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Determination of extremely low concentrations estradiol in human plasma for optimization of breast cancer therapy - Liquid liquid extraction

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Furthermore I wish to thank M. Caillier who allowed me to do my internship in Netherland as well as Erasmus program.

Lastly, I offer my regards and blessings to all of those who supported me during the realization of the project.
Introduction

The determination of extremely low concentrations estradiol in human plasma or serum is required for optimization of breast cancer therapy in order to lower the death rate of this disease.

The goal of my project is to find a liquid liquid extraction method to extract estradiol from human plasma.

To start the aim is to determine which solvent is the more adapted for extract estradiol from plasma.

The second is to find which protocol I can use for extract estradiol from plasma.

Finally, I compare liquid liquid extraction and solid phase extraction (method which is used actually)

During this time, for analyze estradiol, I use HPLC system with UV detection. I must to find good characteristics to quantify estradiol at very low concentrations.
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I. **Avans University**

   a. **History**

      On January 1, 2004 Avans Hogeschool has emerged from union between Hogeschool Brabant and Hogeschool ’s-Hertogenbosch.

      Avans University of Applied Sciences is divided in 19 school located in three cities in the southern part of the Netherlands: Breda, ’s Hertogenbosch and Tilburg.

      The school has 18,000 students and there are 1800 employees.

   b. **Research group**

      During my training period I worked with research group called ASAMPLE. Different projects are studied such as the determination of low concentration of four sex hormones in iguana’s plasma or determination of low concentration of estradiol in human plasma.

      That being so, the research group has many skills as well as very good materials such as HPLC and LC-MS/MS system.

      These qualities enable at research group to work in collaboration with several companies such as animal centers or a hospital for my part and with SMEs regionally as pharmaceutical or chemical industries.

      Furthermore, the students can work together, in various ways such as final project, applied research or a work placement.

   c. **RAAK project**

      The RAAK project is a collaboration project between the clinical Chemistry department of the Amphia Hospital in Breda, several other hospitals spread over the country and the Avans Hogeschool in Breda, specifically the Lectorate Separation technique in the life sciences, a part of research group ASAMPLE.

      The goal of the RAAK project is the development and validation of an LC-MS/MS method for the determination of extremely low concentrations estradiol (<10pM) in human plasma or serum. These low detection limits are required for optimalization of breast cancer therapy.
My objective in this project consists to prepare the sample, in working on a liquid liquid extraction method for extract estradiol from human plasma and quantify at low concentrations.

II. About the project
   a. Estradiol

   It is this hormone that I must quantify after having extracted it from human plasma. This molecule gets a molar mass equal to 272.4 g/mol. It is a crystalline white solid.

   Estradiol is a prevailing sexual hormone in women, forming a part of estrogen’s family and therefore, its key role in human is to maintain fertility and to develop secondary sex characteristics.

   Estradiol is in part used in contraception and for treatment of deficit of estrogen in menopausal women.

   Dosage of this hormone is important for verify the functioning of ovaries in woman. In fact, it is used for exploration of amenorrhea or infertility. It contributes to diagnosis of menopausal status in woman and also for surveillance of medically assisted reproduction. Furthermore, estradiol dosage is used for help of detection of certain tumors.

   Estradiol is in part produced by ovaries. Like other steroids, it is a derived from cholesterol.
b. Breast cancer

Definition:

Cancer begins in cells. Normal cells grow and divide to form new cells as the body needs them. When normal cells grow old or get damaged, they die, and new cells take their place.

Sometimes, this process goes wrong. New cells form when the body doesn’t need them, and old or damaged cells don’t die as they should. The buildup of extra cells often forms a mass of tissue called a tumor. Tumors in the breast can be benign (not cancer) or malignant (cancer). Benign tumors are not as harmful as malignant tumors.

Persons concerned and case numbers:

Ninety nine per cent of breast cancers affect women. Compared to Afro American women, white women are slightly more likely to develop breast cancer, but less likely to die of it.

Furthermore, breast cancers appear most often from 35 years old, when menopause start, and increase to the death.

Breast cancer is second only to lung cancer as a cause of cancer death in women. Each year it is estimated that nearly 200,000 women will be diagnosed with breast cancer and more than 40,000 will die.

Risk factors:

The primary risk factors that have been identified are advanced age, early menarche, late menopause, large size, benign breast diseases, dense breast tissue, obesity after menopause, family history, radiation therapy, some genes mutations, alcohol consumption and history of hormone treatments.

c. Relation between estradiol and breast cancer

Breast cancer risk is increased by early menarche and late menopause, suggesting that the long duration of exposure of breasts to the high levels of ovarian steroids in premenopausal women increases risk.

Recent prospective studies have shown that postmenopausal women who develop breast cancer have significantly greater prediagnostic serum concentrations of estradiol than postmenopausal women who remain healthy.
Serum concentrations of estradiol is probably a major determinant of breast cancer risk, and is important for establish the menopausal status of the patient.

d. Liquid Liquid Extraction (or LLE)

Definition

For extract estradiol from plasma I use a separating technology, the liquid liquid extraction. This technique allows separation of two miscible liquid.

In fact, the principle is to add another immiscible liquid in which solute that we want extract (E₂, for my case) has more affinity.

Generally, one of liquid phases is water and other an organic solvent.

Principle

Principle of LLE is very easy and it is describe below:

![Figure 4: Principle of LLE](image)

Notes

More numbers of extractions are high, more extraction is efficient. The method consists to sequence the extraction with smaller volume than if we do this at one stroke.

Also, solvent choice is very important. In fact, the solvent must

- Be immiscible in initial phase that contain solute
- Be very soluble with the solute
- Have a different density than initial phase

Furthermore, if it’s possible, the solvent must be eco-aware, cheap and not health hazard.
In my case, I have tried two different solvents: **Methyl tert-butyl ether** (MTBE) and **Dichloromethane** (DCM).

- **MTBE**

  MTBE is an organic compound, it is volatile, flammable and colorless liquid that is immiscible with water.

  Molar mass = 88,15 g/mol  
  **Density** = 0,7404 g/mL  
  Melting point = -109°C  
  Boiling point = 55,2°C

- **DCM**

  DCM is also an organic compound, it is also volatile and colorless liquid, furthermore it is immiscible in water.

  Molar mass = 84,93 g/mol  
  **Density** = 1,3266 g/mL  
  Melting point = -96,7°C  
  Boiling point = 40°C

  ![Figure 5: MTBE molecular structure](image)
  ![Figure 6: DCM molecular structure](image)

e. **High Performance Liquid Chromatography (or HPLC)**

  To analyze samples prepared, I use HPLC system. These tools open the possibility of separate and quantify a compound contained in a solution. In this part I describe the functioning of the HPLC system know as inversed phase chromatography as well as essential points.

  **Principle:**

  In fact a sample is pushed, by a liquid called mobile phase, through a column filled stationary phase of high granularity. Each component adopts a rate of migration (called retention phenomena) which is own according to its solubility in the mobile phase and its affinity for the stationary phase.

  In same chromatographic conditions, retention time characterize qualitatively a substance. Amplitude of these peaks allows to measure concentration of each compound in the mixture.
**Instruments:**

HPLC instruments consists of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector:

- **Pumps**

  The pump forces mobile phase to go through column whose stationary phase is very compact. It must maintain high pressure upstream of the injector.

  The pressure depends of different factors such as flow, viscosity, grain size of the stationary phase and geometry column.

  The pump must to provide a high pressure, a stable flow and a resistance to the corrosion whatever the solvent used.

  There are two mode of operation. The first, know as, isocratic for which the mobile phase composition is fixed.

  The second, know as, solvent of gradient for which we can change solvent composition during analysis for improve separation and reduce analysis times. Solvent gradient have need specific pumps for to vary constituents of the mobile phase mixture. This variation is programmable by user.

![A simplified scheme for HPLC](image)
Example of the interest of solvent gradient:

- **Injector**

  Injection should be done very quickly not interfere with the flow of the solvent. We must inject quickly always the same volume. The most common injector is the loop injector (the one I used).

  For this, we use a high pressure valve with multiple channels that allows isolate the circuit loop to fill it then put it in the injection circuit with reversal of flow direction of the solvent in the loop.

  This system allows a good reproducibility provided that loop is completely filled. The filling is thanks to a syringe. We must inject a more volume than the loop and the run off is evacuate.

- **Column**

  It is the active part of the system. The column is a cylinder usually of stainless steel sometimes coupled with an inert material (glass or specific plastic). The standard column length is between 10 and 30 cm and with inside diameter from 4 to 10 mm. It is filled with stationary phase. More information on the stationary phase is described further in this part.

  On each column, there is a note for specifications of the column: bonded phase, column dimension, particle size, pore size.
For protect column, it is often preceded of a guard-column. It filled with the same stationary phase. It is very short (from 0,5 to 1 cm) and its role is to fixe compounds whose affinity, with stationary phase, is too high. This guard-column must be change periodically.

- **Detectors**

There are many types of detectors, but all must have some characteristics to be used, such as: a proportional response to the concentration of the substance detected, high sensibility, but also a lowest inertia and a high stability of signal.

Different detectors usually used are the spectroscopy UV-VIS detector, the fluorescence detector and the refractive index detector.

For my project I use UV detector. It measure absolute absorbance of solvent and solute at a fixed wavelength.

It presents many advantages such as those above-mentioned, and also no much sensible to flow and temperature variation. Furthermore it is easy use and preserve and it can use with gradient solvent mode.

**Silica gels**

It's the more usually stationary phase used in HPLC.

Silica gels is constitute of microspheres. These spheres, in reality, are not homogenous and not regular and it exist gaps called pores.

These pores have a key role because they constitute zones of preferential adsorptions. Surface of microspheres of silica includes silanol groups: Si – OH

The free silanol groups are disturbing for inversed phase chromatography (the one I use), because they are very polar and so they have a great affinity to polar solutes.

Currently, we use silica gels which have grafted a function by covalent binding.

The main grafts that we use in inversed phase chromatography are dimethylsillyle C2, octylsillyle C8, octadecylsillyle C18 (the one I use) and phenylsillyle Φ.

![Figure 11: scheme of silica gel for inversed chromatography](image)
So, the stationary phase is weakly polar or non-polar. The polar components have greater affinity for the mobile phase and are eluted rapidly. Conversely, the weakly polar solute has greater affinity for the stationary phase and slowly eluted.

**Chromatograms analysis**

Computer retrieves all data from detector, trace chromatogram and integrate peak areas. It provides an analytical report showing retention time and area of each peak.

The retention time of a solute is taken as the elapsed time between the time of injection of a solute and the time of elution of the peak maximum of this solute. It is a unique characteristic of the solute and can be used for identification of compound.

A good separation will result in a distinct separation of peaks corresponding to each product.

If you change the composition of the solvent you can adjust the retention time for reduce the times analysis and improve the separation.

For each analysis, we must firm up details such as:

- Column type: mark, nature, diameter, length, support, ...
- Mobile phase characteristics: solvent, composition, flow, detection system
- Quantity injected.
III. **Materials and methods**

   a. **LLE procedure**

   b. **Choice of solvent.**

   For this part, I test two different solvents: MTBE and DCM, the goal is to know which solvent have best characteristics for extraction.

   **Numbers of extraction**

   - **Preparation of stock solution:**

     I prepare mother solution at 800 µg/mL in methanol. I dilute mother solution one hundred times for get a solution (S) at 8 µg/mL still in methanol.

   - **Procedure:**

     In **one test tube**, add 1 mil of water plus 200 µL of (S) and perform LLE procedure above-drawn and perform two extra extractions.

     Don’t collect SOLVENT phase together in only one tube but in different tube for constitute sample numbers 1, 2, 3, 4 and 5.
Moreover, for control, prepare two reference samples:

First sample (100%) consist of 1 mil of water plus 200 µL of (S) dried (in stream air, 60°C), and reconstituted in 1000 µL of ACN/MQ (60/40).

And second (0%) which consist of 1 mL of water plus 200 µL of Methanol dried (with a stream of air, 60°C), and reconstituted in 1000 µL of ACN/MQ (60/40).

I make this protocol with the two solvents: MTBE and DCM.

- **Analysis:**

For a start, I program the system HPLC so follows:

- Injection volume: 20µL
- Column type: Phenomenex, C18, 150×3µm
- Mobile phase: Solvent A: 95%MQ, 5%ACN, 0.1% Formic acid
  Solvent B: 5%MQ, 95%ACN, 0.1% Formic acid
  Mobile phase composition final: 50%A, 50B.
  Flow: 0.2mL/min
  UV detection 210 nm

I inject two times all my samples and I do a graph line which represents the area contingent on sample number. Results are given on page ... *(Result 1)*

**Calibration lines**

- **Preparation of stock solutions:**

With the previous solution (S) at 8 µg/mL, prepare, in test tubes, seven samples of 1 mL at 1000, 750, 500, 250, 100, 50 and 0 ng/mL respectively S1, S2, S3, S4, S5, S6 and Sbl (Blank).

Preparation of solutions is detailed in **appendix 1**.

- **Procedure:**

For each solution perform LLE procedure and reconstitute with 1000µL ACN/MQ (60/40). We get seven samples SOLVENT S1, S2, S3, S4, S5, S6 and Sbl
- **Analysis:**

Characteristics of system HPLC are:

- Injection volume: 20µL
- Column type: Phenomenex, C18, 150×3µm
- Mobile phase:
  - Solvent A: 95%MQ, 5%ACN, 0.1% Formic acid
  - Solvent B: 5%MQ, 95%ACN, 0.1% Formic acid
  - Mobile phase composition final: 50%A, 50B.
  - Flow: 0.2mL/min
  - UV detection 210nm

I inject all my samples, and I do a graph line which represents the area function of estradiol concentrations. Results are given on page ... *(Result 2)*

- **Report**

We can conclude that extraction of estradiol with MTBE is more efficient than DCM. Indeed, calibration lines with MTBE are best and the smaller numbers of extraction have the advantage to reduce the practical time. Consequently, I choose this solvent.

Now I know which solvent I have to used, I can start to work with plasma.

c. **Experimentation with plasma**

- **Preparation of stock solutions:**

I prepare mother solution at 1mg/mL in methanol then I dilute it for obtain S1, S2, S3, S4, S5 and S6 respectively at 20, 10, 2, 1, 0.2 and 0.02 µg/mL.

Details of preparation are attached in **appendix 2**.

- **Procedure:**

Spike 1mL plasma with 50µL of stock solution for get a calibration line CAL0, CAL1, CAL2, CAL3, CAL4, CAL5 and CAL6 at respectively 0, 1, 10, 50, 100, 500, 1000ng/mL.

Then spike 1mL of water with 50µL of stock solution for get a reference line REF0, REF1, REF2, REF3, REF4, REF5 and REF6 at respectively 0, 1, 10, 50, 100, 500, 1000ng/mL.

After spiking, perform LLE with MTBE and reconstitute with 1000µL of ACN/MQ (60/40).
- **Analysis:**

Characteristics of system HPLC are:

- Injection volume: 20µL
- Column type: Phenomenex, C18, 150×3µm
- Mobile phase: Solvent A: 95%MQ, 5%ACN, 0.1% Formic acid  
  Solvent B: 5%MQ, 95%ACN, 0.1% Formic acid  
  Mobile phase composition final: 50%A, 50%B.  
  Flow: 0.2mL/min  
  UV detection 210 nm

I inject all my samples, and I do a calibration line and reference line which represents the area function of estradiol concentrations. Results are given on page ... *(Result 3).*

- **Report**

  We can see liquid liquid extraction method with plasma is working but only with high concentrations because there are so much peak around the estradiol peak and also because estradiol peak is small.

  So, I try with a new procedure that will allow to remove peaks around the estradiol peak and I will make stock solutions with higher concentrations for increase estradiol peak size.

d. **Optimization of the LLE procedure**

  I want to remove interferences of proteins passing estradiol in buffer phase.

  - **Preparation of stock solutions and phosphate buffer**

    Prepare stock solution (S1) at 100µg/mL in methanol and I dilute it for gets S2 and S3 respectively at 10µg/mL and 1µg/mL. Also I prepare Sbl (blank) only with methanol.

    Details of preparation are attached in *appendix 3.*
- **Procedure:**

Spike 1mL of plasma with 100µL of stock solutions for get a calibration sample CAL1, CAL2, CAL3 and CALbl at respectively 10µg/mL, 1µg/mL, 0.1µg/mL and 0µg/mL.

Recover the MTBE phase, evaporate and reconstitute it with 1000µL of mobile phase B/MQ (50/50).

- **Analysis**

I program the system HPLC so follows:

- Injection volume : 20µL
- Column type: Phenomenex, C18, 150×3µm
- Mobile phase: Solvent A: 95%MQ, 5%ACN, 0.1% Formic acid
  Solvent B: 5%MQ, 95%ACN, 0.1% Formic acid
  Mobile phase composition final: 50%A, 50B.
  Flow: 0.2mL/min
  UV detection 210nm

And I inject all my samples.

- **Report**

I don’t have results for this method because estradiol peak don’t appear. So, I verified each step for see where estradiol is. For this, I start again the protocol, just with CAL0 and CAL1.

For the first step, I inject MTBE phase (after drying and reconstituting it in 50%B) and the same concentration of estradiol stock (for reference). Here I can see that estradiol is in MTBE phase, it is right.
Then I continue the protocol and for second step I inject MTBE phase (after drying and reconstituting it in 50%B) and the same concentration of estradiol stock (for reference). I see estradiol peak so I can conclude that estradiol don't goes in buffer phase, my method goes wrong and I don't use it.

I decide to compare LLE and SPE.

e. **Comparison between LLE and SPE.**

**SPE procedure**

<table>
<thead>
<tr>
<th>Conditioning</th>
<th>Loading</th>
<th>Washing</th>
<th>Eluting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mL MeOH</td>
<td>1mL sample</td>
<td>1mL water</td>
<td>1mL 60% ACN</td>
</tr>
<tr>
<td>2 x 0.5mL MeOH (5%)</td>
<td></td>
<td>1mL 30% MeOH</td>
<td></td>
</tr>
</tbody>
</table>

Figure 15: SPE procedure

Choice of percentage of methanol for washing and acetonitrile for eluting is show in page ... *(Result 4)*

**Direct comparison between LLE and SPE**

It is important to mention that these data are measured in human plasma after extraction and evaporation, the estradiol is dansylated. So there are dansylated $\text{E}_2$ measurements instead of the estradiol measured directly in HPLC system. Dansylation is necessary for MS data. I prepare calibration line from 0 ng/mL to 1500 ng/mL.

I inject all my samples in UV and MS detection, and I do a calibration line which represents the area function of estradiol concentrations. Results are given on page ... *(Result 5).*
IV. **Results**

a. **Choice of solvent**

**Result 1**

*Observation:*

Curve with MTBE decrease faster than with DCM. Three extractions are necessary with solvent MTBE while four are necessary with DCM.

**Result 2**

*Observation:*

We can see that calibration line with MTBE is more linear than with DCM. But I can add that estradiol peak is very small and limit of detection is at 250ng/mL.
b. Experimentation with plasma

Result 3

**Observation:**

The method seems to be run but for this analysis, I have met some problems such as size of estradiol peak are very small and it is surround of other peaks:
c. Comparison between LLE and SPE.

Result 4

- SPE procedure
Result 5

- Comparison between SPE and LLE procedure.

<table>
<thead>
<tr>
<th>LOD</th>
<th>UV</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE</td>
<td>50 ng/mL</td>
<td>5-10 ng/mL</td>
</tr>
<tr>
<td>LLE</td>
<td>100 ng/mL</td>
<td>20 ng/mL</td>
</tr>
</tbody>
</table>
Observation and conclusion:

In the UV graph, the two lines are not that far apart from each other, although the SPE gives a somewhat higher recovery. When the experiments are performing, we can noticed that the SPE extract is a lot cleaner than the LLE extract. The proteins that are still in the LLE extract do not interfere in the chromatogram, but sometimes cause the LC-column to clogg (building up pressure). In short, SPE gives a cleaner extract and is therefore first choice in sample pretreatment.

These findings are confirmed by the MS data: the difference between SPE and LLE are much more pronounced here. The recovery of SPE seems to be much higher. But in fact the recovery is only slightly higher, but there is a compound that interferes in the MS detection. Estradiol is not properly ionised (the other compound is easier to ionise in the MS, this process is called ionsuppression) and therefore the estradiol line is lower.

V. Discussion

For conclude, Liquid liquid extraction method is working but only with high concentrations of estradiol. So we can not used for the determination of extremely low concentrations.

But, it will be interesting to investigate about deprotonation of estradiol with basic buffer to remove interferences due to proteins, by trying to change buffer pH or concentration.

Finally, Solid phase extraction is more adapted than Liquid liquid extraction. Indeed, it allows the determination of lower concentrations than liquid liquid extraction, gives a cleaner extract and there for first choice in sample pretreatment.
Conclusion

During my training period I could noticed the difficulties meet in research. Indeed, I think lot of patience and skills are required to carry through a project: Frustration of wrong results must not be view as failure but as an experiment that is always possible to improve.

By three month, I tried to find a liquid liquid extraction method for the determination of low concentrations of estradiol. This goal was not achieves because only high concentrations are detected but I think it is possible to improve my method for next investigations.

Furthermore, this experience gave me an opportunity to grow and acquire new skills in liquid chromatography, and in separation methods such as liquid liquid extraction and solid phase extraction.

Personally, this training period bring me self-confidence, ability for integration and development of my critical mind.
## Appendix 1

### Stock solution $S$ 9.5 $\mu$g/mL

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume of $S$</th>
<th>Volume of MeOH</th>
<th>Concentration</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>105.3 $\mu$L</td>
<td>894.7 $\mu$L</td>
<td>1000 ng/mL</td>
<td>1000 $\mu$L</td>
<td>0 ng/mL</td>
</tr>
<tr>
<td>S2</td>
<td>78.9 $\mu$L</td>
<td>921.1 $\mu$L</td>
<td>750 ng/mL</td>
<td>1000 $\mu$L</td>
<td>0 ng/mL</td>
</tr>
<tr>
<td>S3</td>
<td>52.6 $\mu$L</td>
<td>947.4 $\mu$L</td>
<td>500 ng/mL</td>
<td>1000 $\mu$L</td>
<td>0 ng/mL</td>
</tr>
<tr>
<td>S4</td>
<td>26.3 $\mu$L</td>
<td>973.7 $\mu$L</td>
<td>250 ng/mL</td>
<td>1000 $\mu$L</td>
<td>0 ng/mL</td>
</tr>
<tr>
<td>S5</td>
<td>10.5 $\mu$L</td>
<td>989.5 $\mu$L</td>
<td>100 ng/mL</td>
<td>1000 $\mu$L</td>
<td>0 ng/mL</td>
</tr>
<tr>
<td>S6</td>
<td>5.3 $\mu$L</td>
<td>994.7 $\mu$L</td>
<td>50 ng/mL</td>
<td>1000 $\mu$L</td>
<td>0 ng/mL</td>
</tr>
<tr>
<td>S0</td>
<td>1000 $\mu$L MeOH</td>
<td>0 ng/mL</td>
<td>0 ng/mL</td>
<td>1000 $\mu$L</td>
<td>0 ng/mL</td>
</tr>
</tbody>
</table>
Appendix 2

Stock solution S 1.21mg/mL

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>40µL S + 1960 µL MeOH</td>
<td>24,2 µg/mL</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>750 µL S1 + 750 µL MeOH</td>
<td>12,1 µg/mL</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>400 µL S2 + 1600 µL MeOH</td>
<td>2,42 µg/mL</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>750 µL S3 + 750 µL MeOH</td>
<td>1,21 µg/mL</td>
<td></td>
</tr>
<tr>
<td>S5</td>
<td>400 µL S4 + 1600 µL MeOH</td>
<td>0,242 µg/mL</td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>200 µL S5 + 1800 µL MeOH</td>
<td>0,0242 µg/mL</td>
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</tr>
</tbody>
</table>

Final concentration E$_2$

<table>
<thead>
<tr>
<th>sample</th>
<th>mL plasma</th>
<th>mL water</th>
<th>µL Spike</th>
<th>end [E$_2$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ref0</td>
<td>0</td>
<td>1</td>
<td>50 µL MeOH</td>
<td>0</td>
</tr>
<tr>
<td>ref1</td>
<td>0</td>
<td>1</td>
<td>50 µL S6</td>
<td>1,1524</td>
</tr>
<tr>
<td>ref2</td>
<td>0</td>
<td>1</td>
<td>50 µL S5</td>
<td>11,5238</td>
</tr>
<tr>
<td>ref3</td>
<td>0</td>
<td>1</td>
<td>50 µL S4</td>
<td>57,619</td>
</tr>
<tr>
<td>ref4</td>
<td>0</td>
<td>1</td>
<td>50 µL S3</td>
<td>115,2381</td>
</tr>
<tr>
<td>ref5</td>
<td>0</td>
<td>1</td>
<td>50 µL S2</td>
<td>576,1905</td>
</tr>
<tr>
<td>ref6</td>
<td>0</td>
<td>1</td>
<td>50 µL S1</td>
<td>1152,3809</td>
</tr>
<tr>
<td>cal0</td>
<td>1</td>
<td>0</td>
<td>50 µL MeOH</td>
<td>0</td>
</tr>
<tr>
<td>cal1</td>
<td>1</td>
<td>0</td>
<td>50 µL S6</td>
<td>1,1524</td>
</tr>
<tr>
<td>cal2</td>
<td>1</td>
<td>0</td>
<td>50 µL S5</td>
<td>11,5238</td>
</tr>
<tr>
<td>cal3</td>
<td>1</td>
<td>0</td>
<td>50 µL S4</td>
<td>57,619</td>
</tr>
<tr>
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<td>1</td>
<td>0</td>
<td>50 µL S3</td>
<td>115,2381</td>
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<tr>
<td>cal5</td>
<td>1</td>
<td>0</td>
<td>50 µL S2</td>
<td>576,1905</td>
</tr>
<tr>
<td>cal6</td>
<td>1</td>
<td>0</td>
<td>50 µL S1</td>
<td>1152,3809</td>
</tr>
</tbody>
</table>
Appendix 3

Stock solution S1 105 µg/mL

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S2</td>
<td>100 µL S1 + 900 µL MeOH</td>
<td>10.5 µg/mL</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>100 µL S2 + 900 µL MeOH</td>
<td>1.05 µg/mL</td>
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</tr>
<tr>
<td>Sbl</td>
<td>1000 µL MeOH</td>
<td>0 ng/mL</td>
<td></td>
</tr>
</tbody>
</table>

Final concentration E₂

<table>
<thead>
<tr>
<th></th>
<th>mL plasma</th>
<th>µL Spike</th>
<th>end [E₂]</th>
</tr>
</thead>
<tbody>
<tr>
<td>cal1</td>
<td>1</td>
<td>100 µL S1</td>
<td>10.5 µg/mL</td>
</tr>
<tr>
<td>cal2</td>
<td>1</td>
<td>100 µL S2</td>
<td>1.05 µg/mL</td>
</tr>
<tr>
<td>cal3</td>
<td>1</td>
<td>100 µL S3</td>
<td>0.105 µg/mL</td>
</tr>
<tr>
<td>cal0</td>
<td>1</td>
<td>100 µL Sbl</td>
<td>0 µg/mL</td>
</tr>
</tbody>
</table>