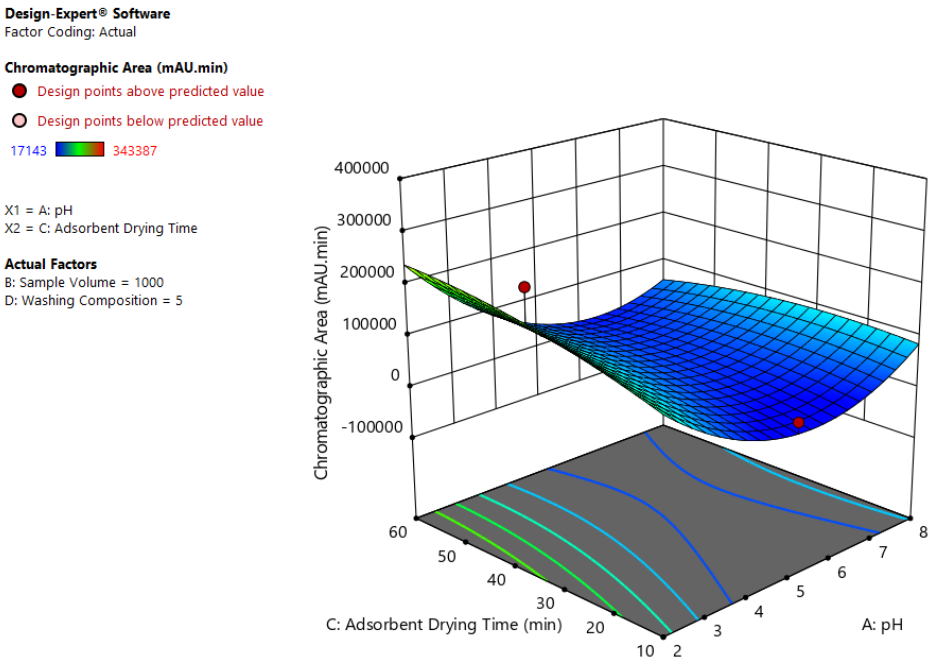

Figure 67. Surface response graphic relating parameters sample adsorbent drying time and pH value.

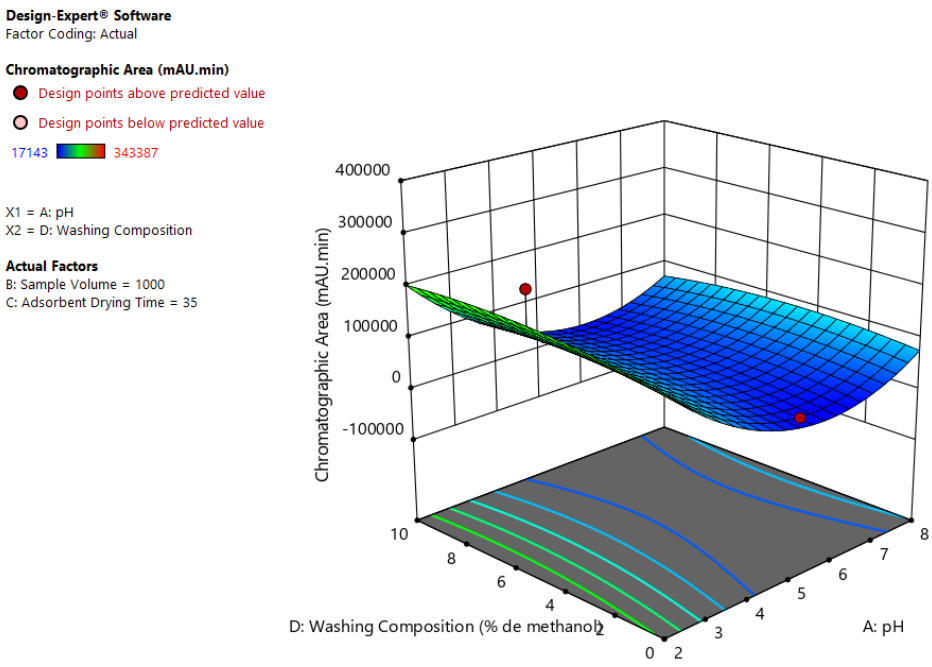

Figure 68. Surface response graphic relating parameters washing composition and pH value.

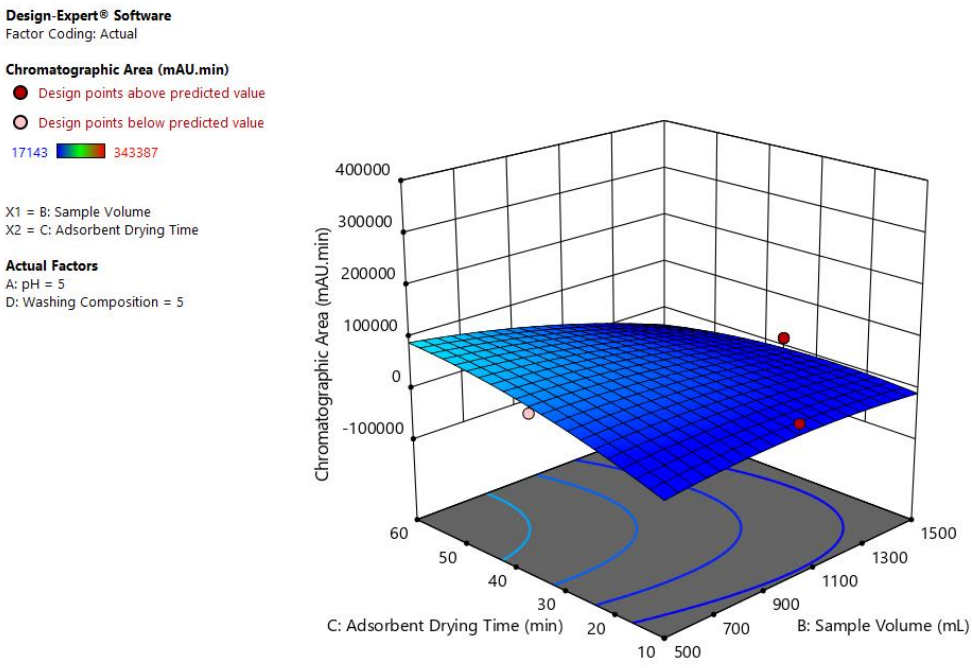

Figure 69. Surface response graphic relating parameters adsorbent drying time and sample volume.

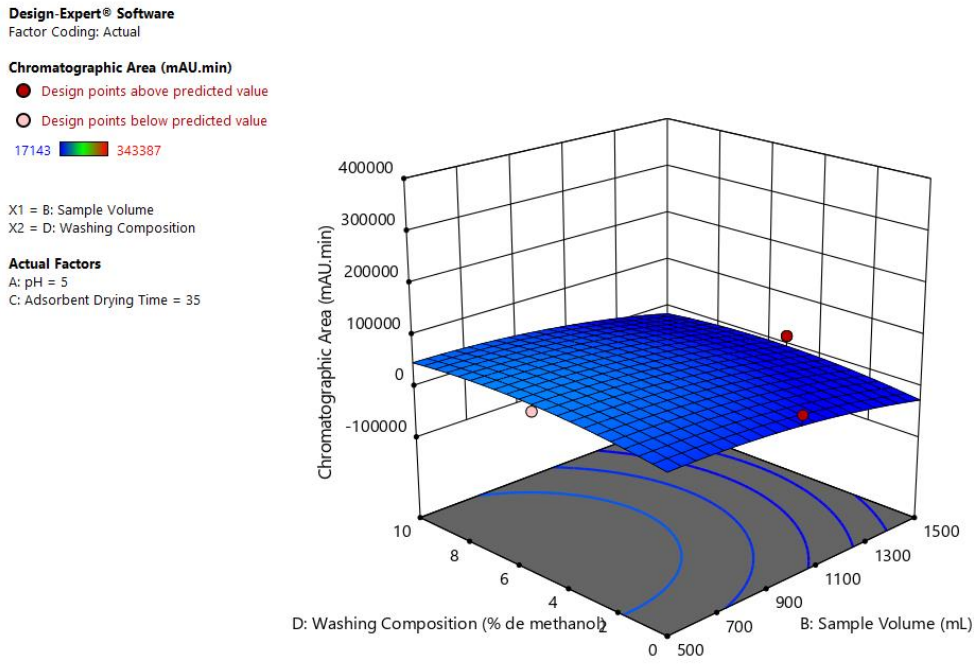

Figure 70. Surface response graphic relating parameters washing composition and sample volume.

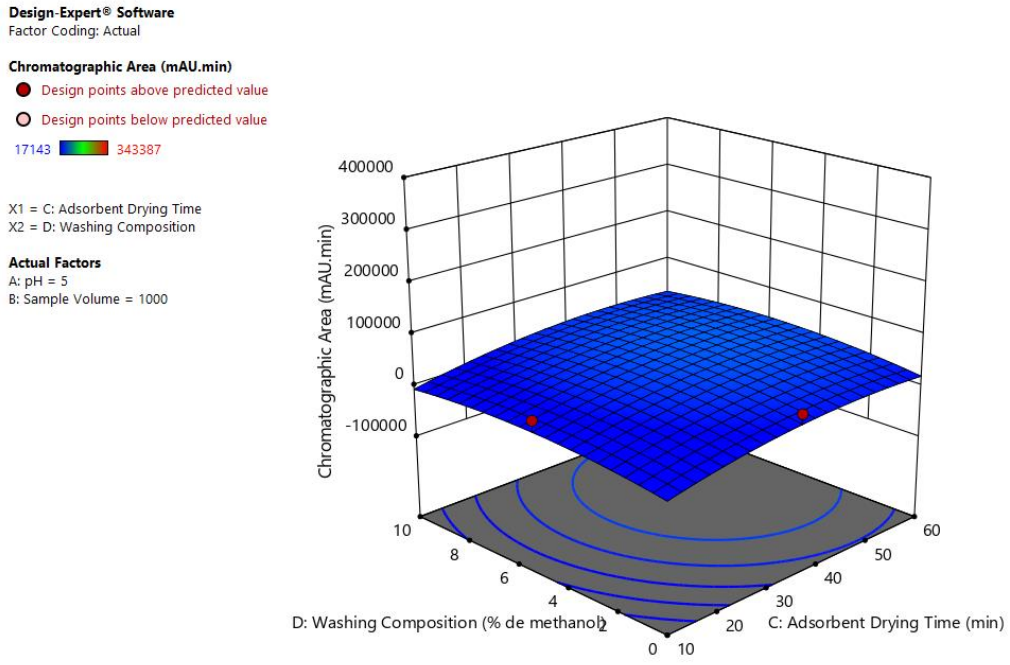

Figure 71. Surface response graphic relating parameters washing composition and adsorbent drying time.

The software used to gather the data of all 27 SPE experiments was the Design-Expert 11, and the quadratic equation obtained by the software relating all four parameters studied is presented next, in equation (5).

$$Y = 42648.93 - 57050.44\,A - 30819.5\,B + 19334.78\,C + 6310\,D + 1.06E{+}05\,A^2 - 15386.72\,B^2 - 19258.22\,C^2 - 17487.22\,D^2 + 19510.63\,AB - 32558.87\,AC - 9030.37\,AD - 33256.25\,BC - 1417\,BD + 765.25\,CD \quad (5)$$

Where A is the pH value parameter, B sample volume, C adsorbent drying time and D washing composition.

Table 31 presents the Analysis of Variance (ANOVA) of the model. The Model F-value of 4,83 implies the model is significant. There is only a 0,48% chance that an F-value this large could occur due to noise. P-values less than 0,0500 indicate model terms are significant, the P-value for the model studied was of 0.0048, meaning that the model is significant.

Table 31. Analysis of Variance – ANOVA for the quadratic model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1,613E+11	14	1,152E+10	4,83	0,0048	significant
A-pH	**5,859E+10**	**1**	**5,859E+10**	**24,55**	**0,0003**	
B-Sample Volume	1,710E+10	1	1,710E+10	7,16	0,0202	
C-Adsorbent Drying Time	6,729E+09	1	6,729E+09	2,82	0,1190	
D-Washing Composition	7,167E+08	1	7,167E+08	0,3003	0,5937	
AB	6,091E+09	1	6,091E+09	2,55	0,1361	
AC	1,696E+10	1	1,696E+10	7,11	0,0206	
AD	1,305E+09	1	1,305E+09	0,5467	0,4739	
BC	1,770E+10	1	1,770E+10	7,41	0,0185	
BD	3,213E+07	1	3,213E+07	0,0135	0,9096	
CD	9,370E+06	1	9,370E+06	0,0039	0,9511	
A^2	2,900E+10	1	2,900E+10	12,15	0,0045	
B^2	6,088E+08	1	6,088E+08	0,2551	0,6227	
C^2	9,537E+08	1	9,537E+08	0,3996	0,5391	
D^2	7,864E+08	1	7,864E+08	0,3295	0,5766	

The effect of low pH on the recovery efficiency of the SPE process was significant and in line with results presented in literature gathered. In lower pH values the interaction between E2 and the stationary phase of the cartridge is increased, allowing a better selective retaining and improving the desorption of E2 [46].

As intended, a number of experiments stood out, presenting a higher response (Figure 64). Run 20 is the experiment with the largest chromatographic area, whose parameters were chosen to be executed in the implementation of the methodology in testing samples from a WWTP. With those parameters being: pH 2, sample volume 500 mL, adsorbent drying time 60 min and 10% of methanol in washing step composition.

4.1.2.2 - VALIDATION OF THE SPE METHODOLOGY OPTMIZED

Once the parameters were defined, them being pH value of 2, sample volume of 500 mL, cartridge drying time of 60 min and 10% of methanol in the washing step, they were validated in two main steps, the first one was to analyze the repeatability and the intermediate precision of the experiment. The second one was to calculate the recovery of E2. The recovery was tested in a sample with non-optimal conditions, in order to evaluate the efficiency of the method with real external interferences, the sample chosen was the effluent completely treated from a WWTP.

First, the procedure Run 20 were performed in triplicate, in two more distinct days. Table 32 presents the peak areas from each of those procedures and their respective CV(%).

Table 32. Run 20 peak areas and its respective CV(%).

SPE DAY	Area 1 (m AU.min)	Area 2 (m AU.min)	Area 3 (m AU.min)	Mean Area (m AU.min)	CV (%)
1	343325	343989	342847	343387	0.17
2	256986	257433	256487	256969	0.18
3	231003	233128	232566	232232	0.47

Table 33. Run 20 intermediate precision and its respective CV(%).

Mean Area 1 (m AU.min)	Mean Area 2 (m AU.min)	Mean Area 3 (m AU.min)	CV (%)
343387	256969	232232	21.03

The CV(%) value is related to the concentration level of the substance in study, in that regard, for solutions with a concentration of 1μg/L (1 ppb), the coefficient of variation allowed is inferior to 45%. Considering that the CV obtained between intermediate precision is 21.03%, the parameters of the experiment Run 20 is satisfactory [70].

Regarding recuperation, this factor was tested using a sample from the WWTP treated effluent. First, it was injected the sample without any prior treatment in the HPLC-UV system, with operating conditions optimized, to verify if there is the presence of E2, and it was verified there is. The chromatographic peak is presented in Figure 72 and its area is 13259 (m AU.min).

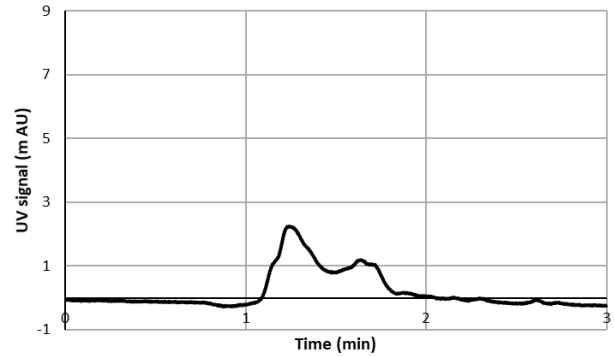

Figure 72. Chromatographic curve of the WWTP treated effluent injected in the HPLC-UV system.

Next, it was prepared three 500 mL of the WWTP treated effluent sample following the guidelines of SPE experiment Run 20. The samples were prepared with a concentration of 10 mg/L and the SPE were made in three distinct days. Then, the solutions from the elution step were injected in the HPLC-UV system in triplicates. Table 34 presents the areas obtained and Table 35 the intermediate precision of the experiment.

Table 34. Area peaks and its respective CV(%) of SPE procedures with 10 mg/L samples.

SPE DAY	Area 1 (m AU.min)	Area 2 (m AU.min)	Area 3 (m AU.min)	Mean Area (m AU.min)	CV (%)
1	1928120	1930843	1926109	1928357	0.12
2	1974887	1979029	1987904	1980607	0.34
3	1983557	1982416	1984022	1983332	0.04

Table 35. 10 mg/L SPE procedure intermediate precision and its respective CV(%).

Mean Area 1 (m AU.min)	Mean Area 2 (m AU.min)	Mean Area 3 (m AU.min)	CV (%)
1928357	1980607	1983332	1.58

For solutions with a concentration of 10 mg/L (10 ppm), the coefficient of variation allowed is inferior to 11%. Considering that the CV obtained between intermediate precision is 1.58%, the parameters of the experiment Run 20 is satisfactory [70].

The recovery was determined using the calibration curve made with HPLC-UV mobile phase of 100% methanol, discounting the peak area of E2 (Figure 72) and it is presented in Table 36.

Table 36. Mean recovery values presented for the SPE procedure.

SPE DAY	Recovery 1 (%)	Recovery 2 (%)	Recovery 3 (%)	Mean Recovery (%)	CV (%)
1	80.4	80.6	80.4	80.5	0.14
2	82.4	82.6	83.0	82.7	0.37
3	82.8	82.7	82.8	82.8	0.07

Table 37 Intermediate precision of the recovery process.

Mean Recovery 1(%)	Mean Recovery 2(%)	Mean Recovery 3(%)	CV (%)
80.5	82.7	82.8	1.59

As observed in Tables 36 and 37, all experiments had a recovery of at least 80%, which means that all of them are between the allowed recovery values (80 - 110%) considering the initial concentration of 10 mg/L of E2. Regarding the CV values, they are

under the 11% allowed, meaning that the parameters optimized admit a satisfactory SPE recovery procedure [70].

4.2 - SPE/HPLC-UV ANALYSIS OF THE WWTP SAMPLES

As mentioned previously, three samples from three different stages of a WWTP were collected. All samples were prepared accordingly to the SPE parameters already established and then, injected in triplicates in the HPLC-UV system with the optimized conditions. It was made three SPE experiments, in different days, for each sample.

Figures 73-75 presents the chromatographic curves from the three different WWTP collection points.

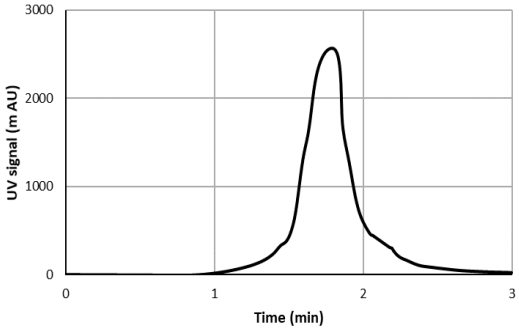

Figure 73. Chromatographic curve of the WWTP sample 1 (entrance).

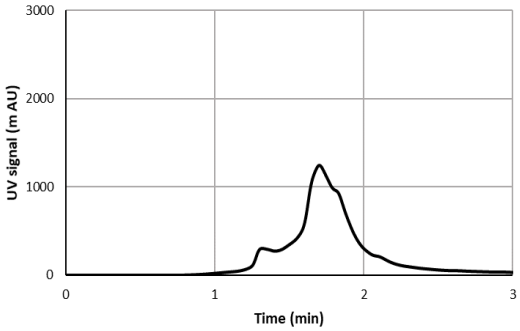

Figure 74. Chromatographic curve of the WWTP sample 2 (aeration tank).

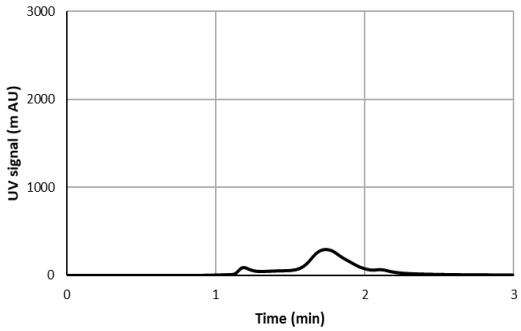

Figure 75. Chromatographic curve of the WWTP sample 3 (treated effluent).

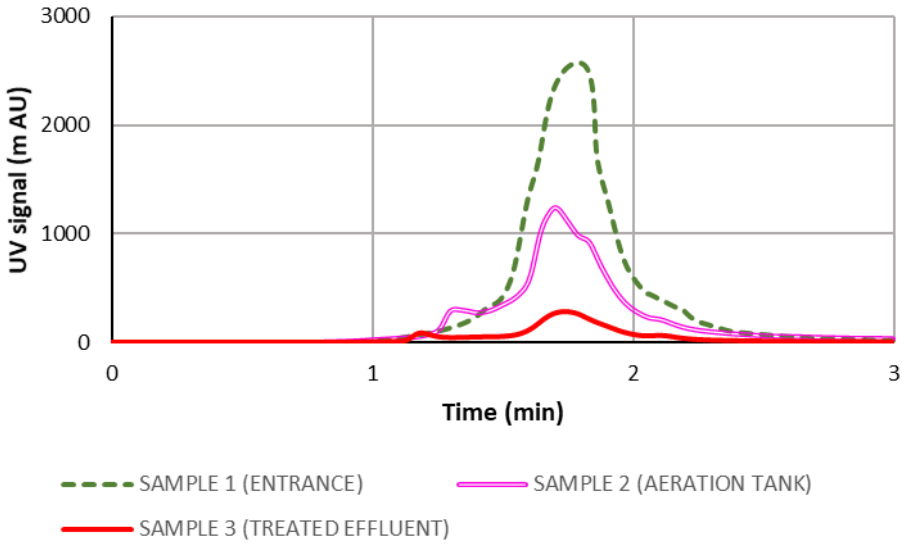

Figure 76. Chromatographic curves of all three WWTP samples overlapped.

It is possible to observe that E2 was detected in all three WWTP samples. Another fact that was noticed, is that the concentration starts higher with the WWTP entrance sample (sample 1), and it gets smaller throughout the collecting points. This analysis, allows to confirm that estrogens are degraded in the primary treatment by partitioning into fat, oil, or sorption into the primary tank, as stated in different literature, however, not completely, considering that primary treatment it is not designed to remove compounds such as estrogens and the fact that still, a considerable concentration of E2 was detected in sample 2 [22].

As expected, the biggest portion of the E2 is degraded after passing through the activated sludge aeration tank, which, in this point are removed by sorption and subsequently biodegraded by bacteria. The presence of bacteria will use estrogens as carbon source for metabolism, confirming that the biodegradation process in the aeration tank has an important role in estrogens removal. Yet, its removal efficiency, as previously mentioned, depends of the effluent retention time in the tank, a higher time implies in a higher removal. Also, the temperature has a direct influence. Considering the samples were collected in the first half of spring, lower temperatures were still registered in the WWTP

region. Those facts can contribute enormously for the detection of E2 in sample 3, the effluent completely treated [63, 22].

In addition to that, still, most WWTP do not have a treatment step focused on the removal of pollutants such hormones, pharmaceuticals or pesticides, justifying the detection of E2 in the treated effluent.

Table 38 presents the chromatographic areas and their respective concentrations. The concentration values presented in the table are already divided by 1000, considering that in the SPE procedure the analysis concentrates the sample 1000 times.

Table 38. Mean areas and its respective mean concentration with standard deviation for all three WWTP samples.

SAMPLE COLLECTING POINT	MEAN AREA (m AU.min)	MEAN CONCENTRATION (mg/L) ± SD
1	31454132	6.61 ± 0.54
2	11598249	2.44 ± 0.53
3	4241414	0.89 ± 0.18

As presented in Table 38, the E2 concentration found in three samples, are all measured in (mg/L), which was not anticipated considering that in the majority of literature gathered, it was found E2 in a large amount of aquatic matrices, but with concentrations such as ng/L or μg/L. Although, not as frequent, there are studies that detected and quantified E2 in rivers in a concentration of g/L [69].

A lot of factors can contribute to the elevated concentrations found in the samples, one of them being the profile of the city that the WWTP is inserted. The city is known for its low temperatures, and in addition to that, the demographic profile of the city indicates a possible influence on the consumption of keen pharmaceuticals such as estrogenic contraceptives.

5 - CONCLUSIONS AND FUTURE WORK

The main objective of the present study was to develop and to validate an experimental methodology to detect and quantify 17β-estradiol in WWTP samples. The analytical methodology developed it was proven adequate and in line with the aims of this work. The first step was to define the operating conditions of the HPLC-UV system and the mobile phase of 100% methanol proved to be the best one. Next, it was the optimization of the SPE parameters with 27 experiments done, which lead to Run 20, the experiment that allowed the highest surface response.

With the analytical methodology defined, the method was implemented by analyzing three WWTP samples. E2 was detected and quantified in all of the three samples, in a concentration higher than expected. Even though there are efforts to decrease the concentration of estrogens in the environment through wastewater treatment plants, E2 is still highly present in WWTP's effluent.

As suggestions of future work, it would be relevant to analyze samples from the same WWTP in all four seasons of the year, in order to verify the influence of the temperature in the removal efficiency of E2. Another suggestion is to identify the estrogenic compounds present in the samples resulted of the degradation of E2. Another important next step would be the development of a E2 removal methodology, using innovative resources as adsorbent materials.

REFERENCES

[1] VEGA-MORALES, T.; SOSA-FERRERA, Z.; SANTANA-RODRÍGUEZ, J. Evaluation of the Presence of Endocrine-Disrupting Compounds in Dissolved and Solid Wastewater Treatment Plant Samples of Gran Canaria Island (Spain). BioMed Research International, 1-15, 2013.

[2] M. Yang, M. S. Park, and H. S. Lee, "Endocrine disrupting chemicals: human exposure and health risks," Journal of Environmental Science and Health C, vol. 24, no. 2, pp. 183-224, 2006.

[3] RACZ, L. & GOEL, R.K. Fate and removal of estrogens in municipal wastewater. J. Environ. Monit., 12(1), 58-70, 2010.

[4] FONSECA, A.P.; CARDOSO, M.; ESTEVES, V. Determination of Estrogens in Raw and Treated Wastewater by HighPerformance Liquid Chromatography-Ultraviolet Detection. Journal of Environmental & Analytical Toxicology, [s. l.], 1-5, 2013.

[5] BELFROID, A.C.; VAN DER HORST, A.; VETHAAK, A.D.; SCHAFER, A.J.; RIJS, G.B.J.; WEGENER, J.; COFINO, W.P. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and wastewater in The Netherlands. The Science of the Total Environment, [S. l.], 101-108, 1999.

[6] Grassi, M., Kaykioglu, G., Belgiorno, V., & Lofrano, G. (2012). Removal of Emerging Contaminants from Water and Wastewater by Adsorption Process. Emerging Compounds Removal from Wastewater, 15-37.

[7] Teixeira, S., Delerue-Matos, C., & Santos, L. (2019). Application of experimental design methodology to optimize antibiotics removal by walnut shell based activated carbon. Science of The Total Environment, 646, 168-176.

[8] KUMAR, M.; BORAH, P.; DEVI, P. Priority and emerging pollutants in water. Inorganic Pollutants in Water, 33-49, 2020.

[9] GODOY, M. & SÁNCHEZ, J. Antibiotics as Emerging Pollutants in Water and Its Treatment. Antibiotic Materials in Healthcare, 221-230, 2020.

[10] GEISSEN, V.; MOL, H.; KLUMPP, E.; UMLAUF, G.; NADAL, M.; VAN DER PLOEG, M.; VAN DE ZEE, S.; RITSEMA, C. Emerging pollutants in the environment: A challenge for water resource management. International Soil and Water Conservation Research, [S. l.], 57-65, 2015.

[11] GAVRILESCU, M.; DEMNEROVÁ, K.; AAMAND, J.; AGATHOS, S.; FAVA, F. Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. New Biotechnology, 32(1), 147-156, 2015.

[12] LIBRALATO, G.; VOLPI G.; AVEZZÙ, F. To centralise or to decentralise: An overview of the most recent trends in wastewater treatment management. Journal of Environmental Management, 94(1), 61-68, 2012.

[13] SCHUG, T.T.; JANESICK, A.; BLUMBERG, B.; HEINDEL, J. J. Endocrine disrupting chemicals and disease susceptibility. The Journal of Steroid Biochemistry and Molecular Biology, 127(3-5), 204-215, 2011.

[14] HILSCHEROVÁ, K.; DUSEK, L.; KANNAN, K.; HOLOUBEK, I. Evaluation of cytotoxicity, dioxin-like activity and estrogenicity of complex environmental mixtures. Central European journal of public health, [S. l.], 9-28, 2000.

[15] MAURÍCIO, R.; DIAS, R.; RIBEIRO, V.; FERNANDES, S.; VICENTE, A.C.; PINTO, M.I.; MANO, A.P. 17α-Ethinylestradiol and 17β-estradiol removal from a secondary urban wastewater using an RBC treatment system. Environmental Monitoring and Assessment, 190(6), 2018.

[16] Loos, R., Marinov, D., Sanseverino, I., Napierska, D. and Lettieri, T., Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List, EUR 29173 EN, Publications Office of the European Union, Luxembourg, 2018, ISBN 978-92-79-81838-7 (print),978-92-79-81839-4 (pdf), doi:10.2760/614367 (online).

[17] CAIS APARECIDA, Thayná. Determinação de hormônios estrogênios em águas superficiais do lago de furnas no município de alfenas-mg. 2016. tese (programa de pós graduação multicêntrico em química de minas gerais) - universidade federal de itajubá, [S. l.], 2016.

[18] LINTELMANN, J.; KATAYAMA, A.; KURIHARA, N.; SHORE, L.; WENZEL, A. Endocrine disruptors in the environment (IUPAC Technical Report). Pure and Applied Chemistry, 75(5), 631-681, 2003.

[19] HUBER, M. M. ET AL. Oxidation of Pharmaceuticals during Ozonation and Advanced Oxidation Processes. Environment Science Technology, v. 37, 1016-1024, 2003.

[20] Zhang, Z., Feng, Y., Gao, P., Wang, C., & Ren, N. (2011). Occurrence and removal efficiencies of eight EDCs and estrogenicity in a STP. Journal of Environmental Monitoring, 13, 1366.

[21] Huang, B., Li, X., Sun, W., Ren, D., Li, X., Li, X., Liu, Y., Li, Q., & Pan, X. (2014). Occurrence, removal, and fate of progestrogens, androgens, estrogens, and phenols in six sewage

treatment plants around Dianchi Lake in China. Environmental Science and Pollution Research, 21, 12898-12908.

[22] Ting, Y.F. & Praveena, S.M. Sources, mechanisms, and fate of steroid, estrogens in wastewater treatment plants: a mini review. Environmental Monitoring and Assessment, 189(4), 2017.

[23] Zeng, Q. L., Li, Y. M., Gu, G. W., Zhao, J. M., Zhang, C. J., & Luan, J. F. (2008). Sorption and biodegradation of 17β- estradiol by acclimated aerobic activated sludge and isolation of the bacterial strain. Environmental Engineering Science, 26(4), 783-790.

[24] Servos, M. R., Bennie, D. T., Burnison, B. K., Jurkovic, A., Mclnnis, R., Neheli, T., Schnell, A., Seto, P., Smyth, S. A., & Ternes, T. A. (2005). Distribution of estrogens, 17βestradiol and estrone, in Canadian municipal wastewater treatment plants. Science of the Total Environment, 336, 155-170.

[25] LEE, Y.M., OLESZKIEWICZ, J.A., CICEK, N.; LONDRY, K. Endocrine Disrupting Compounds (EDC) in Municipal Wastewater Treatment: A Need for Mass Balance. Environmental Technology, 25(6), 635-645, 2004.

[26] Lagana, A., Bacaloni, A., Leva, I., Faberi, A., Fago, G., & Marino, A. (2004). Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. Analytica Chimica Acta, 501, 79-88.

[27] Cui, C. W., Ji, S. L., & Ren, H. Y. (2006). Determination of steroid estrogens in wastewater treatment plant of a contraceptives producing factory. Environmental Monitoring and Assessment, 121, 409-419.

[28] Koh, Y. K. K., Chiu, T. Y., Boobis, A., Cartmell, E., Lester, J. N., & Scrimshaw, M. D. (2007). Determination of steroid estrogens in wastewater by high performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 1173, 81-87.

[29] Zhang, Z., Feng, Y., Gao, P., Wang, C., & Ren, N. (2011). Occurrence and removal efficiencies of eight EDCs and estrogenicity in a STP. Journal of Environmental Monitoring, 13, 1366.

[30] Mohagheghian, A., Nabizadeh, R., Mesdghinia, A., Rastkari, N., Mahvi, A. H., Alimohammadi, M., Yunesian, M., Ahmadkhaniha, R., & Nazmara, S. (2014). Distribution of estrogenic steroids in municipal wastewater treatment plants in Tehran, Iran. Journal of Environmental Health Science and Engineering, 12, 97.

[31] Chang, H., Wan, Y., Wu, S., Fan, Z., & Hu, J. (2011). Occurrence of androgens and progestrogens in wastewater treatment plants and receiving river waters: comparison to estrogens. Water Research, 45, 732-740.

[32] Surujlal-Naicker, S., & Bux, F. (2013). Application of radioimmunoassays to assess the fate of estrogen EDCs in full scale wastewater treatment plants. Journal of Environmental Science and Health, 48, 37-47.

[33] Pessoa, G., Souza, N. D., Vidal, C., Alves, J. A. C., Firmino, P. I. M., Nascimento, R. F., & Santos, A. B. D. (2014). Occurrence and removal of estrogens in Brazilian wastewater treatment plants. Science of the Total Environment, 490, 288- 295.

[34] Layton, A. C., Gregory, B. W., Seward, J. R., Schultz, T. W., & Sayler, G. S. (2000). Mineralization of steroidal hormones by biosolids in wastewater treatment systems in Tennessee U.S.A. Environmental Science and Technology, 34, 3925- 3931.

[35] Stackelberg, P. E., Furlong, E. T., Meyer, M. T., Zaugg, S. D., Henderson, A. K., & Reissman, D. B. (2004). Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. Science of The Total Environment, 329(1-3), 99-113.

[36] Stumpe, B., & Marschner, B. (2010). Dissolved organic carbon from sewage sludge and manure can affect estrogen sorption and mineralization in soils. Environmental Pollution, 158(1), 148-154.

[37] Bai, X., & Acharya, K. (2019). Removal of seven endocrine disrupting chemicals (EDCs) from municipal wastewater effluents by a freshwater green alga. Environmental Pollution, 247, 534-540.

[38] Bianchi, V. D. N., Araujo Silva, M. R. de, Lamim, M. A., Da Silva, C. L., & De Lima, E. C. (2017). Solid phase extraction using molecular imprinting polymers (MISPE) for the determination of estrogens in surface water by HPLC. Ambiente e Agua - An Interdisciplinary Journal of Applied Science, 12(3), 380.

[39] Maurício, Rita; JORGE, Joana; DIAS, Rita; P. NORONHA, João; AMARAL, Leonor. The use of peracetic acid for estrogen removal from urban wastewaters: E2 as a case study. Environ Monit Assess, [S. l.], p. 114-192, 9 jan. 2020.

[40] Ben, W., Zhu, B., Yuan, X., Zhang, Y., Yang, M., & Qiang, Z. (2017). Transformation and fate of natural estrogens and their conjugates in wastewater treatment plants: Influence of operational parameters and removal pathways. Water Research, 124, 244-250.

[41] Kibambe, M. G., Momba, M. N. B., Daso, A. P., Van Zijl, M. C., & Coetzee, M. A. A. (2020). Efficiency of selected wastewater treatment processes in removing estrogen compounds and reducing estrogenic activity using the T47D-KBLUC reporter gene assay. Journal of Environmental Management, 260, 110135.

[42] Verbinnen, R. T., Nunes, G. S., & Vieira, E. M. (2010). Determinação de hormônios estrógenos em água potável usando CLAE-DAD. Química Nova, 33(9), 1837-1842.

[43] De Liz, M. V., do Amaral, B., Stets, S., Nagata, N., & Peralta-Zamora, P. (2016). Sensitive Estrogens Determination in Wastewater Samples by HPLC and Fluorescence Detection. Journal of the Brazilian Chemical Society.

[44] Välitalo, P., Perkola, N., Seiler, T.-B., Sillanpää, M., Kuckelkorn, J., Mikola, A., … Schultz, E. (2016). Estrogenic activity in Finnish municipal wastewater effluents. Water Research, 88, 740-749.

[45] Brasil Bernardelli, J. K., Liz, M. V., Belli, T. J., Lobo-Recio, M. A., & Lapolli, F. R. (2015). Removal of estrogens by activated sludge under different conditions using batch experiments. Brazilian Journal of Chemical Engineering, 32(2), 421-432.

[46] Vallejo-rodríguez, Ramiro; Lopez-lopes, Alberto; Saldarriaga-Noreña, Hugo; Alfonso murillo tovar, Mario; Hernández-mena, Leonel. Optimization of Analytical Conditions to Determine Steroids and Pharmaceuticals Drugs in Water Samples Using Solid Phase-Extraction and HPLC. American Journal of Analytical Chemistry 02(08), [S. l.], p. 863-870, 3 jan. 2011.

[47] Fang, T. Y., Praveena, S. M., Aris, A. Z., Norkhadijah Syed Ismail, S., & Rasdi, I. (2018). Quantification of selected steroid hormones (17β-Estradiol and 17α-Ethynylestradiol) in wastewater treatment plants in Klang Valley (Malaysia). Chemosphere.

[48] Sun, J., Wang, J., Zhang, R., Wei, D., Long, Q., Huang, Y., … Li, A. (2017). Comparison of different advanced treatment processes in removing endocrine disruption effects from municipal wastewater secondary effluent. Chemosphere, 168, 1-9.

[49] Belhaj, D., Baccar, R., Jaabiri, I., Bouzid, J., Kallel, M., Ayadi, H., & Zhou, J. L. (2015). Fate of selected estrogenic hormones in an urban sewage treatment plant in Tunisia (North Africa). Science of The Total Environment, 505, 154-160.

[50] Carvalho, A. R., Cardoso, V., Rodrigues, A., Benoliel, M. J., & Duarte, E. (2016). Fate and Analysis of Endocrine-Disrupting Compounds in a Wastewater Treatment Plant in Portugal. Water, Air, & Soil Pollution, 227(6).

[51] Diniz, M. S., Maurício, R., Petrovic, M., De Alda, M. J. L., Amaral, L., Peres, I., … Santana, F. (2010). Assessing the estrogenic potency in a Portuguese wastewater treatment plant using an integrated approach. Journal of Environmental Sciences, 22(10), 1613-1622.

[52] Rocha, M. J., Madureira, T. V., Venade, C. S., Martins, I., Campos, J., & Rocha, E. (2019). Presence of estrogenic endocrine disruptors in three European estuaries in Northwest Iberian Peninsula (Portugal). Toxicological & Environmental Chemistry, 101(3-6), 244-264.

[53] Vidal, Carla B. et al. Multiresidue Determination of Endocrine Disrupting Compounds in Sewage Treatment Plants (SPE-HPLC-DAD). J. Braz. Chem. Soc. [online]. 2020, vol.31, n.12, pp.2518-2530. Epub Dec 14, 2020.

[54] Jardim, I. C. S. F. Extração em Fase Sólida: Fundamentos Teóricos e Novas Estratégias para preparação de Fases Sólidas. Química Nova, v. 2, n. 1, p. 13-25, 2010.

[55] Hernández-Borges, J. et al. Sample treatments prior to capillary electrophoresis-mass spectrometry. Journal of Chromatography A, v. 1153, p. 214-226, 2007.

[56] Chaves, K. S. Determinação dos desreguladores endócrinos bisfenol-A, 17b-estradiol, 17ª-etilnilestradiol e estrona no Rio Paraíba do Sul. 2015. 133p. Dissertation (Masters in Science) - Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, 2016.

[57] Ribani, M. et al. Validação em métodos cromatográficos e eletroforéticos. Química Nova, v. 27, n. 5, p. 771-780, 2004.

[58] Amarante, O. P. J. et al. Validação de método analíticos: uma breve revisão. Caderno de Pesquisa, v. 12, n. 1/2, p. 116-131, 2001.

[59] Saini, S. S. (2018). A Microextraction Coupled with HPLC-UV for a Sensitive Detection of Estrogens in Water. Analytical Chemistry Letters, 8(5), 606-621.

[60] WANG, L., CAI, Y., HE, B., YUAN, C., SHEN, D., SHAO, J., & JIANG, G. (2006). Determination of estrogens in water by HPLC-UV using cloud point extraction. Talanta, 70(1), 47-51.

[61] Yilmaz, B., & Kadioglu, Y. (2017). Determination of 17 β-estradiol in pharmaceutical preparation by UV spectrophotometry and high performance liquid chromatography methods. Arabian Journal of Chemistry, 10, S1422-S1428.

[62] Nováková, L., Solich, P., Matysová, L., & Šícha, J. (2004). HPLC determination of estradiol, its degradation product, and preservatives in new topical formulation Estrogel HBF. Analytical and Bioanalytical Chemistry, 379(5-6), 781-787.

[63] Esperanza, M., Suidan, M. T., Marfil-Vega, R., Gonzalez, C.,Sorial, G. A., McCauley, P., & Brenner, R. (2007). Fate of sex hormones in two pilot scale municipal wastewater treatment plants: conventional treatment. Chemosphere, 66, 1535-1544.

[64] HOLLER, F.J.; SKOOG, D.A.; CROUCH, S.R. Introdução às Separações Cromatográficas. In: Princípios de Análise Istrumental. Porto Alegre: Bookman, Ed. 6, 776-801, 2009.

[65] JARDIM, I. C. S. F.; COLLINS, C. H.; GUIMARÃES, L. F. L. Cromatografia líquida de alta eficiência. In: Fundamentos de cromatografia. Campinas: UNICAMP, cap. 9, p. 273-397, 2006.

[66] BRESSOLLE, F., BROMET-PETIT, M., AUDRAN, M. Validation of liquid chromatographic and gas chromatographic methods. Application to pharmacokinetics. J. Chromatogr. B, v. 686, p. 3-10, 1996.

[67] Shah, V.P., Midha, K.K., Dighe, S. et al. Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies. Pharm Res 9, 588-592 (1992).

[68] Miller, J. N. Basic statistical methods for Analytical Chemistry. Part 2. Calibration and regression methods. A review. The Analyst, 116(1), 3, 1991.

[69] De Lima, E. C., & Daniel, M. D. S. (2014). Determinação simultânea de estriol, b-estradiol, 17a-etinilestradiol e estrona empregando-se extração em fase sólida (SPE) e cromatografia líquida de alta eficiência (HPLC). Ambiente e Agua - An Interdisciplinary Journal of Applied Science, 9(4).

[70] BRITO, N. M. et al. Validação de métodos analíticos: estratégia e discussão. Pesticidas: R.Ecotoxicol. e Meio Ambiente, v. 13, p. 129-146, 2003.

[71] NORMAN Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances. [S. l.], 5 abr. 2021. Disponível em: https://www.norman-network.net/?q=node/19. Acesso em: 9 jul. 2021.

[72] Sandoval R., Maria. (2017). Extraction of Phorbol Esters (PEs) from Pinion cake using computationally-designed polymers as adsorbents for Solid Phase Extraction.

APPENDICES

APPENDIX A

In order to try to identify the other peaks present in the chromatograms presented, it was also injected in the HPLC-UV system: 100% methanol, 100% acetonitrile, ultrapure water with pH variation of (2, 5 and 8), 1 mg/L solutions of estradiol in ultrapure water with pH variation (2, 5 and 8). Also, to confirm whether there is degradation of estradiol in methanol, a solution of 1 mg/L concentration of estradiol in methanol prepared 1 month ago was also injected into the HPLC-UV system and compared with the chromatographic curve obtained when the standard was first prepared. The respective chromatographic curves are shown next

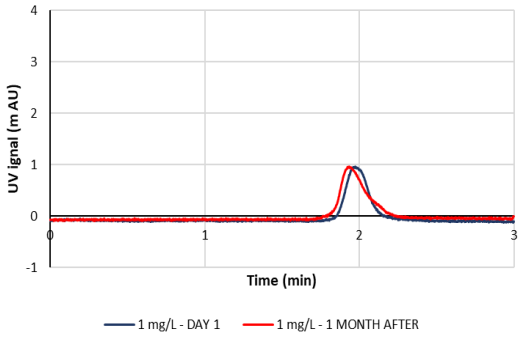

Figure A77. Chromatographic curve of 2 solutions of 1 mg/L of E2 in 100% methanol, injected with a difference of 1 month between them.

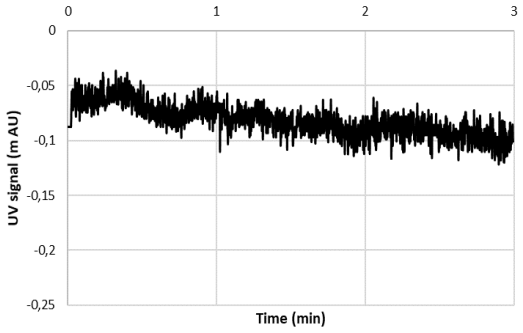

Figure A78. Chromatographic curve of 100% methanol.

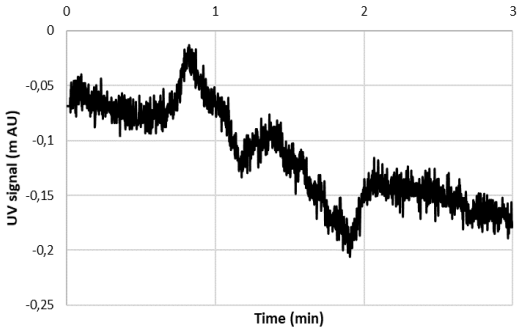

Figure A79.Chromatographic curve of 100% ACN.

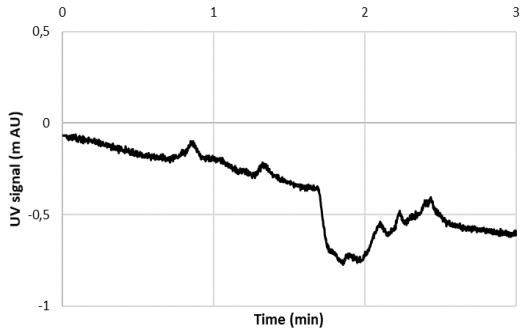

Figure A80. Chromatographic curve of ultrapure water with a pH value of 5.

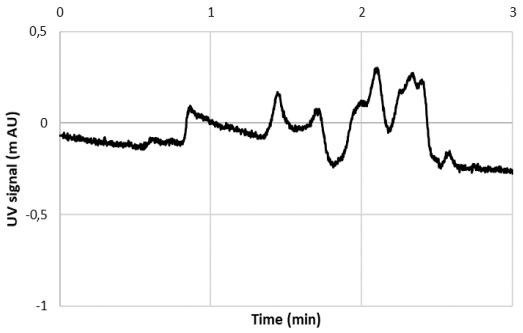

Figure A81. Solution of 1 mg/L of E2 in ultrapure water pH 5.

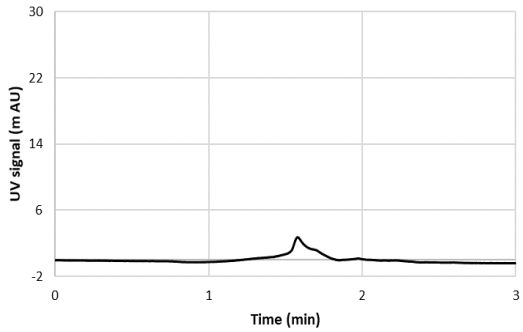

Figure A82. Chromatographic curve of ultrapure water with a pH value of 2.

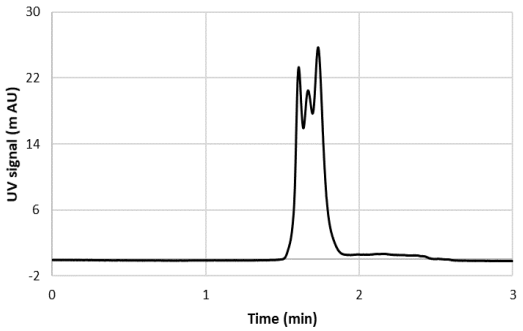

Figure A83. Solution of 1 mg/L of E2 in ultrapure water pH 2.

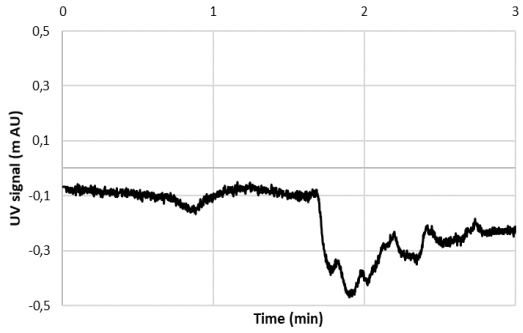

Figure A84. Chromatographic curve of ultrapure water with a pH value of 8.

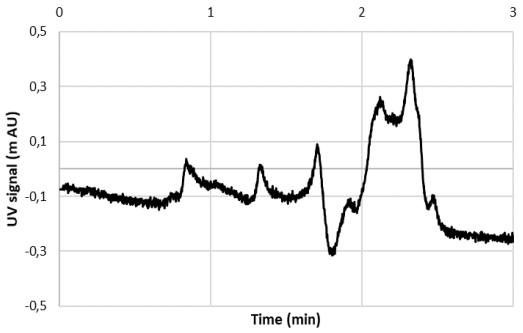

Figure A85. Solution of 1 mg/L of E2 in ultrapure water pH 8.

As seen in Figures A77-A85, the peak with a retention time of approximately 1.3 min has not been identified, which meant that those conditions were not the responsible for its appearance. After observation, the hypothesis was raised that estradiol was undergoing

degradation in the adsorption process during the SPE experiments, with that being the possible reason for its presence. In order to further identify the origin of the peak in question, three solutions of estradiol with a concentration of 1 mg/L in ultrapure water were prepared, varying the pH value between 2, 5 and 8. These solutions did not undergo the procedure of SPE. They passed directly to the evaporation process, once the evaporation was completed, they were reconstituted in 10 mL of acetonitrile, these 10 mL were passed through the second evaporation process and then, the samples were reconstituted in 0.5 mL of methanol and injected in the HPLC-UV system. Figures A86-A88 show the chromatograms referred to the mentioned processes.

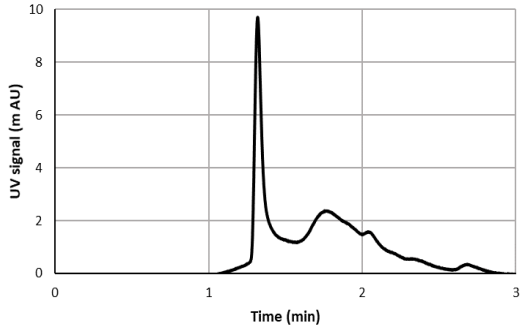

Figure A86. Chromatographic curve of the reconstituted samples prepared in ultrapure water with a pH value of 2.

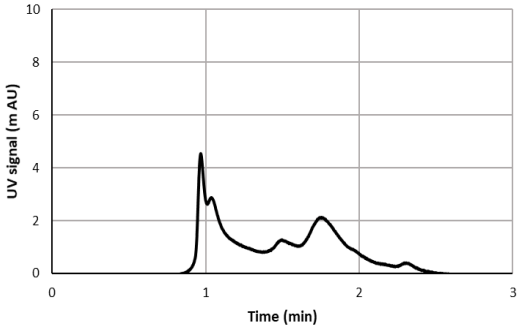

Figure A87. Chromatographic curve of the reconstituted samples prepared in ultrapure water with a pH value of 5.

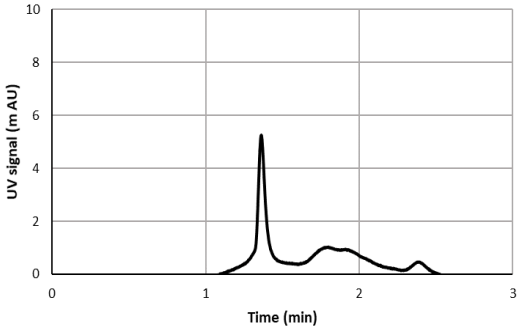

Figure A88. Chromatographic curve of the reconstituted samples prepared in ultrapure water with a pH value of 8.

When looking at Figures A86-A88, it is possible to see that the peak with a retention time of approximately 1.3 min still continues to appear, and it can be concluded that they are not resulted from the SPE process. The next hypothesis raised was that this peak is caused by the heating of the sample that occurs in the evaporation step to accelerate it. This heating degrades the sample, causing the peak in question to appear.

To verify this hypothesis, it was necessary to perform the SPE process, using the optimal conditions (Run 20), eliminating the heating/ evaporation step of the sample. By eliminating the sample heating/evaporation step, the methanol reconstitution step is consequently eliminated. Thus, in order to make it possible to identify estradiol in the HPLC-UV system, the concentration of the charge must be higher than the LOD. The concentration chosen was 10 mg/L and the sample volume was 500 mL. The elution step was performed with 10 mL of acetonitrile and injected, immediately afterwards, in the HPLC-UV system. Its respective chromatogram is shown in Figure A89.

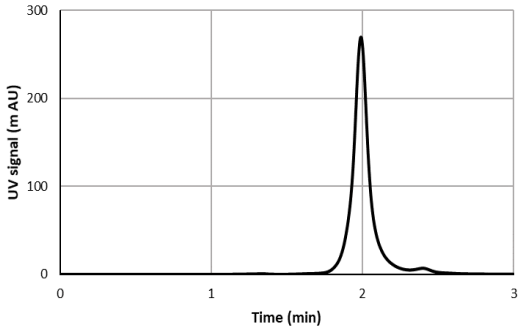

Figure A89. Chromatographic curve of the elution step in 10 mL of ACN.

As seen in Figure A89, there is no peak in the retention time of approximately 1.3 min, confirming that it is possible that it occurs due to the heating of the sample, causing its degradation.