



Norwegian University of  
Science and Technology

# LC-MS/MS Analysis of Thyroid Hormones in Blood Serum: Instrumental Method Development and Optimization of Extraction from Bovine Serum

**Ole Christian Bråtveit**

Chemical Engineering and Biotechnology

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Supervisor: Alexandros Asimakopoulos, IKJ

Co-supervisor: Rudolf Schmid, IKJ

Norwegian University of Science and Technology  
Department of Chemistry



## Abstract

Thyroid hormones are a class of hormones that are essential to mammals for maintaining normal cell functions. In this study, we developed an instrumental method for the analysis of four thyroid hormones: L-Thyroxine (T<sub>4</sub>), 3,3',5'-Triiodo-L-thyronine (rT<sub>3</sub>), 3,3',5-Triiodo-L-thyronine (T<sub>3</sub>), and 3, 3'-Diiodo-L-thyronine (T<sub>2</sub>), using liquid chromatography (LC)–tandem mass spectrometry (MS/MS). For optimal chromatographic separation we evaluated two LC columns, Atlantis T3 3 $\mu$ m (2.1 x 150mm) and Kinetex 1.7 $\mu$ m C18 (2.1 x 150mm) column. The performance characteristics of the instrumental method were evaluated, including, repeatability, ion ratio, retention and relative retention time, correlation coefficients, linear range, and limits of detection and quantification. To effectively analyze these thyroid hormones in bovine blood serum, a Liquid-Liquid extraction (LLE), two Solid-Phase Extraction (SPE), and two Hybrid Solid-Phase Extraction (Hybrid SPE-PPT) protocols were evaluated for extraction purposes. The Hybrid SPE-PPT cartridge is a novelty in the field of bioanalysis, and still, its applications are not fully explored. The Hybrid SPE cartridge is consisted of a zirconium packed-bed/low porosity filter/0.2 m hydrophobic frit assembly, where phospholipids are retained specifically to the zirconium sorbent, while proteins are retained non-specifically to the low porosity filter of the assembly. The absolute and relative recoveries (to the D<sub>2</sub>-T<sub>4</sub> internal standard) were assessed. Moreover, the matrix effects that derived from every sample preparation protocol and impacted LC-MS/MS analysis, were evaluated. The instrumental calibration range for each thyroid hormone ranged from 0.25 to 100 ng/mL and showed excellent correlation coefficients linearity ( $r > 0.99$ ). The instrumental detection and quantification limits ranged from 0.16-0.77 and 0.52-2.54 ng/mL, respectively. The selection of the optimal extraction method was made based on time-efficiency, optimal recoveries, and low matrix effects.

## **Preface**

This master thesis in Chemistry, within the field of Analytical Chemistry, was carried out as part of the study program in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU). The work was carried out during the fall semester of 2017.

The master thesis has given me valuable laboratory experience within the field of Analytical Chemistry, it has shown me the importance of hard work, dedication and to never give up, and it has given me added motivation to keep pursuing a career in Chemistry.

I would like to thank my supervisor, Associate Professor Dr. Alexandros G. Asimakopoulos, co-supervisor, Associate professor Dr. Rudolf Schmid, and finally but not least, the PhD student, Ms. Kristine Vike, for without their help this master thesis would not be realized.

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## **Abbreviations**

3 –  $T_1$ : 3-Iodothyronamine

3,5 –  $T_2$ : (3,5)-Diiodothyronine

AC/DC: Alternating Voltage

APCI: Atmospheric-pressure chemical ionization

APPI: Atmospheric Pressure Photoionization

$\vec{E}$ : Electric vector field

EI: Electron ionization

ESI: Electron Spray Ionization

eV: Electron voltage

HCL: Hydrochloric Acid

HETP: height equivalent to a theoretical plate

HPLC: High Pressure Liquid Chromatography

Hybrid SPE-PPT: Hybrid Solid Phase Extraction-Phospholipid Precipitation Technology

i.e.: in essence

$L$ : Column length

LC: Liquid Chromatography

LC-MS/MS: Liquid Chromatography Tandem Mass Spectroscopy

LC-QTOF-MS: Liquid Chromatography Quadrupole Time of Flight Tandem Mass Spectroscopy

LLE: Liquid Extraction

MALDI: Matrix-assisted laser desorption/ionization

$m$ : Mass

$m/z$ : Mass-to-Charge Ratio.

MS: Mass Spectroscopy

MS/MS: Tandem Mass Spectroscopy

$N_2$ : Nitrogen gas



RIA: Radioimmunoassay

$rT_2$ : 3, 5- Diiodothyronine

$rT_3$ : 3, 3', 5'- Triiodothyronine

SIS: Sodium-Iodide Symporter

SPE: Solid Phase Extraction

SPE-LC-MS/MS:

TGB: Thyroxine binding globulin

$T_0$ : Thyronine

$T_1$ : Iodothyronine

$T_2$ : Diiodothyronine

$T_3$ : Triiodothyronine

$T_4$ : Thyroxine

$T_4-^2H_5$ : Thyroxine deuterated.

T.A.: Target Analyte

$U$ : Voltage

$\vec{v}$ : Speed

v/v: Volume/Volume

w/v: Weight/Volume



## 1. Introduction

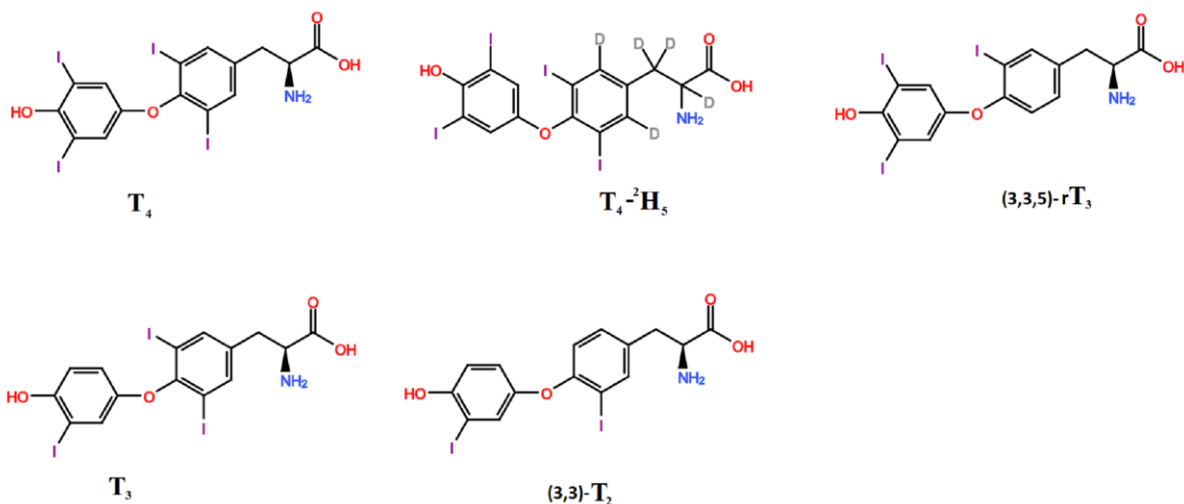
### 1.1. Scope and objectives of Thesis

The scope of this thesis was to develop an optimal Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) method to extract 4 target thyroid hormones, namely, L-Thyroxine ( $T_4$ ), 3,3',5'-Triiodo-L-thyronine ( $rT_3$ ), 3,3',5-Triiodo-L-thyronine ( $T_3$ ), and 3, 3'-Diiodo-L-thyronine ( $T_2$ ) from bovine blood serum (**Fig. 1**). Here-in this work five different extraction protocols were evaluated based on Solid Phase Extraction (SPE), Liquid Liquid Extraction (LLE), and Hybrid SPE-Protein Precipitation (PPT) Techniques. The experiments were performed by using bovine pool blood serum. The objectives of the thesis were: a) develop a LC-MS/MS chromatographic system for the analysis of Thyroid hormones and b) develop an optimized sample preparation protocol for the extraction of thyroid hormones from blood.

### 1.2. Thyroid Hormones

Thyroid hormones are specific hormones that regulate cell replication and tissue growth [1]. The thyroid hormones are produced and secreted from the thyroid gland of mammals, with  $T_4$  being the main product of the synthesis, and  $T_3$  the by-product. The ratio between the  $T_4$  and  $T_3$  changes between mammal species, i.e., for humans the thyroid gland produces about 80%  $T_4$  and 20%  $T_3$ , and a ratio of 50%/50% of  $T_4$  and  $T_3$  in sea cows [2].

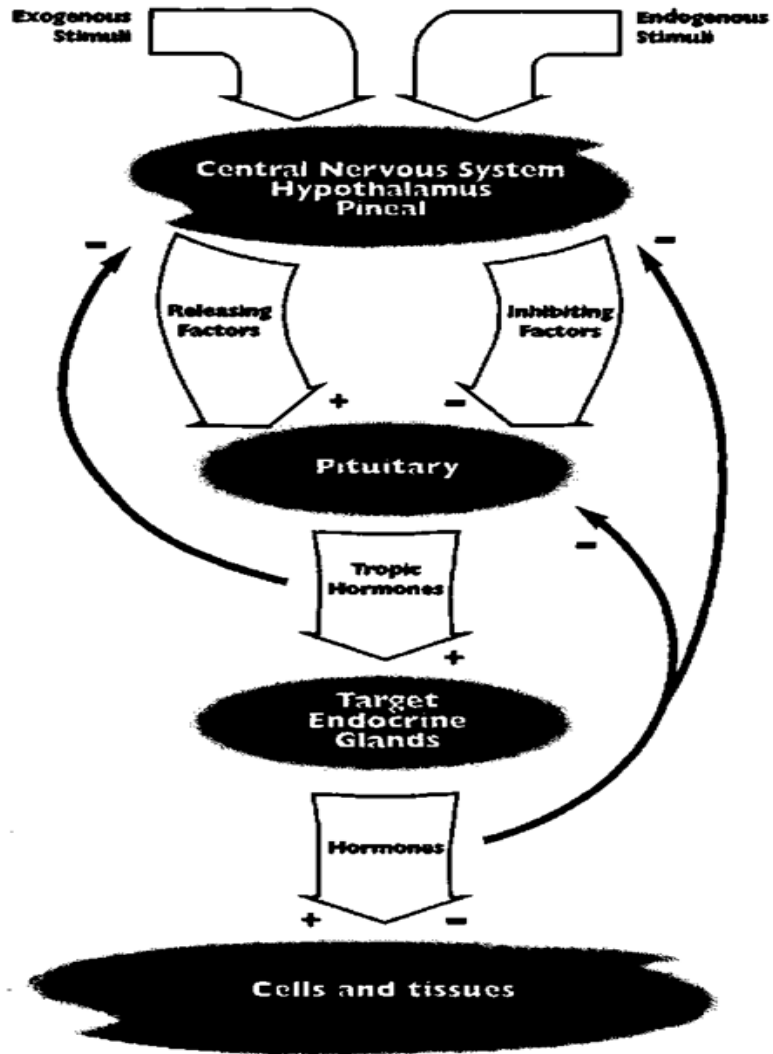
As show in **figure 1**, the thyroid hormones consist of two aromatic rings connected to each other by a single oxygen ligand. Another common characteristic, is that the thyroid hormones have an amino acid group attached to one of the aromatic rings. The other aromatic ring of their structure has an alcohol group in the para position, and an amino acid structure on the right carbon ring in the para position. The difference between the derivatives is on the number of Iodine elements attached to the two rings of the thyroid hormones and their relative position on the rings.



**Figure 1:** Target thyroid hormones.

### 1.3. The Endocrine System of Mammals

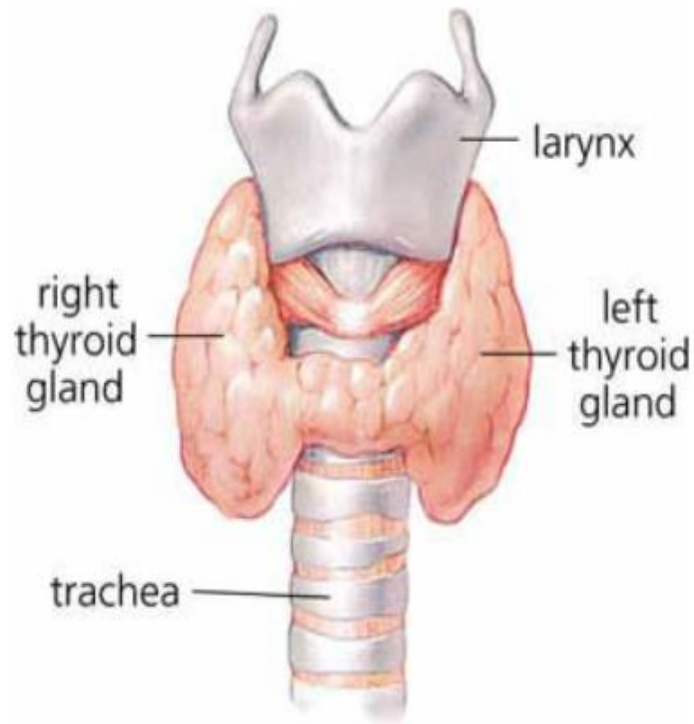
The endocrine system is a collection of organs in mammals, which secretes hormones in order to perform specific “tasks” in the body of the mammals, including the regulation of the concentration of salts and sugars, cell regulation, and cell replication [3]. An illustration of the endocrine system in sea mammals is demonstrated in **figure 2**, where the arrows illustrate the negative feedback response of the endocrine system in sea mammals. The secretion of the hormones by the endocrine systems is regulated by a negative feedback loop. This loop is a mechanism in which a “stimulus” causes an opposite “response stimulus” to regulate and maintain the ideal concentrations of hormones, sugars salts, and others, in the mammal’s body [3]. In general, the endocrine system consists of the thyroid gland, hypothalamus, the pineal gland, bi-kidneys, the testicles (males) and the ovaries (females) in mammals.



**Figure 2:** Illustration of endocrine hormone system in sea mammals [4].

#### 1.4. Thyroid Gland in Mammals

The thyroid gland is the gland which produces and releases thyroid hormones into the blood stream [1]. It is usually located in front of the throat, below the larynx and above the Triclia. An illustration of a thyroid gland in mammals is demonstrated in figure 3 [5].

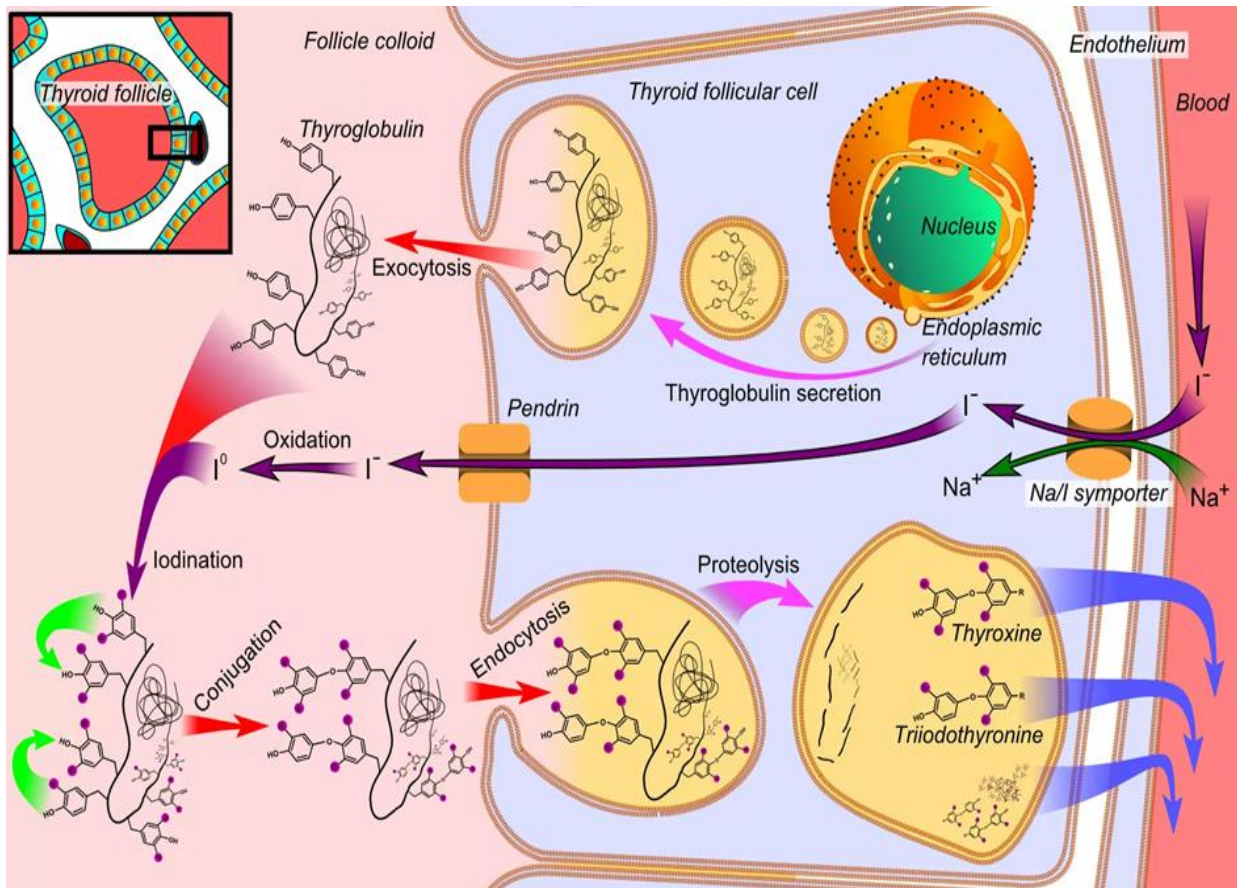


**Figure 3:** Anatomy of the thyroid gland in mammals [5].

The gland has a butterfly like shape, and consists of two lobes located on each side of the trachea. Connecting the two lobes is the isthmus. The thyroid gland varies in size and weight from species to species. As an example, the average thyroid gland in adult's seals, is between 5-6 grams in weight, while the thyroid gland is 15-16 grams in adult humans [6].

### 1.5. Synthesis of Thyroid hormones

The synthesis of the thyroid hormones, involves processes that are about intracellular and extracellular in the follicular cells [1]. Illustration of the synthesis of  $T_3$  and  $T_4$  is given in **figure 4** [7].



**Figure 4:** Illustration of thyroid hormone synthesis [7].

The steps for synthesizing and transporting the thyroid hormones from the thyroid gland is described below:

1. Trapping of iodine from the diet intake, and absorbed from the blood stream. This process is an example of secondary active transport.
2. Thyroid peroxidase oxidizes the iodide ion into iodine.
3. Thereafter, thyroglobulin is synthesized in the cell, and stored in a secretory vehicle. The vehicle travels through the secretory pathways to the membrane, where the thyroglobulin is released into the follicle colloid.
4. Once the iodine is released into the colloid, it reacts with the tyrosine attached to the thyroglobulin that forms  $T_1/T_2$ . In the last step of the  $T_3/T_4$  synthesis,  $T_1$  and  $T_2$  combines to form  $T_3$  and  $T_4$ . A structure backbone with integrated  $T_3$  and  $T_4$  is the “storing place”, where the hormones will be stored until they are needed by the organism [7].  $rT_3$  is an isomer of  $T_3$ , and is the third-most abundant iodothyronine the thyroid gland releases into the bloodstream, of which 0.9% is  $rT_3$ ,  $T_4$  constitutes 90% and  $T_3$  is 9%.

### **1.6. Trace level analysis-An overview**

Analysis of biological samples, is often a complicated procedure, which involves many steps. These steps often culminate in the use of chromatographic separation coupled to a suitable detector [8]. Screening for hormones in a complex biological matrix, requires high performance from the analytical instruments, as well as adequate sample preparation protocols. In general, biological samples cannot be analyzed without some preliminary sample preparation because they obtain too complex sample matrices (high concentration of endogenous components) and efficient chromatographic separation and detection of analytes cannot be achieved at low detection levels. Sample pre-treatment is therefore an essential part of the chromatographic procedure [9]. The objective of pre-treatment is to provide a sample fraction enriched with all the analytes of interest, and as “clean” as possible from other interfering matrix components. But, before implementing any strategy, it is important to consider the strong interdependence of the various steps of the whole analytical procedure; i.e. sample handling, separation and detection of analytes [9]. Sample pre-treatment mainly depends on the nature of the solutes to be determined (e.g. volatility, polarity, molecular weight, etc.), on the nature of the matrix and on the level of concentration required. Interference removal is a critical step which depends strongly on the concentrations of the analytes of interest and the nature of the analyzed media. The sample pre-treatment step is in general the time-determining step of the whole analytical procedure.

### **1.7. Sampling Strategy**

Obtaining a useful sample of the matrix is often down prioritized or overlooked but is vital for any analysis. Failure to properly collect a sample can invalidate any results obtained. The main objective in any sampling strategies is to obtain a representative portion of the sample. A detailed plan on how to perform the sampling is required [11]. Therefore, planning of sampling strategy is an important part of the overall analytical procedure as the consequences of a poorly defined sampling strategy, could well lead to getting misleading results, as well as increased time and money costs. A successful sampling strategy should begin with a thorough plan and established protocols. In general, there are three prerequisites that need to be usually addressed before the experimental analytical part of the work is initiated:

1. Selection of the sampling method to obtain a representative sample.



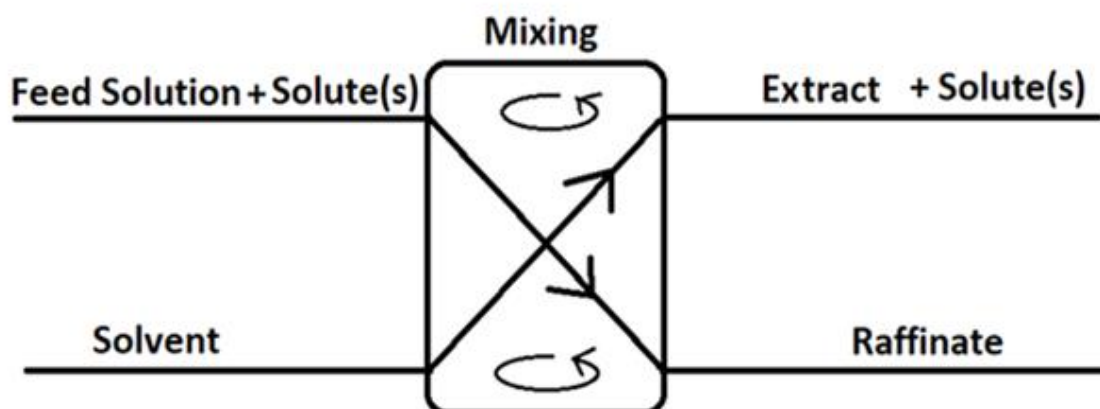
2. Determination of the sampling quantity needed to meet the minimum limits of the analytical method.
3. Identification of safety measures that need to be taken.

### **1.8. Sampling Preparation and Instrumental Analysis**

Performing trace analysis of biological samples is always challenging due to the complexity and diversity of the sample matrix. Matrix effects might have a profound impact on the performance of the method, such as the limit of detection, limit of quantification, accuracy, precision and linearity. Sample pre-treatment involving isolation of analytes, purification of extracts and pre-concentration are required [12]. In addition, concentrations of hormones in bio samples are usually found in the low ng/mL range, rendering extraction, preconcentration, and cleanup prior to detection very important prior to analysis [13]. There are several goals of sample extraction prior to analysis [14-16]. Firstly, there is the necessity to separate the target analytes from a complex matrix such as blood and urine samples to remove interfering components. The second task, is the enrichment of the target analytes in the biological sample, especially when analytes in trace levels are to be determined. Sample extraction and concentration are applied steps used to improve instrumental sensitivity. Lastly, the compatibility between the sample matrix and the instrumental analysis must be considered. The most common detection technique for analysis of organic compounds and biological samples, are based on mass spectroscopy analysis [17]. This technique has become the preferred analytical method in bioanalysis and environmental analysis, due to its high sensitivity. The dominating modern analytical technique for quantitative and qualitative analysis is mass spectroscopy tailored to a chromatographic technique, such as gas chromatography (GC) and High-Performance Liquid Chromatography (HPLC). The capability to analyze polar analytes, without the need for any derivation steps, is the main reason that the LC techniques have become popular. The extremely high selectivity and sensitivity of LC-MS/MS techniques in particular, allows trace concentration of complex mixtures to be determined. Due to the matrix complexity of the samples, and the polar character of the thyroid hormones, the LC-MS/MS technique was used in this thesis.

### 1.8.1. Liquid-Liquid Extraction

Liquid-Liquid extraction (LLE) is a classic extraction technique, used to extract organic compounds from a liquid sample [18]. The choice of solvent is critical when doing the LLE separation. In general, good selectivity and little miscibility with the sample matrix, are highly desirable attributes of the extraction solvent. The general procedure of LLE, is to separate compounds based on their relative solubility in two different immiscible liquids, often water and an organic solvent. The LLC method is an extraction of a substance, from one liquid into another. One or more solute(s) that are contained in the initial solution (aqueous phase) is extracted to the other immiscible liquid/solvent. Not all organic compounds can be extracted, from the aqueous phase. This is mainly due to that some chemicals are strongly hydrophilic. However, we can increase the extraction efficiency of very polar chemicals by adding high concentrations of salt in the aqueous phase; the polar compounds are introduced to the organic phase through the “salting-out” effect. An illustration of the principle of mixing is seen in figure 5.



**Figure 5:** Illustration of the LLE mixing principle (own figure).

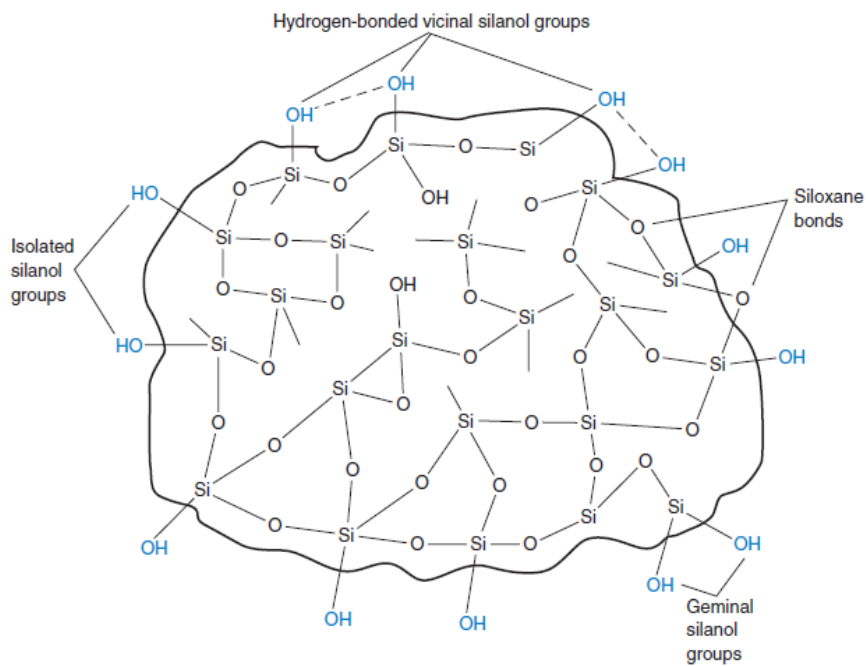
The usage of LLE to extract thyroid hormones from biologicals sample matrices, has been performed previously. Kunisue et al. [69] vortexed the samples (thyroid gland) and performed extraction using methanol. Escobar et al. [19] performed LLE using chloroform and methanol in a mixture of 2:1 (v/v) on rat embryonic tissues. Simon et al. [20] used for the same matrix as Escobar et al. [19], a methanol and water (1:1, v/v) mixture, followed by hexane in the extraction process. Another method for extraction of thyroid hormones using LLE, has been performed by Villanger et al. [21], where they used ethanol in the extraction process.

### 1.8.2. Solid-Phase Extraction

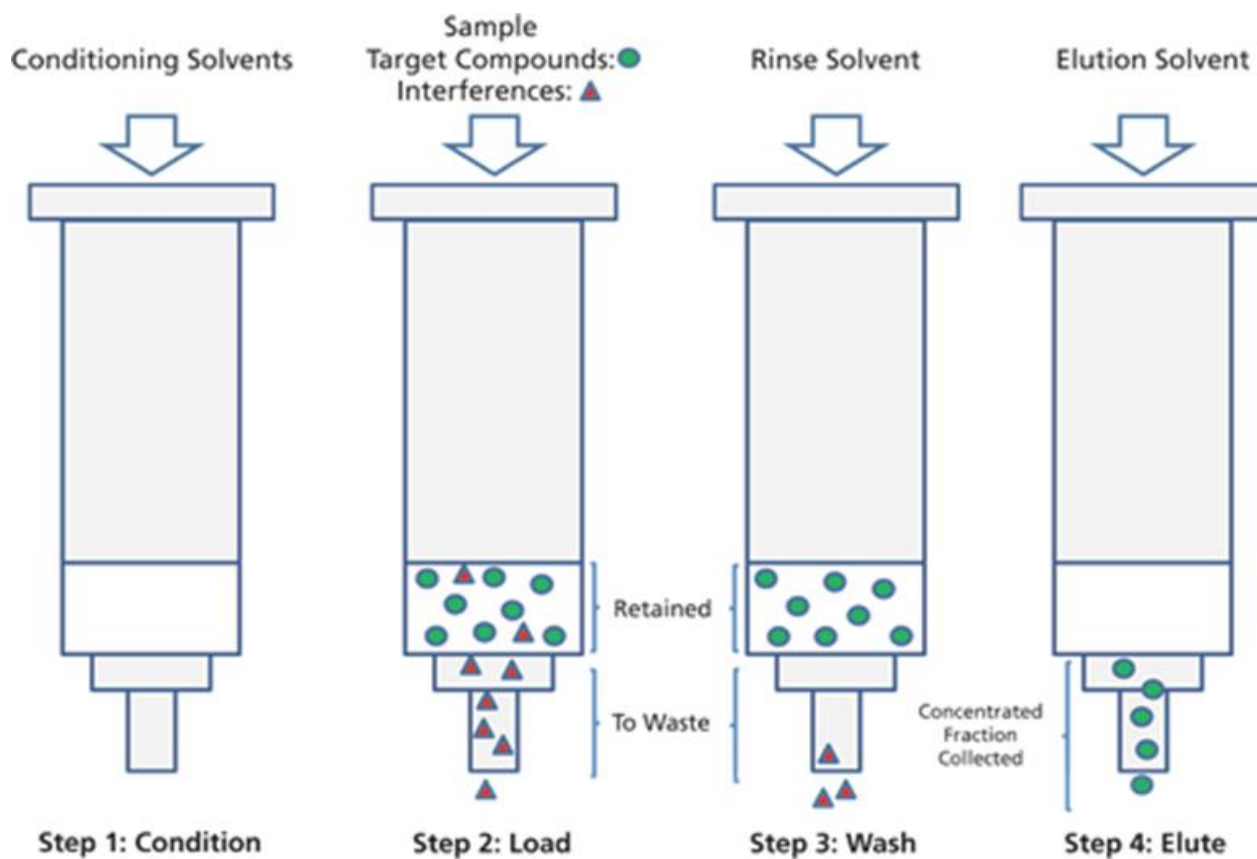
The SPE method requires a measured volume of the liquid state sample to be passed through a cartridge tube packed with a suitable solid adsorbent material. The chemicals in the sample are adsorbed onto the solid surface from which they are eluted by a properly selected solvent. The sample is applied at the top of the tube and drawn through the bed by a syringe or vacuum, maintaining a flow rate of 1–2 drops/second. Alternatively, larger pore size particles may be used to allow fast flow rates for large volume samples. The tube is washed with a nonpolar solvent for polar analytes, and with a polar solvent for non-polar analytes. The SPE provides a good alternative to LLE [22]. Compared to LLE, SPE is a more modern extraction technique which has become increasingly common when performing sample preparation for trace level analysis [23]. This is mainly attributed to that the SPE technique is characterized by a lower solvent consumption. Other advantages of SPE is that it eliminates emulsions, and it is considered to improve selectivity, specificity and reproducibility. The most common SPE packing materials used for trace level analysis of organic compounds, consists of nonpolar pores that obtain a chemical bound silica, with a carbon chain length from C-8 to C-18. The head of the carbon chain usually obtains an ion-exchange head (e.g., OASIS HLB cartridge) (**figure 6**) [25].

The general procedure of SPE consists of four steps (**figure 7**) [26]:

1. Condition: Solvation of the silica cartridge. This is done to increase the efficiency of the cartridge.
2. Loading: Adding the sample with the target compound and the remaining sample matrix onto the cartridge.
3. Washing: Use of solvent/solution to remove unwanted endogenous matrix components from the sample.
4. Elution: Use of solvent to elute the target chemicals from the cartridge.



**Figure 6:** Illustration of the SPE cartridge packing material [25].



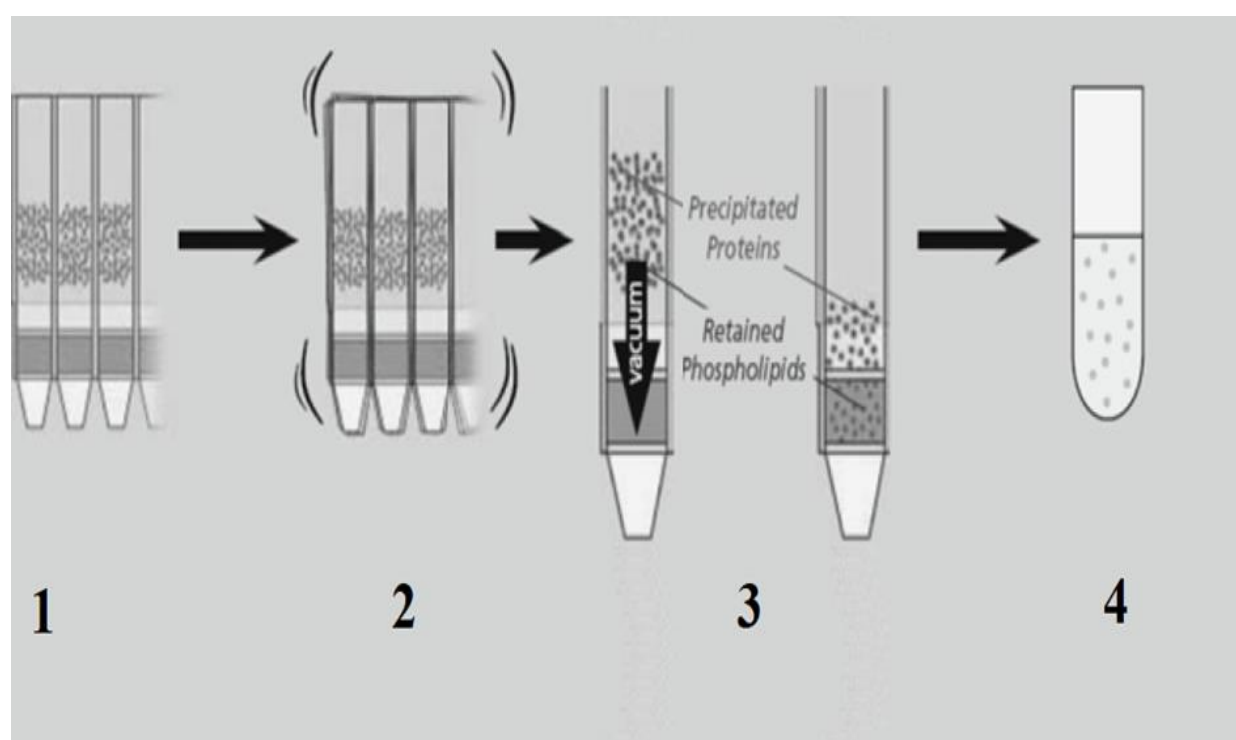
**Figure 7:** The four steps in the SPE procedure [27].

SPE extractions are also considered easier to perform than the LLE for the extraction of thyroid hormones. Soldin et al. [28] extracted the thyroid hormone derivatives from human blood serum and plasma, centrifuged at 2900 rpm, before SPE extraction. Centrifugation was applied to remove the high concentration of endogenous proteins from the samples so that they do not interfere with the SPE extraction. Noyes et al. [29] extracted the thyroid hormones from plasma by initially incubating for one hour (covered on ice) to allow for equilibration of endogenous and labeled thyroid hormones with plasma proteins. The incubation medium also contained an antioxidant/reducing solution containing of ascorbic acid, citric acid, and DTT to prevent deiodination of thyroid hormones in the incubation samples. The samples were vortexed before and after adding standards and antioxidant solutions. After this equilibration step, a 1-mL volume of hydrochloric acid was added, vortexed, and samples incubated covered for 60 min in a 50 °C water bath oscillating at 180 rpm to allow for denaturation of plasma proteins and release of protein-bound hormones. Thyroid hormones were isolated from extracts using a solid-phase extraction (SPE) procedure with SampliQ OPT polymer cartridges. Other examples of SPE methods for extracting thyroid hormones using SPE, is described by Simon et al. [20] whirl-mixed plasma samples from mammals, sonicated them in an ultrasonic bath for 10 min, and stored them in the dark for 50 min at room temperature. Subsequently, 2 to 3 mL (depending on the species) of water/2-propanol (4:1, v/v) were added to each sample, and the resulting mixture was sonicated for another 10 min. Finally, to reduce the influence of the organic solvent on the SPE procedure, the samples were diluted with water until the organic solvent percentage (isopropanol and solvent of the spiking mixture) was less than 5% v/v. When the organic solvent is less than 5 % v/v, then when the diluted sample is loaded on the SPE cartridge, there is no risk of having our target analytes lost during the loading step. In another method described by Hansen et al. [30] the thyroid hormones were extracted by vortexing the serum or plasma aliquots, then the samples were cooled down for 1 hour on ice. Thereafter, urea solution was added, vortexed, and incubated for another hour at 50 °C. The SPE cartridges were placed on a vacuum manifold and preconditioned using 1.0 mL methanol, 1.0 mL ultrapure water, and 1.0 mL 1 % v/v ammonia hydroxide. Thereafter, samples were loaded and extracted, and cartridges were immediately washed with 2.0 mL aliquots of 1 % ammonia hydroxide, ultrapure water, 1 % formic acid, and again ultrapure water. Using the vacuum pump, a 1-min drying period was used, followed by a final wash with

0.50 mL 3 % v/v methanol, to remove more lipophilic residues. Analytes were eluted from the SPE cartridge with 1.50 mL methanol.

### 1.8.3. Hybrid SPE-PPT Extraction

One of the major causes of signal ion suppression in bioanalysis is the presence of phospholipids during LC-MS analysis. Hybrid SPE dramatically reduced the levels of residual phospholipids in biological samples, leading to significant reduction in suppression effects derived from the interfering matrix components. This new procedure that combines the simplicity of precipitation of proteins with the selectivity of SPE to remove phospholipids, allows to obtain much cleaner extracts than with the conventional procedures (1.7.2. and 1.7.3.). Hybrid SPE-precipitation procedure provides significant improvement in bioanalysis and a practical and fast way to ensure the avoidance of phospholipids-based matrix effects [31]. The illustration of the Hybrid SPE-PPT method is demonstrated in **figure 8**.



**Figure 8:** Illustration of the hybrid SPE-PPT extraction procedure [31].

The general procedure for the Hybrid SPE-PPT technique (**Figure 8**) is described below:

1. 100-300  $\mu\text{L}$  plasma or serum is added to the hybrid SPE-PPT plate, followed by either a 1 % v/v formic acid in 300-600  $\mu\text{L}$  acetonitrile or 1 % w/v ammonium formate in 300-600  $\mu\text{L}$  methanol.

2. The samples are sealed and mixed by shaking/vortexing the hybrid plate.
3. After the mixing, vacuum is applied, to push the solvent, through the filter, while retaining the proteins and phospholipids.
4. The eluent is collected, free of proteins and phospholipids, and can be analyzed directly, or further work-up can be performed.

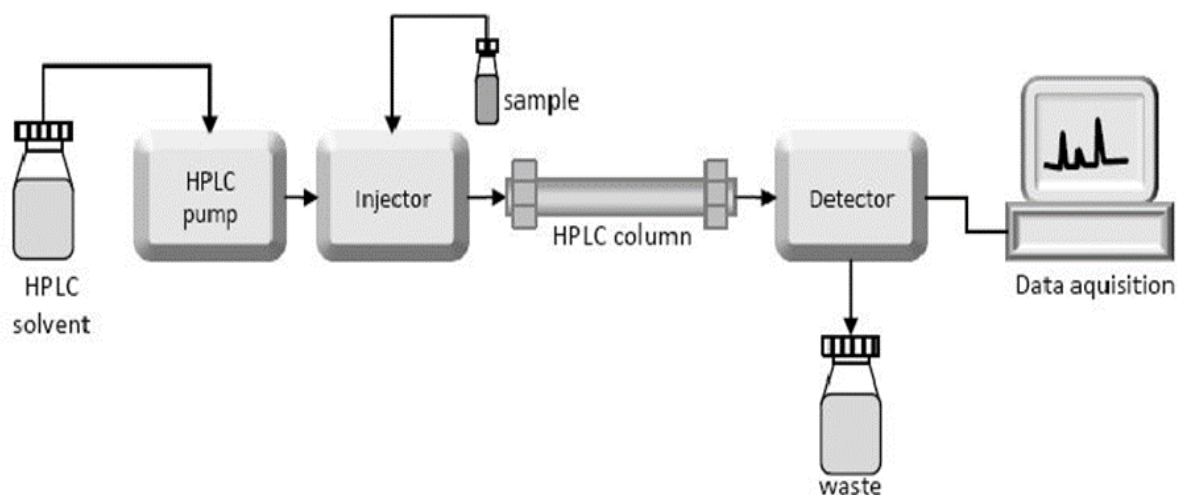
The use of the hybrid SPE-PPT plate is not a necessity. The samples can be centrifuge instead for the removal of proteins; centrifugation was performed here-in this work.

Methods like liquid-liquid extraction (LLE) and solid phase extraction (SPE) are commonly used for extracting thyroid hormones from biological samples, but the usage of hybrid SPE-PPT for extracting thyroid hormones has not be explored extensively. In general, there are few research articles relevant to hybrid SPE-PPT and trace level bioanalysis. Alvarez et al. [32] used the hybrid SPE-PPT for the extraction of thyroid hormones, while Asimakopoulos et al. [33] was the first to use it on trace level analysis of endocrine disruptors. In the work of Alvarez, the analysis of thyroid hormones, the deproteinization of serum was performed by mixing serum with acetonitrile containing 3% (v/v) of formic acid. The samples were vortexed, sonicated, and kept frozen. Thereafter, the deprotonated fraction was removed using centrifugation and the precipitate was extracted twice with ethyl acetate acidified with 3% (v/v) formic acid. Supernatants and extracts were combined and loaded onto the Hybrid SPE cartridges mounted onto a vacuum manifold. The resulting eluate was collected in amber glass vials, evaporated to dryness and the residue dissolved in mobile phase. In the work Asimakopoulos et al. [33], pool serum sample were thawed, equilibrated to room temperature and vortex mixed for 1 min. The eluents were evaporated to near-dryness under a gentle stream of nitro-gen gas (N<sub>2</sub>), reconstituted and analyzed.

### **1.9. High Performance Liquid Chromatography**

The central pillar of the HPLC analysis system is the chromatographic column where the actual separation occurs (**Figure 9**). The ability to separate compounds in a sample using chromatography is an essential step in analyzing compounds in complex matrixes like biological samples. The efficient separation of different target analytes from each other, and from the matrix

components is essential to avoid or reduce background noise obtained in analysis. It also diminishes the risk of false positive or negative results [34]. The HPLC separates the compounds contained within a sample based on their difference in polarity.



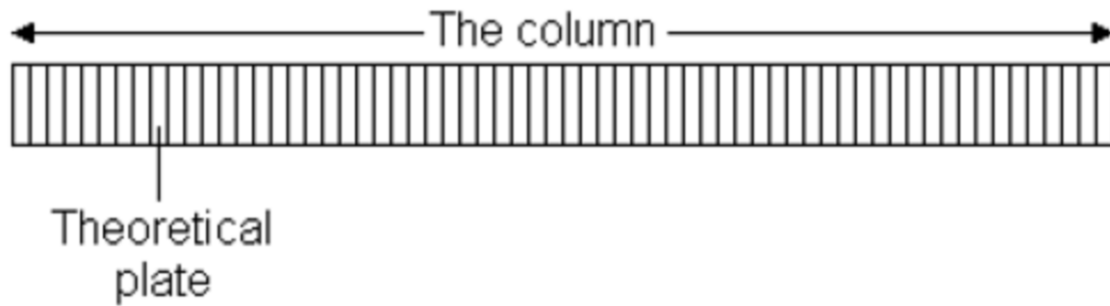
**Figure 9:** Illustration of a general HPLC analysis system [35].

The HPLC system consist of different components, which perform different tasks in the HPLC system (**Figure 9**) [35]. At the start, a carrier stream of solvent (the mobile phase) is pumped into an injector. From the injector the sample is introduced. The sample is dissolved in the mobile phase or in a similar solvent. After the sample has been introduced into the carrier stream (mobile phase), the sample goes through the column, where the separation of the individual components (analytes) are performed, based on the polarity of every different component. After the sample has been divided into its individual components, the different components are detected. The ability of the column to separate each component from each other, is based on the plate theory (**see 1.8.1.**) and rate theory (**see 1.8.2.**). Separation is specific for each column design/type and the separated peaks elute flowing into the detector.

### 1.9.1. Plate Theory

The plate theory is a theoretical zone, in which two different phases, the solid and liquid phase, establishes an equilibrium with each other, often referred to as the theoretical tray [25, 36].





**Figure 10:** Illustration of the plate theory concept [37].

The use of theoretical plates is important; as the number of plates in a column increases, so does the height equivalent to a theoretical plate (HETP, see equation 1.1), and consequently, does the separation of components in the column. The plate theory also describes the elution curve or the chromatogram of a solute. Based on this, the volume and the column efficiency can be obtained.

$$HETP = \frac{L}{N} \quad (1.1)$$

Where  $L$  = the column length and  $N$  = the number of theoretical plates.

### 1.9.2. Rate Theory

In contrast to the plate theory, the rate theory describes the migration of molecules in a column [25]. The rate theory follows the Van Deemter equation (equation 1.2.), which gives the most accurate prediction of the dispersion in a liquid chromatography column

$$H = A + \frac{B}{u} = Cu \quad (1.2)$$

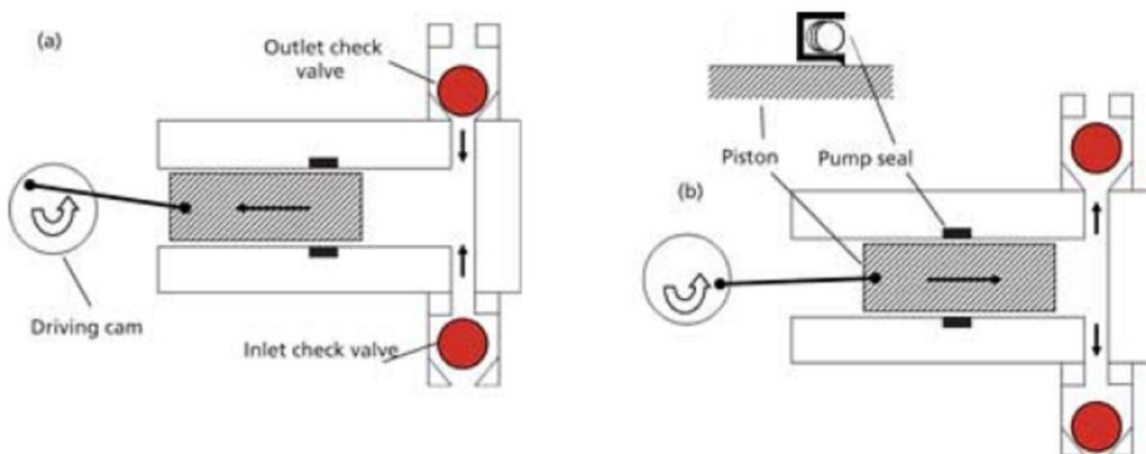
Where  $A$  = Eddy-diffusion,  $B$  = Longitudinal diffusion,  $C$  = Mass transfer and  $u$  = Linear velocity of the solid phase through the column; it considers the various pathways that a sample must travel through a column. By using the Van Deemter equation, it is possible to obtain the optimal velocity and minimum plate height needed for the HPLC column to achieve the desired separation.

### 1.9.3. Mobile Phase

The mobile phase is the carrier stream, in which the sample to be analyzed is injected into [25]. The choice of the mobile phase is mainly relative to the column's polarity, and is usually a mixture of a polar and a non-polar liquid component. With a C18 HPLC column (reversed phase column), a mixture of water-acetonitrile or water-methanol, is usually applied. The HPLC solvent(s) are usually degassed before use so that chromatographic separation is optimal.

### 1.9.4. HPLC Pump

The HPLC-pump is a pumping system, which is based on the piston pump design seen in figure 11 [38].

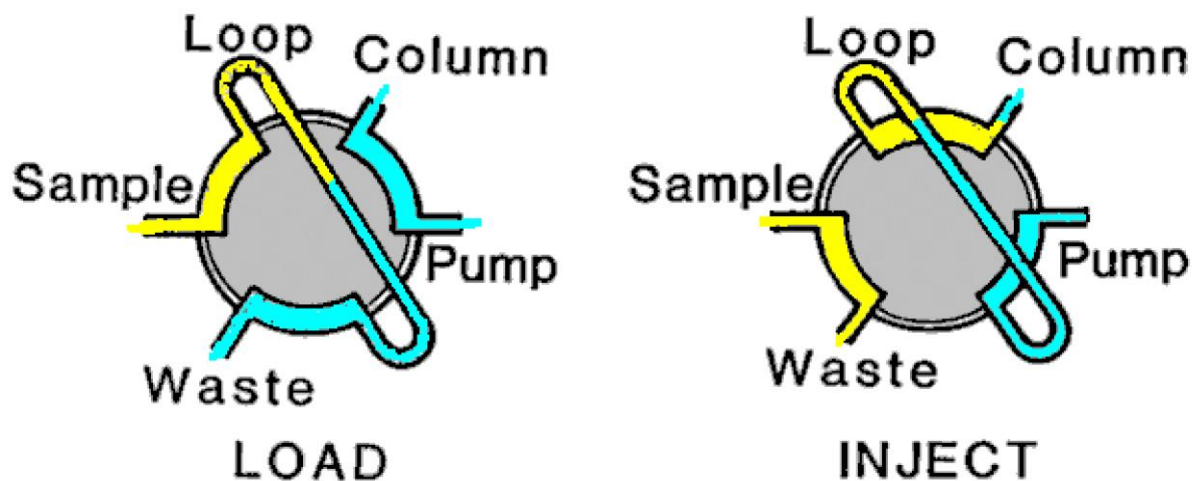


**Figure 11:** Illustration of an HPLC pump, with a moving piston (a) and (b) [38].

The principle of the LC-pump, consists of a cylindrical pump chamber in which the piston resides, a drive cam, a pair of check valves and a pump seal. As the motor rotates the piston moves in and out of the pump chamber. The check valves are used to control the flow and direction of the mobile phase through the system. In the bottom of the chamber is a sapphire seat. The ruby ball moves up and down on the sapphire seat, in response to gravity and fluid pressure (see figure 11).

### 1.9.5. Sample Injector

The sample injector in HPLC, is the part of the HPLC set up, where the samples are injected into the carrier liquid (the mobile phase) [25]. The sample can be injected automatically using an injection loop or manually using a syringe (see figure 12).

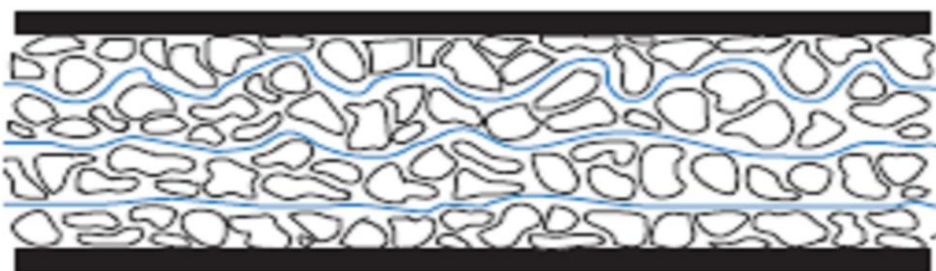


**Figure 12:** Illustration of a manual injector site [39].

### 1.9.6. HPLC Column

Standing as the central component in the HPLC system, the column, is the critical part of the HPLC system which separates the different components in a sample [25]. A typical column is a long tube, with an internal diameter ( $d_i$ ), e.g., 4.5 mm. The tube is packed with small silica particles, which have an average diameter, e.g., 3.5  $\mu\text{m}$ . Illustration of the flow of mobile phase through the column is demonstrated in figure 13.

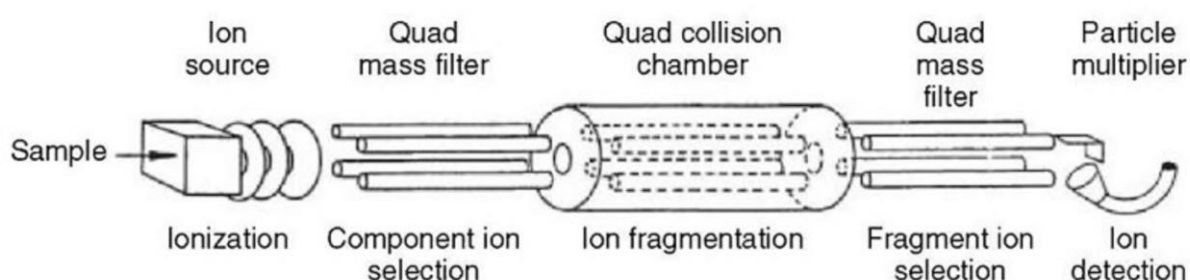
The particles in the column act upon the different components in the injected sample differently, based on the polarity of the individual component. When the sample moves through the column, the target analytes with the highest polarity are retained the longest in the column, while the target analytes with the least polarity will exit the column first. This leads to the separation of every component in the sample, and every separated component corresponds to a specific retention time. Retention time, is the time between the sample injection into the mobile phase until the separated component is observed by the specific detector.



**Figure 13:** Cross section illustration of an eluent system moving through column packed with small particles [25].

### 1.10. Mass Spectrometry

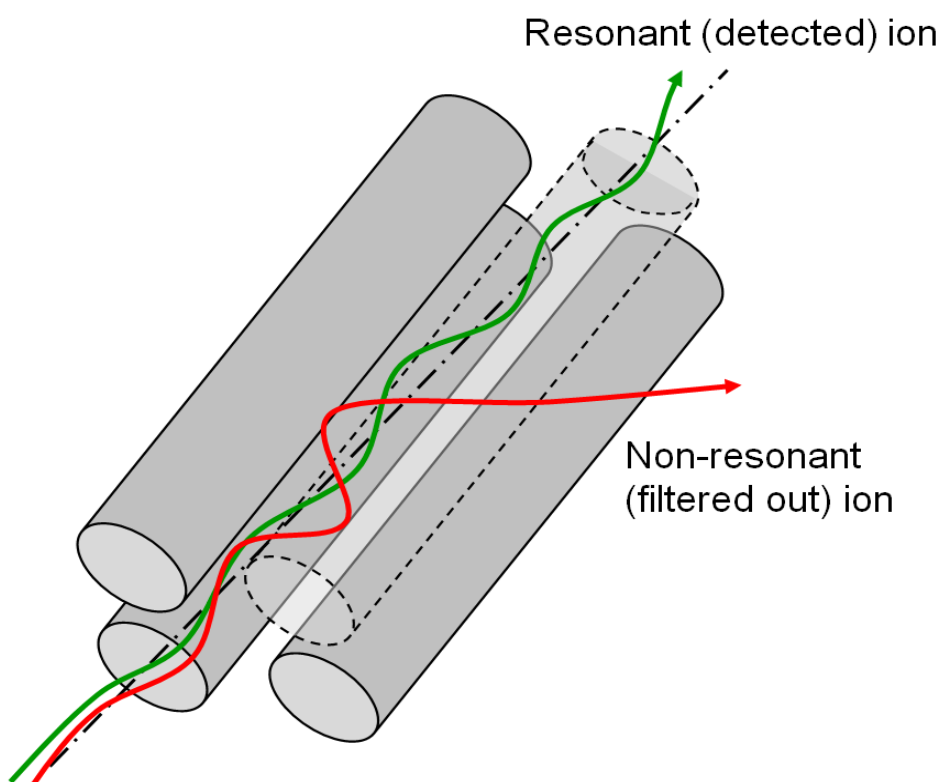
Here-in this work, a tandem mass spectrometer (triple quadrupole) were used for the detection and quantification of the target analytes. The quadrupole is probably the most widely used MS/MS instrument and consists of three sets of quadrupole rods in series. In the first stage of Tandem MS, the initial quadrupole is used to isolate an ion of interest [40, 41]. The second quadrupole is not used as a mass separation device, but as a collision cell, where the fragmentation of ions transmitted by the first set of quadrupole rods is carried out, and is used as a device for focusing any product ions into the third quadrupole where we isolate the target fragments of interest. Other types of tandem MS instruments tailored to HPLC, include, the quadrupole-time-of-flight instrument (QTOF), and the ion-trap instrument [42-45]. An illustration of the different components in a triple quadrupole MS system, is demonstrated in figure 14.



**Figure 14:** Illustration of the components in a triple quadrupole MS system [42].

Once the eluent from the HPLC column enters the MS interface, it is vaporized and ionized through heat and an electrical current, respectively. After this, the ionized molecules are accelerated and separated based on their charge-to-mass ratio ( $m/z$ ), which is then detected by the detector.

The quadrupole is a type of trap which uses dynamic electrical fields to trap the charged particles of the samples. Invented by Wolfgang Paul, which obtained the Nobel Prize for his work in 1989 [45]. An illustration of the quadrupole mass analyzer is given in **figure 15**.



**Figure 15:** Concept illustration of Quadrupole mass analyzer [47].

The quadrupole consists of a stream of ions that moves in-between four cylindrical rods. The four rods have charge, and are either positive or negative charged. By changing the strength of the field in the quadrupole, it is possible to obtain only the desired ionized molecule.

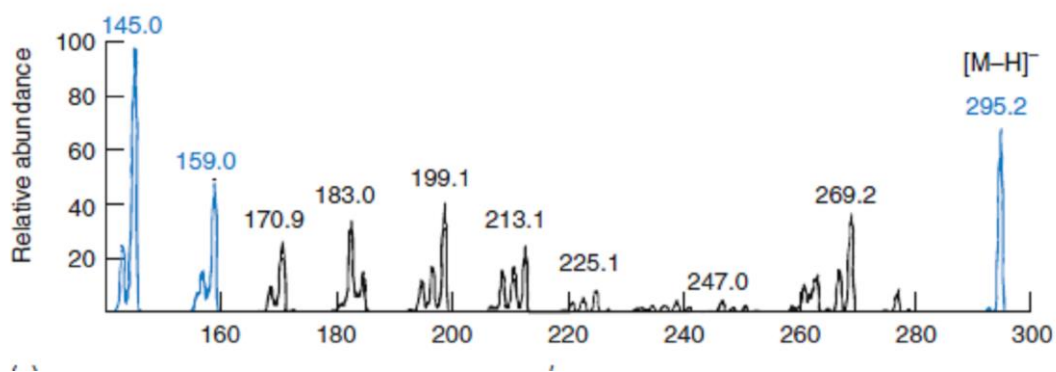
### 1.10.1. Vaporization

The sample is first injected into the vaporization chamber, where the sample is heated, until it becomes an aerosol/vapor [25]. This part of the mass spectroscopy apparatus, is critical, in

order to totally vaporize the HPLC eluent. The type of vaporization chamber depends on the phase of the sample, and the complexity of the sample. For liquid phases, the Electrospray (ESI) or Atmospheric pressure chemical ionization (APCI) is usually used [25].

### 1.10.2. Detection

When the target analytes have entered the deflection part of the MS, the ionized molecules become subject to an electrical field, which separates the different compounds based on their mass to charge ratio [25]. The final component of the MS apparatus, is the detector. The charged ions are recorded. The signal produced in the detector during the scan will produce a mass spectrum as demonstrated in **figure 19** [25].



**Figure 19:** Example of a common MS spectra [25].

In the detector, typically, some type of electron multiplier is used to amplify the signal. This is because the number of ions observed by the detector at any instant is typically quite small, and due to this amplification is often necessary to get a usable signal, which is used to produce a MS chromatogram spectrum of the components.

### 1.11. Hard Vs. Soft Ionization

In mass spectrometry, ionization refers to the production of gas phase ions suitable for resolution in the mass analyzer [48-51]. There are several ion sources available, each has advantages and disadvantages for different applications. For example, electron ionization (EI) gives a high degree of fragmentation, yielding highly detailed mass spectra which when skillfully analyzed can provide important information for structural elucidation/characterization and facilitate identification of unknown compounds by

comparison to mass spectral libraries obtained under identical operating conditions. However, EI is not suitable for coupling to HPLC, i.e. LC-MS, since at atmospheric pressure, the filaments used to generate electrons burn out rapidly. Thus, EI is coupled predominantly with GC, i.e. GC-MS, where the entire system is under high vacuum. Hard ionization techniques are processes which impart high quantities of residual energy in the subject molecule invoking large degrees of fragmentation [52]. Resultant ions tend to have  $m/z$  lower than the molecular mass. The most common example of hard ionization is electron ionization (EI). Soft ionization refers to the processes which impart little residual energy onto the subject molecule and as such, results in little fragmentation. Examples include fast atom bombardment (FAB), APCI, ESI and matrix-assisted laser desorption/ionization (MALDI).

### **1.12. Interfacing Liquid Chromatography and Mass Spectroscopy**

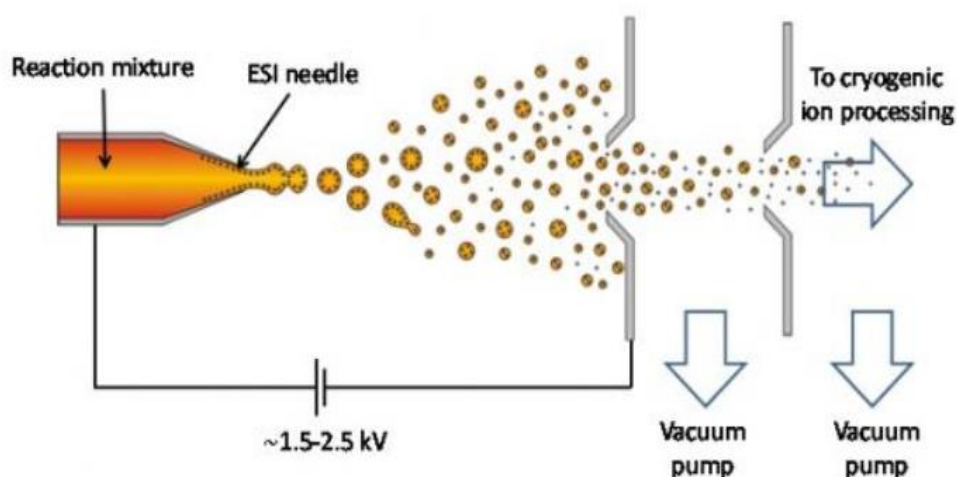
Once the target analytes have been adequately chromatographically separated through the LC column, they move into the MS (or tandem MS) detector for the detection and measurement. The coupling of LC to MS (or tandem MS) has always been a demanding task, since there are major incompatibilities between the operating characteristics of the two techniques [53]. In general, the target analytes need a liquid mobile phase in order to get separated with LC, but thereafter they need to be transferred into a gas phase in order to be drawn into the MS (or tandem MS) detector; these objectives are achieved by the interfaces of the mass spectrometers that serve as junction couplings between the two techniques. Today there are a large variety of interfaces for LC-MS/MS on the market.

The LC eluent that derives from the chromatographic column contains the separated target analytes, the elution solvent, and numerous other dissolved components (i.e., buffers). Consequently, solvents and buffers must be evaporated, and the target analytes need to be ionized efficiently to be measured successfully by the MS (or tandem MS) analyzer. Modern LC-MS and LC-MS/MS instruments are mainly equipped with atmospheric pressure interfaces (API) in which mobile phase solvent is removed under normal atmospheric pressure (i.e., non-vacuum) conditions as the samples are being ionized. The two most applicable interfaces for analysis are ESI and APCI; atmospheric pressure photoionization (APPI) is also used for a number of chemical classes [53-60]. In all three cases, a “soft ionization” is performed; this term refers to the formation of ions without breaking any chemical bonds in other words, all covalent interactions are kept intact during the ionization process [53-60]. The interfaces are

operated under positive or negative ionization mode depending on the fact that the target analyte(s) is prone to accept (positive analyte ion formation; cation) or lose (negative analyte ion formation; anion) a proton, respectively. Ideally, the interface should not cause any reduction in LC performance, any uncontrolled chemical modification of the target analyte(s), low sample transfer to the MS or tandem MS instrument, and high background noise during analysis. The operation of the interface should be compatible with all chromatographic conditions including flow rates (from 20 nL/min to 2 mL/min), mobile phase composition, and gradient elution programs [25, 53-60].

### 1.12.1. ESI

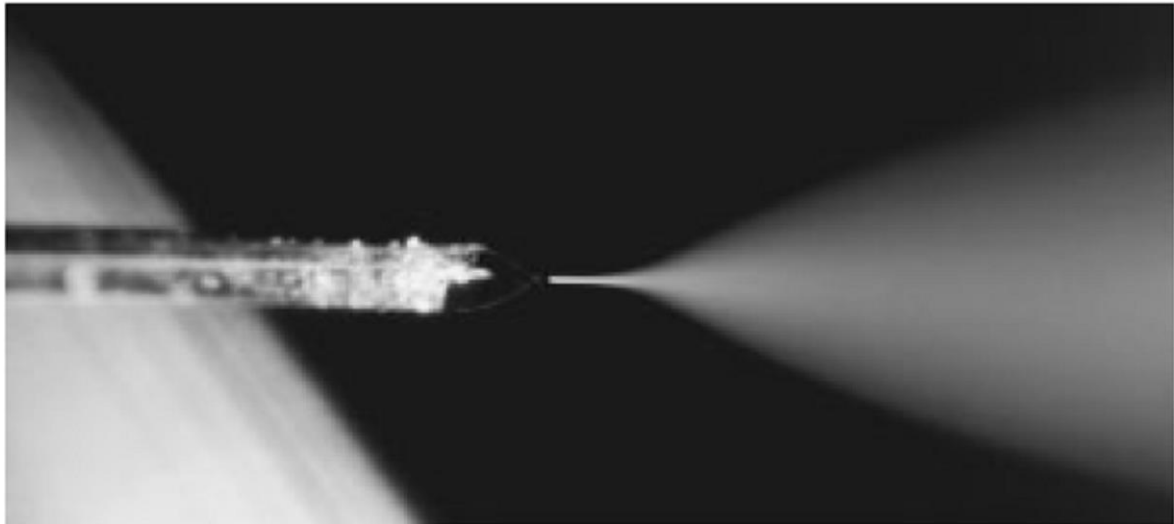
ESI is a technique which predominately creates ion species in solvent and converts them into gas phase ion species by passing the LC eluent through a capillary that is maintained at high voltages, typically on the order of a positive (+) or negative (-) potential of 2-5 kV. The high voltage disperses the LC eluent stream, forming a charged droplet (Taylor Cone) that undergoes further de-solvation and emits a mist of highly charged spray (droplets and ions) (figure 20). In the majority of MS and tandem MS instruments, the application of a stream of gas (nitrogen) and temperature (typically on the order of 250-350 °C) is used to assist in the de-solvation and nebulization of the formed spray [56].



**Figure 20:** Illustration of the ESI system used in LC-MS/MS [56].

The sample is ejected through a needle at the end of the metal ESI needle, which is connected to the LC apparatus (figure 21) [25].

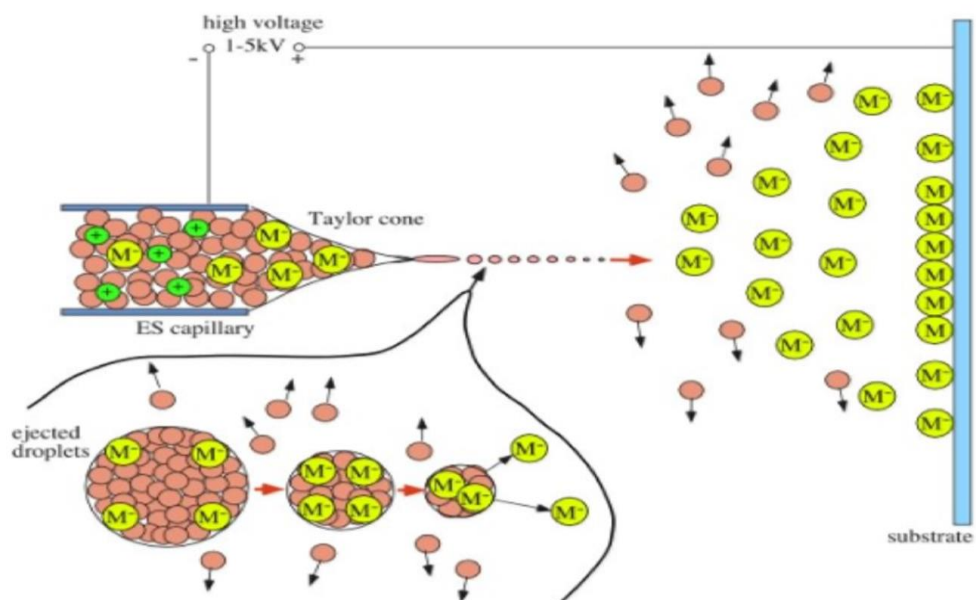




**Figure 21:** Photo of an ESI needle [25].

### 1.12.2. Taylor Cone Formation

The Taylor cone is a cone shaped jet of charged particles observed in electro spraying and electrospinning techniques [57-60]. The jet of charged particles occurs when a small volume of liquid is exposed to an electrical field. Due to the surface tension in the liquid, the shape of the charged volume is changed into the shape of a cone. Illustration of this is seen in **figure 22** [59].



**Figure 22:** Illustration of the Taylor cone [59].

When a certain amount (a threshold) of voltage is reached, the Taylor cone inverts and emits a jet of charged particles, called a cone-jet. This is the beginning of the electrospray process, in which the charged particles from the cone-jet are transferred to the gas phase. The charged ionic gas is then transferred to the rest of the mass spectrometer sections.

### 1.12.3. APPI

APPI is an ionization method where a UV light source emits a light in the UV band (320-100 nm) [61, 62]. The light source can be anything that emits a wave length in the UV band, normally either a lamp or a laser. This process is therefore called single photon ionization, and is the basic principle of the APPI function. For sufficiently high-power densities, absorption of at least two photons in a rapid sequence can occur. This process is called multi-photon ionization (MPI). The laser light sources used in APPI have power densities which allow multiphoton ionization. The required power density must be sufficiently high, so that in the lifetime of the first reached electronic state, which is in the range of a few nanoseconds, a second photon can be absorbed with a reasonable probability. At which point a radical cation is formed (see figure 23) [63]. Most of the organic molecules which are favorable for a photoionization method have ionization potentials smaller than approximately 10 eV. Thus, APPI utilizes light with a photon energy of around 5 eV which corresponds to a wavelength of about 250 nm, which is in the ultraviolet (UV) part of the electromagnetic spectrum.

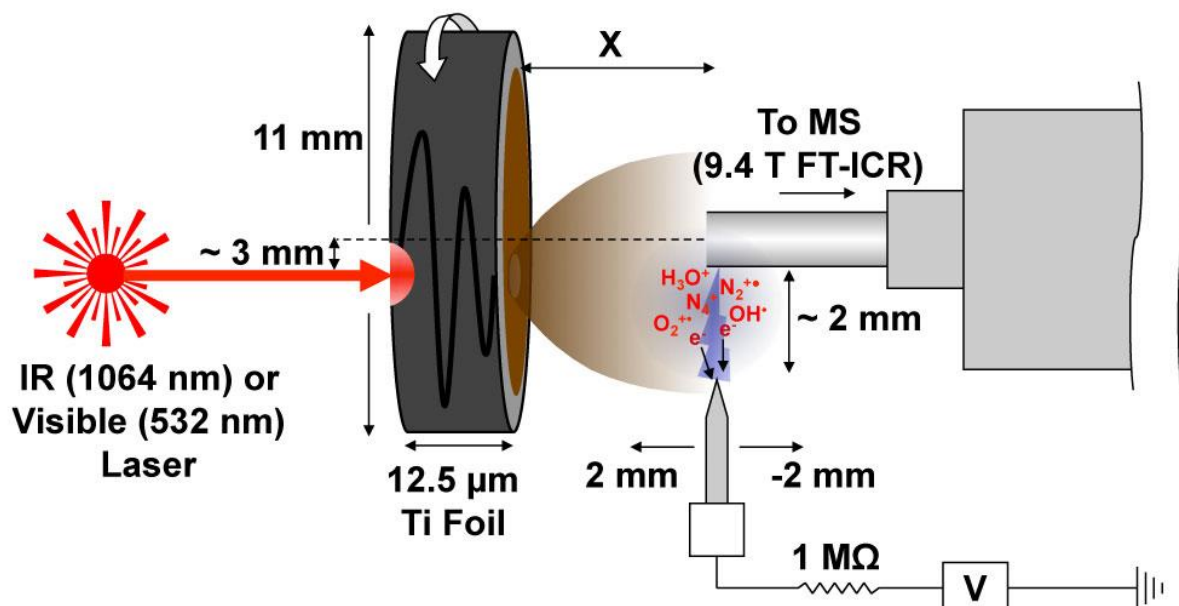
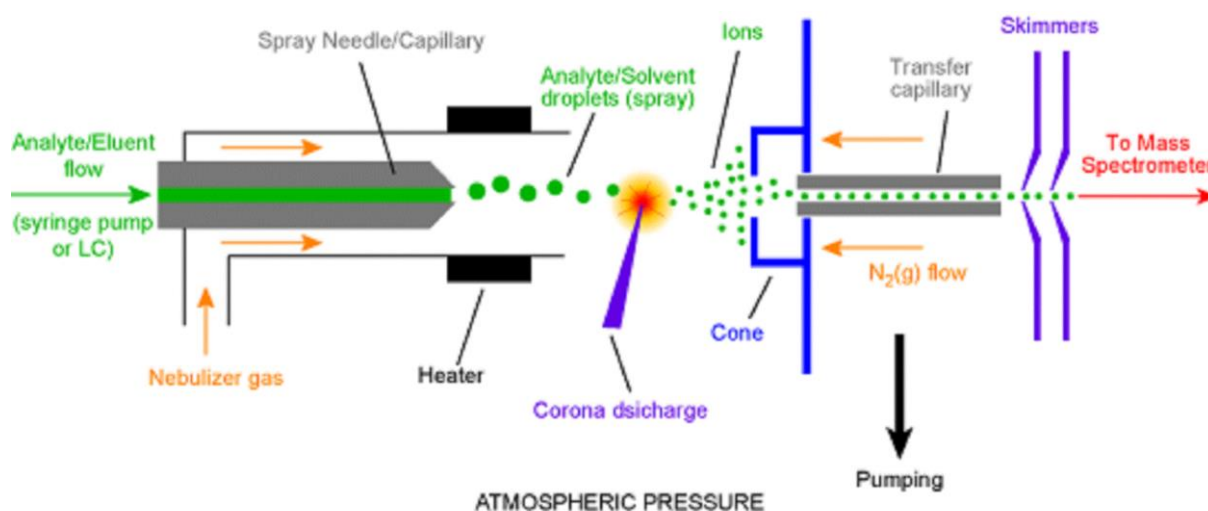


Figure 23: Illustration of the APPI interface [63].

#### 1.12.4. APCI

APCI is an ionization method utilized in mass spectrometry analysis [64]. The technique utilizes gas-phase ion-molecule reactions at standard atmospheric pressure (1 atmosphere). APCI is an ionization method like chemical ionization where the ions are produced by discharging aerosol into a corona, which ionizes the aerosol particles. The application of APCI coupled with HPLC has gained a lot of popularity over the years, given the methods ability to perform trace analysis detection of molecules such as steroids, pesticides and drug metabolites.



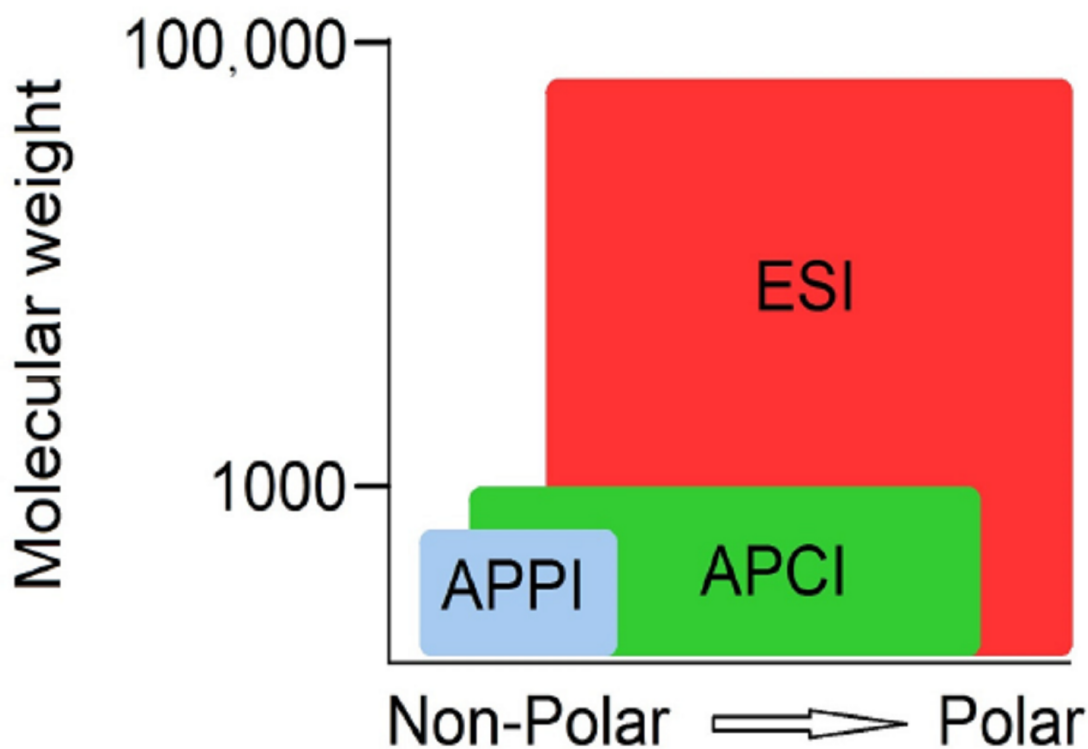
**Figure 24:** Illustration of the APCI principle [65].

A typical APCI interface (see figure 24), consists usually of three main parts: a nebulizer probe which can be heated between 350-500°C, an ionization area with a corona discharge needle, and an ion-transfer area in vacuum. The analyte in solution is introduced from a direct inlet probe or a liquid chromatography (LC) eluate into a pneumatic nebulizer. In the heated nebulizer, the analyte coaxially flows with nebulizer N<sub>2</sub> gas to produce an aerosol. Once the aerosol stream arrives in the ionization region under atmospheric pressure, molecules are ionized at the corona. After the ionization, the sample ions pass through a small orifice skimmer into the ion-transfer region, and into a mass analyzer for subsequent mass analysis.

#### 1.12.5 When to Choose Between ESI, APPI and APCI ?

The most important parameters which determine the choice of interface, between APCI, APPI and ESI, is the molecular weight of the target analytes and the polarities of these analytes [66]. As demonstrated in figure 25, there is an overlapping region between the three interfaces, in

which more than one interface may be applicable. When choosing the interface, the general rule is that ESI is applied to high molecular weight target analytes as these compounds may be thermally labile, APCI is applied to volatile and thermally stable compounds, and APPI is applied to compounds that are poorly ionizable with ESI and APCI.



**Figure 25:** Illustration of the three interface criteria [66].

Dealing with target analytes that have their molecular weight below 900 g/mole, and the target analyte maintains non-polarity to low polarity, the APPI is employed as an interface. If the target analytes are around 1000 g/mole or below, and have low to medium polarity, then the APCI interface is used, and when the polarity of the target analytes is between low and high, and have a molar weight below 100 000 g/mol, then the ESI interface is applied (**see figure 25**).

### 1.13. What has been done thus far for thyroid hormones analysis in mammals?

In **table 1**, an overview of the most relevant previous works on thyroid hormone analysis in mammals, are presented. The methods used for analysis vary from isotope analysis using Radioimmunoassays (RIA) to LC-MS/MS. The articles in **table 1**, cover more than three decades from the publication of Escobar et al. [19] in 1985, to the publication of Alvarez et al.

**[32]** in 2017. The articles incorporate the analysis of thyroid hormones and derivatives, involving mainly  $rT_3$ ,  $T_4$  and  $T_3$ . From **table 1**, it can be observed that 11 of the 14 methods published use LC-MS/MS as the method for analysis. And, out of these 11 articles, a total of 9 articles use ESI. Regarding the column used for the liquid chromatography system (LC-system) some variations of the classical C-18 column is used in 6 out of the 11 LC-MS/MS systems.

Regarding the mass spectrometry part, the usage of a triple quadrupole is present in 7 out of the 11 articles. Some trends due to publication year of the articles is also observed, where the older articles like Escobar et al. **[19]** (published in 1985) uses RIA and not LC-MS/MS, while in newer articles, like Bussy et al. **[68]** and Richards et al. **[69]** which were both published in 2017, favor the LC-MS/MS technique (triple quadrupole) using ESI as an interface. Literature indicates that there is a clear trend towards the use of a triple quadrupole LC-MS/MS system, with ESI being as a more favored approach when analyzing thyroid hormones. Based on the literature on method development for the analysis of thyroid hormones in biological media, there were two challenges indicated, that I took into consideration in the experimental part of this work:

- 1) Instability of target analytes during sample preparation due to the potential deiodination of thyroid hormones; and
- 2) Potential low LC-MS/MS instrumental sensitivity.

**Table 1:** Overview of analytical methodologies for determination of thyroid hormones.

| Analytes   | Analytical technique(s) | Triple Quadrupole | Column                         | Interface | Method Limit of Detection (LOD)   | Method Limit of Quantification (LOQ)  | References |
|--|-------------------------|-------------------|--------------------------------|-----------|---|---|------------|
| T <sub>1</sub><br>3,3' - T <sub>2</sub><br>3,5 - T <sub>2</sub><br>rT <sub>3</sub> , T <sub>3</sub> , T <sub>4</sub>                         | LC-MS/MS                | Yes               | ZORBAX Extend-C <sub>18</sub>  | ESI       | 0.16 ng/mL<br>0.20 ng/mL<br>0.20 ng/mL<br>0.33 ng/mL                                    | 0.53 ng/mL<br>0.70 ng/mL<br>0.70 ng/mL<br>1.1 ng/mL                                       | [70]       |
| T <sub>3</sub><br>T <sub>4</sub><br><sup>12</sup> C <sub>12</sub> -T <sub>4</sub>  | RIA and LC-MS/MS        | No                | Synergi Polar RP               | ESI       | 0.24 ng/mL<br>0.42 ng/mL<br>0.42 ng/mL  | 0.79 ng/mL<br>1.39 ng/mL<br>1.39 ng/mL  | [29]       |
| 3,3', 5 - T <sub>3</sub><br>3,3 - T <sub>3</sub><br>T <sub>4</sub>   | LC-MS/MS                | Yes               | Waters BEH C <sub>18</sub>     | ESI       | 1.0 pg/mL<br>1.0 pg/mL<br>1.0 pg/mL   | 10.0 pg/mL<br>10.0 pg/mL<br>10.0 pg/mL  | [68]       |
| T <sub>3</sub><br>T <sub>4</sub>   | LC-MS                   | No                | ACE Excel C <sub>18</sub> -PPF | ESI       | 0.4 µg/L<br>0.4 µg/L  | 1.0 µg/L<br>1.4 µg/L  | [32]       |
| rT <sub>2</sub><br>T <sub>2</sub><br>rT <sub>3</sub><br>T <sub>3</sub><br>T <sub>4</sub>   | (SPE)-LC-MS/MS          | No                | Waters BEH C <sub>18</sub>     | ESI       | 0.16 pg/mL<br>0.32 pg/mL<br>0.59 pg/mL<br>0.20 pg/mL<br>0.39 pg/mL                      | 1.73 pg/mL<br>1.31 pg/mL<br>0.98 pg/mL<br>1.43 pg/mL<br>1.32 pg/mL                        | [71]       |
| T <sub>4</sub><br>T <sub>3</sub><br>rT <sub>3</sub><br>3,3' - T <sub>2</sub><br>3,5 - T <sub>2</sub><br>3 - T <sub>1</sub><br>T <sub>0</sub> | LC-MS/MS                | Yes               | -                              | ESI       | 3.5 pg/mL<br>1.5 pg/mL<br>2.9 pg/mL<br>1.7 pg/mL<br>2.3 pg/mL<br>0.3 pg/mL<br>7.5 pg/mL | 11.6 pg/mL<br>5.0 pg/mL<br>9.6 pg/mL<br>5.6 pg/mL<br>7.6 pg/mL<br>1.0 pg/mL<br>25.0 pg/mL | [30]       |

|                                 |               |     |                            |      |              |              |             |
|---------------------------------|---------------|-----|----------------------------|------|--------------|--------------|-------------|
| <sup>125</sup> I-T <sub>3</sub> | RIA           | No  | —                          | —    | 1.5 pg/mL    | 4.9 pg/mL    | <b>[19]</b> |
| T <sub>3</sub>                  |               |     |                            |      | 1.5 pg/mL    | 4.9 pg/mL    |             |
| <sup>131</sup> I-T <sub>4</sub> |               |     |                            |      | 2.5 pg/mL    | 8.3 pg/mL    |             |
| T <sub>4</sub>                  |               |     |                            |      | 2.5 pg/mL    | 8.3 pg/mL    |             |
| <sup>125</sup> I-T <sub>3</sub> | RIA           | No  | —                          | —    | 0.75 pg/mL   | 2.49 pg/mL   | <b>[21]</b> |
| T <sub>3</sub>                  |               |     |                            |      | 0.75 pg/mL   | 2.49 pg/mL   |             |
| <sup>125</sup> I-T <sub>4</sub> |               |     |                            |      | 2.50 pg/mL   | 8.30 pg/mL   |             |
| T <sub>4</sub>                  |               |     |                            |      | 2.50 pg/mL   | 8.30 pg/mL   |             |
| T <sub>3</sub>                  | LC-MS/MS      | Yes | Eclipse XDB-C <sub>8</sub> | ESI  | 2.5 pg/mL    | 8.3 pg/mL    | <b>[72]</b> |
| T <sub>3</sub>                  | LC-MS/MS      | Yes | Sephadex® LH-20            | ESI  | 0.20 pg/mL   | 1.43 pg/mL   | <b>[73]</b> |
| T <sub>4</sub>                  |               |     |                            |      | 0.39 pg/mL   | 1.32 pg/mL   |             |
| T <sub>4</sub>                  | LC-MS/MS      | Yes | μPorasil NP-HPLC           | ESI  | 0.016 μmol/L | 0.053 μmol/L | <b>[20]</b> |
| T <sub>4</sub> *                |               |     |                            |      | 0.016 μmol/L | 0.053 μmol/L |             |
| T <sub>3</sub>                  | LC-MS/MS      | Yes | C-18 Column                | APCI | 2.5 pg/mL    | 8.3 pg/mL    | <b>[28]</b> |
| T <sub>4</sub>                  |               |     |                            |      | 2.5 pg/mL    | 8.3 pg/mL    |             |
| T <sub>3</sub>                  | LC-MS/MS      | No  | Zorbax SB C <sub>18</sub>  | APCI | 2.5 pg/mL    | 8.3 pg/mL    | <b>[74]</b> |
| T <sub>4</sub>                  |               |     |                            |      | 2.5 pg/mL    | 8.3 pg/mL    |             |
| T <sub>3</sub>                  | RIA and Cobra | No  | —                          | —    | 0.022 nmol/L | 0.073 nmol/L | <b>[75]</b> |
| T <sub>4</sub>                  |               |     |                            |      | Auto-Gamma,  | 0.341 nmol/L |             |

## 2. Methods and Materials

In this chapter, the materials and methods used in this thesis are presented.

### 2.1. Chemicals and Instrumentation

Methanol HPLC grade ( $\geq 99.9\%$ ), Acetonitrile (99.8%), formic acid (95-97%), ammonium formate ( $\geq 98\%$ ), ammonium acetate, ( $\geq 98\%$ ), ammonium hydroxide, ( $\geq 25\%$ ) and hydrochloric Acid (37%) were obtained from Sigma Aldrich. Ethyl acetate (analytical grade) was purchased from VWR Chemicals.

The thyroid hormones, D<sub>2</sub>-Thyroxine (Internal Standard), 99% (neat), 3,3',5-Triiodo-L-thyronine (T<sub>3</sub>) solution, 100  $\mu\text{g}/\text{mL}$  in methanol with 0.1 N NH<sub>3</sub>, 3,3',5'-Triiodo-L-thyronine (rT<sub>3</sub>) solution 100  $\mu\text{g}/\text{mL}$  in methanol with 0.1N NH<sub>3</sub>, L-Thyroxine solution 100  $\mu\text{g}/\text{mL}$  in methanol with 0.1N NH<sub>3</sub>, and 3,3'-Diiodo-L-thyronine (T<sub>2</sub>), 98% (neat), were obtained from Sigma Aldrich Norway. Standard stock solutions (10  $\mu\text{g}/\text{mL}$ ) of all target analytes were prepared in methanol and were stored at  $-20\text{ }^{\circ}\text{C}$  for up to 3 months. A standard stock solution (10  $\mu\text{g}/\text{mL}$ ) for the internal standard was also prepared in methanol, and was also stored at  $-20\text{ }^{\circ}\text{C}$ . The calibration standards were prepared from stock solutions through serial dilutions with methanol / Milli-Q water (1:1, v/v). Polypropylene Eppendorf® tubes (Sarstedt, Nümbrecht, Germany) were used during sample preparation. Matrix serum standards were prepared from bovine serum pool samples provided by the Department of Biology. The pools were stored in polypropylene tubes of 15 mL (120 mm  $\times$  17 mm, Sardstedt, Nümbrecht, Germany) at  $-20\text{ }^{\circ}\text{C}$ .

The Milli-Q water was obtained from Sigma Aldrich, LC-MS Grade (LiChrosolv). The samples were shaken using a IKA Shaker HS 501 digital Ident. No. 0002527001. A Hettich EBA III Centrifuge-2008, was used for centrifugation purposes.

All SPE cartridges were obtained from Sigma Aldrich Norway: 1) The Hybrid SPE-PPT cartridges (55261-U SUPELCO), bed wt. 30 mg, volume 1 mL, pk of 100; 2) The Strata- X- CW cartridges, 60 mg/3 mL Tubes (Part No; 8B-S035-UBJ, Scribent Lot: S305-0062, Prod Lot: S17-001658); and 3) The Oasis HLB 60 mg/3 mL Tubes (Part No: WAT 094226, Sorbent Batch No: 150A, MfG Lot NO: 150A37181A). The SPE extraction was performed through a Visiprep™ SPE Vacuum Manifold DL (Disposable Liner), 24-port model obtained from Sigma Aldrich. Disposable liners (from PTFE) were used to eliminate any possibility of cross contamination during SPE. The LC-MS/MS analyses was carried out using a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters) connected to Xevo TQS (Triple quadrupole; Waters)



instrument. Two HPLC columns were supplied: 1) Atlantis T3 3 $\mu$ m column (2.1 x 150mm) from Waters; and 2) Kinetex 1.7 $\mu$ m C18 column (2.1 x 150mm) from Phenomenex.

Three organic single channel pipettes (Eppendorf Research plus) with variable volume were obtained from VWR: 1) 2 - 20  $\mu$ L (code 613-0869); 2) 10 - 100  $\mu$ L (code 613-0864); and 3) 100-1000  $\mu$ L (code 613-0866). Conical autosampler vials of 1.5 mL with an assembled screw cap (with hole and PTFE/silicone septum) and vial inserts (size 200 L) were supplied by Sigma–Aldrich Norway. Statistical treatment was performed with Excel (Microsoft, 2010).

## **2.2. Sample preparation/Extraction**

Three different sample preparation techniques were assessed for optimal thyroid hormones extraction: LLE, SPE, and Hybrid-SPE PPT. Throughout these experiments, we prepared the following samples:

- 1) Reagent Blanks: These are samples that contain only the solvents used for extraction throughout the process. These samples are spiked only with the internal standard. The target analytes are not spiked. They are used so that we can monitor and control potential cross contamination.
- 2) Sample Blanks: Matrix spikes fortified with a known amount of internal standard prior to extraction. They are used to determine the endogenous concentration of the samples.
- 3) Pre-extraction Spikes: Matrix spikes fortified with a known amount of internal standard and an appropriate amount of target analytes prior to extraction. They are used to determine the losses we have through the extraction process (when compared to the Post-extraction Spikes).
- 4) Post-extraction Spikes: Matrix spikes fortified with a known amount of internal standard and an appropriate amount of target analytes post to extraction and prior to instrumental analysis. They are used to determine the suppression of the signal due to the matrix (when compared to the solvent standards or post-extraction spikes in Milli-Q water).
- 5) Solvent Standards: Standards prepared in solvent.
- 6) Instrumental Blanks: These contain solvent solution and spiked only with the internal standard.

### 2.2.1. LLE

An aliquot of 250  $\mu\text{L}$  of matrix (serum or Milli-Q water) was transferred into a 15-mL polypropylene (PP) tube. The samples were fortified with 300  $\mu\text{L}$  of 1.0 M ammonium acetate. All blanks reagent and sample blanks) were spiked with a known amount of internal standard before extraction. Three pre-extraction matrix spikes in water and another three pre-extraction matrix spikes in serum were prepared by fortification of a known amount of internal standard and an appropriate amount of target analytes. Thereafter, the samples were extracted 3 times with 3 mL of ethyl acetate each time ( $3 \times 3\text{mL}$ ). For each successive extraction, the mixture was shaken in a shaker at 90 rpm for 45 minutes, and then centrifuged. The supernatants were combined, and 1 mL of Milli-Q water was added. The mixture was centrifuged again, and the supernatant was transferred into a PP tube and concentrated to near-dryness under a gentle nitrogen stream ( $\text{N}_2$ ). Finally, 250  $\mu\text{L}$  of methanol/Milli-Q water was added, vortex mixed, and transferred into an auto sampler vial for LC-MS/MS analysis. For the calculation of recoveries and matrix effects, 2 post-extraction matrix spikes in water and another 2 post-extraction matrix spikes in serum were prepared by fortification of a known amount of internal standard and an appropriate amount of target analytes into the final extracts prior to instrumental analysis.

Overall, the samples below were prepared for the evaluation of the extraction from Milli-Q water matrix (N=7 in total samples):

- 1) 1 Reagent blank- Fortified with 50 ng/mL internal standard;
- 2) 1 Sample blank- Fortified with 50 ng/mL internal standard;
- 3) 3 Pre-extraction Spikes-Fortified with 50 ng/mL internal standard and 50 ng/mL target analytes; and
- 4) 2 Post-extraction Spikes-Fortified with 50 ng/mL internal standard and 50 ng/mL target analytes

Overall, the samples below were prepared for the evaluation of the extraction from serum matrix (N=7 in total samples):

- 1) 1 Reagent blank- Fortified with 25 ng/mL internal standard;
- 2) 1 Sample blank- Fortified with 25 ng/mL internal standard;
- 3) 3 Pre-extraction Spikes-Fortified with 25 ng/mL internal standard and 25 ng/mL target analytes; and

4) 2 Post-extraction Spikes-Fortified with 50 ng/mL internal standard and 50 ng/mL target analytes.

In total, 14 samples were prepared for the evaluation of the LLE.

### 2.2.2. SPE

Isolation of the target analytes from serum and Milli-Q water was also accomplished by Oasis HLB, and Strata- X-CW cartridges.

An aliquot of 150  $\mu$ L of matrix (serum or Milli-Q water) was transferred into a 15-mL polypropylene (PP) tube. The samples were fortified with 300  $\mu$ L of 1.0 M ammonium acetate. All blanks reagent and sample blanks) were spiked with a known amount of internal standard before extraction. Three pre-extraction matrix spikes in water and another three pre-extraction matrix spikes in serum were prepared by fortification of a known amount of internal standard and an appropriate amount of target analytes. Oasis HLB and Strata- X-CW cartridges were conditioned by passage of 3 mL of methanol and 3 mL of acidified Milli-Q water (adjusted to pH < 3, acidified with HCl solution). Thereafter, the samples were passed through the cartridges, and the cartridges were washed with 3 mL of Milli-Q water and then dried under a vacuum for 10 min (8–10 psi). The target analytes were eluted with 3 mL of methanol and 3 mL of methanol with 2% (v/v) ammonium hydroxide from the Oasis HLB and Strata-X-CW cartridges, respectively. All eluents were evaporated to near-dryness under a gentle stream of nitrogen. The solvents were reconstituted with a 300  $\mu$ L of methanol/Milli-Q water (1:1 v/v), vortex-mixed, and transferred into an autosampler vial for LC-MS/MS analysis. For the calculation of recoveries and matrix effects, 2 post-extraction matrix spikes in water and another 2 post- extraction matrix spikes in serum were prepared by fortification of a known amount of internal standard and an appropriate amount of target analytes into the final extracts prior to instrumental analysis.

Overall, the samples below were prepared for the evaluation of the extraction from Milli-Q water matrix (N=7 in total samples for the evaluation of Oasis HLB cartridges, and N=7 in total samples for the evaluation of Strata-X-CW cartridges):

- 1) 1 Reagent blank- Fortified with 50 ng/mL internal standard;
- 2) 1 Sample blank- Fortified with 50 ng/mL internal standard;
- 3) 3 Pre-extraction Spikes-Fortified with 50 ng/mL internal standard and 50 ng/mL target analytes; and

4) 2 Post-extraction Spikes-Fortified with 50 ng/mL internal standard and 50 ng/mL target analytes

Overall, the samples below were prepared for the evaluation of the extraction from serum matrix (N=7 in total samples for the evaluation of Oasis HLB cartridges, and N=7 in total samples for the evaluation of Strata-X-CW cartridges):

1) 1 Reagent blank- Fortified with 50 ng/mL internal standard;

2) 1 Sample blank- Fortified with 50 ng/mL internal standard;

3) 3 Pre-extraction Spikes-Fortified with 50 ng/mL internal standard and 50 ng/mL target analytes; and

4) 2 Post-extraction Spikes-Fortified with 50 ng/mL internal standard and 50 ng/mL target analytes

In total, 28 samples were prepared for the evaluation of the SPE.

### 2.2.3. Hybrid SPE-PPT

The sample preparation procedure was developed based on previous work [32, 33]. 150  $\mu$ L of sample was pipet transferred to a 15-mL polypropylene tube, 450  $\mu$ L of methanol with 1 % v/v formic acid or 450  $\mu$ L of acetonitrile containing 1% w/v ammonium formate was added to each sample; two precipitation agents were evaluated (methanol with 1 % v/v formic acid and 450  $\mu$ L of acetonitrile containing 1% w/v ammonium formate). All blanks reagent and sample blanks) were spiked with a known amount of internal standard before extraction. Three pre-extraction matrix spikes in water and another three pre-extraction matrix spikes in serum were prepared by fortification of a known amount of internal standard and an appropriate amount of target analytes. The samples were manually mixed for 30 seconds and centrifuged (2000rpm) for 15 min. The supernatants were collected and passed through the Hybrid-SPE cartridges (the vacuum on the SPE manifold was kept between 8–10 psi). The extracts, approximately 450  $\mu$ L in volume, were received into an autosampler vial for LC-MS/MS analysis. For the calculation of recoveries and matrix effects, 2 post-extraction matrix spikes in water and another 2 post- extraction matrix spikes in serum were prepared by fortification of a known amount of internal standard and an appropriate amount of target analytes into the final extracts prior to instrumental analysis.

Overall, the samples below were prepared for the evaluation of the extraction from Milli-Q water matrix (N=7 in total samples for the evaluation of methanol with 1 % v/v formic

acid, and N=7 in total samples for the evaluation of acetonitrile containing 1% w/v ammonium formate):

- 1) 1 Reagent blank- Fortified with 33.3 ng/mL internal standard;
- 2) 1 Sample blank- Fortified with 33.3 ng/mL internal standard;
- 3) 3 Pre-extraction Spikes-Fortified with 33.3 ng/mL internal standard and 33.3 ng/mL target analytes; and
- 4) 2 Post-extraction Spikes-Fortified with 33.3 ng/mL internal standard and 33.3 ng/mL target analytes

Overall, the samples below were prepared for the evaluation of the extraction from serum matrix (N=7 in total samples for the evaluation of methanol with 1 % v/v formic acid, and N=7 in total samples for the evaluation of acetonitrile containing 1% w/v ammonium formate):

- 1) 1 Reagent blank- Fortified with 33.3 ng/mL internal standard;
- 2) 1 Sample blank- Fortified with 33.3 ng/mL internal standard;
- 3) 3 Pre-extraction Spikes-Fortified with 33.3 ng/mL internal standard and 33.3 ng/mL target analytes; and
- 4) 2 Post-extraction Spikes-Fortified with 33.3 ng/mL internal standard and 33.3 ng/mL target analytes

In total, 28 samples were prepared for the evaluation of the Hybrid SPE-PPT.

## 2.3. LC-MS/MS Method Development

### 2.3.1. Determination of the precursor and product ions with loop injection experiments

Loop injection experiments were performed by a FIA (Flow Injection Analysis) system coupled to a loop, providing a continuous flow of mobile phase (methanol/Milli-Q water, 50:50 v/v %) at a flow rate of 0.2mL/min into the ion source. A solution of 1 µg/mL was prepared for every target chemical in order to be injected into the ion source and determine the precursor and product ions. The chemicals were found to have optimal signal intensity in the positive ESI mode. In **Table 2**, the parameters for the precursor and product ions are being demonstrated. For every target analyte, two product ions were used for their monitoring (a quantifier ion: the one that presented the highest intensity was used for quantification; and a qualifier ion: the one that presented the second highest intensity was used for confirmation)

**Table 2:** Precursor and product ions of the target hormones and the deuterated internal standard.

| Compounds                      | Precursor (m/z) | Product Ions (m/z) | Collision energy | Cone voltage | Quantifier/Qualifier |
|--------------------------------|-----------------|--------------------|------------------|--------------|----------------------|
| T <sub>4</sub>                 | 777.97          | 732.11             | 18               | 50           | Qual.                |
|                                |                 | 324.19             | 54               |              | Quant.               |
| T <sub>3</sub>                 | 652.10          | 197.54             | 24               | 32           | Qual.                |
|                                |                 | 606.23             | 56               |              | Quant.               |
| rT <sub>3</sub>                | 652.03          | 603.23             | 24               | 30           | Qual.                |
|                                |                 | 508.32             | 22               |              | Quant.               |
| T <sub>2</sub>                 | 526.16          | 382.40             | 18               | 6            | Qual.                |
|                                |                 | 480.27             | 14               |              | Quant.               |
| D <sub>2</sub> -T <sub>4</sub> | 782.67          | 736.58             | 22               | 46           | Quant.               |

The transitions from every precursor ion to a respective product ion is termed SRM (Selected Reaction Monitoring), and for every target analyte two SRMs were monitored, the quantification and the confirmation SRM.

### 2.3.2. Optimization of the electrospray parameters

For the optimization of the electrospray ionization parameters, the same loop injection experimental setting was used. For every target analyte, the optimal ESI parameters were found and then a compromise was performed in order to conclude on the ESI parameters for the developed method. The parameters did not differ significantly between the target analytes because they all belong to the same chemical class; they are all thyroid hormones. Thus, the capillary cone voltage was set at +2.8 kV, the desolvation temperature 500 °C, the desolvation gas flow 800 L/hr, the cone gas flow 150 L/hr, and the source temperature 150 °C.

The accurate mass and the fragments were determined by the IntelliStart software, part of the MassLynx V4.1 software package. Integration and data processing was done with MassLynx V4.1 software.

### 2.3.3. Determination of the chromatographic gradient

According to literature (see references in Table 1), the mobile phase that provides optimal signal intensity is Milli-Q water (0.1 % formic acid) and methanol. Chromatographic separation was achieved on the Acquity UPLC from Waters, using the Acquity Column Manager, the Acquity Sample Manager and the Acquity Binary Solvent Manager. Solvent gradient for the

duration of the run can be found in **Table 3**. The complete chromatographic run was 8 minutes, and was applied to both HPLC columns that were evaluated, Atlantis T3 3 $\mu$ m column (2.1 x 150mm) and Kinetex 1.7 $\mu$ m C18 column (2.1 x 150mm). For this experiment, a mixture of target analytes (50 ng/mL) were injected through the column(s).

**Table 3:** Solvent gradient; Flow was set to 0.2 mL/min.

| Time [min] | % A v/v - Milli-Q water (0.1 % formic acid) | % B v/v - Methanol |
|------------|---|--------------------|
| 0.0        | 50  | 50                 |
| 4.0        | 20  | 80                 |
| 6.0        | 0   | 100                |
| 8.0        | 50  | 50                 |

## 2.4. Calculations

### 2.4.1. Retention Time and Relative Retention Time

The retention time (RT) is a measure of time needed for an analyte to be introduced in the mobile phase, until the analyte is eluted through the chromatography column [77]. The RT for a compound is not fixed; it is dependent on the chromatographic system that is applied, and it can fluctuate even between consecutive injections. These fluctuations can be caused, but they are not limited to:

- The instability of the mobile phase flow rate;
- Instability in the column Temperature;
- Column degradation;
- Air bubble(s) in the mobile phase (not efficiently degassed); and
- Column length differences

These factors can make it difficult to compare absolute RTs [25]. Even if the same sample is analyzed within a few days' time, there can be small differences in the retention time of a compound. Because of this, the relative retention time (RRT) is a more robust parameter because it reduces the effects of these variables that affect the RT. To measure RRT, a sample matrix is made up by mixing the sample with an internal standard (IS). RRT is an expression of the RT of the target analyte relative to the internal standard's RT (equation 2.2.).

$$RRT = \frac{RT \text{ of target analyte}}{RT \text{ of Internal standard}} \quad (2.1)$$

### 2.4.2. Relative Response

The relative response (RR), is a factor commonly used in LC-MS/MS analysis. This factor is used to compensate for variations in the signal intensity of a target analyte due to variabilities in instrumental response and variations during sample preparation (e.g., loss of sample volume). The ratio between a signal intensity produced by an analyte and the signal intensity produced by an internal standard, compensates for these variations. The relative response ratio is given in equation 2.2 [25, 76].

$$\text{Relative Response} = \frac{\text{Area Target Analyte}}{\text{Area Internal Standard}} \quad (2.2)$$

One of the main reasons to use response factors is to compensate for the variations of injecting the samples into the liquid chromatography system (LC). In this thesis an injection volume of 2  $\mu\text{L}$  were used. Slight differences in the volume of injected analyte leads to differences in the areas of the peaks in the chromatogram and any quantitative results can be considered not reliable. To compensate for all the above, including this uncertainty in the injection volume, a known amount of an internal standard is always added to all samples for analysis. By applying the internal standard method, the ratio of the area of the analyte in a specific concentration and that of the internal standard will remain constant between injections.

### 2.4.3. Ion Ratio

To verify that we do not have a false positive in our analysis, the Ion Ratio always calculated. The ion ratio is a specific ratio unique for each compound in a sample matrix or even among sample matrices. The ratio is calculated using equation 2.3.

$$\text{Ion Ratio \%} = \frac{\text{Confirmation Area}}{\text{Quantification Area}} \times 100\% \quad (2.3)$$

In equation 2.3, the confirmation area is the area of an analyte in the confirmation SRM, while the quantification area is the area of an analyte in the quantification SRM. The presence of a target analyte is confirmed in a sample only when the Ion Ratio % falls in a predetermined value, that is usually calculation from different concentrations of the calibration curve.



#### 2.4.4. Repeatability

Repeatability is the variation in measurements taken on the same concentration, under the same conditions, and in a short period of time [78]. A measurement is repeatable when this variation is smaller than a pre-determined acceptance criterion. Repeatability can be calculated using the standard deviation formula (STD; formula 2.4) or the relative standard deviation (RSD %; formula 2.6). The mean value is calculated using the equation 2.5.

$$STD = \sqrt{\frac{\sum(x-\bar{x})^2}{N-1}} \quad (2.4)$$

$$\bar{x} = \frac{\sum_{i=1}^N x_i}{N} \quad (2.5)$$

Where the  $\{x_1, x_2 \dots x_n\}$  are the observed values for the repeated test,  $\bar{x}$  is the mean of the data sample,  $N$  is the number of samples and  $N - 1$  is the degree of freedom. Another important factor when calculating repeatability is the relative standard deviation (RSD%) also known as coefficient of variation. The RSD % is given as equation 2.6.

$$RSD\% = \frac{STD}{\bar{x}} \quad (2.6)$$

And  $\bar{x}$  is the mean of the data sample, and STD is the standard deviation of the data set. Replicate analyses are defined as the measurement of two or more standard solutions which are independently carried through all steps of sample preparation and instrumental analysis in an identical manner.

#### 2.4.5. Absolute and Relative Recovery

Recovery is divided into two sub-categories, absolute and relative recovery [25]. Recovery is the measure of how much of the analyte(s) in the sample is extracted through the sample preparation protocol(s) for analysis. The absolute recovery, is given by equation 2.7 and the relative recovery, is given by equation 2.8.

$$Absolute Recovery \% = \frac{Area\ of\ target\ analyte\ in\ the\ pre-extraction\ spike\ sample}{Area\ of\ target\ analyte\ in\ the\ post-extraction\ spike\ sample} * 100\% \quad (2.7)$$

$$Relative Recovery \% = \frac{\frac{Area\ of\ target\ analyte\ in\ the\ pre-extraction\ spike\ sample}{Area\ of\ internal\ standard\ in\ the\ pre-extraction\ spike\ sample}}{\frac{Area\ of\ target\ analyte\ in\ the\ post-extraction\ spike\ sample}{Area\ of\ internal\ standard\ in\ the\ post-extraction\ spike\ sample}} * 100\% \quad (2.8)$$

The absolute recovery gives us the “real” recovery, but it generally is provided with higher uncertainty than the relative recovery that gives us the “corrected” recovery and is provided with smaller uncertainty. The relative recovery is corrected with a specific internal standard.

The absolute recovery % for each target analyte at a specific fortification level was calculated from the response (area of quantification MRM peak) of the analyte in the pre-extraction matrix matched spiked standard solution divided by the response of the analyte in the post-extraction matrix matched spiked standard solution, and multiplied by 100.

The relative recovery % (to internal standards) for each target analyte at a specific fortification level was calculated from the ratio of the analyte response to the internal standard response in the pre-extraction matrix matched spiked standard solution divided by the same ratio in the post-extraction matrix matched spiked standard solution, and multiplied by 100.

#### 2.4.6. Instrumental Limit of Detection and Quantification

In analytical chemistry, the detection limit or limit of detection (LOD), is the lowest quantity of a substance that can be distinguished from the noise in a blank sample, within a specified deviation range [25]. The quantification limit or limit of quantification (LOQ), is the lowest quantity of a substance that can be quantified with a specific certainty. There are many ways presented in literature to calculate the LODs and LOQs, and needs to “fit the purpose” of the analytical method. One way of calculation is that the LOD and LOQ can be calculated for every target analyte as three and ten times the signal from the baseline noise (S/N ratio). Another way of calculation is that the LOD and LOQ can be calculated as three and ten times, respectively, the standard deviation of the lowest concentration of the calibration curve and dividing by the slope of the calibration curve (equations 2.9 and 2.10).

$$LOD = \frac{3 \times STD}{Slope} \quad (2.9)$$

$$LOQ = \frac{10 \times STD}{Slope} \quad (2.10)$$

Depending on the way of calculation, the LODs and LOQs can differ significantly. However, in this work, it was considered appropriate to calculate them with equations 2.9 and 2.10, and verify them visually on the chromatograms.

### 2.4.7. Matrix Effect

In analytical chemistry, the matrix is considered the endogenous components in a sample which are not the desired analytes [25]. The matrix can suppress the signals from the analytes, an effect also known as the matrix suppression effect. The matrix also can enhance the signals from the target analyte, also known as the matrix enhancement effect. This is often the case with complex samples such as biological samples. The most common approach for accounting for matrix effects is to compare on a specific concentration, the response area between the extract from the target matrix (e.g., serum) and the extract from a Milli-Q solution or pure solvent. When dealing with complex or unknown matrices, and there is a lack of C13-labeled internal standards, then the standard addition method must be used for quantification purposes instead of the common solvent calibration curve. Matrix enhancement and suppression is frequently observed in LC-MS/MS methods.

In order to estimate the matrix effect (ME %), we need first to calculate the matrix factor (MF). The MF is the ratio between the area of the target analyte in the post-extraction spike in serum matrix with the post-extraction spike in Milli-Q matrix (or standard solvent), as demonstrated in equation 2.11:

$$MF = \frac{\text{Area of target analyte in the post-extraction spike in serum matrix}}{\text{Area of target analyte in the post-extraction spike in Milli-Q matrix}} * 100\% \quad (2.11)$$

The concentration of analyte in both the sample standards should be the same. If the MF is close to 1, this indicates absence of matrix influence on the analyte signal. While the MF value of less than 1 indicates suppression of the analyte, and if the MF is larger than 1, this indicates possible matrix enhancement.

The ME % is calculated by the equation 2.12:

$$ME\% = (MF - 1) * 100\% \quad (2.12)$$

The advantages of this definition are that negative values indicate suppression of analytes, while positive values indicate matrix enhancement of the analyte signal. Ideally, the value of equation 2.12 should be zero, indicating the absence of matrix effect.

#### 2.4.8. Linear regression

Linear regression is the most basic type of regression and commonly used in data analysis [78]. In linear regression, the relationship between Y and x are modeled based on the linear functions, where a linear regression curve is estimated from the dataset, known as linear model. The simplest form of the equation with one dependent and one independent variable is defined by the formula 2.13.

$$\hat{y} = mx + b \quad (2.13)$$

Where  $\hat{y}$  = estimated dependent score, b = constant, m = regression coefficients, and x = independent variable.

#### 2.4.9. Internal standard method

When using the internal standard method, a calibration curve is constructed for every target analyte from the ratio of the analyte response to the internal standard response in every measured standard solution (solvent or matrix matched), plotted against the concentration (amount) of the spiked analyte. With the internal standard method, all validation criteria (i.e. reproducibility, accuracy) are calculated in accordance to this ratio.

The internal standard is a compound that is very similar, but not identical to the chemical species of interest in the samples. The internal standard used needs to provide a signal that is like the analyte signal in most ways but sufficiently different so that the two signals are readily distinguishable by the instrument. If the concentration of standard is known, the corresponding concentration of analyte can be derived. Internal standards remain one of the most widely used analysis method, and are especially useful in LC-MS/MS analysis. Internal standards are also desirable when the potential of sample loss can occur during sample preparation steps, prior to analysis. This is because the ratio of standard to analyte remains constant because the same fraction of each species is lost in any operation.

### 3. Results and Discussion

#### 3.1. LC column selection

Two different LC columns, Atlantis T3 3 $\mu$ m column (2.1 x 150mm) and Kinetex 1.7 $\mu$ m C18 column (2.1 x 150mm), were evaluated under the same chromatographic conditions. Atlantis T3 is an alkyl reverse-phase column designed to retain hydrophilic compounds in highly aqueous mobile phases (including 100% water). The Atlantis T3 surface chemistry (steric protection of the bonded phase) is designed to allow the alkyl phase to remain fully accessible in highly aqueous mobile phases. It prevents it from “phase dewetting” (whereby an alkyl chain of the hydrophobic C18 bonded phase collapses). The main advantage of this column is that it can be used with highly aqueous mobile phases and provides better retention of small polar compounds like the thyroid hormones than does a typical C18 column. The Kinetex 1.7 $\mu$ m C18 column is a typical C18 core-shell phase column. With both column, excellent chromatographic separations were achieved (**Figures 26 and 27**). However, signal intensity was higher for all compounds with the Atlantis T3 3 $\mu$ m column. This was attributed to the fact that with Atlantis T3, where we have less silanol activity the peaks are “narrower” due to less tailing. Therefore, the assessment of the instrumental method performance and the experiments on sample preparation were performed with the Atlantis T3 3 $\mu$ m column.

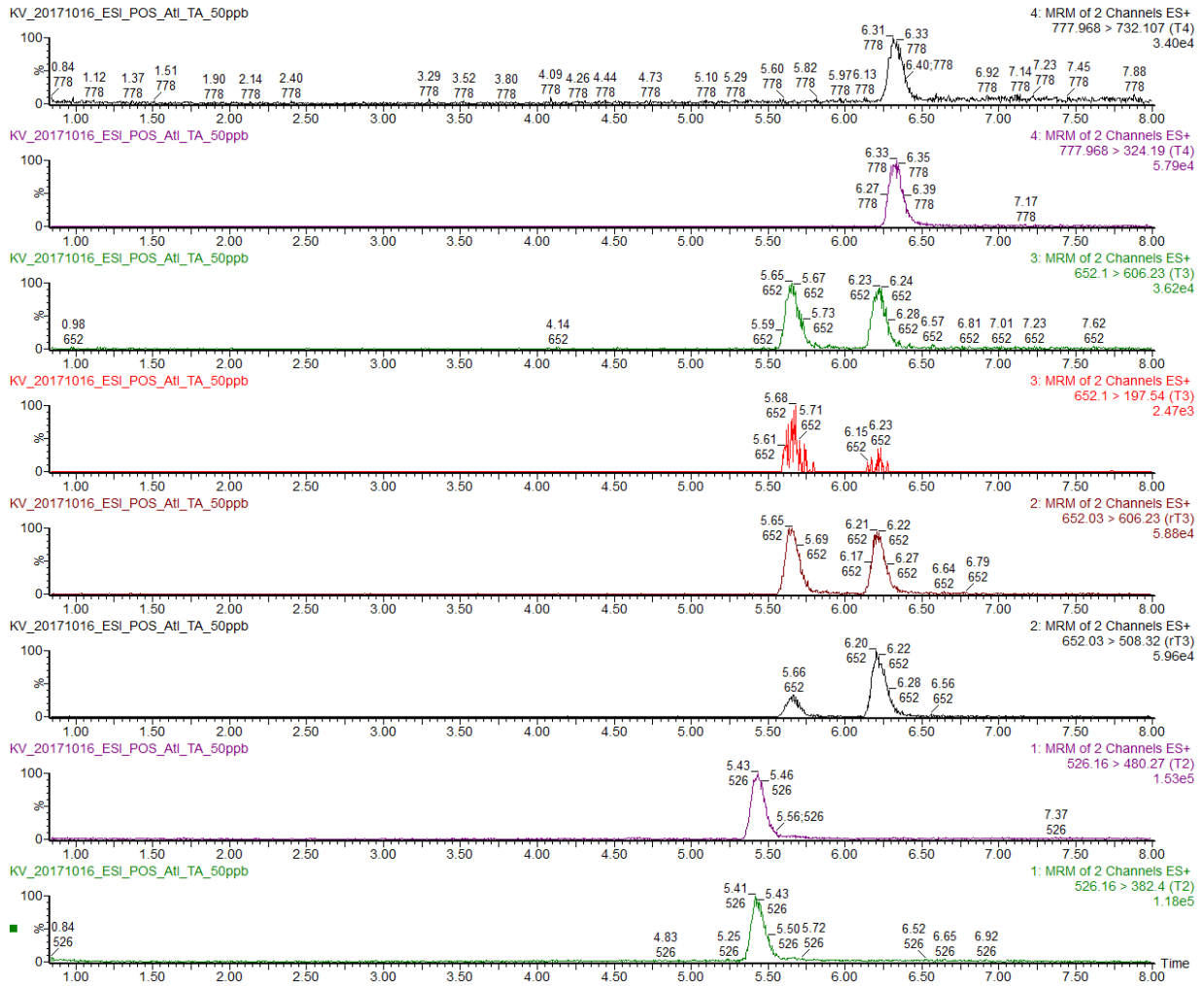
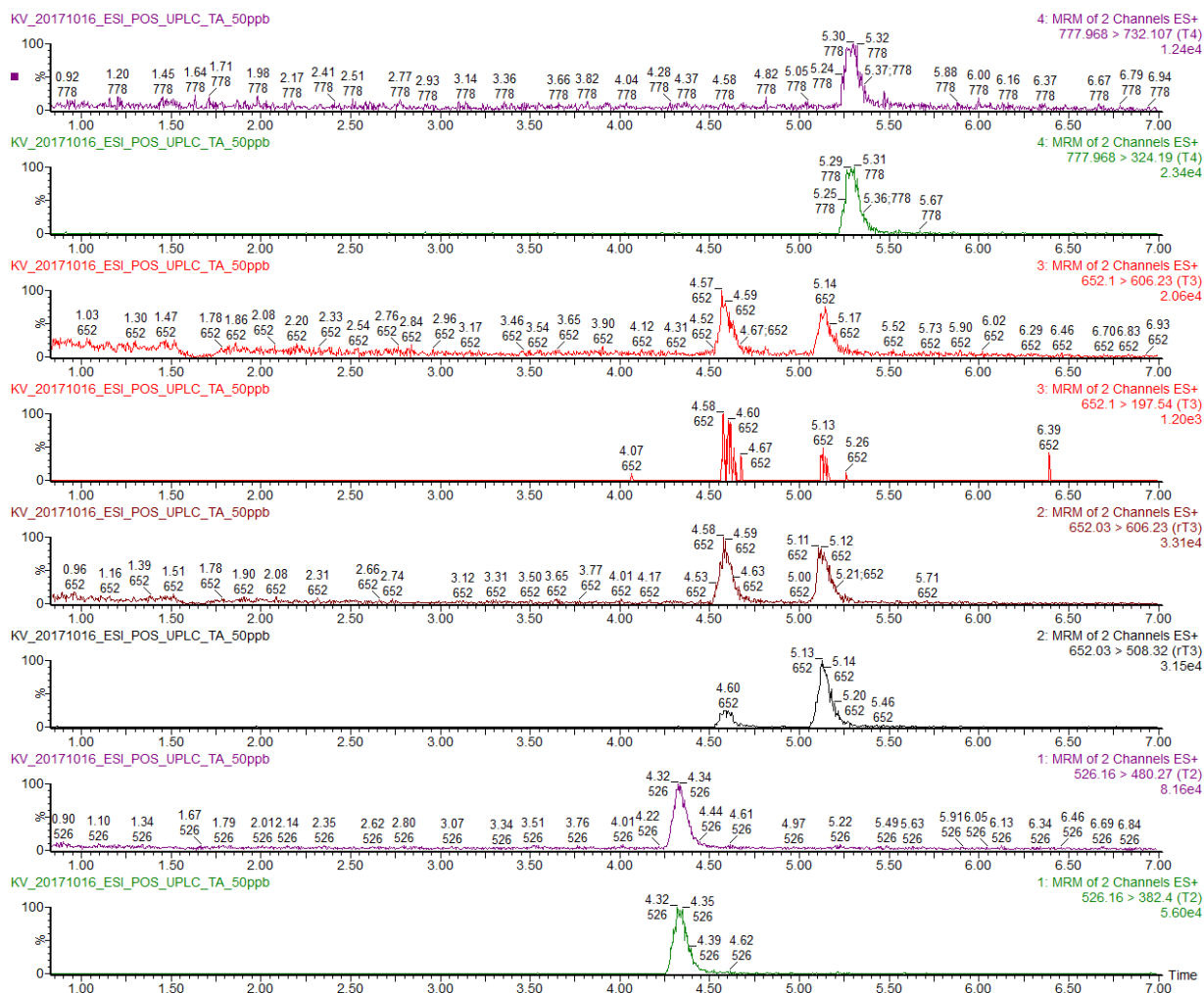


Figure 26: 50 ng/mL standard solution injected on the Waters Atlantis T3 3µm column



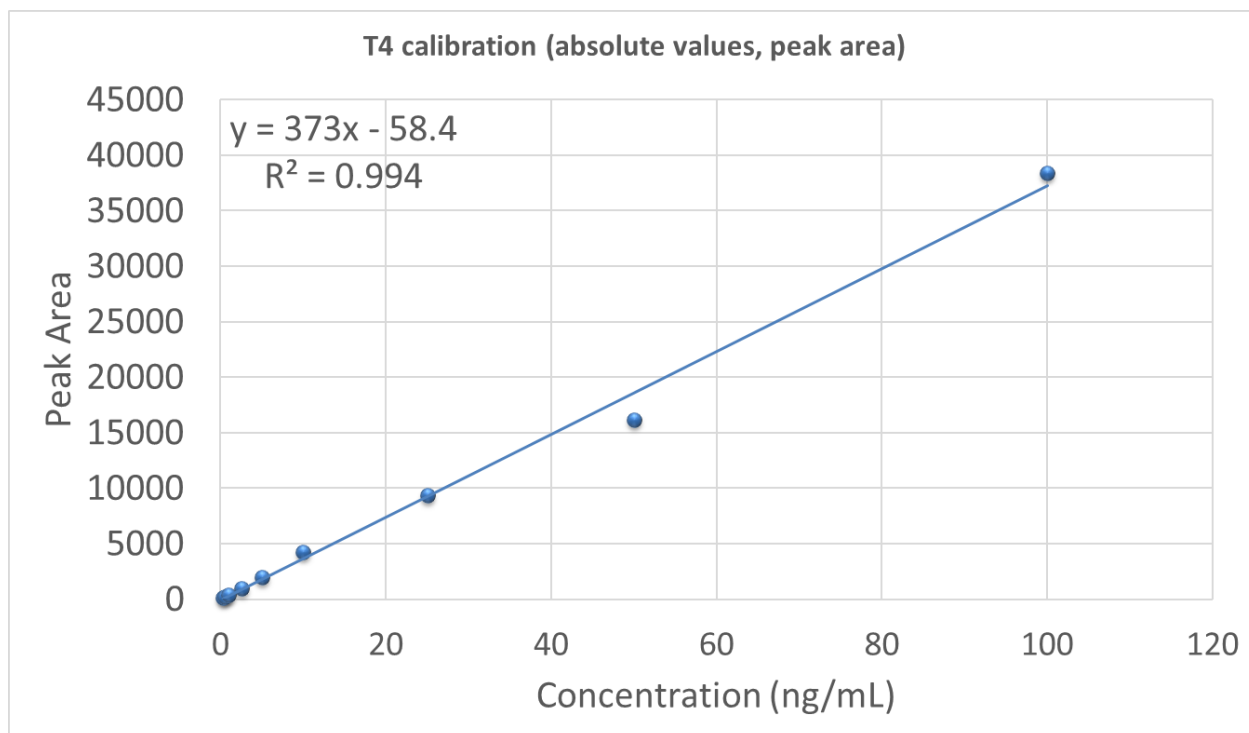
**Figure 27:** 50 ng/mL standard solution injected on the Kinetex 1.7µm C18 column

### 3.2. Calibration curves

Calibration standards were prepared by preparing standard solvent solutions in methanol/milli-Q water (50:50 v/v). The concentrations that were prepared for the target analytes were 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL. The internal standard was fortified at 10 ng/mL in all standard solvent solutions. For every target analyte, two calibration curves were prepared, one with absolute values (area of target analyte), and another with relative response values (area of target analyte divided by the area of the IS).

The calibration curves were obtained for the hormones T<sub>2</sub>, T<sub>3</sub>, rT<sub>3</sub> and T<sub>4</sub>, with absolute and relative values (see Appendix A.1; and figure 28), The correlation coefficients R<sup>2</sup> have a value between 0.994 to 0.999. The slope of the calibration curves correlates to the sensitivity of the method, where a higher slope value means a higher sensitivity. From the calibration curves derived from the absolute values, a slope range of 263 (T<sub>3</sub>) to 1075 (T<sub>2</sub>), which indicates

that the derivatives demonstrate different sensitivities; the hormones, rT<sub>3</sub>, T<sub>3</sub> and T<sub>4</sub> provide a slope range of 263-376.



**Figure 28 (A.1):** The calibration curve for T<sub>4</sub> hormone, using the absolute values of peak area (y-axis), and concentration (x-axis).

For the relative response, the same trend is demonstrated, with the values of the rT<sub>3</sub>, T<sub>3</sub> and T<sub>4</sub> being close to each other in range (slope range 0.045- 0.064), and the T<sub>2</sub> slope having a value of 0.184; this indicates that the T<sub>2</sub> hormone is more sensitive.

An example of the calibration curves, is shown in **figure 28**, which show the correlation between the concentration (x-axis) and the relative response signal (y-axis).

### 3.3. RT and RRT

The RRT values presented in **Table 4**, were calculated using the equation 2.1. The confirmation criteria for the detection of the analytes comprised of their relative retention time (RRT), which should correspond to that of the daily standard solution at a tolerance of 2.5 % (RSD %). As demonstrated in **Table 4**, all the relative retention time values are below 2.5 %.



**Table 4:** Retention Time (RT) and Relative Retention time (RRT) of the target analytes in this thesis.

| Target Analytes | rT <sub>3</sub> |      | T <sub>3</sub> |      | T <sub>4</sub> |      | T <sub>2</sub> |      |
|-----------------|-----------------|------|----------------|------|----------------|------|----------------|------|
|                 | RT              | RRT  | RT             | RRT  | RT             | RRT  | RT             | RRT  |
| Mean (N=10*)    | 6.10            | 0.98 | 5.57           | 0.90 | 6.21           | 1.00 | 5.35           | 0.86 |
| RSD % (N=10*)   | 0.27            | 0.25 | 0.17           | 0.15 | 0.075          | 0.14 | 0.088          | 0.12 |

\*Replicates performed in a standard solvent solution 25 ng/mL

### 3.4. Ion Ratio

The ion ratio was calculated using the equation 2.3, and the results of these calculations are demonstrated in **Table 5**. The ion ratio for every target analyte ranged within the tolerance limits given by the EU Decision 2002/675: rT<sub>3</sub> demonstrated an ion ratio of 104 % and an RSD % = 7.15 (maximum limit by the Directive 2002/675 is 20 %); T<sub>3</sub> demonstrated an ion ratio of 3.36 % and an RSD % = 12.2 (maximum limit by the Directive 2002/675 is 30 %); T<sub>4</sub> demonstrated an ion ratio of 56.7 % and an RSD % = 7.04 (maximum limit by the Directive 2002/675 is 20 %); and T<sub>2</sub> demonstrated an ion ratio of 63.1 % and an RSD % = 5.41 (maximum limit by the Directive 2002/675 is 20 %).

The ion ratio for the rT<sub>3</sub> hormone, which is an isomer of the T<sub>3</sub> hormone, should have approximately the same ion ratio as the T<sub>3</sub> hormone, but as demonstrated in **table 5**, the ion ratio of rT<sub>3</sub> is 104, and for T<sub>3</sub> the ion ratio is 3.36. The hormone rT<sub>3</sub> and T<sub>3</sub> are isomers, and the difference in ion ratio should not be this high. This indicates that the fragmentation pathway of the rT<sub>3</sub> is different than the fragmentation pathway for the T<sub>3</sub> hormone.

**Table 5:** Ion ratio of the target hormones

| Target Analytes | rT <sub>3</sub> | T <sub>3</sub> | T <sub>4</sub> | T <sub>2</sub> |
|-----------------|-----------------|----------------|----------------|----------------|
| Mean (N=5)      | 104             | 3.36           | 56.7           | 63.1           |
| RSD % (N=5)     | 7.15            | 12.2           | 7.04           | 5.41           |

\*Replicates performed in a standard solvent solution 50 ng/mL

### 3.5. Repeatability

The repeatability values are demonstrated in **Tables 6 and 7**, and were calculated using the equations 2.4, 2.5 and 2.6. For the repeatability experiments (intra-day precision), a sample was fortified at three concentrations of target analytes, and five replicate analyses (N=5) were performed for each concentration. The low, medium, and high fortification level for all target analytes, , was 0.25, 0.50, and 1.00 ng/mL, respectively. The general trend of the repeatability is that the absolute and relative RSD% decreases with the increase in concentration.

**Table 6:** Repeatability of the T<sub>3</sub> and T<sub>4</sub> target hormones.

| Target Analytes                         | rT <sub>3</sub> |      |          |      |       |      | T <sub>2</sub> |      |          |      |       |      |
|---|-----------------|------|----------|------|-------|------|----------------|------|----------|------|-------|------|
|   | 0.25 ppb        |      | 0.50 ppb |      | 1 ppb |      | 0.25 ppb       |      | 0.50 ppb |      | 1 ppb |      |
| Concentration levels (ng/mL)            | Abs.            | Rel. | Abs.     | Rel. | Abs.  | Rel. | Abs.           | Rel. | Abs.     | Rel. | Abs.  | Rel. |
| Instrumental Repeatability (RSD %, N=5) | 28.7            | 15.8 | 15.3     | 13.2 | 9.33  | 17.4 | 35.6           | 8.4  | 8.32     | 23.8 | 12.9  | 18.2 |

**Table 7:** Repeatability of the rT<sub>3</sub> and T<sub>2</sub> target hormones.

| Target Analytes                         | T <sub>4</sub> |      |          |      |       |      | T <sub>3</sub> |      |          |       |       |       |
|---|----------------|------|----------|------|-------|------|----------------|------|----------|-------|-------|-------|
|   | 0.25 ppb       |      | 0.50 ppb |      | 1 ppb |      | 0.25 ppb       |      | 0.50 ppb |       | 1 ppb |       |
| Concentration levels (ng/mL)            | Abs.           | Rel. | Abs.     | Rel. | Abs.  | Rel. | Abs.           | Rel. | Abs.     | Rel.  | Abs.  | Rel.  |
| Instrumental Repeatability (RSD %, N=5) | 34.1           | 17.3 | 8.91     | 23.4 | 8.20  | 9.00 | 39.1           | 23.0 | 7.40     | 13.24 | 7.22  | 13.23 |

At lower concentrations such as at 0.25 ppb, the instrument error (systematic error) becomes larger, than at higher concentration such as 1 ppb. The usage of the internal standard D<sub>2</sub>-T<sub>4</sub> is not improving the RSD%. This is due to two reasons:

1. D<sub>2</sub>-T<sub>4</sub> is deuterated analogue of T<sub>4</sub>, and therefore obtains different physicochemical properties than T<sub>4</sub>. D<sub>2</sub>-T<sub>4</sub> is more polar than T<sub>4</sub> due to the deuterium; chromatographically D<sub>2</sub>-T<sub>4</sub> elutes slightly earlier than T<sub>4</sub>. Due to the above, D<sub>2</sub>-T<sub>4</sub> could not improve the repeatability of T<sub>4</sub>.
2. D<sub>2</sub>-T<sub>4</sub> has even larger physicochemical differences when compared to rT<sub>3</sub>, T<sub>3</sub>, and T<sub>2</sub>.

It was demonstrated, that to improve the analytical method, the usage of  $^{13}\text{C}$ -isotopes specific to target analytes, should be used. A  $^{13}\text{C}$ -isotope has no physicochemical differences from the target analyte.

### 3.6. LODs and LOQs

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the equations 2.9 and 2.10, respectively. The STD at the 0.5 ng/mL concentration was used for the calculations. The results are demonstrated in **Tables 8 and 9**. The concentration of the LODs and LOQs are ranging in the low ng/mL area; the calculations of the LODs and LOQs are based on absolute and relative response values. The differences that were observed based on the calculations with the absolute and relative response values, are contributed to the differences in the STD of the replicates. The instrumental LODs and LOQs were compared to previous literature [29, 70], and were found in the same order of magnitude.

**Table 8:** The LOD and LOQ values for  $rT_3$  and  $T_2$

| Target Analytes       | $rT_3$ |      | $T_2$ |      |      |      |      |      |
|-----------------------|--------|------|-------|------|------|------|------|------|
|                       | LOD    | LOQ  | LOD   | LOQ  |      |      |      |      |
|                       | Abs.   | Rel. | Abs.  | Rel. | Abs. | Rel. | Abs. | Rel. |
| Concentration (ng/mL) | 0.30   | 0.41 | 1.00  | 1.34 | 0.17 | 0.77 | 0.56 | 2.54 |

**Table 9:** The LOD and LOQ values  $T_3$  and  $T_4$

| Target Analytes       | $T_3$ |      | $T_4$ |      |      |      |      |      |
|-----------------------|-------|------|-------|------|------|------|------|------|
|                       | LOD   | LOQ  | LOD   | LOQ  |      |      |      |      |
|                       | Abs.  | Rel. | Abs.  | Rel. | Abs. | Rel. | Abs. | Rel. |
| Concentration (ng/mL) | 0.16  | 0.44 | 0.52  | 1.45 | 0.17 | 0.69 | 0.56 | 2.28 |

### 3.7. Extraction and purification of samples

For the LLE protocol, the organic solvent ethyl acetate is commonly used in bioanalytical applications. The addition of high concentration of ammonium acetate was performed with the basic aims to increase the extraction of the polar thyroid hormones through the “salting-out” effect. The addition of Milli-Q water after the extraction of the samples and the centrifugation, was performed to remove the excess of salt from the sample; this could have led to severe ion suppression during LC-MS/MS analysis.

Both two polymeric sorbents (Strata-X CW, and Oasis HLB) exhibit hydrophobic,  $\pi$ - $\pi$ , and hydrophilic intermolecular forces on the target analytes. Due to the polar group of N-vinylpyrrolidone present in Oasis® HLB sorbent, there is an increased contribution of hydrogen bonding, and hydrophilic chemical moieties are strong. The Strata-X CW cartridge is designed to increase the retention of basic compounds by strong cationic exchange through its deprotonated sulfonyl moieties. The classes with an amine group, such as thyroid hormone derivatives, can be potentially positively charged in an acidic environment and, consequently, promote cation exchange interactions with the cartridge. Thyroid hormones that obtain also a conjugated molecular system are prone to be strongly retained by the cartridges through  $\pi$ - $\pi$  interactions.

On the application of the Hybrid SPE-PPT methodology, the serum sample was subjected to centrifugation, after the addition of a protein precipitation agent, isolating the sample from the endogenous gross amounts of proteins. Then, the obtained supernatant fluid from the centrifugation step was directly subject to SPE using the Hybrid SPE cartridge. Making use of this cartridge, the SPE step demanded less than a minute for completion avoiding the time-consuming steps of “classic SPE” that needed a matter of a few hours for completion, and consequently, correlates to low sample throughput, higher workload and higher costs of analysis. The Hybrid SPE cartridge is consisted of a zirconium packed-bed/low porosity filter/0.2 m hydrophobic frit assembly. Phospholipids are retained specifically to the zirconium sorbent and the remaining proteins from the centrifugation step are retained non-specifically to the low porosity filter of the assembly. The obtained eluent is eventually filtered through the cartridge frit, and thereafter it is ready for analysis. Ultimately, three processes are performed simultaneously in one.

### 3.8. Recoveries

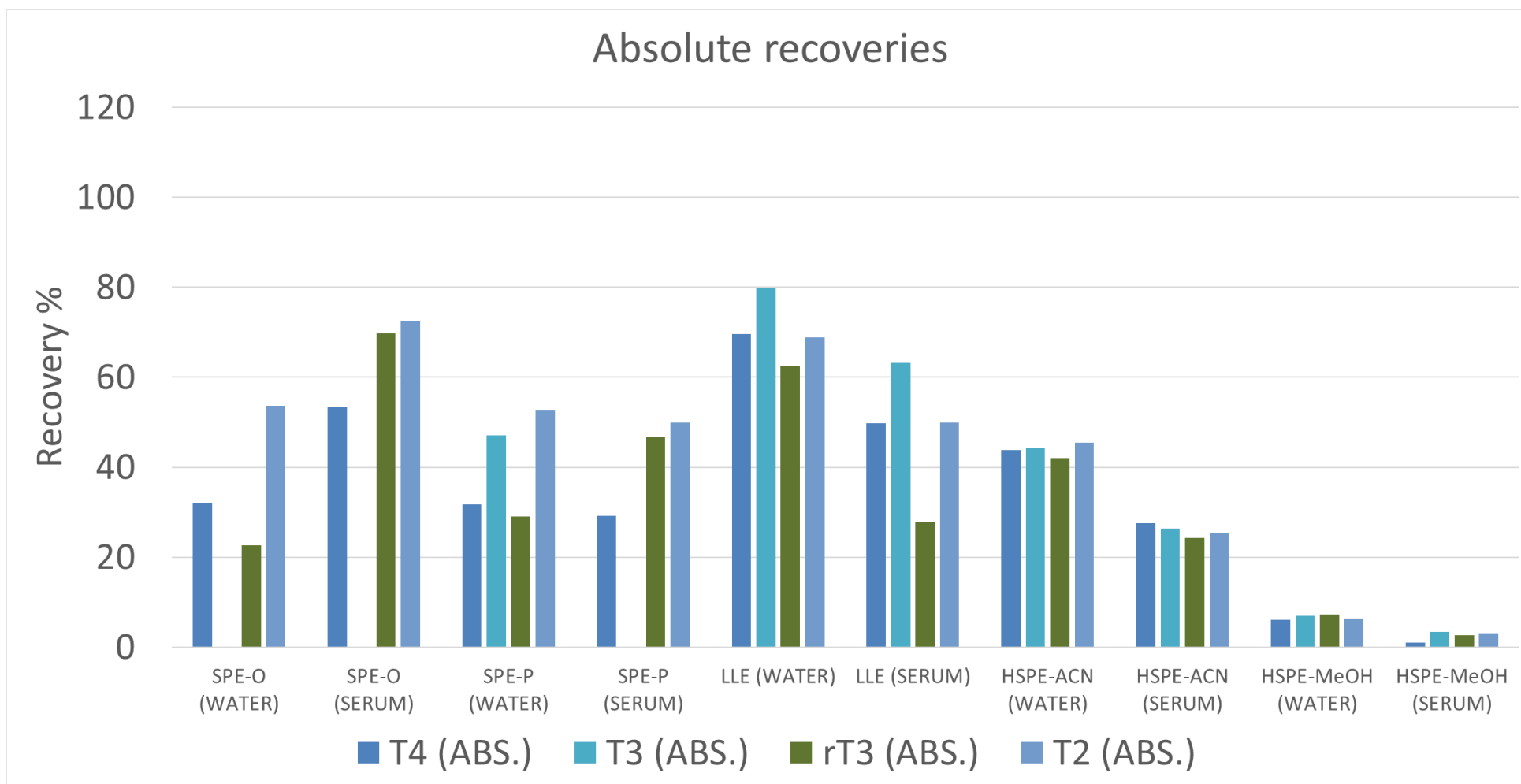
The absolute recoveries were calculated from the **equation 2.7.**, and the results are presented in **figure 29**. The relative recoveries were calculated from the **equation 2.8.**, and the results are presented in **figure 30**. The recoveries were calculated by subtracting the reagent or sample blank (depending on which obtained higher concentrations of target analytes) from the respective triplicate of pre-extraction matrix spikes and the duplicate of post-extraction matrix spikes, and thereafter, performing a comparison between the derived pre-extraction with the post-extraction values. It was observed that the Oasis HLB cartridge demonstrated absolute recoveries of 32% for T<sub>4</sub>, 22.7% for rT<sub>3</sub> and 53.7 % for T<sub>2</sub> in the Milli-Q water matrix, while the absolute recoveries of 53.4% for T<sub>4</sub>, 69.7% for rT<sub>3</sub> and 72.4% for T<sub>2</sub>, were demonstrated in serum matrix, which were higher than the Milli-Q water samples. T<sub>3</sub> obtained high reagent blank values in both Milli-Q and serum samples. The lower recoveries from the Milli-Q water samples was attributed on the higher losses during their evaporation to near-dryness; the flow on the samples was harsher, and eventually, the reagent blanks of the Milli-Q water samples demonstrated contamination for all target analytes. It was observed that the Strata-X CW cartridge demonstrated absolute recoveries of 31.8% for T<sub>4</sub>, 29% for rT<sub>3</sub> and 52.7 % for T<sub>2</sub> in the Milli-Q water matrix, while the absolute recoveries of 29.2% for T<sub>4</sub>, 46.7% for rT<sub>3</sub> and 49.9% for T<sub>2</sub>, were demonstrated in serum matrix; the recoveries were equal in both matrices. Again, T<sub>3</sub> obtained high reagent blank values in both Milli-Q and serum samples. Overall, there were not observed any significant differences between the two types of cartridges. Due to the polar character of the thyroid hormones, they possibly break-through during the loading step or washing step of the SPE process rather than stay absorbed on the sorbent of the cartridge.

In the LLE method, all thyroid hormones were extracted from the Milli-Q water and the serum matrix. It was observed that the LLE method demonstrated absolute recoveries of 69.6% for T<sub>4</sub>, 79.8 % for T<sub>3</sub>, 62.4% for rT<sub>3</sub> and 68.8 % for T<sub>2</sub> in the Milli-Q water matrix, while the absolute recoveries of 49.8% for T<sub>4</sub>, 63.2 % for T<sub>3</sub>, 27.8% for rT<sub>3</sub> and 49.9 % for T<sub>2</sub>, were demonstrated in serum matrix, which were lower than the Milli-Q water samples. The lower recoveries are expected in a more complex matrix like serum. Again, it was observed a cross-contamination for T<sub>3</sub>, but it was not so severe as observed in SPE.

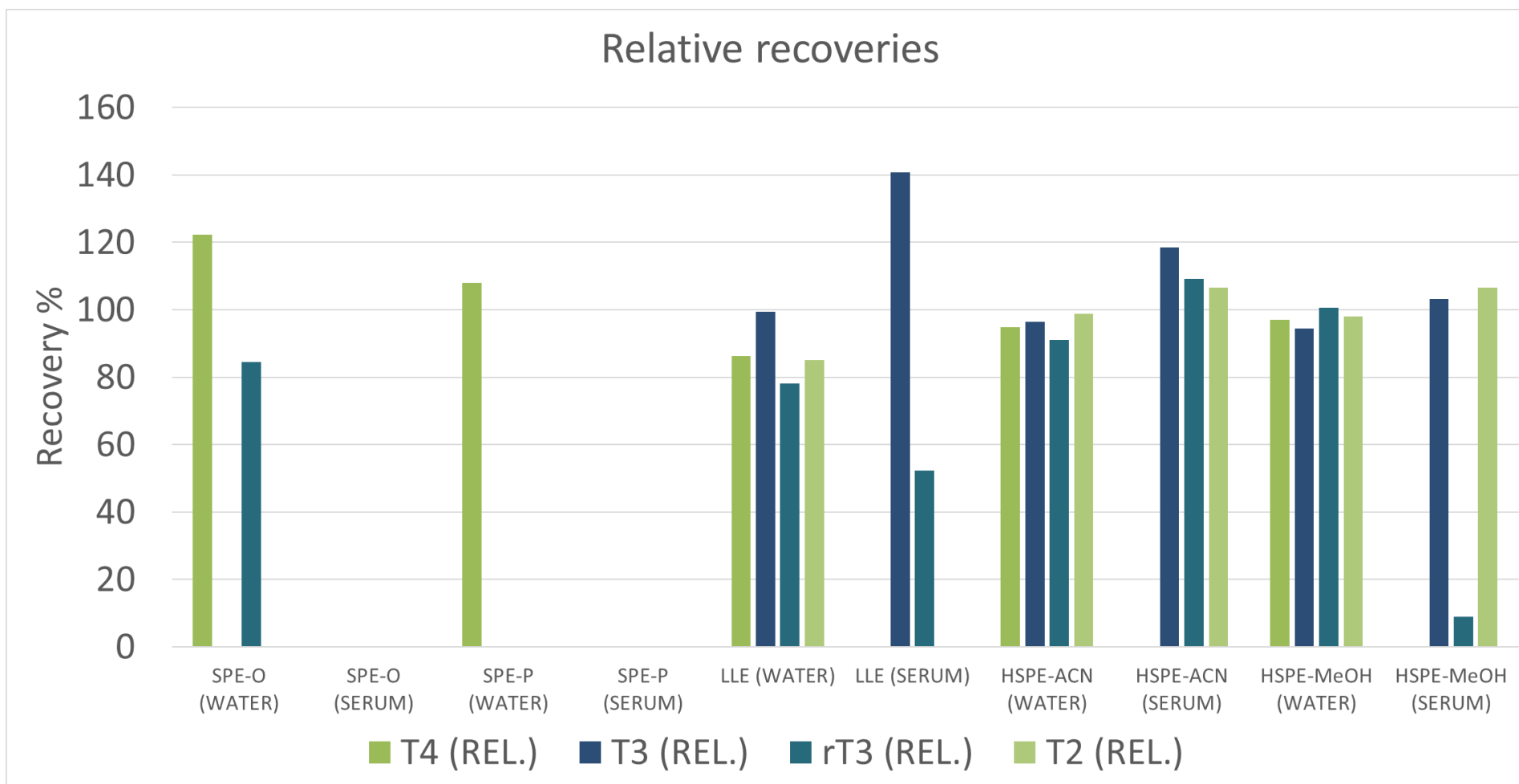
For the hybrid SPE-PPT technique, the best results were obtained with the acetonitrile (1 % v/v formic acid) as the precipitation agent. It was observed that this precipitation agent demonstrated absolute recoveries of 43.9% for T<sub>4</sub>, 44.3 % for T<sub>3</sub>, 41.9% for rT<sub>3</sub> and 45.4 % for

T<sub>2</sub> in the Milli-Q water matrix, while the absolute recoveries of 27.6 % for T<sub>4</sub>, 26.4 % for T<sub>3</sub>, 24.3% for rT<sub>3</sub> and 25.2 % for T<sub>2</sub>, were demonstrated in serum matrix, which were lower than the Milli-Q water samples. The second precipitation agent that was assessed, methanol (1 % w/v Ammonium Formate), provided recoveries < 8 % for all thyroid hormones. The reagent blanks did not demonstrate any contamination. The advantage of the hybrid SPE-PPT is that it demonstrated low STD between replicates (**Appendix; Table A.1.**), compared to the other two techniques, LLE and SPE. Another advantage of the Hybrid SPE-PPT protocol that was used here-in, was that an evaporation step was not incorporated to decrease the volume of solvent prior to analysis; due to this, contamination was not experienced.

As far as the relative recoveries are concerned (**figure 30**), D<sub>2</sub>-T<sub>4</sub> did not perform well to compensate for target analyte absolute variabilities, even for T<sub>4</sub>. For the SPE methods, values for T<sub>3</sub> were not calculated due to the high background noise. For the other derivatives that are not presented in figure 30, they demonstrated inconsistent relative recovery > 200 % and/or high uncertainty values. The detailed results are demonstrated in the **Appendix, Table A.2.**



**Figure 29:** The absolute recovery values (average from N=3 pre-extraction spikes) from the different extraction methods tested (SPE-O: SPE with Oasis HLB; SPE-P: SPE with Strata-X CW; LLE: Liquid-Liquid Extraction; HSPE-ACN: Hybrid SPE-PPT with the precipitation agent of acetonitrile (1 % v/v Formic Acid); and HSPE-MeOH: Hybrid SPE-PPT with the precipitation agent of methanol (1 % w/v Ammonium Formate).

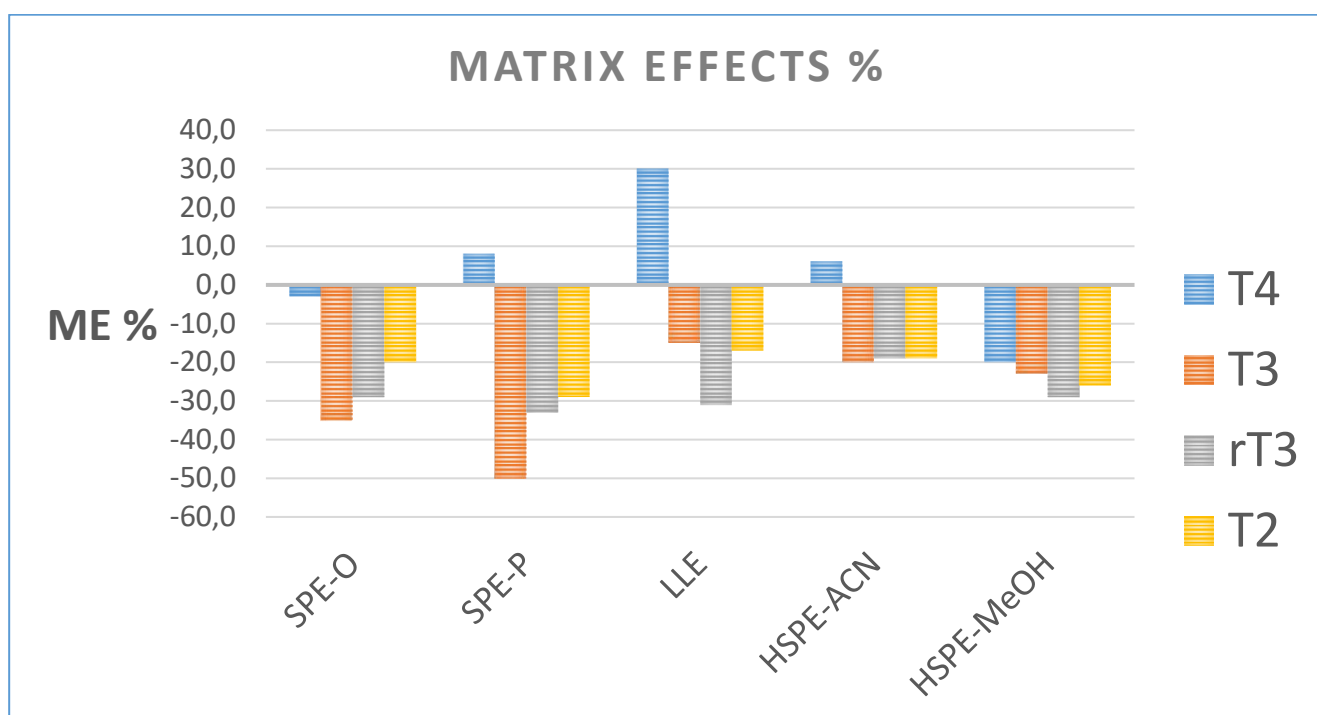


**Figure 30:** The relative recovery values (average from N=3 pre-extraction spikes) from the different extraction methods tested (SPE-O: SPE with Oasis HLB; SPE-P: SPE with Strata-X CW; LLE: Liquid-Liquid Extraction; HSPE-ACN: Hybrid SPE-PPT with the precipitation agent of acetonitrile (1 % v/v Formic Acid); and HSPE-MeOH: Hybrid SPE-PPT with the precipitation agent of methanol (1 % w/v Ammonium Formate)).



### 3.9. Matrix effects

The MF of the target analytes ranged from 0.50-1.30, with most target analytes experiencing ion suppression rather than ion enhancement. The ME % were calculated for all target analytes. T4 was the only target analyte where ion enhancement was observed. For the remaining derivatives, there was ion suppression. The Hybrid SPE – PPT combination with the precipitation agent of acetonitrile (1 % v/v Formic Acid), gave a good compromise; the ion suppression was minimum compared to the other 4 sample preparation protocols.



**Figure 30:** The ME % calculated by comparing a duplicate post-extraction spike in serum at 50 ng/mL with a duplicate post-extraction spike in Milli-Q water at the same concentration. (SPE-O: SPE with Oasis HLB; SPE-P: SPE with Strata-X CW; LLE: Liquid-Liquid Extraction; HSPE-ACN: Hybrid SPE-PPT with the precipitation agent of acetonitrile (1 % v/v Formic Acid); and HSPE-MeOH: Hybrid SPE-PPT with the precipitation agent of methanol (1 % w/v Ammonium Formate)).

#### 4. Conclusion: Choosing the Optimal Extraction Method

The optimal extraction method was proven to be the one with the Hybrid SPE – PPT technique when the precipitation agent of acetonitrile (1 % v/v Formic Acid) is used. First, this extraction protocol was more rapid than LLE or common SPE. With Hybrid SPE – PPT technique the eluent passes through the cartridge in a few seconds. On the contrary, SPE and LLE, need much more time to be completed. Time is a critical parameter for thyroid hormones analysis; the more time the sample preparation takes, the chances for deiodination are higher. Secondly, the absolute recoveries were low, but they obtained an extremely low STD. This low STD is due to the rapidness of the protocol and the avoidance of evaporation to dryness, while for the LLE and the common SPE, the STD of the recoveries were high, in some cases extremely high. The recoveries with the LLE protocol were the highest, but the values obtained a high uncertainty. One possible explanation can be that the harsher and time-consuming the conditions of extraction (e.g. use of strong organic solvent, ethylacetate) are, the higher the potential of deiodination can be. Low absolute recoveries in thyroid hormones bioanalysis is considered normal due to the binding to proteins. Here-in this work, D<sub>2</sub>-T<sub>4</sub> was assessed as a cheaper alternative, but at the end, it was found unsuitable. However, the low absolute recoveries can be always corrected with <sup>13</sup>C- internal standards specific to the target analytes. When having <sup>13</sup>C- internal standards for all target analytes, then quantification can be performed with the internal standard method through calibration curves in solvent, and there is no need to perform standard addition in serum matrix. Thirdly, the Hybrid SPE – PPT technique when the precipitation agent of acetonitrile (1 % v/v Formic Acid) presented the lower suppressions from matrix.

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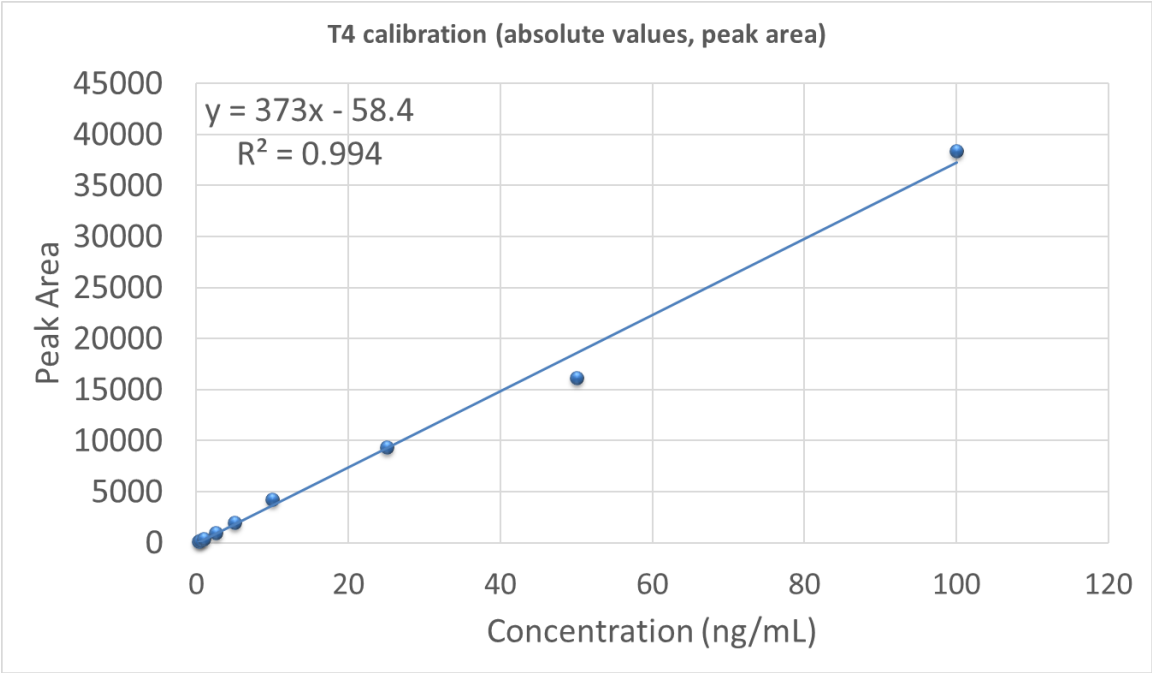


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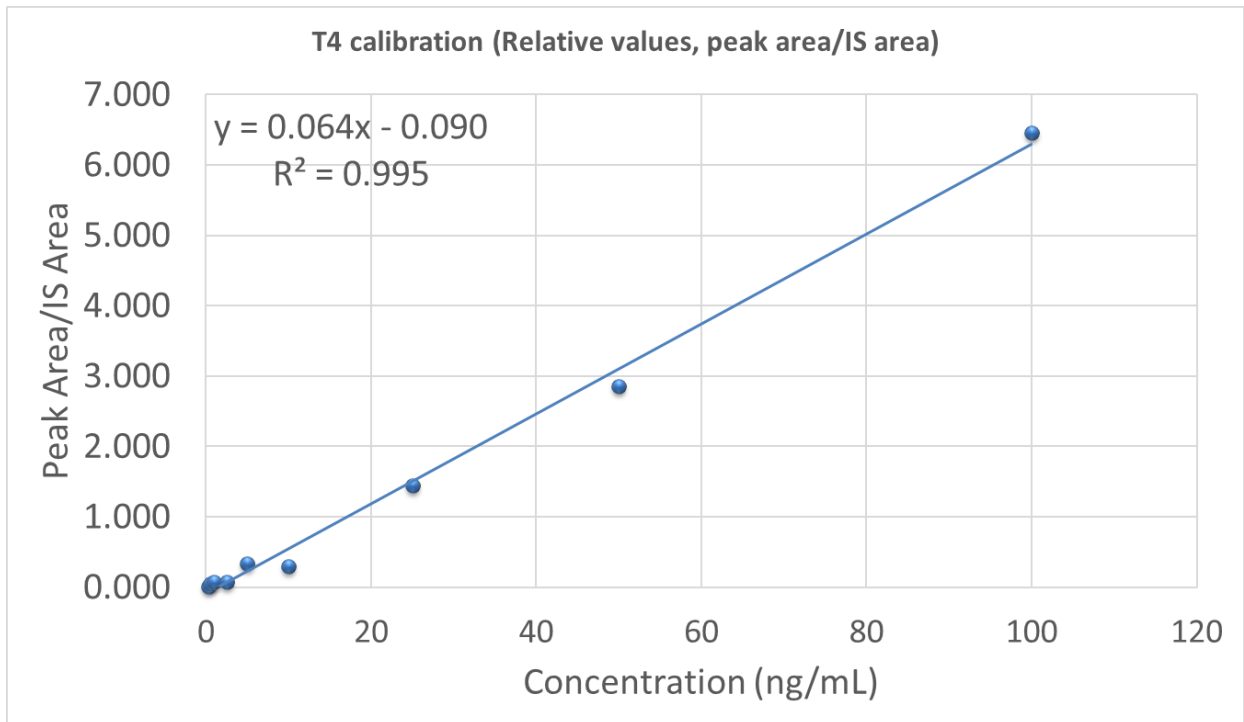
Appendix

The Calibration Curves of the Thyroid Hormones

- T<sub>4</sub> Hormone

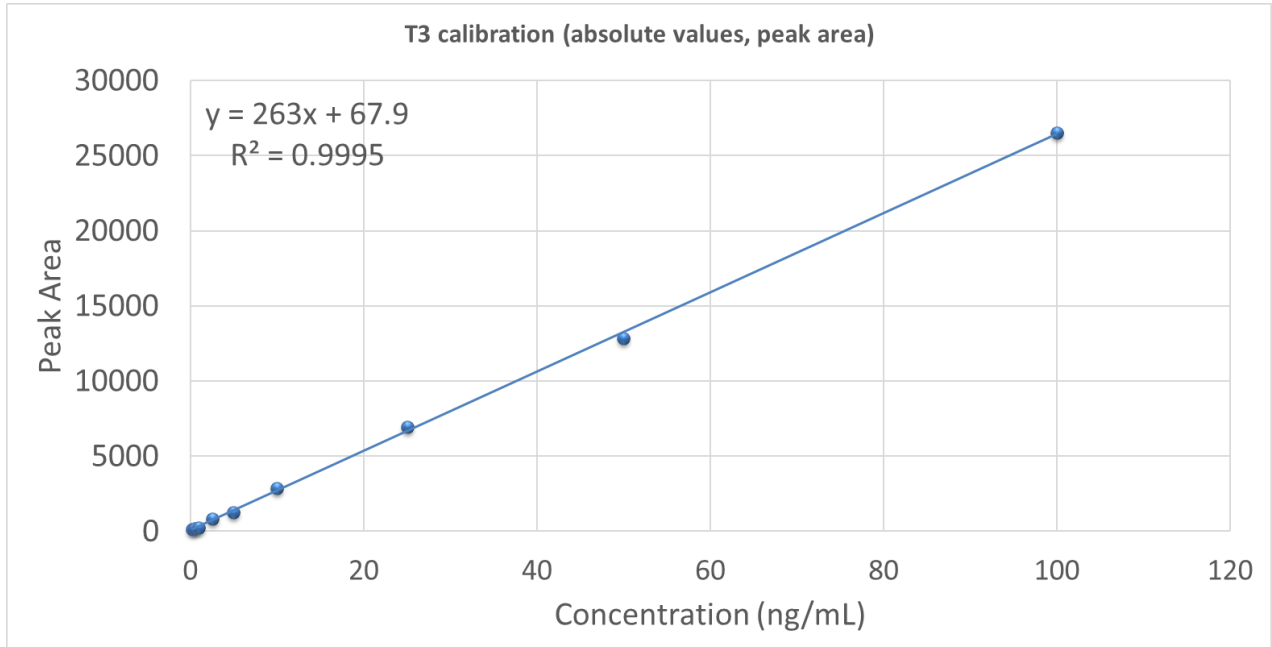


**Figure A.1:** The calibration curve for T<sub>4</sub> hormone, using the absolute values of peak area (y-axis), and concentration (x-axis).

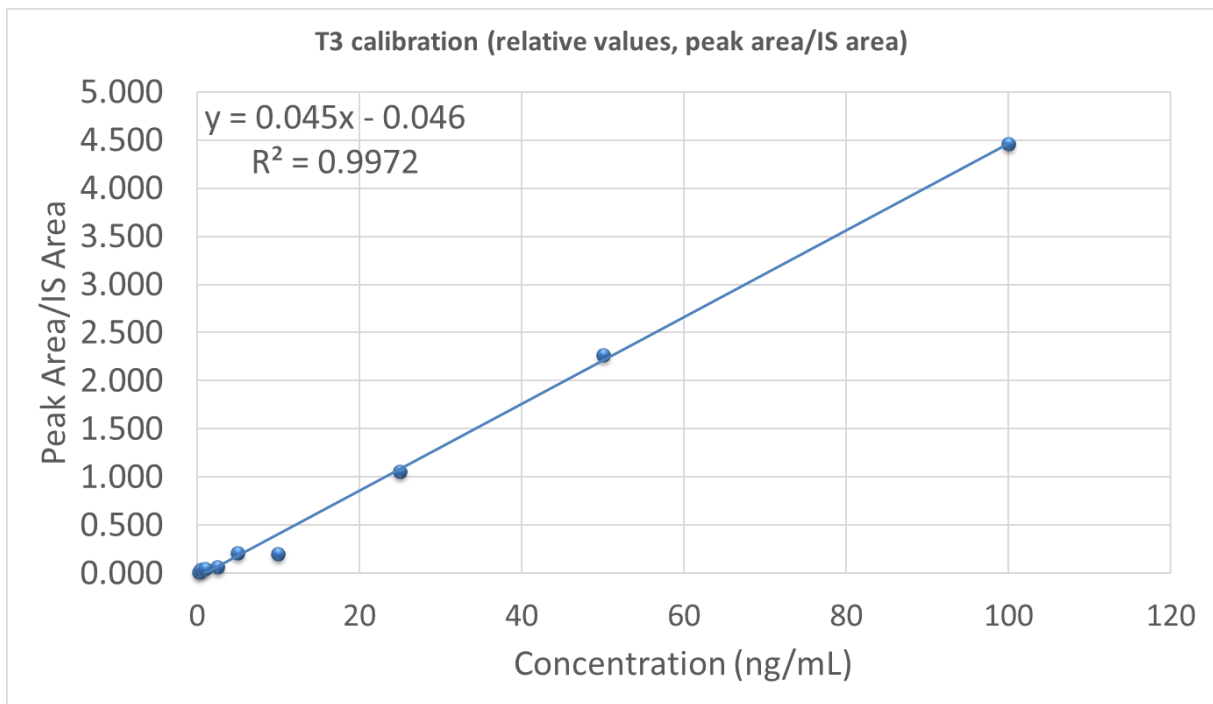


**Figure A.2:** The calibration curve for T<sub>4</sub> hormone, using the relative response values (y-axis), and concentration (x-axis).

- T<sub>3</sub> Hormone

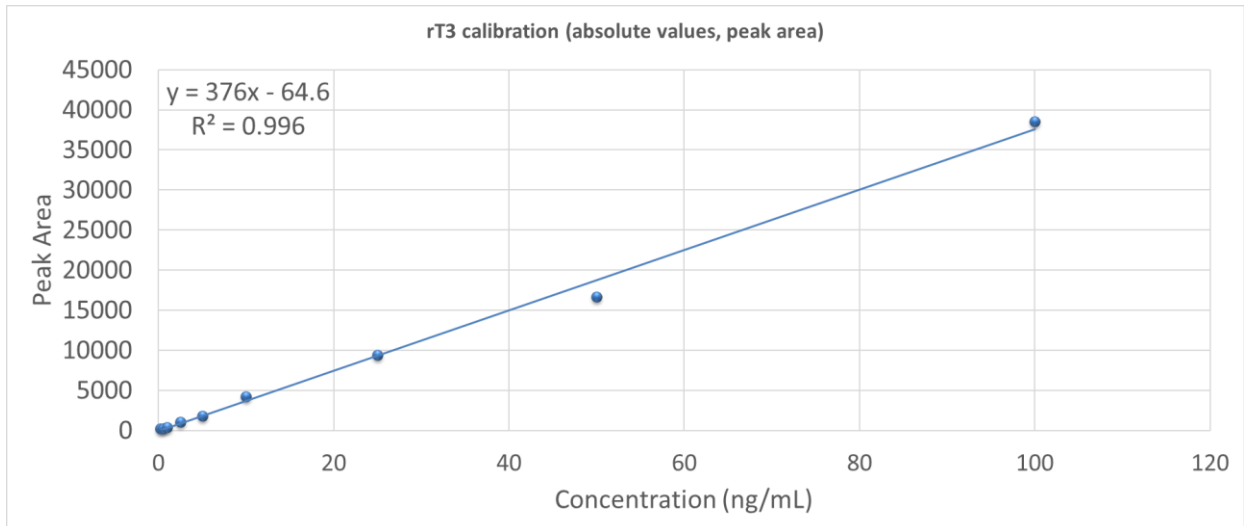


**Figure A.3:** The calibration curve for T<sub>3</sub> hormone, using the absolute values of peak area (y-axis), and concentration (x-axis).

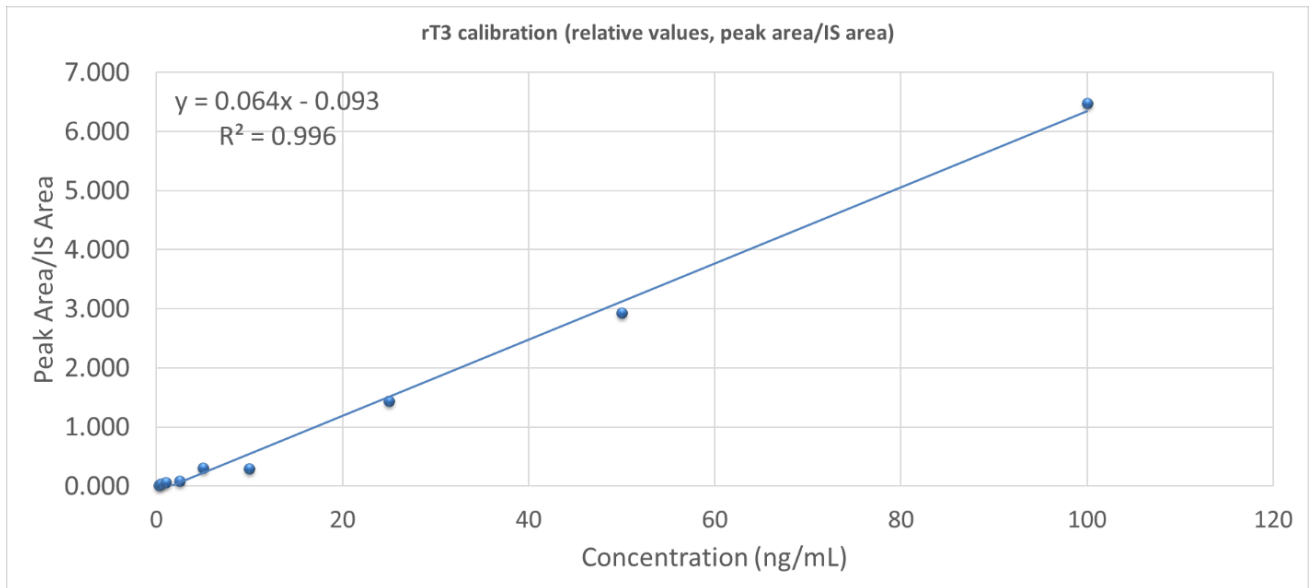


**Figure A.4:** The calibration curve for T<sub>3</sub> hormone, using the relative response values (y-axis), and concentration (x-axis).

- rT<sub>3</sub> Hormone

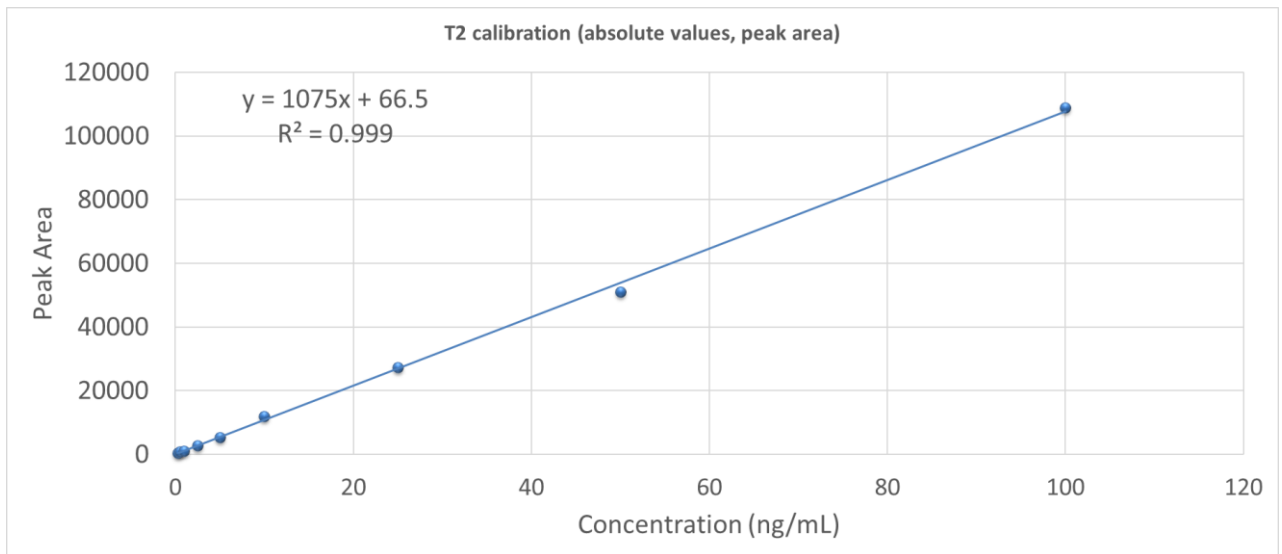


**Figure A.5:** The calibration curve for rT<sub>3</sub> hormone, using the absolute values of peak area (y-axis), and concentration (x-axis).

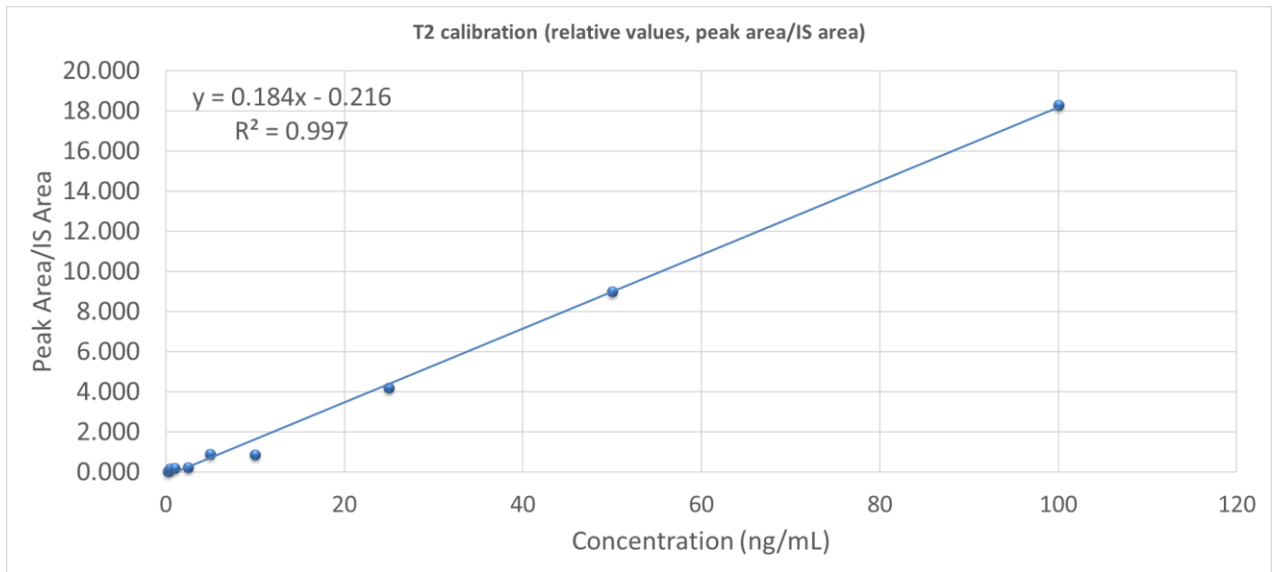


**Figure A.6:** The calibration curve for rT<sub>3</sub> hormone, using the relative response values (y-axis), and concentration (x-axis).

- T<sub>2</sub> Hormone



**Figure A.7:** The calibration curve for T<sub>2</sub> hormone, using the absolute values of peak area (y-axis), and concentration (x-axis).



**Figure A.8:** The calibration curve for T<sub>2</sub> hormone, using the relative response values (y-axis), and concentration (x-axis).

**Table A.1.** Absolute recoveries for the thyroid hormones.

|                   | T4 (ABS.) | STD T4 (ABS.) | T3 (ABS.) | STD T3 (ABS.) | rT3 (ABS.) | STD rT3 (ABS.) | T2 (ABS.) | STD T2 (ABS.) |
|-------------------|-----------|---------------|-----------|---------------|------------|----------------|-----------|---------------|
| SPE-O (WATER)     | 32.0      | 14.9          | 0         | 0             | 22.7       | 8.47           | 53.7      | 20.8          |
| SPE-O (SERUM)     | 53.4      | 4.75          | 0         | 0             | 69.7       | 3.87           | 72.4      | 1.77          |
| SPE-P (WATER)     | 31.8      | 3.17          | 47.0      | 43.6          | 29.0       | 2.83           | 52.7      | 4.64          |
| SPE-P (SERUM)     | 29.2      | 9.23          | 0         | 0             | 46.7       | 3.79           | 49.9      | 3.99          |
| LLE (WATER)       | 69.6      | 4.56          | 79.8      | 18.0          | 62.4       | 3.42           | 68.8      | 6.24          |
| LLE (SERUM)       | 49.8      | 11.77         | 63.2      | 7.34          | 27.8       | 2.12           | 49.9      | 3.99          |
| HSPE-ACN (WATER)  | 43.9      | 2.18          | 44.3      | 1.03          | 41.9       | 1.75           | 45.4      | 1.00          |
| HSPE-ACN (SERUM)  | 27.6      | 4.51          | 26.4      | 1.47          | 24.3       | 2.77           | 25.2      | 2.06          |
| HSPE-MeOH (WATER) | 6.08      | 1.32          | 6.98      | 0.49          | 7.22       | 0.64           | 6.44      | 1.45          |
| HSPE-MeOH (SERUM) | 1.02      | 5.59          | 3.36      | 2.98          | 2.68       | 2.10           | 3.07      | 3.13          |

\*The absolute recovery values (average from N=3 pre-extraction spikes) from the different extraction methods tested (SPE-O: SPE with Oasis HLB; SPE-P: SPE with Strata-X CW; LLE: Liquid-Liquid Extraction; HSPE-ACN: Hybrid SPE-PPT with the precipitation agent of acetonitrile (1 % v/v Formic Acid); and HSPE-MeOH: Hybrid SPE-PPT with the precipitation agent of methanol (1 % w/v Ammonium Formate).

**Table A.2.** Relative recoveries for the thyroid hormones.

|                   | T4 (REL.) | STD T4 (REL.) | T3 (REL.) | STD T3 (REL.) | rT3 (REL.) | STD rT3 (REL.) | T2 (REL.) | STD T2 (REL.) |
|-------------------|-----------|---------------|-----------|---------------|------------|----------------|-----------|---------------|
| SPE-O (WATER)     | 122       | 43.2          | 0         | 0             | 84.5       | 16.8           | -         | -             |
| SPE-O (SERUM)     | #         | -             | 0         | 0             | -          | -              | -         | -             |
| SPE-P (WATER)     | 108       | 9             | 0         | 0             | -          | -              | -         | -             |
| SPE-P (SERUM)     | -         | -             | 0         | 0             | -          | -              | -         | -             |
| LLE (WATER)       | 86.2      | 15.5          | 99.3      | 7.79          | 78.0       | 17.05          | 85.1      | 13.92         |
| LLE (SERUM)       | -         | -             | 140.6     | 15.8          | 52.2       | 3.65           |           |               |
| HSPE-ACN (WATER)  | 94.8      | 9.03          | 96.3      | 14.7          | 91.1       | 15.68          | 98.8      | 16.62         |
| HSPE-ACN (SERUM)  | -         | -             | 118       | 4.74          | 109        | 11.71          | 106.6     | 9.02          |
| HSPE-MeOH (WATER) | 96.9      | 3.21          | 94.5      | 7.31          | 101        | 5.26           | 97.9      | 0.85          |
| HSPE-MeOH (SERUM) | -         | -             | 103       | 13.3          | 8.95       | 5.22           | 106       | 9.02          |

\*The relative recovery values (average from N=3 pre-extraction spikes) from the different extraction methods tested (SPE-O: SPE with Oasis HLB; SPE-P: SPE with Strata-X CW; LLE: Liquid-Liquid Extraction; HSPE-ACN: Hybrid SPE-PPT with the precipitation agent of acetonitrile (1 % v/v Formic Acid); and HSPE-MeOH: Hybrid SPE-PPT with the precipitation agent of methanol (1 % w/v Ammonium Formate).

# inconsistent relative recovery > 200 % and/or high uncertainty values.



**Table A.3.** Matrix Factor calculated for every thyroid hormone with different sample preparation protocol

|           | T4   | T3   | rT3  | T2   |
|-----------|------|------|------|------|
| SPE-O     | 0.97 | 0.65 | 0.71 | 0.80 |
| SPE-P     | 1.08 | 0.50 | 0.67 | 0.71 |
| LLE       | 1.30 | 0.85 | 0.69 | 0.83 |
| HSPE-ACN  | 1.06 | 0.8  | 0.81 | 0.81 |
| HSPE-MeOH | 0.80 | 0.77 | 0.71 | 0.74 |

\*SPE-O: SPE with Oasis HLB; SPE-P: SPE with Strata-X CW; LLE: Liquid-Liquid Extraction; HSPE-ACN: Hybrid SPE-PPT with the precipitation agent of acetonitrile (1 % v/v Formic Acid); and HSPE-MeOH: Hybrid SPE-PPT with the precipitation agent of methanol (1 % w/v Ammonium Acetate).