Universidade de Aveiro Departamento de Química
2014

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Determinação de hormonas em bivalves por HPLC-FL

HPLC-FL for determination of hormones in bivalves

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Dissertação apresentada no Departamento de Química da Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Química - Especialidade em Química Analítica e Qualidade, realizada sob a orientação científica do Doutor Armando da Costa Duarte, Professor Catedrático do Departamento de Química da Universidade de Aveiro, e da Doutora Regina Maria Brandão de Oliveira Duarte, Equiparada a Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro

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Palavras-chave

Cromatografia líquida de alta eficiência; fluorescência; desenvolvimento de métodos; bivalves; ostras; hormonas

Resumo

O principal objetivo deste trabalho foi a aplicação de um método de cromatografia líquida de alta eficiência (HPLC, sigla inglesa para *High-Performance Liquid Chromatography*) acoplada a um detetor de fluorescência para encontrar as condições mais adequadas para a separação isocrática das hormonas 17β-estradiol (E2) e 17-α-etinilestradiol (EE2), em amostras de bivalves. Foi utilizada uma coluna com uma fase reversa (Kromasil 100 C18, 5µm, 15 x 0,21 cm) e um detetor de fluorescência operando a comprimentos de onda de excitação e de emissão de 280nm e 305nm, respectivamente. Obtiveram-se curvas de calibração com bom coeficiente de correlação tendo-se contudo verificado problemas de contaminação durante as análises de amostras de bivalves. Estes problemas de contaminação tiveram origem, principalmente, no vasilhame de vidro usado, na preparação dos padrões e durante o procedimento de extração das hormonas E2 e EE2 das amostras. Assim, e numa primeira fase, procedeu-se à eliminação dos vários fatores potencialmente na origem dos problemas de contaminação de modo a ser possível obter os menores limites de quantificação possíveis para E2 e EE2, bem como proceder à respetiva quantificação nas amostras de bivalves. Assim, todo o material utilizado teve de ser cuidadosamente lavado com acetonitrilo e fase móvel utilizada na separação cromatográfica, posteriormente ao processo de lavagem habitualmente aplicado no laboratório. Depois deste processo de lavagem, e a utilização de uma pré-coluna no procedimento de separação cromatográfica, conseguiu-se identificar e quantificar a hormona E2 nos cromatogramas obtidos para as amostras de bivalves. Obteve-se um valor médio de 24.59 ng/g para as amostra de bivalves recolhidas num local de aquacultura em Aveiro.

Keywords High performance liquid chromatography; fluorescence; method development; bivalves; oysters; hormones

Abstract

The main objective of this work was the application of an isocratic high performance liquid chromatography (HPLC) method coupled with a fluorescence detector to find the best conditions for the separation and quantification of 17β-estradiol (E2) and 17α-ethinyl estradiol (EE2) hormones in bivalve samples. A reversed phase column (Kromasil 100 C18, 5µm, 15 x 0.21cm), was employed with a fluorescence detector operating at excitation and emission wavelengths of 280nm and 305nm, respectively. A calibration curve was obtained with a good correlation coefficient for each compound. During the chromatographic analysis of the bivalve extracts, there were contamination problems, originated from glassware containers, E2 and EE2 standards preparation, and during hormones extraction from the samples. Thus, in a first step, a special attention was given to the elimination of these contamination problems in order to obtain the lowest limits of quantification for both E2 and EE2, and achieve their quantification in oyster samples. Therefore, after the washing process routinely applied in the laboratory, all glassware employed in samples preparation were washed with acetonitrile followed by the mobile phase used in the chromatographic analysis. After this washing procedure, and the use of a pre-column in the chromatographic separation process, the identification and quantification of hormone E2 was successfully accomplished for the oyster samples. An average value of 24.59 ng/g was obtained for oyster samples collected at an aquaculture location in Aveiro.

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List of Abbreviations

ACN	Acetonitrile
E1	Estrone
E2	17β-estradiol
EE2	17α-ethinyl estradiol
EDCs	Endocrine-disrupting compounds
EEM	Excitation-Emission Matrix
EtOH	Ethanol
ESI	Electrospray Ionisation
FL	Fluorescence
GC	Gas Chromatography
H_3PO_4	Orthophosphoric Acid
HLB	Hydrophilic-Lipophilic-Balanced
HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
MeOH	Methanol
MP	Mobile Phase
MS	Mass Spectrometry
NaOH	Sodium Hydroxide
R^2	Correlation Coefficient
RP	Reversed Phase
RSD	Relative Standard Deviation
RT	Retention Time

SD	Standard Deviation
SPE	Solid Phase Extraction
SPK	Spike
STP	Sewage Treatment Plant
$\boldsymbol{\lambda_{em}}$	Emission Wavelength
$\boldsymbol{\lambda_{exc}}$	Excitation Wavelength

1. Introduction

In this research work, two steroid hormones have been analyzed in oyster samples, to be more specific: the 17β-estradiol (E2) and 17α-ethinyl estradiol (EE2). They are biologically active compounds synthesized from cholesterol, which have in common a cyclopntan-o-perhidrophenanthreno ring. The hormone E2 is a natural estrogenic steroid, while the hormone EE2 is a synthetic steroid. Natural estrogenic steroid have higher solubility than the synthetics. The solubility of E2 in water is of 13 mg/l at 20ºC while the water solubility of EE2 is reduced to 4.8 mg/l at 20ºC according to *Ying et al.* (2002).

Estrogens, such as estradiol, are predominantly female hormones, being important to the health of reproductive tissues, breasts, skin and brain in human and animal. The estrogens play an important role in their differentiation, development and reproduction in wildlife. In invertebrates, the steroid control of reproduction is unclear.

Steroid hormones can enter the environment through sewage discharge and animal waste disposal, since all humans and animals can excrete the hormones from their bodies in different amounts, depending on gender, age, state of health, diet or pregnancy in the case of the females. Animal waste and biosolids as well as recycled wastewater have been increasingly applied to agricultural land; therefore, it is vital to estimate the input of steroids and their possible movement into surface and ground water through runoff and leaching. These steroids have a moderate binding on sediments and are reported to degrade rapidly in soil and water.

Steroids have been detected in effluents of sewage treatment plants (STPs) and surface water. They may interfere with the normal functioning of endocrine systems, thus affecting reproduction and development of wildlife (*Jobling et al.*, 1998). The steroids of concern for the aquatic environment due to their endocrine disruption potential are mainly estrogens and contraceptives, which include E2 and EE2.

E2 and EE2 are analyzed because their importance in the environment. The presence of E2 causes the feminization of the males according to *Metcalfe et al.*, (2001).

They observed gonadal intersex and high prevalence of the female phenotype in fish populations in urbanized areas. Environmental estrogens discharged in sewage treatment plant effluents may be responsible for feminization of fish but many compounds with the potential to induce these responses occur in the effluents, including natural and synthetic estrogen hormones.

Steroids are also active signal transmitters in vertebrates. As E2 has shown some physiological activities in the oyster and as estrogens or estrogen-like molecules can be present in water, *Le Curieux-Belfond et al*. (2005) have investigated the bioaccumulation and metabolism of this estrogen *in vivo* in the oyster *Crassostrea gigas*. They concluded that oyster is able to take in charge estradiol as a potential contaminant in seawater. Therefore, its bioaccumulation and transformation into estrone could be studied as potential biomarker of endocrine disruption.

In this study, for the analysis of E2 and EE2 in oyster samples, a high performance liquid chromatography (HPLC) coupled with a fluorescence (FL) detector was used. HPLC is a technique for the separation of mixtures, where the sample is normally carried in a mobile phase through a stationary phase. The constituents of the sample are separated based on their affinity between the mobile phase and the stationary phase. There are two different modes of mobile phase elution in HPLC: a) isocratic elution, where the mobile phase composition is kept constant through the whole procedure; and b) gradient elution, where the composition of the mobile phase can be programed during the separation. Our goal is to get an isocratic method for separating the hormones E2 and EE2. In this work, a chromatography column with a reversed phase (RP) stationary phase was used for separating the hormones. RP chromatography is any chromatographic method that uses a hydrophobic (non-polar) stationary phase, and can bind quite polar molecules. In RP chromatography, water is usually the base solvent. Other organic solvents, such as methanol, acetonitrile or tetrahydrofuran are added in fixed (isocratic elution) or varying proportions (gradient elution).

More than one detection method can be used at the same time (usually known as hyphenated detector arrangement), in order to obtain information-rich detection for both identification and quantification compared to that using a single detector. Fluorescence is the detection technique chosen for this research work. Fluorescence detection is very

selective and sensitive of either natively fluorescent or derivatizable analytes. In the case of hormones E2 and EE2, it is not necessary to derivatize these compounds as they are natively fluorescent. By definition, fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation. It also occurs when molecules return to ground state after being excited to higher electronic states by energetic electron bombardment.

The hormones E2 and EE2 have been also analyzed by other methods. For example, *Ronan et al.* (2013) used a sensitive method combining liquid chromatography and tandem mass spectrometry, with electrospray ionisation in negative mode (LC/ESI-MS/MS), for determining estrone (E1), E2 and EE2 at concentrations between 0.07 and 60 ng/L in seawater, and between 0.4 and 200 ng/g wet weight in mussels (*Mytilus* spp.). According to the authors, this method is suitable for the detection of E1, E2 and EE2 at biologically relevant concentrations and, due to the specificity offered, is not subject to potential interferences from endogenous E1 and E2 that often complicate the interpretation of estrogenic biomarker assays. The method developed in this report was successfully employed for the simultaneous detection of steroid estrogens (E1, E2 and EE2) in water and biota at concentrations above 0.4 to 0.9 ng/g in biota, and 0.07 to 0.14 ng/L in seawater.

E2 an EE2 can also be analyzed by gas chromatography (GC) coupled to mass spectrometry (GC-MS). *Petrovic et al.* (2001) presented an overview of the analytical methods for target endocrine-disrupting compounds in freshwater sediments. In this work, the estrogens were Soxhlet-extracted using methanol and subsequently derivatized for further GC–MS analysis.

2. Materials and methods

2.1. Reagents and standards

The steroid hormones E2 (97.5%) and EE2 (99.5%) were supplied by Dr. Ehrenstofer, in methanol at a concentration of 100 mg/L. HPLC-plus gradient ethanol (CH_3CH_2OH, EtOH) and HPLC-GOLD-ultragradient methanol (CH_3OH, MeOH) were obtained from Carlo Erba, HPLC gradient grade acetonitrile (CH_3CN, ACN) from Fisher scientific, and orthophosphoric acid (H_3PO_4) (85%) from Panreac.

Each standard solution of E2 and EE2 were further diluted to a stock solution of 6 ppm concentration, using methanol to analyze the fluorescence spectra and different method of HPLC. After choosing the method, a 5 ppm concentration stock solution was prepared by dilution with the mobile phase (MP) consisting of 50% (v/v) ACN to get a 1 ppm concentration to make the standard solutions for the calibration curve.

2.2. Instrumentation and HPLC method conditions

In a first step, it was necessary to select the most appropriate excitation and emission wavelengths (λ_{exc} and λ_{em}, respectively) for detecting E2 and EE2 in the samples extracts. This was done by recording the excitation-emission matrix (EEM) fluorescence spectra of both E2 and EE2 on a fluorescence spectrophotometer JASCO, model FP-6500. The chromatographic analysis of both E2 and EE2 was performed on a JASCO chromatographic system equipped with a JASCO isocratic HPLC pump (model PU-2080), a Rheodyne injection valve (model 7725i) equipped with a 20 µl loop, and a JASCO fluorescence detector (FP-2020 Plus) operating at λ_{exc} / λ_{em} = 280/305 nm. For the separation of E2 and EE2, it was used a Kromasil 100 C18 column (diameter 2.1 mm; length 150 mm; particle size 5 µm; pore diameter 100Å). The mobile phase consisted of 50% (v/v) ACN, with a flow rate set at 0.4 ml/min.

2.3. Glassware washing procedures

In order to avoid contamination problems, the use of properly cleaned material is important in any research work. These contaminations can interfere with the analysis of the compounds of interest, thus deteriorating the results. In this research work, due to the high sensitivity of the fluorescence detector, the glassware washing process becomes even more important.

For processing the samples, all the material was washed with deionized water, soaked in detergent (Derquim, alkaline) for a few hours and again washed with deionized water. The material was then immersed in sodium hydroxide (NaOH 1M) and thoroughly washed, first with deionized water and then with Elix water. To finish the washing process, the material was washed with ACN followed by the MP (50% (v/v) ACN) and then dried.

2.4. Extraction of E2 and EE2 from oyster samples

For E2 and EE2 extraction, 1g of homogeneous oyster sample was placed into a Teflon centrifuge tube with 20 ml MeOH and shaken for 1 minute with a Vortex. Afterwards, this mixture was extracted in an ultrasonic bath for 5 minutes. Then, the samples were centrifuged at a speed of 4000 r/min for 5 minutes. With this procedure, the liquid and solid phases were separated, and the liquid phase was transferred into a round bottom flask. The remaining residue was subjected again to the solid-liquid extraction procedure, and the obtained liquid phase was transferred into the previous round bottom flask. The MeOH extract was evaporated to dryness and redissolved with 10 ml of ultrapure water.

Sample clean-up was conducted using an Oasis hydrophilic-lipophilic-balanced (HLB) extraction cartridge (6cc, 150mg). The cartridges were rinsed and conditioned with 10 ml MeOH and 10 ml ultra-pure water before the addition of the sample extract. The cartridges were then rinsed with 5 ml 5% (v/v) MeOH in ultrapure water, and the adsorbed compounds were eluted with 4 ml MeOH under a gentle vacuum. The eluates were then

evaporated to dryness and redissolved in 2 ml of MP. The obtained sample extract was filtered through HPLC certified syringe filters of 0.22 μm pore size before analysis by means of the HPLC-FL method previously described.

3. Results and discussions

3.1. Selection of λ_{exc} and λ_{em}

The first step entailed the selection of the most appropriate λ_{exc} / λ_{em} pair for detecting the hormones E2 and EE2. This λ_{exc} / λ_{em} pair was selected based upon the EEM fluorescence spectra of both hormones, diluted in the MP. The compounds were diluted to a concentration of 0.1ppm using 55% (v/v) MeOH and 45% water acidified with H_3PO_4 (10mM). As shown in Figure 1 and Figure 2, each hormone has two distinct λ_{exc} due to the resonance forms that both E2 and EE2 may undertake, both emitting at λ_{em} 305 nm. In this study, it was decided to select the λ_{exc} / λ_{em} pair of 280/305 nm because the obtained fluorescence peak is much better defined. According to *Yoon et al.* (2003), both E2 and EE2 are excited at a λ_{exc} of 280 nm, and they emit at a λ_{em} of 310 nm. The results obtained in this study are very similar to those reported in the literature.

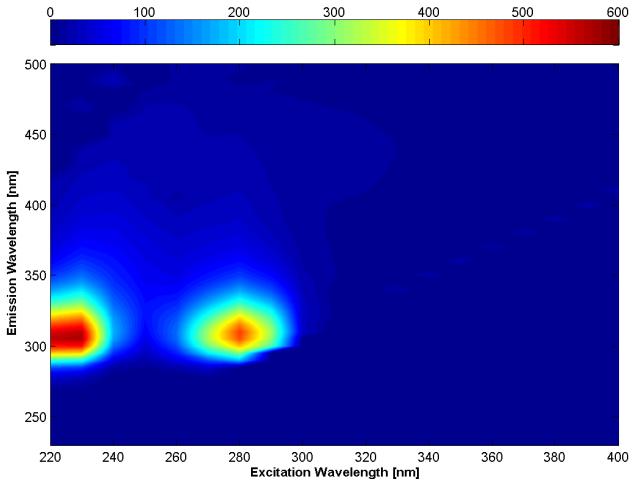

Figure 1- Excitation-emission matrix (EEM) fluorescence spectra of EE2 (0.1ppm).

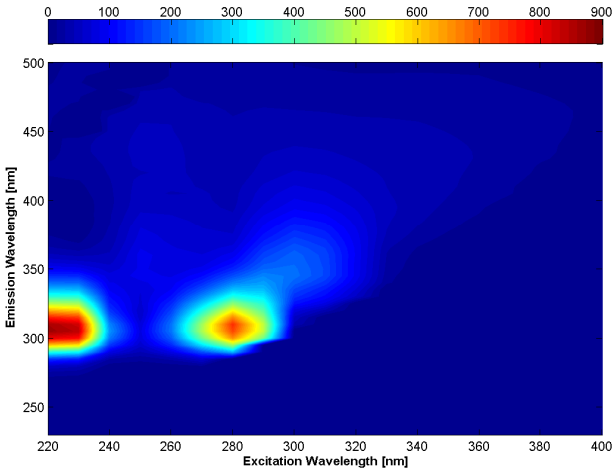

Figure 2- Excitation-emission matrix **(**EEM) fluorescence spectra of E2 (0.1ppm).

3.2. Selection of the best HPLC separation conditions

Different HPLC conditions were tested in order to achieve the separation and further quantification of the E2 and EE2 hormones. The first tested method was that of *Yoon et al.* (2003) for analyzing E2, EE2 and other compounds on powered activated carbon. A reversed phase column (Kromasil 100-5 C18 15cm x 4.6mm) was used for the isocratic elution with a MP composition consisting of 55% (v/v) MeOH in 10mM H_3PO_4, at a constant flow rate of 0.8 ml/min. It was decided to use a MP with MeOH instead of ACN, which is known to be a common organic solvent used in the chromatographic separation of E2 and EE2. The advantages of using MeOH include the fact that it is cheaper than ACN and it is a more ecological solvent.

As shown in Figure 3 and Figure 4, there are a few unknown peaks before a retention time of 5 minutes, while the E2 and EE2 elute between the retention time of 27 and 40 minutes. Each compound gave more than one non-Gaussian overlapping peaks, being impossible to identify each compound using these separation conditions. Another drawback is that it took a long time to elute each hormone, thus suggesting the need for using a different MP composition in order to decrease the time of analysis.

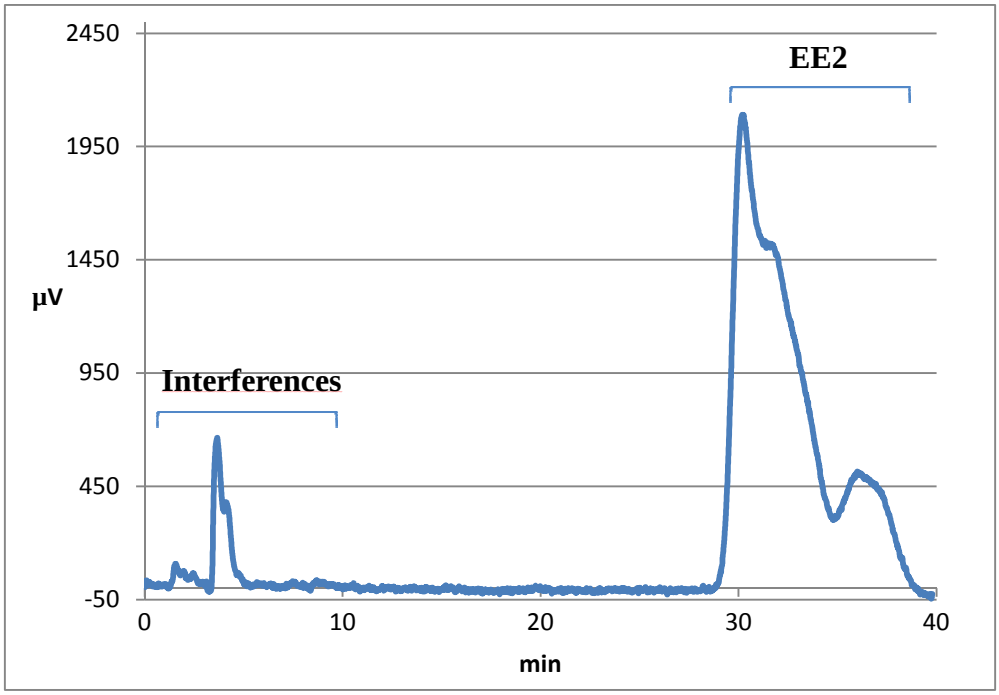

Figure 3- Chromatogram of EE2 (1ppm), using a MP composition consisting of 55% (v/v) MeOH in 10mM H_3PO_4 and a flow rate of 0.8ml/min**.**

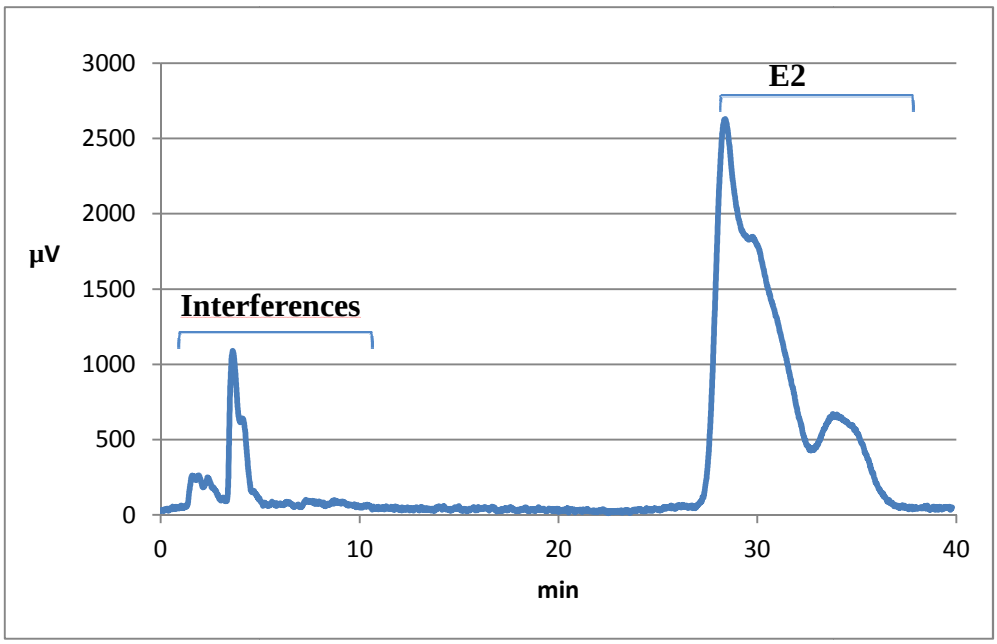

Figure 4- Chromatogram of a solution of E2 (1ppm) using in 55% (v/v) MeOH in 10 mM H_3PO_4 and a flow rate of 0.8ml/min**.**

This work uses a reversed phase column, which has a hydrophobic (non-polar) stationary phase that can bind quite polar molecules. Knowing this, it was decided to use a less polar mobile phase to reduce the retention time of each hormone. As such, in a second stage, the elution was performed using a MP composition consisting of 50% (v/v) EtOH at a lower flow rate, 0.5 ml/min, due to the high backpressures that could damage the column packing material. Figure 5 and Figure 6 illustrate the obtained chromatograms, where the retention times of both E2 and EE2 were lower than those obtained using MeOH in the MP. Both E2 and EE2 elute around minute 12, almost 10 minutes less than those obtained using MeOH in the MP However, with this new MP composition, each hormone is eluted as two partially resolved peaks, and both E2 and EE2 appear at the same retention time, which do not allow to separate and distinguish between both compounds in a mixture. In these chromatograms, it also appears unknown peaks before minute 10, but they do not interfere with the chromatographic peaks of compounds E2 and EE2.

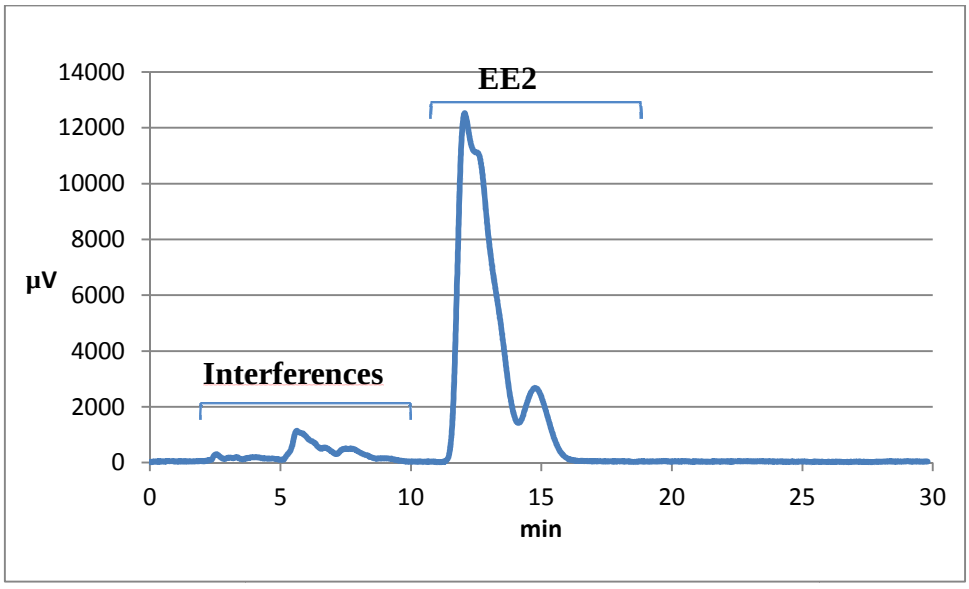

Figure 5- Chromatogram of a solution of EE2 (1ppm) using in 50% (v/v) EtOH and a flow rate of 0.5 ml/min**.**

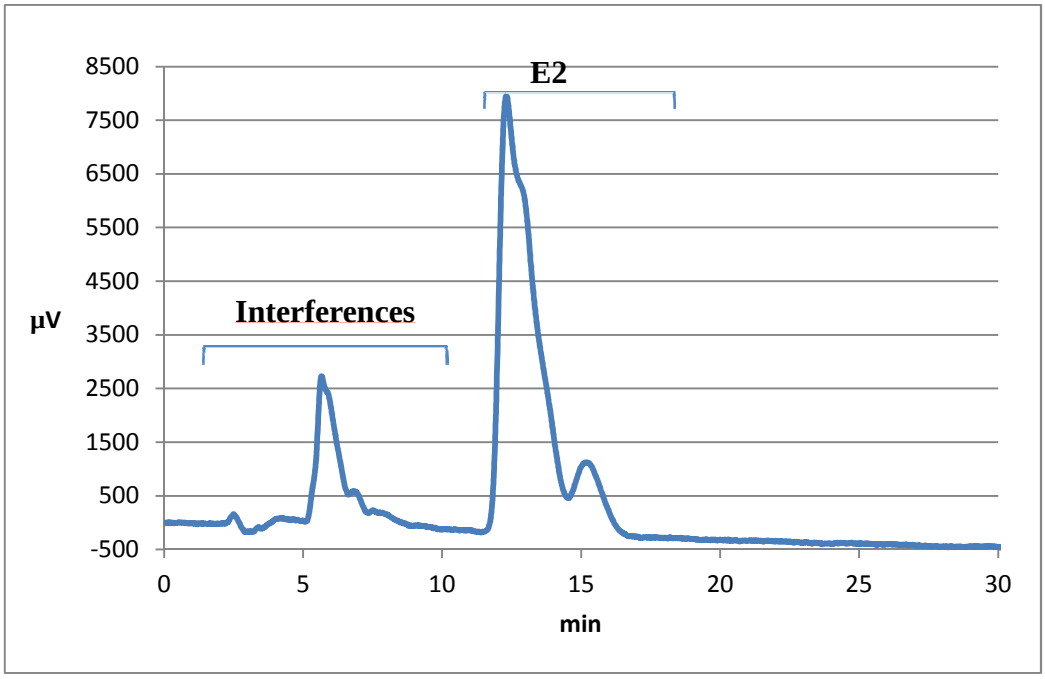

Figure 6- Chromatogram of a solution of E2 (1ppm) using in 50% (v/v) EtOH and a flow rate of 0.5 ml/min**.**

In order to solve this elution problem where each hormone is eluted as two partially resolved peaks, a new MP composition was tested, now using acidified water instead of ultrapure water. Usually, the retention time of polar compounds change with the pH of the MP. In this case, the new MP composition consisted of 50% (v/v) EtOH in 10mM H_3PO_4, with a pH of 2.33, and the flow rate was set at 0.5ml/min. Using acidified water and EtOH, no major differences were verified in the retention times of both E2 and EE2, also eluting around minute 12, as seen in Figure 7 and Figure 8. Each compound is eluted as two partially resolved non-Gaussian peaks. Using this MP composition, the unknown peaks eluting before minute 10 still appear in the chromatograms.

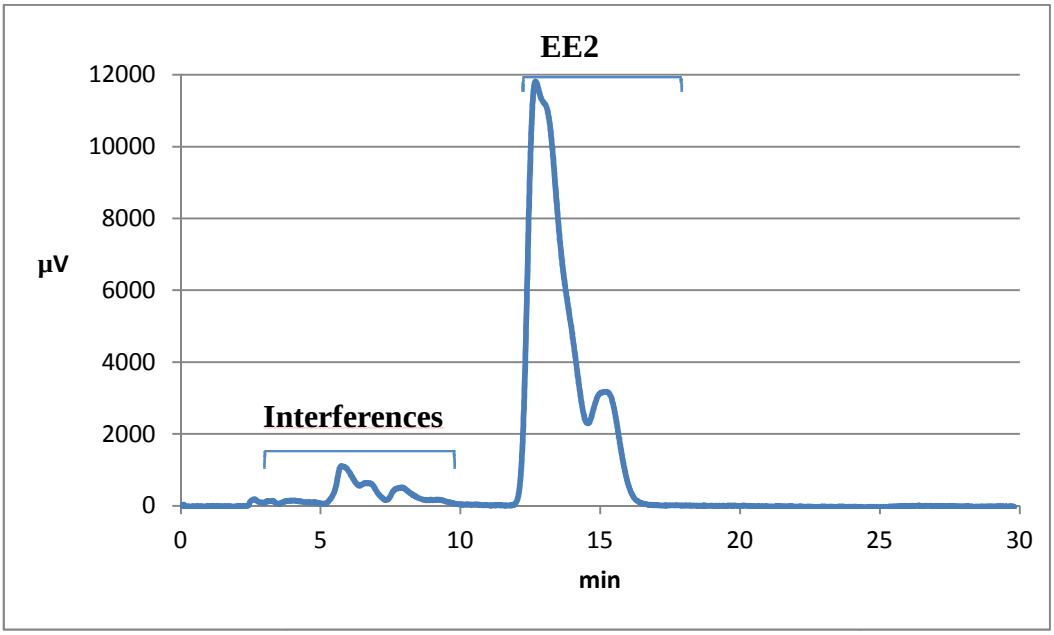

Figure 7- Chromatogram of a solution of EE2 (1ppm) in 50% (v/v) EtOH in 10 mM H_3PO_4 and a flow rate of 0.5ml/min.

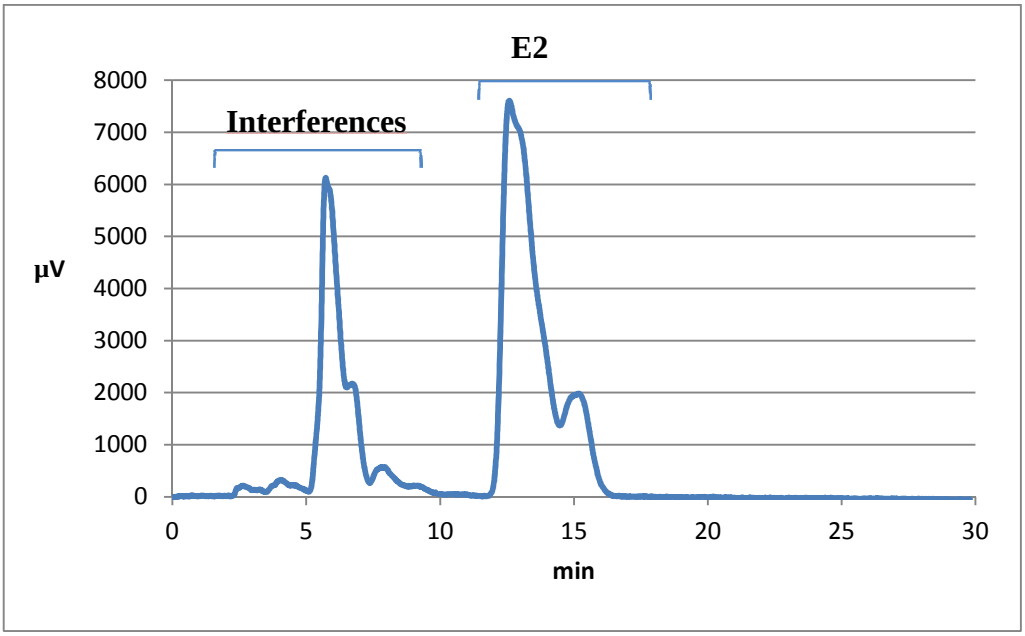

Figure 8- Chromatogram of a solution of E2 (1ppm) in 50% (v/v) EtOH in 10 mM H_3PO_4 and a flow rate of 0.5ml/min.

Finally, the typical MP composition based on 50% (v/v) ACN was used. In this case, for analyzing the hormones, a standard solution containing both E2 and EE2 was prepared, with a concentration of 0.5 ppb. The flow rate was set at 0.2 ml/min because of the high backpressure in the Kromasil 100 C18 5µm (15cm x 4.6mm) column. As seen in Figure 9, under these conditions, each compound was eluted as two partially resolved peaks which are not completely separated. For this reason, it was decided to replace the column by another similar column but with a smaller inner diameter, Kromasil 100 C18 5µm (15 x 0.21cm). After replacing the column, each compound eluted as one single peak (Figure 10); the peak with the lower retention time correspond to the compound E2, while the signal of the compound EE2 appear later. These peaks are well separated, which facilitates the integration for subsequent analysis .

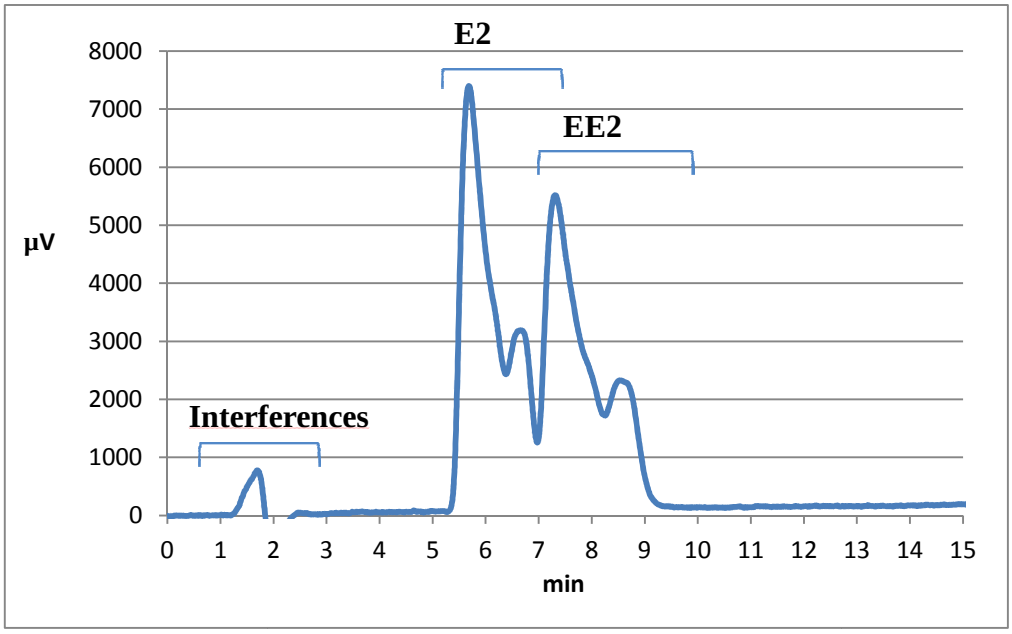

Figure 9- Chromatogram of E2 and EE2 (0.5ppm) in 50% (v/v) ACN and a flow rate of 0.2ml/min, using Kromasil 100-5 C18 (15cm x 4.6mm) column.

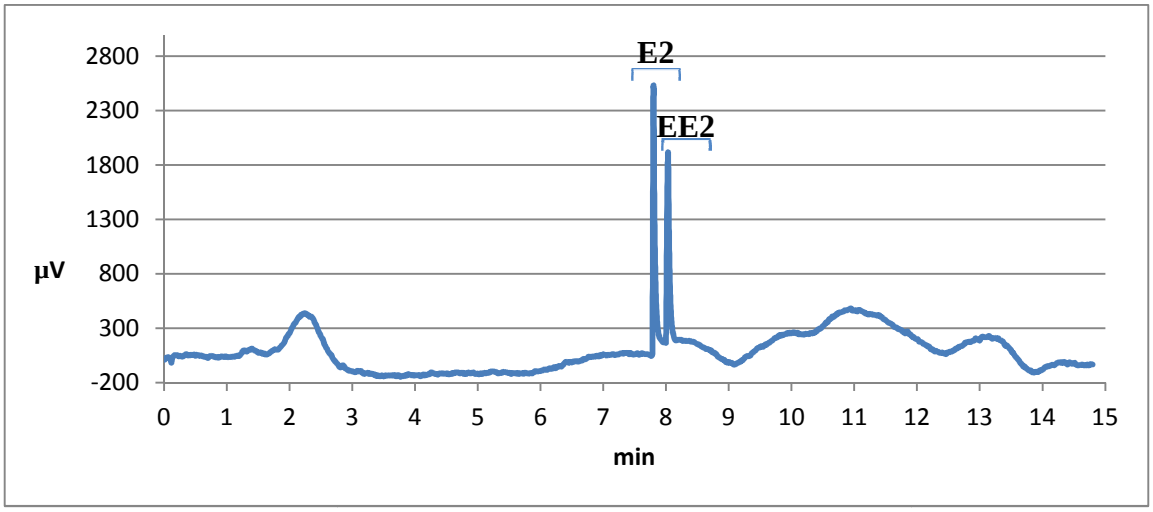

Figure 10- Chromatogram of E2 and EE2 (0.5ppm) in 50% (v/v) ACN and a flow rate of 0.2ml/min, using Kromasil 100 C18 5µm (15 x 0.21cm) column.

Since the flow rate used until now was very low, it would be advisable to increase the flow rate. Therefore, different values of the flow rate were tested, namely 0.2ml/min, 0.3ml/min, 0.4ml/min, and finally 0.5ml/min. The following figures (Figure 11, Figure 12 and Figure 13) show the influence of the flow rate in the chromatographic separation.

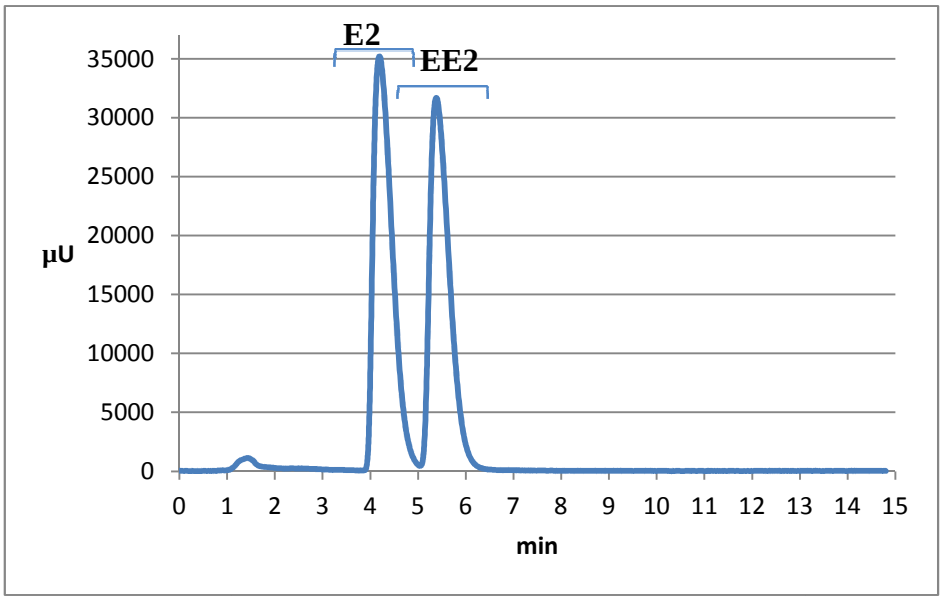

Figure 11- Chromatogram of E2 and EE2 (0.5ppm) in 50% (v/v) ACN and a flow rate of 0.3ml/min.

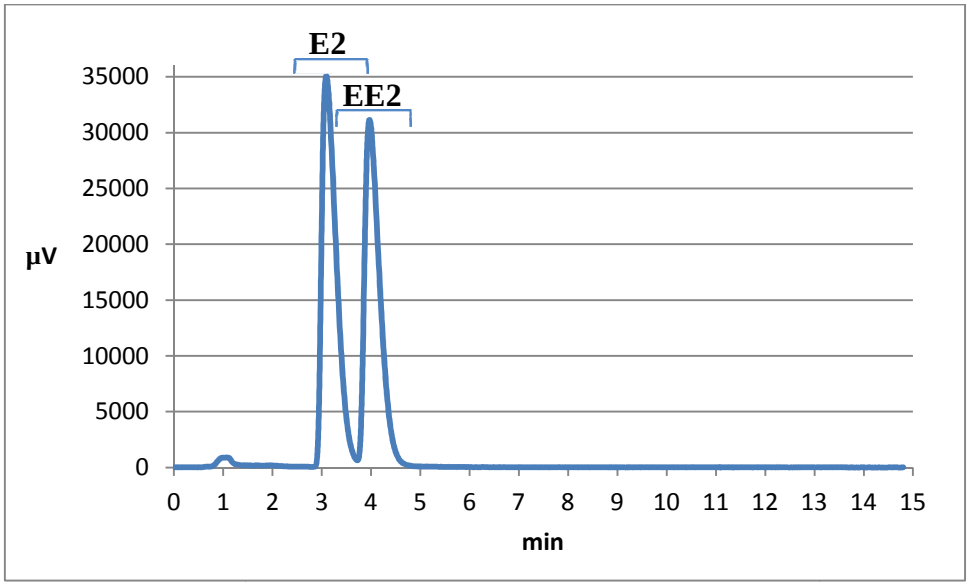

Figure 12- Chromatogram of E2 and EE2 (0.5ppm) in 50% (v/v) ACN and a flow rate of 0.4ml/min.

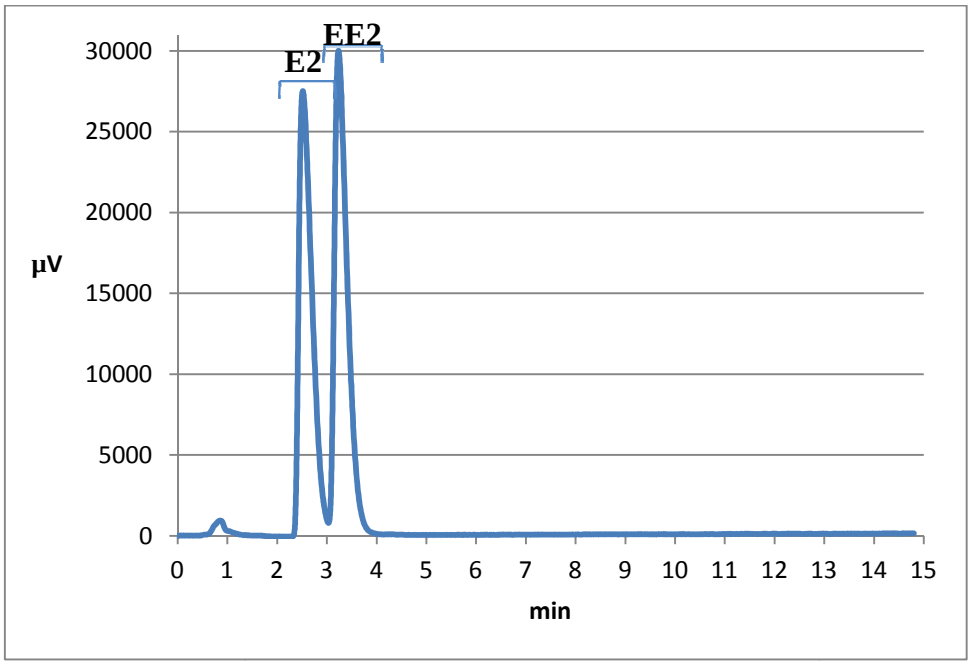

Figure 13- Chromatogram of E2 and EE2 (0.5ppm) in 50% (v/v) ACN and a flow rate of 0.5ml/min.

By increasing the flow rate, the peak separation was found to be better and the time of chromatographic analysis was lower, thus getting lower retention times. After analyzing these chromatograms obtained at different flow rates, it was decided to set the flow rate at 0.4 ml/min as the most appropriate for achieving hormones separation. With this flow rate, the first compound, hormone E2, elutes after 3 minutes, and only before minute 4 it appeared the second hormone EE2.

The best separation conditions (best separation between peaks, Gaussian peaks) were therefore established, with the MP composition consisting of 50% (v/v) ACN and a flow rate of 0.4 ml/min, also was the method with lowest RTs.

3.3. Figures of merit for the quantification of E2 and EE2

For the quantification of E2 and EE2, two calibration curves were obtained from five standards of different concentrations between 5 ppb and 25 ppb: 5 ppb, 10 ppb, 15 ppb, 20 ppb and 25 ppb. These standards were prepared from a 1ppm stock solution, by diluting an appropriate amount in the MP (50% (v/v) ACN).

Each standard was injected three times over three days. It was necessary to repeat the process to see the reproducibility and repeatability of the acquired data. These chromatograms were obtained using the best separation conditions previously chosen: Kromasil 100 C18 (5µm, 15 x 0.21 cm) column, MP consisting of 50% (v/v) ACN, and a flow rate of 0.4 ml/min. A 7 minutes chromatography was enough, considering that the peaks appear from minute 3 to minute 5 with this method. Figure 14 shows the chromatograms of the E2 and EE2 standards, where it is possible to see an increase in the area of each peak proportionally to the standard concentration.

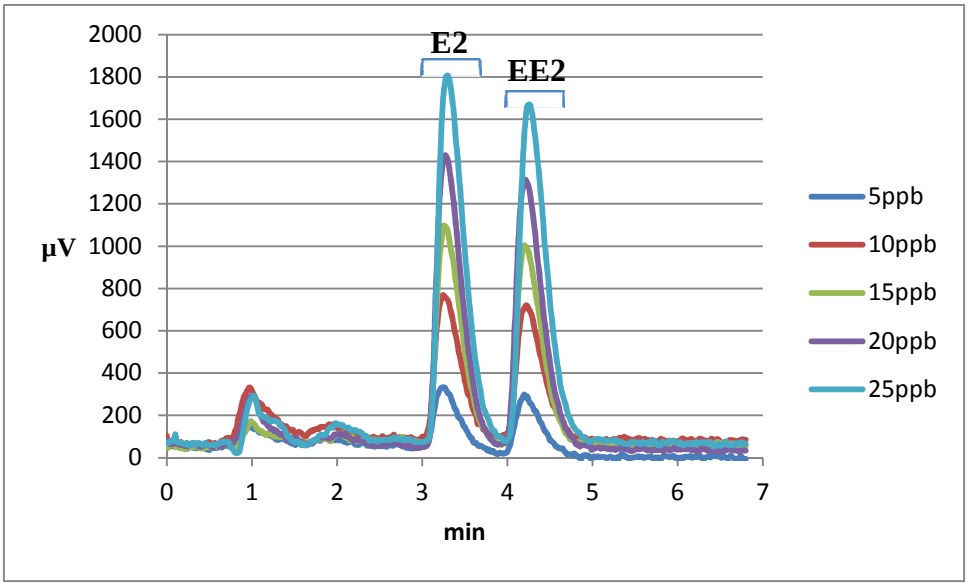

Figure 14- Chromatogram of the different standards in 50% (v/v) ACN and a flow rate of 0.4 ml/min.

A calibration curve for each hormone was calculated for each day of analysis, using the average values of the peak areas from 3 injections, where the peak area was obtained by integration. The average values of the areas were plotted against standard concentration for obtaining the calibration curve. First, it was assumed that straight line calibration graphs take the algebraic form defined as shown in Equation (1) (*Miller & Miller*, 2000):

$$y = a + bx \quad (1)$$

where *b* is the slope of the line and *a* is the intercept on the *y*-axis. Applying this Equation (1), and analyzing the data displayed in Table 1 and Table 2 below, it was possible to apply Equation (2) because the confidence interval of intercept includes the zero.

$$y = bx \quad (2)$$

When the calibration curve passes through the origin, the value 0 is within the interval between the "lower 95%" and "upper 95%" values as in this present study. Therefore, it was assumed that the curve cross the origin, and Equation (2) was applied to calculate the figures of merit for each hormone.

Table 1- Data analysis of the calibration curve of compound EE2.

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	88.37	433.60	0.20	0.85	-1291.55	1468.29
X Variable 1	1339.35	26.15	51.22	1.64E-05	1256.14	1422.56

Table 2- Data analysis of the calibration curve of compound E2.

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-96.26	508.77	-0.19	0.86	-1715.39	1522.88
X Variable 1	1431.08	30.68	46.64	2.17E-05	1333.44	1528.72

The calibration curves obtained for each compound are shown in Figure 15 and Figure 16. Figure 15 shows the calibration curve obtained for EE2 and Table 3 shows the statistical data obtained for this calibration curve. On the other hand, Figure 16 shows the calibration curve obtained for E2 and Table 4 summarizes the statistical data obtained for this calibration curve. The obtained calibration curves have a good regression coefficient (R^2), apart from the calibration curve we obtain a low standard deviation (STD) as it is shows in these figures (Figure 15,Figure 16).

Table 3- Data analysis of the calibration curve of compound EE2

Regression Statistics	
Multiple R	0.9999
R Square	0.9998
Adjusted R Square	0.7498
Standard Error (S_E)	360.5057
Observations	5

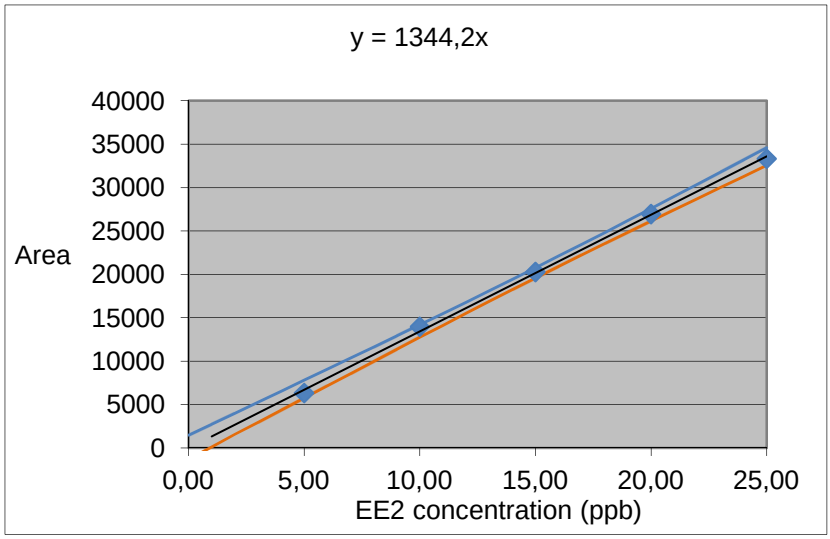

Figure 15- Calibration curve obtained for the compound EE2 on the second day (Orange and blue lines are the STD of the calibration curve).

Table 4- Data analysis of the calibration curve of the compound E2.

Regression Statistics	
Multiple R	0.9999
R Square	0.9997
Adjusted R Square	0.7497
Standard Error (S_E)	422.6015
Observations	5

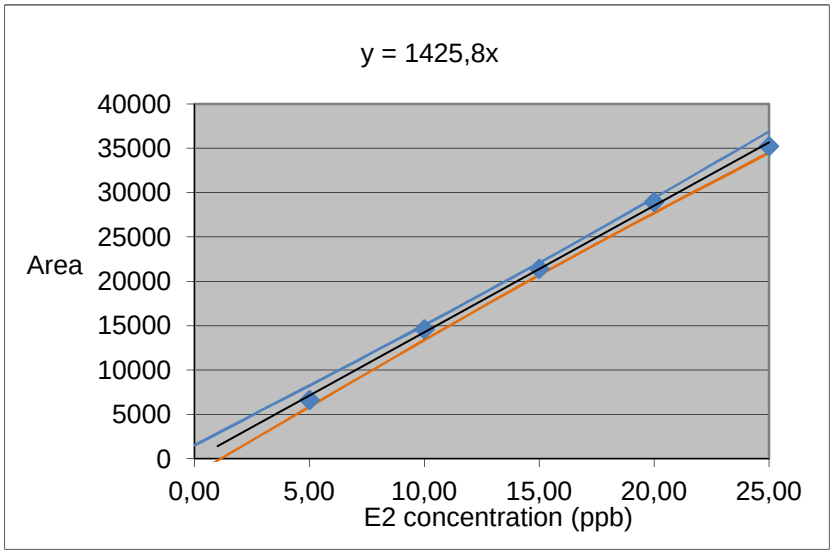

Figure 16- Calibration curve obtained for the compound E2 on the second day (Orange and blue lines are the STD calibration curve).

For each hormone, the calibration curve did not vary much from one day to another, as shown by the slope values in Table 5 and Table 6. The average value of the slope as well as the standard deviation and relative standard deviation are presented in these tables.

Table 5- Slope values obtained for EE2 in the three days of calibration.

	Slope	**Slope mean value**	**STD**	**RSD**
1st Day	1354.99			
2nd Day	1344.17	1344.30	10.63	0.79
3rd Day	1333.73			

Table 6- Slope values obtained for E2 in the three days of calibration.

	Slope	**Slope mean value**	**STD**	**RSD**
1st Day	1431.96			
2nd Day	1425.83	1421.53	13.12	0.92
3rd Day	1406.80			

The retention time of each hormone is another value we got from the chromatograms, which are summarized in Table 7 and Table 8 for the hormone EE2 and hormone E2, respectively. Each table also shows the average of the retention times obtained for each standard and the standard deviation. As seen, the chromatographic peaks appear at similar retention times for the different injections, which is supported by the low value of the RSD. This feature helps to identify each hormone when analyzing the samples. As such, EE2 is the first compound appearing in the chromatogram with an average retention time of 3.12 minutes, while E2 appears at a retention time of 3.99 minutes.

Table 7- Retention times (RT) obtained for EE2 during the three days of analysis.

EE2 concentration	RT of EE2 (min)			RT mean value of EE2 (min)	STD (min)	RSD (%)
	1st Day	2nd Day	3rd Day			
5ppb	4.19	3.93	3.88	3.99	0.17	4.23
10ppb	4.22	3.86	3.91			
15ppb	4.23	3.86	3.93			
20ppb	4.23	3.86	3.86			
25ppb	4.20	3.81	3.86			

Table 8- Retention times (RT) obtained for E2 during the three days of analysis.

E2 concentration	RT of E2 (min)			RT mean value of E2 (min)	STD (min)	RSD (%)
	1st Day	2nd Day	3rd Day			
5ppb	3.24	3.09	3.06	3.12	0.10	3.38
10ppb	3.26	3.03	3.07			
15ppb	3.27	3.03	3.11			
20ppb	3.28	3.03	3.04			
25ppb	3.25	3.00	3.04			

The limits of detection and quantification were calculated for each compound. The limit of detection (LOD) is the concentration which gives an instrumentation signal significantly different from the blank or background signal, being calculated with the following equation:

$$LOD = 3.S_E/Slope \quad (3)$$

The LOD is calculated as three times the value of the standard error (S_E) divided by the slope value. The values of S_E and slope were obtained from the statistical data analysis of the calibration curve. Moreover, the limit of quantification (LOQ) is the concentration at which quantitative results can be reported with a high degree of confidence and was calculated with Equation (4). LOQ is ten times S_E divided by the slope value. The values of LOD and LOQ obtained for EE2 and E2 are in Table 9 and Table 10, respectively.

$$LOQ = 10.\ S_E/Slope \quad (4)$$

For EE2, the obtained LOD were between 0.81ppb and 0.97ppb, while the LOQ were between 2.68ppb and 3.26ppb. In the case of E2, the obtained LOD were between 0.89ppb and 1.33ppb, while the LOQ were between 2.96ppb and 4.44ppb.

Table 9- Limits of detection and quantification obtained for EE2 on the three days of analysis.

EE2	**Limit of Detection (LOD) (ppb)**	**Limit of Quantification (LOQ) (ppb)**
1st day	0.97	3.23
2nd day	0.81	2.68
3rd day	0.85	2.83

Table 10- Limits of detection and quantification obtained for E2 on the three days of analysis.

E2	**Limit of Detection (LOD) (ppb)**	**Limit of Quantification (LOQ) (ppb)**
1st day	1.06	3.53
2nd day	0.89	2.96
3rd day	1.33	4.44

3.4. Contamination problems

3.4.1. Flasks contamination

The problems with contamination began when injecting the standards for obtaining the calibration curve. The first standard injected was that of 5 ppb concentration, followed by that of 25 ppb concentration. The chromatogram of the 25 ppb standard exhibit unknown peaks that despite not interfere with the peaks of interest, but may cause some analytical problems by the proximity (Figure 17). This situation was not verified for the 5 ppb standard, and it was assumed that the 25 ppb standard was contaminated. A new standard of 25 ppb was prepared, but when this new standard was injected, the contamination reappeared.

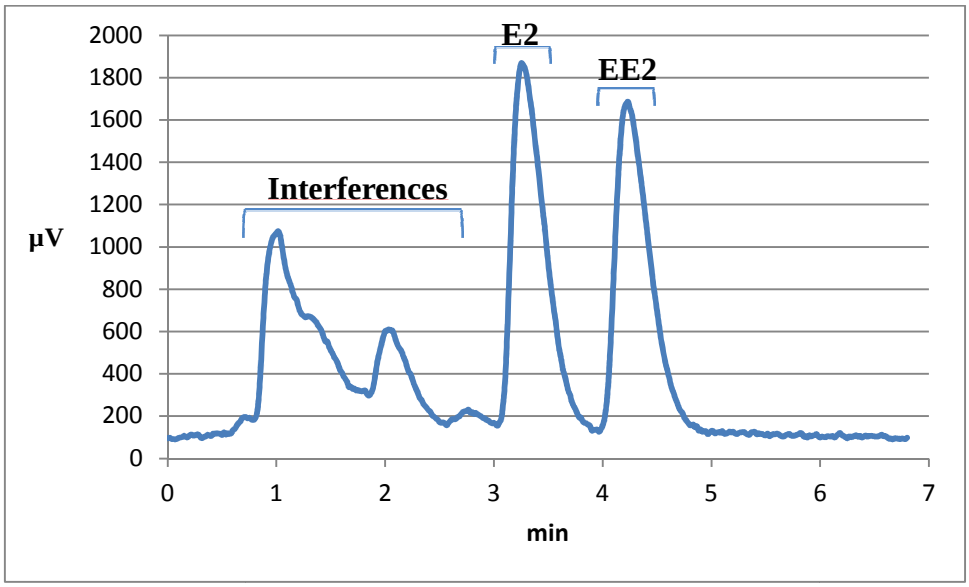

Figure 17- Chromatograms of E2 and EE2 (25ppb), using MP consisting of 50% (v/v) ACN and a constant flow rate of 0.4ml/min.

Afterwards, another EE2 and E2 standard was injected to check if these interferences are only verified for the 25 ppb standard or if it is a problem common to all standards. The 15 ppb standard was therefore analyzed, and the obtained chromatogram

also exhibits a similar interfering problem that overlaps the peaks of E2 and EE2, as seen in Figure 18.

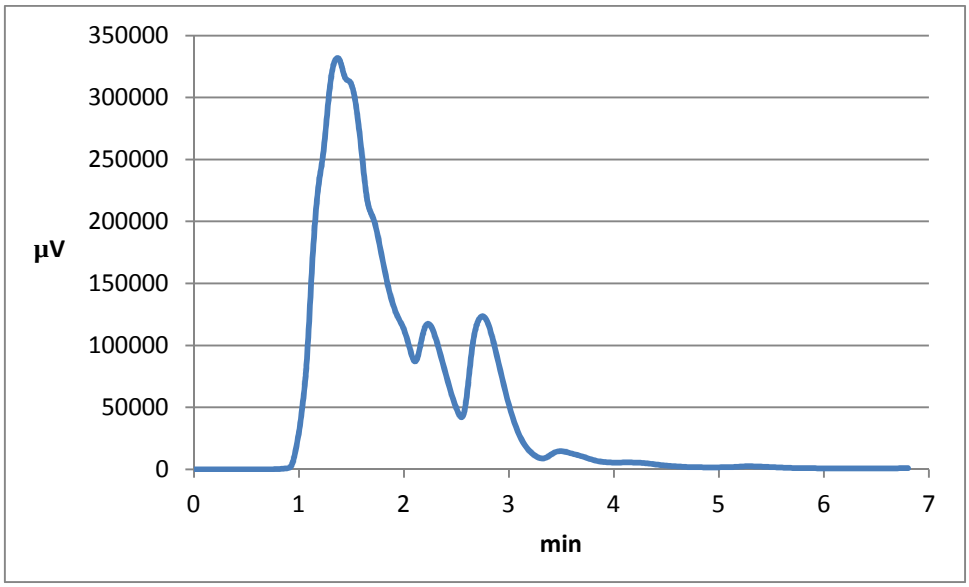

Figure 18- Chromatogram of E2 and EE2 (15ppb) showing an interfering peak, obtained with a MP consisting of 50% (v/v) ACN and a constant flow rate of 0.4ml/min.

Being a general contamination problem, it could originate from any material or product that had been used in standard preparation. First, the MP was checked, getting the chromatogram of Figure 19. This chromatogram does not show the huge signal that appears in the previous chromatograms of the standards. This results suggests that the contamination does not come from the MP. As the standards solutions contains MP and stock solution of 1ppm, it was necessary to check whether or not the stock solution was contaminated. For this, the stock solution of 1ppm was injected, and as seen in the chromatogram of Figure 20, the stock solution was free from contaminations.

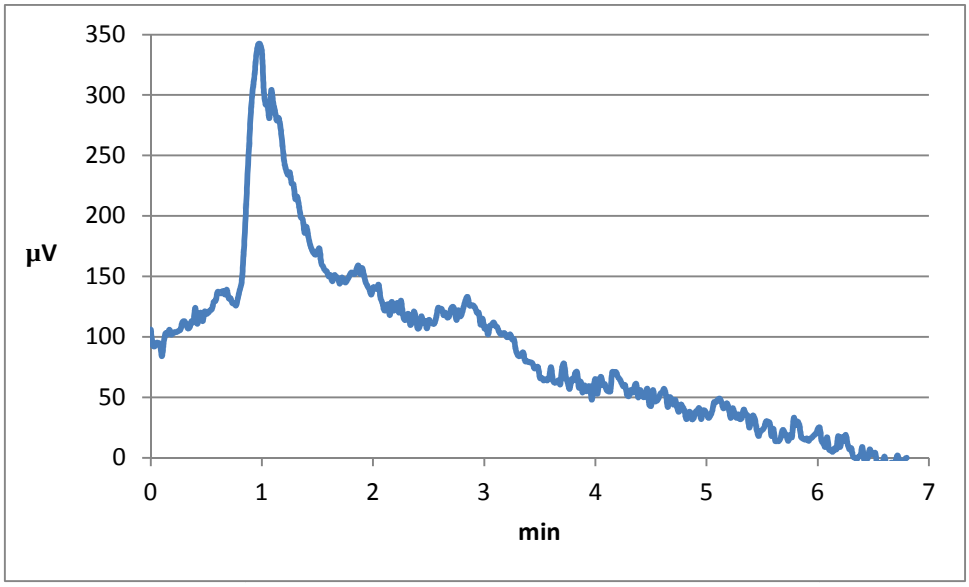

Figure 19- Chromatogram of the MP consisting of 50% (v/v) ACN, and acquired at a constant flow rate of 0.4ml/min.

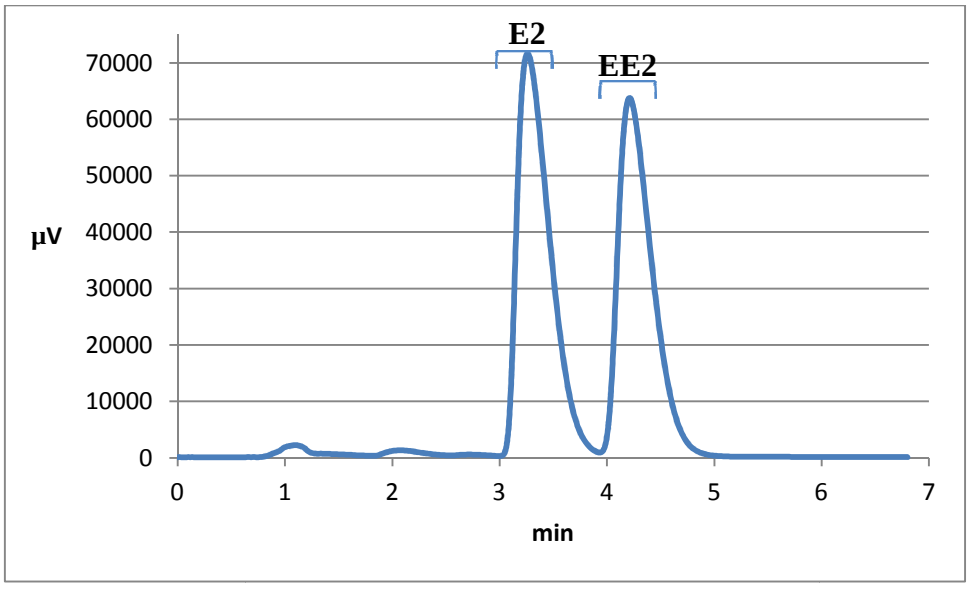

Figure 20- Chromatogram of E2 and EE2 (1ppm stock solution) using a MP consisting of 50% (v/v) ACN, and acquired at a constant flow rate of 0.4ml/min.

Since the contamination did not come from the standard stock solution, the material used in the laboratory for this study had to be analyzed. The first one to be analyzed was the 5ml micro reaction vessels used to prepare the standards. Therefore, the MP was

transferred into these flasks and analyzed and the chromatograms obtained is shown in Figure 21, meaning that interferences came from these flasks. Considering these results it was decided to test different flasks (4ml). Therefore, two flasks of 4ml were cleaned by different procedures: one was washed with NaOH (1M) and HCl (25%), whereas the other was washed only with NaOH. Figure 22 and Figure 23 show the chromatograms of the MP after being stored in these two flasks.

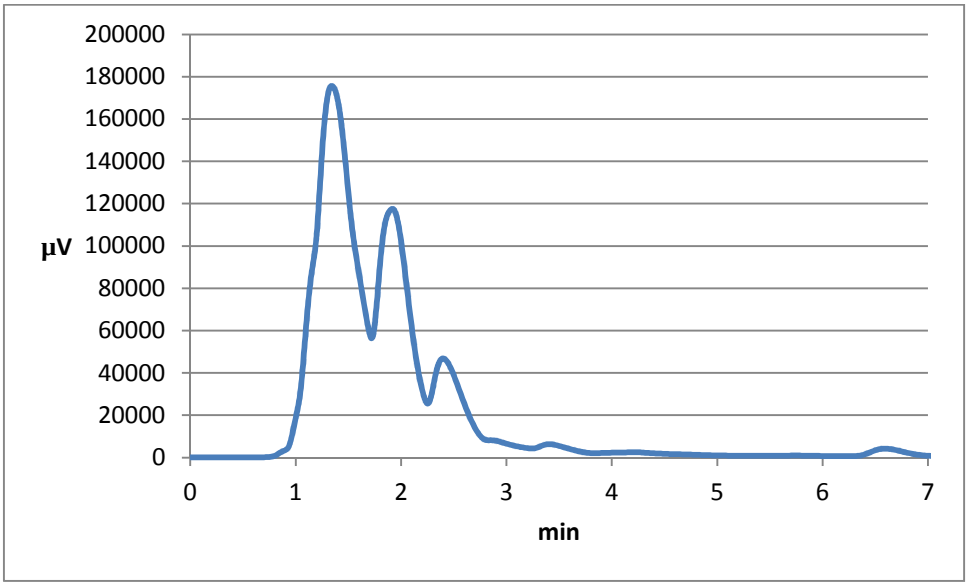

Figure 21- Chromatogram of the MP after being stored in a 5 ml micro reaction vessels.

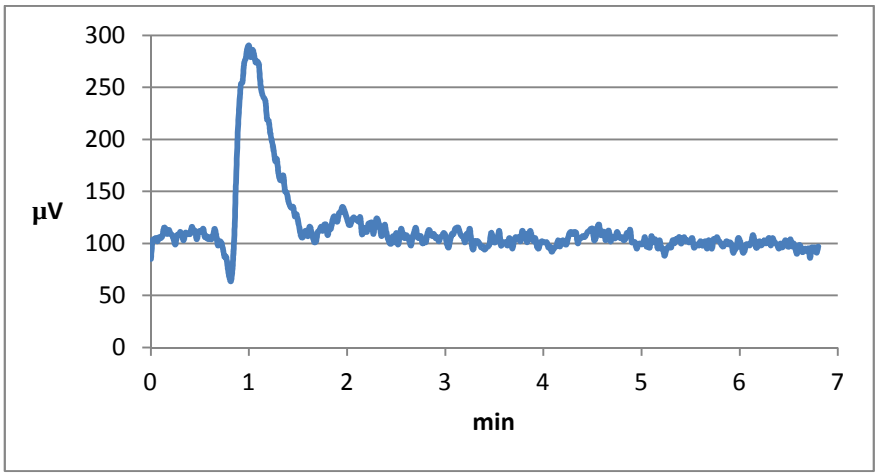

Figure 22- Chromatogram of the MP after being stored in a 4ml flask washed with NaOH (1M)

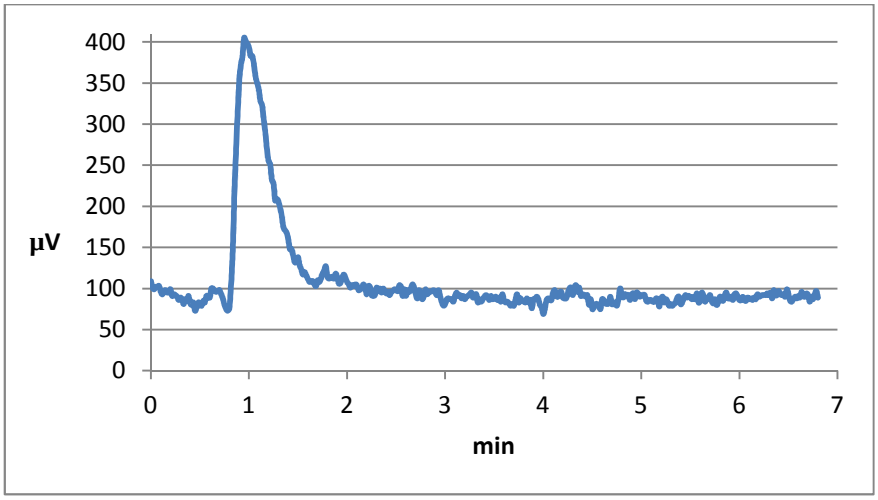

Figure 23- Chromatogram of the MP after being stored in a 4ml flask washed with NaOH (1M) and HCl (25%).

The chromatogram of the solution stored in the new flasks exhibit a signal at a retention time near 1 minute, which do not interfere with the signals of the hormones. Although it could be concluded that the contamination came from the flasks, the chromatogram of the solution stored in those flasks that were not washed with acid exhibit a similar profile, which hinders any conclusion about the source of the contamination.

However, it was further observed a contamination in some of the 4 ml flaks that interfere with the compounds of interest as shown in Figure 24. Considering this, it was decided to clean all the flask with ACN and MP flask after being washed with NaOH and acid. Several flasks were tested and for this, 1ml of the MP was transferred into the flasks and stored before being injected. An example of a chromatogram obtained is illustrated in Figure 25. As can be observed in the chromatogram, the contamination decreased using this procedure.

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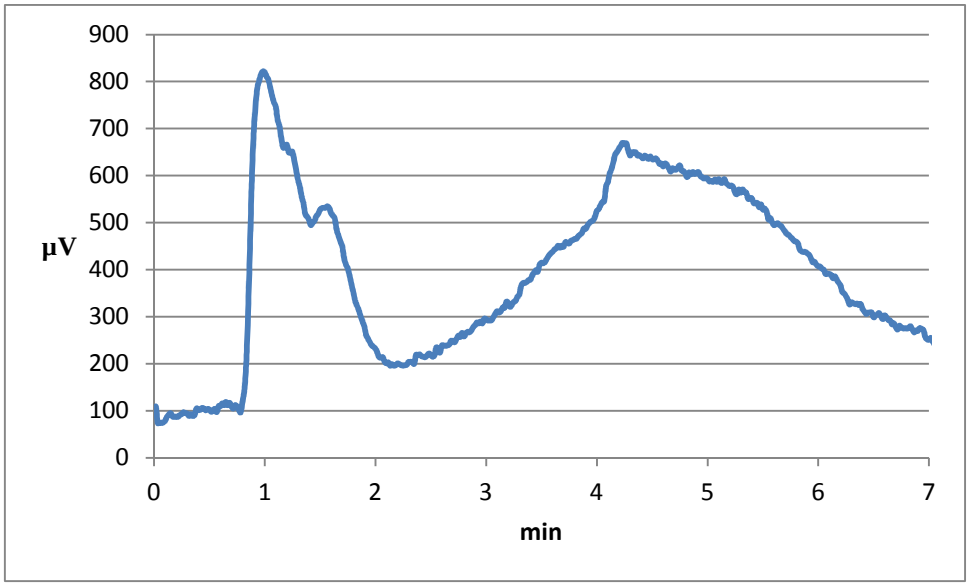

Figure 24- Chromatogram of the MP after being stored in a 4ml flask with some interferences.

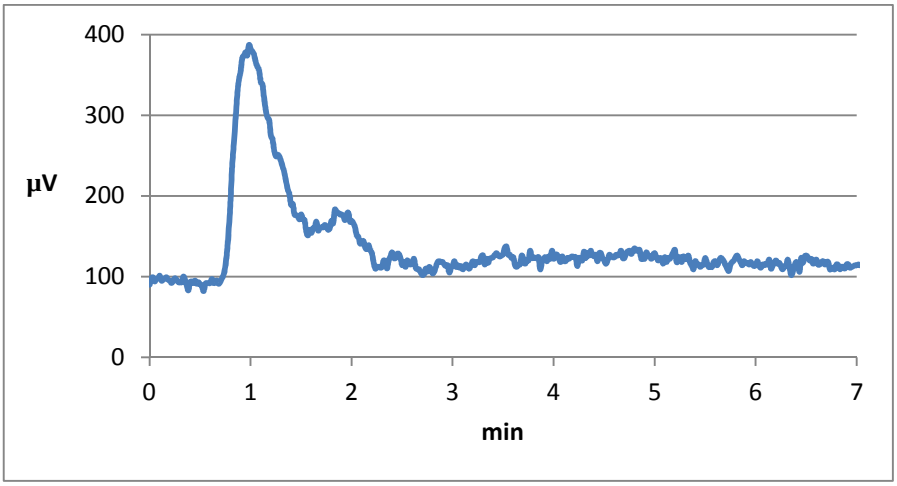

Figure 25- Chromatogram of the MP after being stored in a 4ml flask washed with acid, ACN and the MP.

Washing the flasks with ACN followed by the MP, decrease drastically the signal of the potential interferences in the chromatogram. This result suggests that all the material has to be washed with ACN and MP after the first washing process. In the Figure 26, the chromatograms of EE2 and E2 (25ppb) exhibiting different levels of interference are compared; the interfering signal does not disappear completely for the new standard, but do not overlap the signal of the hormones.

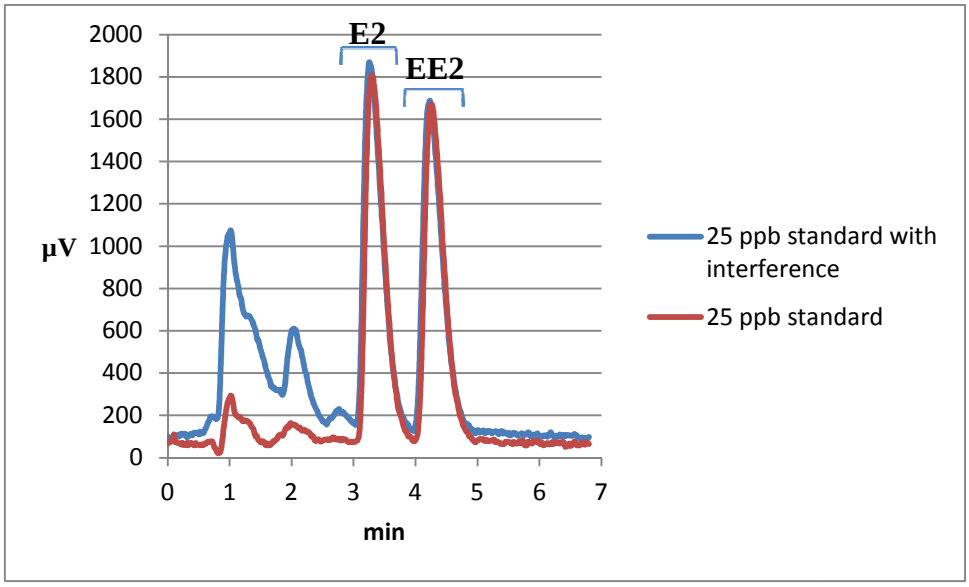

Figure 26- Chromatograms of E2 and EE2 (25ppb) with interference and without interference, using MP consisting of 50% (v/v) ACN and a constant flow rate of 0.4ml/min.

3.4.2. Contamination problems in the extraction procedure

Once the calibration curves were obtained, the replicates of the oyster sample were analyzed, and a new problem of contamination emerged. In light of these results, it was decided to make recovery studies of the SPE procedure.

Two spike (SPK) solutions were prepared with a concentration of 25 ppb for both EE2 and E2, and each solution was injected twice. The obtained chromatograms are illustrated in Figure 27 and Figure 28. As it can be seen in these chromatograms, the intensity of the peak of the hormone E2, which has the lower retention time, is higher than that of the 25ppb standard and it might overlap the EE2 peak. These results suggest that the SPK solutions could also be subjected to contamination problems. This situation affects mostly the signal of hormone E2, which explains why the percentages of recovery for E2 are much higher than 100%. On the other hand, the percentages of recovery for EE2 are in the range of 95-110%, as shown in Table 11.

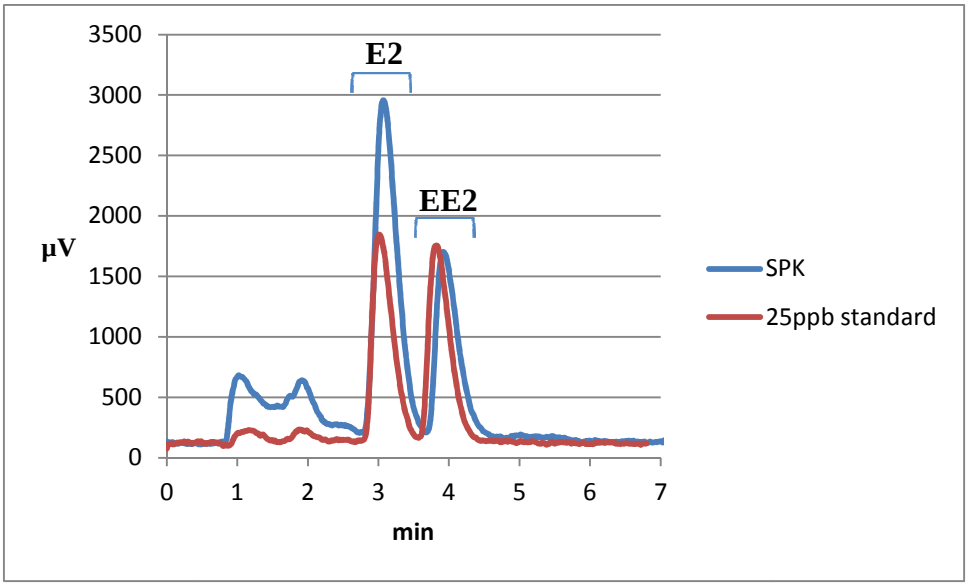

Figure 27- Chromatograms of the first SPK solution (25ppb) in comparison with a chromatogram of EE2 and E2 standard (25ppb), using a MP consisting of 50% (v/v) ACN and acquired at a constant flow rate of 0.4ml/min.

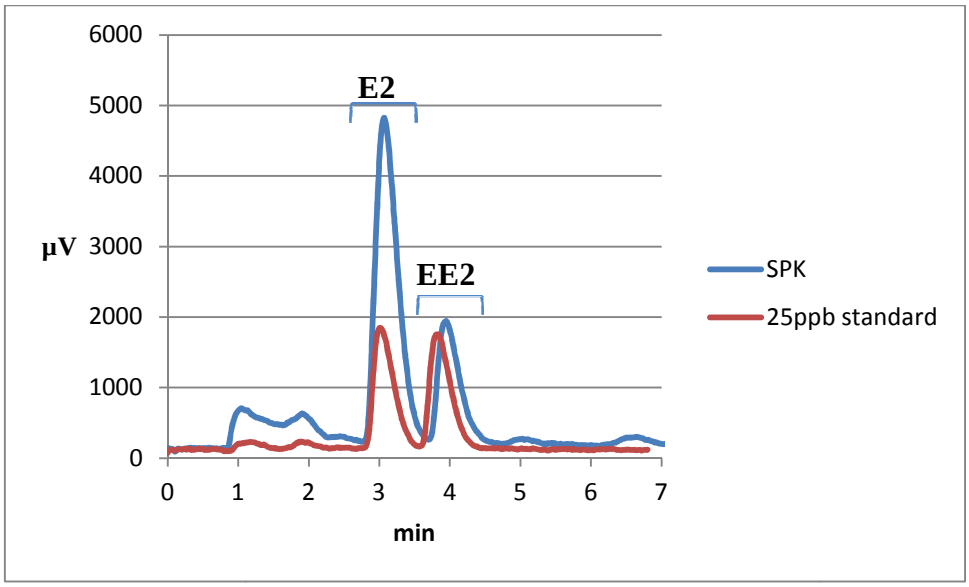

Figure 28- Chromatogram of the second SPK solution (25ppb) in comparison with a chromatogram of EE2 and E2 standard (25ppb), using a MP consisting of 50% (v/v) ACN and acquired at a constant flow rate of 0.4ml/min.

Table 11- Percentages of recoveries for E2 and EE2 in the SPK solutions.

	E2 recovery (%)	EE2 recovery (%)
SPK-A_R1	173	96
SPK-A_R2	173	95
SPK-B_R1	292	110
SPK-B_R2	285	109

All the material used in this study was analyzed for assessing the presence of sources of contamination. For this, the procedures of solid-liquid extraction and SPE were assessed using blank samples.

The blanks of the solid-liquid extraction part were analyzed after transferring 20 ml of MeOH to a centrifuge tube, shake it with a vortex for 1minute, and transferred into a round bottom flask. All this process was repeated twice, storing 40ml of MeOH in the round bottom flask. Then, the MeOH was evaporated until dryness, re-dissolved with 2ml of MP and injected after being filtered. Figure 29 shows the obtained chromatogram, and it can be concluded that the extraction procedure originate signals that interfere with the signals of the compounds.

To prepare blanks of the SPE procedure, the cartridges were rinsed and conditioned with 10 ml MeOH and 10 ml ultrapure water before the addition of the sample, in this case 10ml of ultrapure water. The cartridges were then rinsed with 5 ml 5% (v/v) MeOH, and the samples were eluted in 4 ml MeOH under a gentle vacuum. The sample was then reduced to dryness and re-dissolved in 2 ml of MP. The solution was injected after being filtered with a glass syringe. The SPE procedure also originates signals that interfere with the signals of the compounds, as shown in Figure 30.

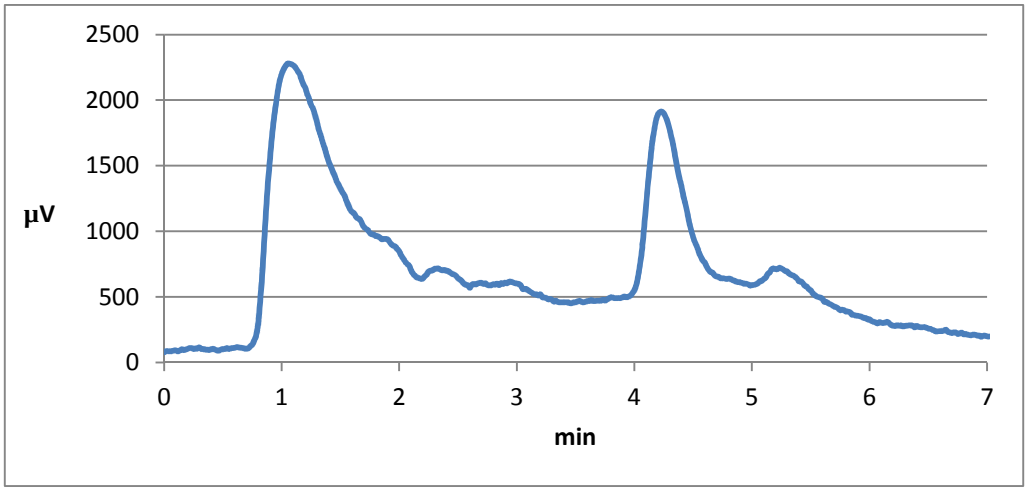

Figure 29- Chromatogram of a blank sample of the solid-liquid extraction procedure.

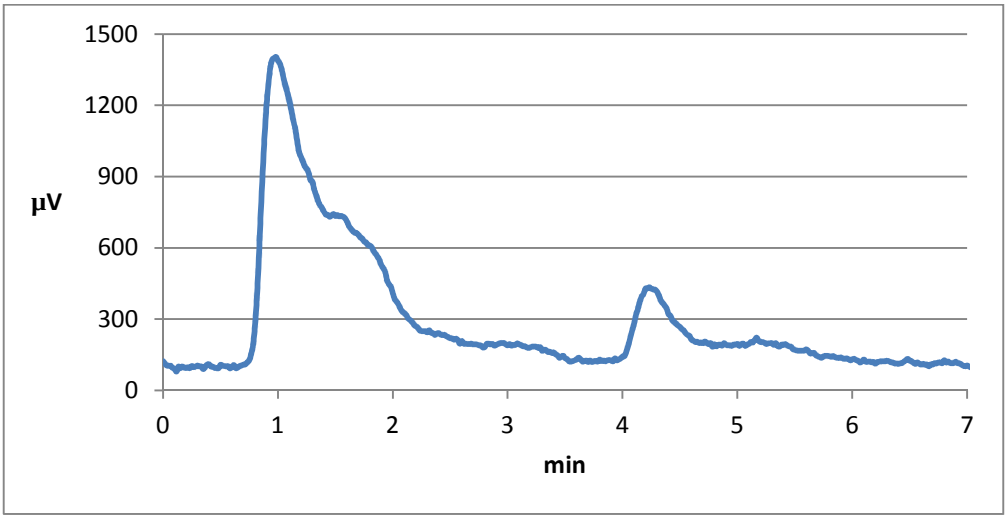

Figure 30- Chromatogram of a blank sample of the SPE procedure.

Both solid-liquid extraction and SPE procedures originate contamination signals. In the SPE, the cartridges are likely to be responsible for originating such signals. The cartridges used were made of plastic, so the contamination could come from this material; such cartridges were used because those of glass are much more expensive. In the solid-liquid extraction procedure, the contamination could originate from the centrifuge tube or from the round bottom flask, and therefore these materials were tested separately. To test the centrifuge tube, 5ml of MeOH were transferred into the tube, shaken in a Vortex for 1 minute, and then transferred to a flask and dried. The final residue was dissolved with 2ml

of MP and injected after being filtered. As shown in Figure 31, the centrifuge tubes did not give any contamination signal; the contamination did not come from this material. These centrifuge tubes were made of Teflon, so it was unlikely to be the cause of contamination.

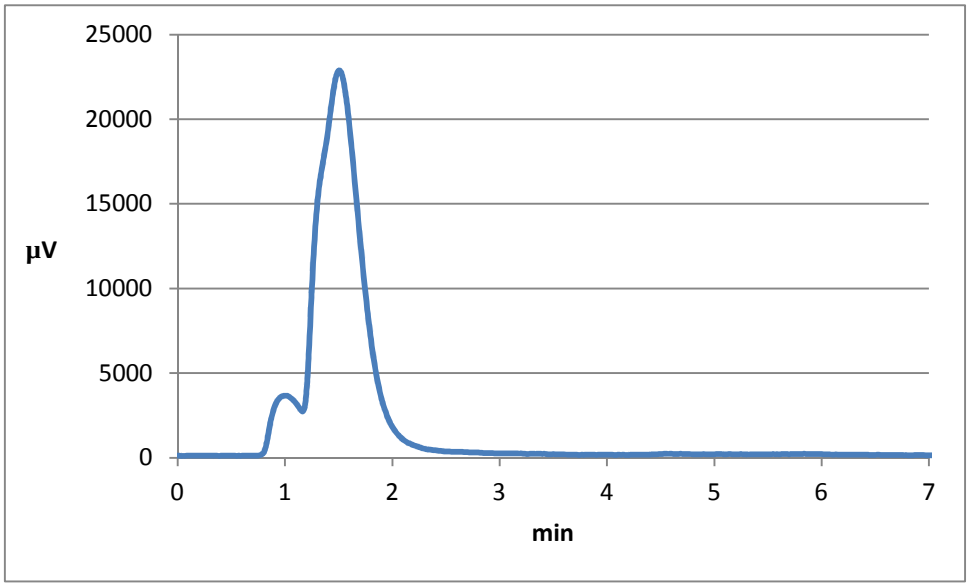

Figure 31- Chromatogram of a blank sample of the centrifuge tube using a MP consisting of 50% (v/v) ACN and acquired at a constant flow rate of 0.4ml/min.

The next step entailed testing the round bottom flasks. For this, 40ml of MeOH were transferred directly into the round bottom flask, and evaporated until dryness. The final residue was dissolved with 2ml of MP and filtered before being injected. As shown in the chromatogram of Figure 32, the round bottom flasks originated signals which interfere with both E2 and EE2. Afterwards, new round bottom flasks were tested, after being appropriately washed. These blank samples were treated in the same way as above described. With the new round bottom flasks, the contamination still appears (Figure 33).

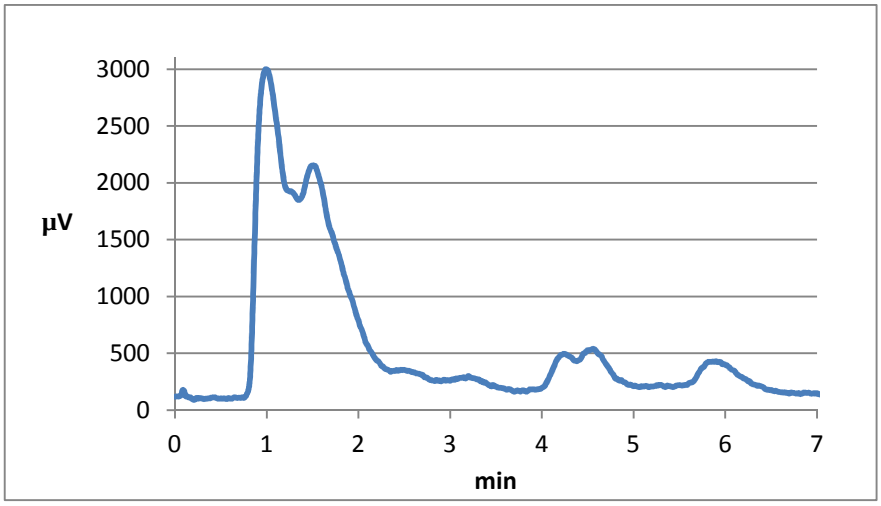

Figure 32- Chromatogram of a blank sample of a round bottom flask, using a MP consisting of 50% (v/v) ACN and acquired at a constant flow rate of 0.4ml/min.

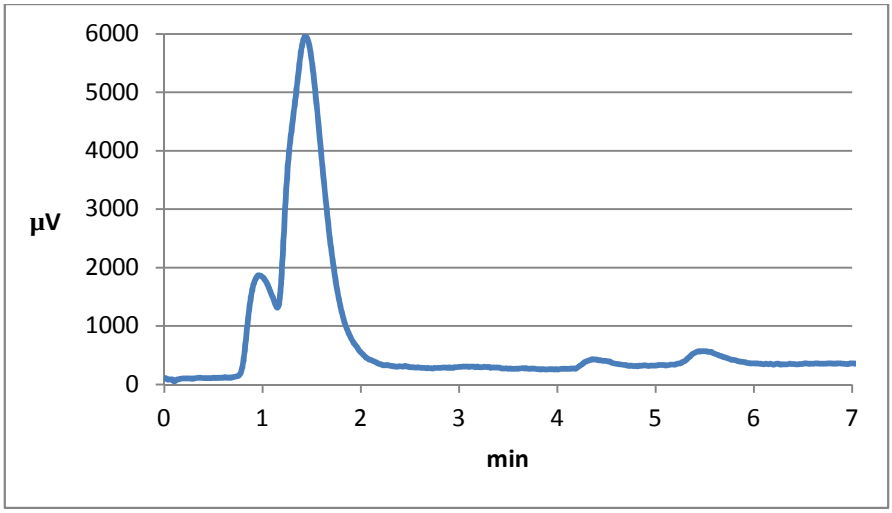

Figure 33- Chromatogram of a blank sample of a new round bottom flask, using a MP consisting of 50% (v/v) ACN and acquired at a constant flow rate of 0.4ml/min.

As MeOH was used in this process, these new round bottom flasks were cleaned using complete washing process, plus a final step with MeOH. However, with this procedure, the intensity of the interference increases even further, as shown in Figure 34. This result suggests that MeOH could actually be the source of contamination, thus pointing to the fact that future efforts should be devoted to the use of fluorescence grade MeOH.

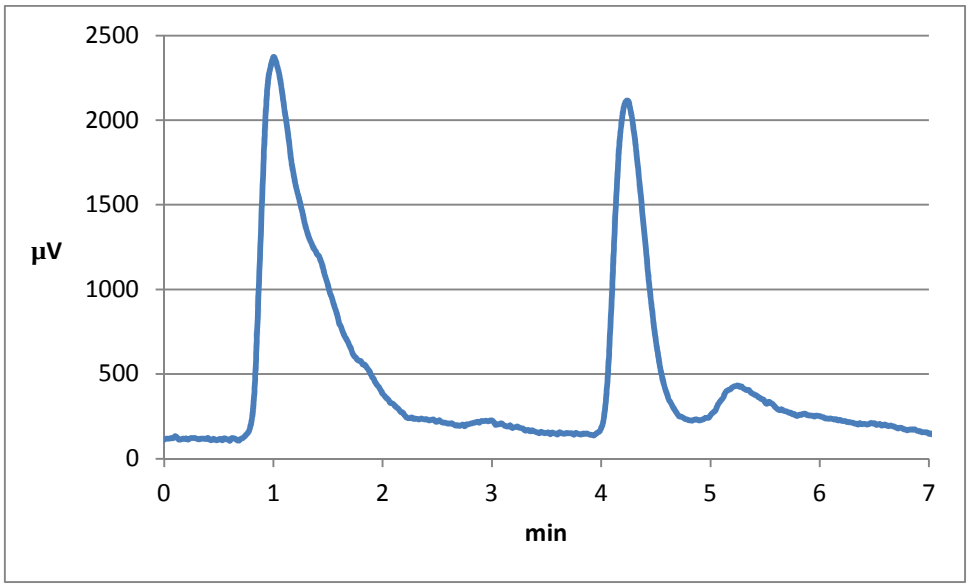

Figure 34- Chromatogram of a blank sample using a new round bottom flask after being washed with MeOH. The MP consists of 50% (v/v) ACN and the flow rate was 0.4ml/min.

3.5. Recovery studies of the extraction procedure

SPK solutions were prepared using E2 and EE2 standards with a concentration of 10 ppb; they were designated as blank SPK. Three blank SPK solutions were prepared and each solution was injected three times. As shown in Figure 35, only the peak of E2 could be integrated to obtain the corresponding area for further estimative of its concentration. The percentage of recoveries of E2 obtained for the different replicates of SPK solutions are shown in Table 12; the recoveries were between 96% and 103%.

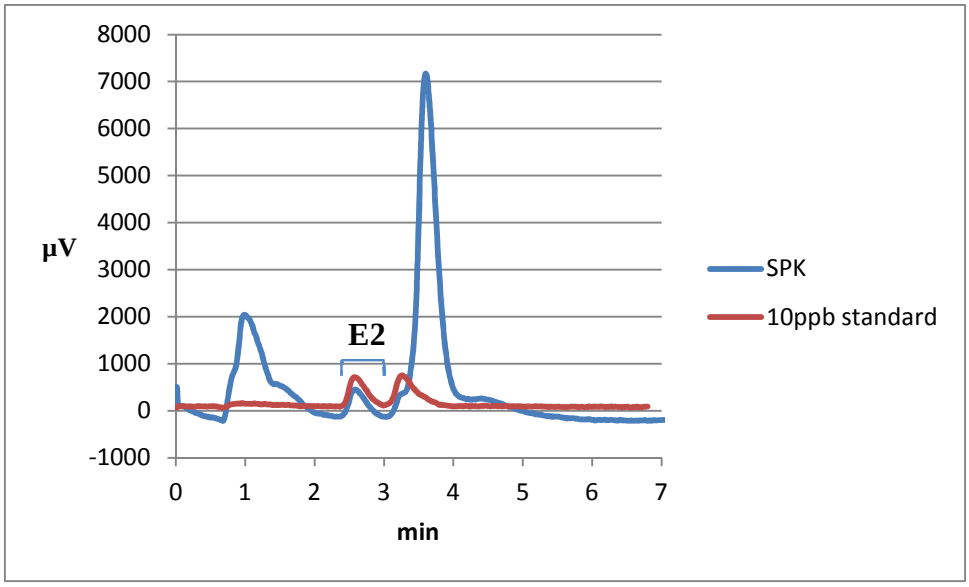

Figure 35- Chromatogram of a SPK solution (10ppb) in comparison with a chromatogram of EE2 and E2 pattern (10ppb), in 50% acetonitrile and 50% water mobile phase at a constant flow rate of 0.4ml/min.

Table 12- Percentage of recoveries obtained for E2 in the analysis of the SPK solutions (10ppb standard).

	E2 recovery (%)	STD	RSD
SPK 1	96.2	11.4	11.8
SPK 2	103	9.02	8.78
SPK 3	98.5	16.3	16.6

3.6. Sample extraction

The sample treatment was based on an article published by *Ronan and McHugh* (2013) and on the application note (*Zhai & Zou*, 2009). The treatment consists in two parts, the solid-liquid extraction of the hormones from the oyster tissues and the (SPE) purification. Three blank samples together with a bivalve sample collected at Aveiro (2 replicates) were extracted using the procedure described in *Materials and methods* section, and analyzed under the HPLC conditions previous developed. The chromatogram obtained for one of the blank samples is shown in Figure 36, while Figure 37 shows the chromatogram obtained for the oyster sample and that of a 5ppb standard for comparison.

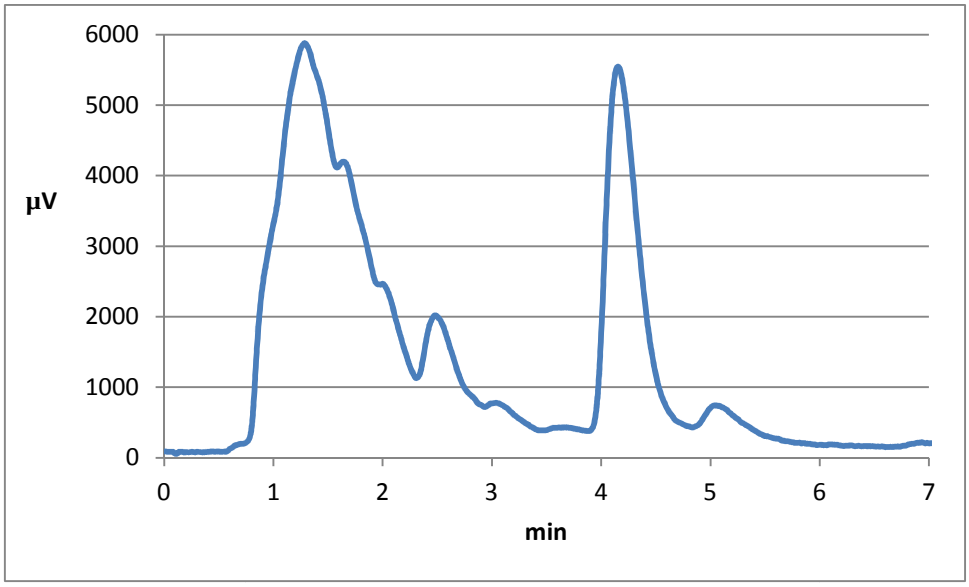

Figure 36- Chromatogram of the blank sample obtained with a MP consisting of 50% (v/v) ACN at a constant flow rate of 0.4ml/min.

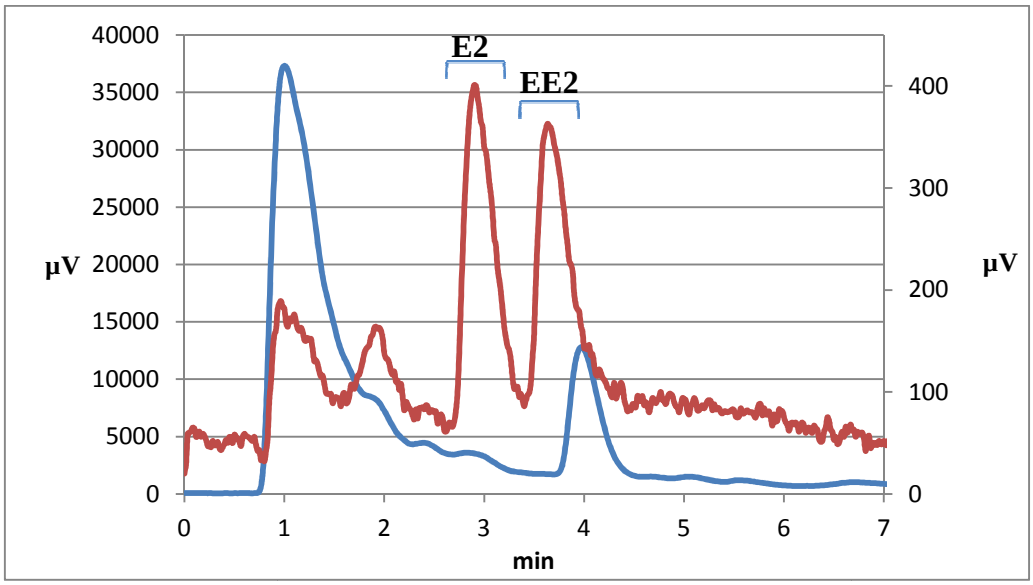

Figure 37- Chromatogram of the oyster sample and of the 5ppb standard obtained with a MP consisting of 50% (v/v) ACN at a constant flow rate of 0.4ml/min.

It was expected that the chromatogram of the blank sample, in Figure 36, should be free from any type of signal, but the chromatogram reveal many unknown peaks. These peaks are likely to interfere with the chromatographic peaks of E2 and EE2, as these elute

between 3 and 5 minutes. For the oyster sample (Figure 37), there are also several of unknown peaks that interfere with those of E2 and EE2, preventing their quantification in the sample. also shows the chromatogram of the 5ppb standard in order to assist in the identification of both compounds in the chromatogram of the oyster sample. The chromatographic peak of the E2 can be identified in the chromatogram of the sample but it is partially overlapped by unknown peaks, while the peak of EE2 is totally overlapped. This interference can originate from a contamination problem, being therefore extremely important to find the source of contamination in order to eliminate or reduce this problem, so it does not interfere with the quantification of both E2 and EE2.

After these analyses, and to prevent any damage of the HPLC analytical column, a pre-column was added into the HPLC instrumentation, which increased the backpressure of the analytical column and also the retention times of the compounds. After adding the pre-column, the sample was re-analyzed (Figure 38). In this case, the EE2 was also completely overlapped and it was impossible to identify and quantify this hormone in the sample. In the case of E2, it was possible to identify and integrate the chromatographic peak, which allowed the estimative of the concentration of this hormone in the oyster samples from Aveiro. The average concentration obtained for the oyster samples was 24.59 ng per gram of sample (Table 13). To ensure that the identified peak really corresponds to the E2 hormone, a 15ppb standard was added to the sample extract (Figure 39). By increasing the concentration of E2, the chromatographic peak intensity also increases, thus confirming that the identified peak really belongs to the E2 hormone.

Table 13- Concentrations of E2 obtained in the oyster samples from Aveiro.

Sample	Concentration of E2 (ng/g)	Average concentration of E2(ng/g)	STD	RSD
AV-A	22.11	24.59	3.51	14.29
AV-B	27.08			

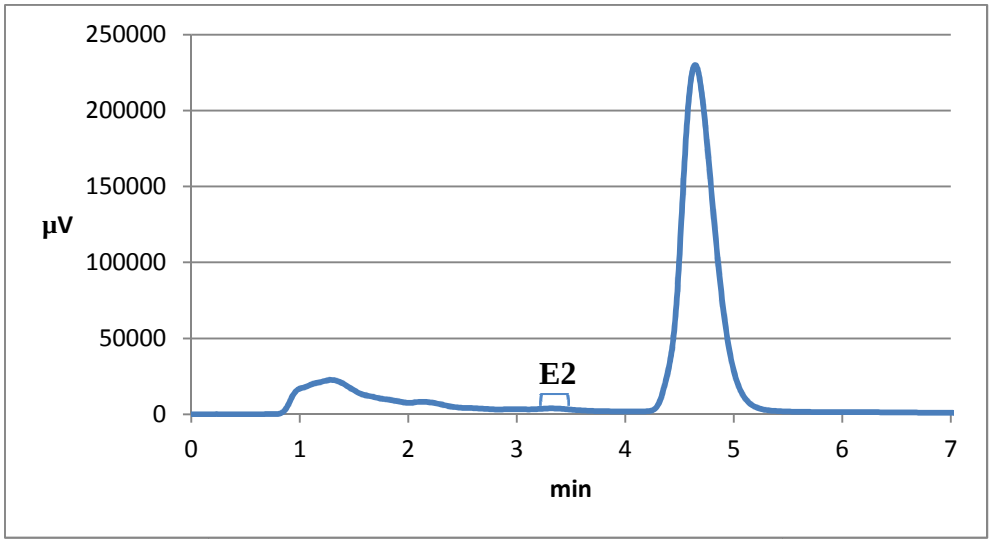

Figure 38- Chromatogram of the oyster sample from Aveiro obtained with a MP consisting of 50% (v/v) ACN at a constant flow rate of 0.4ml/min, after adding a pre-column.

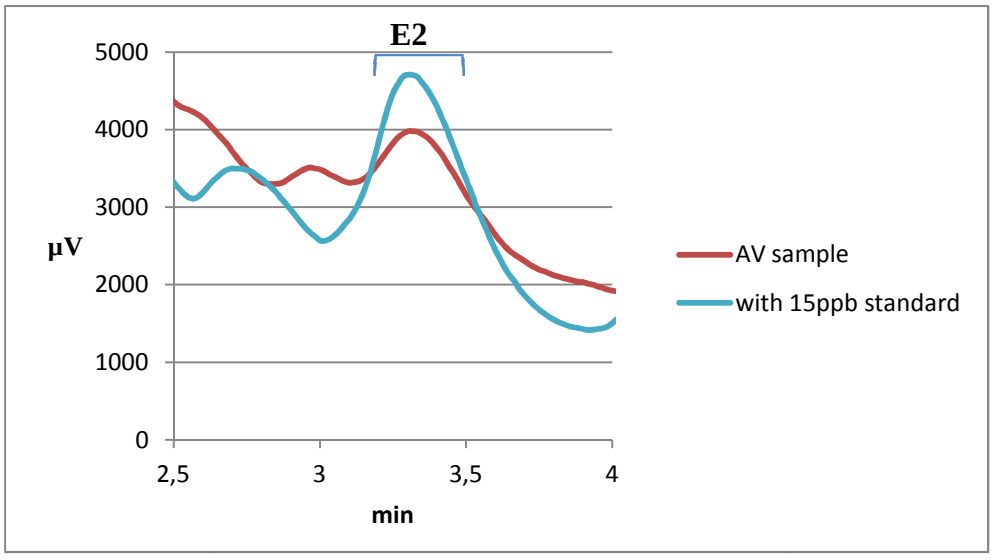

Figure 39- Comparison between the chromatogram of the oyster sample and a chromatogram of the oyster sample after the addition of 15 ppb standard. The MP consists of 50% (v/v) ACN and the flow rate was 0.4ml/min. The chromatograms were obtained after installing the pre-column.

4. Conclusions

In this research, an HPLC-FL system was used to analyze the hormones E2 and EE2 in oyster samples. The fluorescence detector is very sensitive, demanding a thoroughly cleaning procedure of all the material used for preparing the standards and the samples.

The problems with contamination decreased drastically after washing the material with ACN. After being washed with detergent (Derquim, alkaline) and NaOH, all the material had to be washed with ACN and MP. With this procedure, the contamination problems disappeared from all the material, except for the round bottom flasks and micro reaction vessels (5ml). When the round bottom flasks were used, the contamination signal interfered with the signal of EE2. This contamination could originate from the MeOH used in the extraction process. It should be checked whether or not the contamination comes from this organic solvent by using fluorescence grade MeOH.

The calibration curves obtained for both E2 and EE2 were good and the peaks appeared properly separated, thus enable integration of the peaks area. For the chromatograms of the samples extracts, it was not possible to integrate the peak of the EE2 due to the interference of unknown signals. However, for the hormone E2, it was possible to calculate its concentration in the oyster samples collected at Aveiro. Although we were able to estimate the concentration of E2, additional estimates should be performed to confirm the obtained value. Blank SPK solutions of 10ppb were analyzed, but this procedure should be applied to blank SPK solutions with other concentration levels. Also, oyster samples with addition of SPK solutions should also be analyzed.

In conclusion, the LOD calculated for the EE2 should not be considered a true value, because it was not possible to calculate the true concentration of EE2 in both the SPK solutions and oyster samples. The contamination that hinders a proper quantification of this hormone has to be eliminated or has to be reduced until the peak of EE2 is free from interferences. In this research, it was possible to quantify the concentration of E2 in the oyster samples collected in Aveiro using a HPLC-FL system and a MP consisting of 50%

(v/v) ACN at a constant flow rate of 0.4ml/min. The average concentration of E2 obtained for the oyster samples was 24.59 ng/g.

5. References

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