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Report

Suitability and Efficacy of the newly HBM4EU sample preparation procedure for Effect-Directed Analysis

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Figure 1. (HBM4EU, 2018)

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Acronyms

ACN	Acetonitrile
AhR	Aryl Hydrocarbon Receptor
AR	Androgen receptor
CAS	Chemical Abstracts Service
CEC	Chemical of Emerging Concern
CLP	Classification, Labelling and Packaging
DMSO	Dimethylsulfoxide
EC (50)	Effect Concentration (50%)
EC	European Commission
ECHA	European Chemicals Agency
EDA	Effect-Directed Analysis
EU	European Union
Er	Estrogen receptor
ESI	Electron Ionization Spray
FITC	Fluorescein Isothiocyanate
HBM	Human Biomonitoring
HBM4EU	Human Biomonitoring Initiative for the European Union
HPC	High-precision calibration
IC	Inhibitory concentration
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Limit of detection
MeOH	Methanol
MS	Mass spectrometer/spectrometry
No.	Number
OHBDE	Hydroxylated Polybrominated diphenyl ethers metabolites
OHCB	Hydroxylated Chlorinated biphenyl metabolites
OPFR	Organophosphorus Flame Retardants
PAHs	Polycyclic aromatic hydrocarbons
PB	Procedure Blank
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyl
PFAS	Polyfluoroalkyl Substances
PS	Polystyrene
Q-ToF	Quadruple-Time of Flight
R^2	Square of the correlation coefficient
REACH	Registration, Evaluation, Authorisation or Restriction of Chemicals
RF	Relative fluorescence
Rpm	Rounds per minute
RRHD	Rapid Resolution High Definition
RT	Retention time
SB	Serum Blank
SD	Standard Deviation
SPE	Solid Phase Extraction
SRM	Standard Reference Material
SS	Spiked Serum
SVHC	Substance of Very High Concern
T_4	Thyroxine
TBBPA	Tetrabromobisphenol A
TBP	Tributyl phosphate
TH	Thyroid Hormone
THDC	Thyroid Hormone Disrupting Compound
TRIS	Trisaminomethane
TTR	Transthyretin
UPLC	Ultra Performance Liquid Chromatography
VU	Vrije Universiteit van Amsterdam
v/v	Volume per volume

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1. Introduction

1.1 Problem Statement

An increasing number of diverse potentially toxic substances has been produced since the beginning of industrialisation, leading to the contamination of the environment and consequently to exposure via their usage in various applications. Recently, there has been a growing concern over the rise in the contamination of the environment by Chemicals of Emerging Concern (CECs). The term 'Chemicals of Emerging Concern' is used to describe the newly introduced contaminants, as well as toxicants that have persisted in the environment for years but their toxicity has not been described yet (Shareef, Kookana, Kumar, & Tjandraatmadja, 2008). Most CECs are synthetic and are likely to significantly alter the metabolism of living beings (Sauvé & Desrosiers, 2014).

In line with the increasing numbers of CECs and the possible exposure to these chemicals in everyday lives, there is a concern of their effect on the human health. Therefore, it is important to monitor human exposure to such chemicals, in order to assess the chemicals' impacts on health. This is known as Human Biomonitoring (HBM) (Gavrilescu, Demnerova, Aamand, Agathos, & Fava, 2014).

With the aim of tracking and identifying the presence of CECs in human matrices, as well as prioritizing and regulating these based on their effects on human health (HBM), the European Environment Agency and the European Commission started the HBM4EU initiative, which is a joint effort of 28 countries. The HBM4EU initiative proposed a framework that incorporates Effect-Directed Analysis (EDA). EDA allows the identification of biologically active compound in samples by combining chemical analysis (chromatography) and bio-testing (bio-assay) (Brack, Bandov, & Streck, 2008). So far, the Direct-Effect-based approach for identification of CECs and their toxicity has rarely been applied in the human biomonitoring context (Lamoree & Vinggaard, 2017). EDA has mainly been used in other research fields including environmental sciences (abiotic compartment), pharmaceutical sciences and food safety (Kool & Niessen, 2015a; Groh & Muncke, 2017). Nevertheless, the application of EDA in human samples, such as blood, could have a great potential to provide insight into which CECs can be found in the human body and their adverse effects on health. Additionally, EDA of biological samples can provide useful information on, for instance, bioaccumulation and possible metabolization and this approach seems to be much more (eco) toxicologically relevant than abiotic compartments (Simon, Lamoree, Hamers, & de Boer, 2015). Therefore, EDA may be used as a promising integrated approach for human biomonitoring (HBM) (Lamoree & Vinggaard, 2017).

Considering the fact that several different institutes in different countries are involved in the HBM4EU initiative, it is important to harmonize and standardize sample preparation and measurement workflows as much as possible for the sake of acquiring consistency and comparable results. The critical step before effect-directed analysis is the sample preparation, which involves extraction of the compounds of interest from the given matrix, making the samples suitable for both bioassay testing and chemical analysis in EDA. Therefore, a new generic sample preparation method is proposed by the HBM4EU initiative to perform enhanced throughput EDA studies on serum samples. This sample preparation procedure was made under the premise of using low volumes of sample and use as little steps as possible to ensure a minimal loss of compounds during the procedure. The preparation method was tested successfully with an LC-MS system. However, the suitability and effectiveness of this sample preparation procedure for EDA is not known.

1.2 The purpose of this research

My goal in the exertion of this project is to determine whether the new serum sample preparation method developed by the HBM4EU project is suitable for EDA and how its efficacy compares to an existing (VU) sample preparation method.

The first step will be a Proof-of-Concept experiment of the selected EDA method that includes LC-Q-ToF-MS, LC-fractionation and TTR-FITC-T_4 bioassay. This step takes advantage of a Standard Mixture (MiSSe, 2018) of seven Thyroid Hormone Disrupting Compounds (THDCs). The second step will be to determine if an extraction step is suitable for the selected EDA analysis. Therefore, a Solid Phase Extraction (SPE) method will be performed on Standard Reference material (SRM) 2585 dust sample for the preparation step, followed by the

EDA procedure. Once these two experiments give positive results on the functionality of the EDA combined with an extraction method, the main objective of the present study can be performed; I will perform two complete identical EDA procedures on serum samples to identify biologically active thyroid hormone disrupting compounds. I will use the HBM4EU sample preparation method for one EDA procedure, as well as the existing (VU) sample preparation method for the other. The two sample preparation methods will be evaluated against each other for EDA suitability and efficacy by a (TTR-FITC-T_4) bioassay and Liquid Chromatography quadruple Time of Flight mass spectrometer (LC-Q-ToF-MS), measuring spiked Thyroid Hormone Disrupting Compounds (THDCs) in serum samples.

This research is subject to the following research question:

How suitable and effective is the newly proposed sample preparation procedure by HBM4EU for use in EDA, compared to an existing sample preparation method?

- *How qualitatively-well does the HBM4EU sample preparation recover the spiked THDCs in the EDA products, i.e. chromatograms and bioassay spectra? How do these EDA products compare to the ones retrieved from an existing sample preparation method?*
- *What is the ratio of the recovered THDCs between the HBM4EU sample preparation method and the existing (VU) sample preparation method?*

2. Theoretical background

2.1 Human biomonitoring (HBM) in Europe

Contaminants are ubiquitous in the environment. They can be found in water, air, soil as well as in industrial products, consumer goods and food products. Exposure to environmental pollutants occurs through different routes, such as inhalation (air), ingestion (food), and dermal absorption (through skin), and with the combined exposure via all routes known as the aggregate exposure. Uptake of contaminants can consequently reach and be stored in human tissues such as blood or fat, or they can be metabolized and excreted via the urine (Plaßmann, Brack, & Krauss, 2014). In addition, exposure to these compounds may lead to adverse health effects in humans (Ouyang, et al., 2017). As humans are substantially exposed to contaminants in their everyday lives, it is important to monitor their exposure to such chemicals, to identify contamination sources, and to assess the chemicals' impacts on health.

An important tool for detecting environmental chemicals in the human body and their possible effects on human health exposure is Human Biomonitoring (HBM). HBM reflects the total body burden or biological effect of a compound as it considers all the relevant sources, all routes of uptake (absorbed dose), timing of exposure, the physical and chemical properties of the contaminants and the individual factors (metabolism and excretion rates) (World Health Organization Regional Office for Europe, 2015).

The European Commission's 2012 Communication on the combination effects of chemicals identifies a lack of knowledge on "*where, how often and to what extent humans and the environment are exposed to certain chemical mixtures and how exposure may change over time*" (European Commission, 2012). The Communication recognises the need to build Europe-wide understanding of the chemical mixtures to which the human populations are actually exposed (Ganzleben, et al., 2017). Additionally, the 7th Environmental Action Programme (European Commission, 2016) called for the development of a Union Strategy for a non-toxic environment (Goldenman, et al., 2017). In recognition of this need, the HBM4EU initiative was created in 2017 and runs until 2021. The initiative represents a joint-European effort of 28 countries, the European Environment Agency and the European Commission, and it is co-funded under the European Union Framework Program for Research and Innovation (Horizon 2020). The European Human Biomonitoring (HBM4EU) initiative aims at coordinating and advancing a sustainable human biomonitoring program across Europe (HBM4EU, 2018) by including continued harmonization in HBM procedures (Joas, Schwedler, Choi, & Kolossa-Gehring, 2016). This will allow facilitation to generate robust knowledge on the exposure of European citizens to chemicals and on the possible impacts of chemical exposure to human health (Ganzleben, et al., 2017). This requires, among others, development and improvement of sample preparation procedures and techniques.

HBM4EU can thereby provide evidence of the actual exposure of citizens to chemicals and the possible health effects, in order to inform the policy makers in the EU. EU policy makers will contribute to better regulation of the internal market, while striking a balance between the interests of industrial competitiveness and the safe management of chemicals in Europe for public health (European Commission, 2018). The initiative intends to establish dialogue and collaboration among the several Commission services, EU agencies, national representatives, stakeholders and scientists involved, demonstrating how research funding can build bridges between the research and policy worlds (Joas, Schwedler, Choi, & Kolossa-Gehring, 2016). It is anticipated that HBM4EU will contribute to the overarching goal of ensuring that EU chemical policies minimise the adverse effects of chemicals on human health. In addition, HBM4EU is generating exploratory human exposure data that can guide the prioritisation of substances for monitoring and research under future human biomonitoring activities. The initiative prioritizes human biomonitoring for twelve substances including CECs, Aniline family, Bisphenols, Cadmium and chromium VI, Chemical mixtures, Flame retardants, PAHs, Per-/poly-fluorinated compounds, Phthalates and Hexamoll® DINCH, Acrylamide, Aprotic solvents, Arsenic, Di-isocyanates, Lead, Mercury, Mycotoxins, Pesticides and Benzophenones (Vicente & Ganzleben, 2018; HBM4EU, s.d.). In this study, focus is brought to the CECs.

2.1.1 HBM4EU for Chemicals of Emerging Concern

Chemicals of Emerging Concern (CECs) can reach human tissues via direct usage of consumer products or uptake via the environment and food. The term "Chemicals of Emerging Concern" is used to describe the newly introduced contaminants, as well as toxicants that have persisted in the environment for years but their toxicity has not been described yet (Shareef, Kookana, Kumar, & Tjandraatmadja, 2008). Most CECs are synthetic and may alter the metabolism of a living being (Sauvé & Desrosiers, 2014). These chemicals are currently not included in routine human monitoring programmes at the European level, partially due to the lack of analytical methods to measure the chemical or its metabolites in human specimens. There is overall a lack of knowledge about general population exposure to CECs and potential health impacts (HBM4EU, s.d.; Gavrilescu, Demnerova, Aamand, Agathos, & Fava, 2014).

Nevertheless, the CECs may be candidates for future regulation, depending on research on their (eco)toxicity, potential health effects and on monitoring data regarding their occurrence in the various environmental compartments (van Houten & Alphenaar, 2016). So far, too little is known about the occurrence, the actual risks and the approach to formulate appropriate policy and legislation (van Houten & Alphenaar, 2016). Therefore, knowledge and awareness are necessary to properly understand how to deal with CECs.

The HBM4EU work on CECs aims at providing early warning of their potential human health effects, to support current EU health and environment policy making in order to properly regulate and reduce the actual exposure and health effects of a population to CECs (World Health Organization Regional Office for Europe, 2015). The HBM4EU initiative proposed a human biomonitoring framework that incorporates Effect-Directed Analysis (EDA) that allows the identification of (non-target) biologically active compounds in human samples (Lamoree & Vinggaard, 2017). The evidence on the adverse effects of CECs and their identification retrieved from the HBM4EU surveys can be used to support the process of prioritising substances for further risk assessment, such as the identification of Substances of Very High Concern (SVHC) under the European Regulation REACH ((EC) No 1907/2006), or prioritising measures for future policy making.

2.2 Effect-Directed Analysis (EDA)

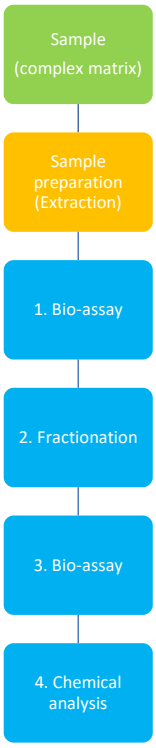


***Figure 2**. General steps of EDA*

Effect-directed analysis (EDA) is a promising integrated tool, enabling the identification of compounds in complex matrices, which may be responsible for causing adverse effects (Simon E. , 2013). The major components of EDA are chemical tools (fractionation via chromatography for a reduced complexity of the sample to be tested and compound identification using mass spectrometry) together with biological tools (bioassays to focus on active compounds) (Froment, 2017; HBM4EU, s.d.).

The main EDA procedure includes: 1) a bio-test on a chosen *in vitro* bio-assay related to a specific toxicological endpoint (e.g. TTR-FITC-T_4 assay for thyroid hormone disruptors) that will determine the amount of biologically active compounds in a sample, 2) a fractionation method is applied to reduce the matrix complexity and to discriminate non-toxicants from the matrix 3) the collected fractions are tested with the same bioassay to detect bioactive compounds 4) mass spectrometry is used for the identification of the compounds that showed a response in the bioassay (Burgess, Ho, Brack, & Lamoree, 2013), see figure 2.

Performing analysis of biological samples is always challenging due to the diversity and complexity of the sample matrix (high concentration of endogenous components, lipids, proteins etc.) (Weiss & Reemtsma, 2005). Accordingly, matrix effects might have profound impact on chemical analysis, hampering the efficient chromatographic separation and detection of analytes at low detection levels, i.e. limit of detection (LOD) (Weiss & Reemtsma, 2005). Matrix complexity is also hindering the identification of the active compounds in bio-testing. Therefore, sample preparation is essential for chromatographic and bio-testing procedures (Weiss & Reemtsma, 2005). The sample preparation separates and removes the target analytes as 'clean' as possible from other interfering matrix components (Weiss & Reemtsma, 2005), commonly via extraction. This preparation 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 concentrations required.

The first and very critical step before applying an EDA method is the sample preparation, which involves extraction of the toxicants from the given matrix, making the samples suitable for both bioassay testing and chemical analysis (Simon E. , 2013). Therefore, a robust sample preparation method is proposed by the HBM4EU initiative to perform enhanced throughput EDA studies. Protein denaturation is used as an extraction step for the HBM4EU sample preparation. The protein denaturation procedure is selected by the HBM4EU initiative under the premise of using low volumes of serum sample, i.e. approximately 1 to 3mL, and use as little steps as possible to ensure a minimal loss of compounds during the procedure. In contrast, a protein denaturation combined with a Solid Phase Extraction (SPE) method is selected for the existing VU sample preparation. The method includes more steps than the HBM4EU sample preparation method, allowing an increased possibility of compound-loss. Nevertheless, SPE has a high selectivity, specificity and reproducibility (Weiss & Reemtsma, 2005). Moreover, the method requires short sample preparation time (World Health Organization Regional Office for Europe, 2015).

For this study, an *in vitro* TTR-FITC-T_4 assay is used for bio-testing, an LC combined with a fraction collector is used for fractionation and LC-Q-ToF-MS is used for chemical analysis. These instruments and techniques are discussed in this chapter.

2.2.1 Solid Phase Extraction (SPE)

The primary separation that is applied before EDA is an extraction step separating the toxicants from other interfering compounds forming the sample matrix. The latter may include large biogenic organic molecules such as proteins, lipids and polysaccharides (Brack, Ulrich, & Bataineh, 2011). In other words, extraction allows isolation of the analytes of interest.

Solid Phase Extraction (SPE) utilises a liquid-solid extraction separation principle in which a large particle sized sorbent (chromatographic solid stationary phase packing material) is sealed into a small chromatographic column, cartridge tube. Based on the features (e.g. polarity) of the compound of interest to be extracted and the sample matrix, different SPE sorbents may be selected. The SPE method requires a measured volume of the liquid state sample to be passed through the cartridge tube. 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 (Weiss & Reemtsma, 2005). The desired analytes (cation or an anion) in a liquid state are separated from a complex sample matrix onto the sorbent and then selectively removed from the column either before or after elimination of the interfering compounds using an adequate (polar or non-polar) solvent (Hichrom, 2018). As a result, the analyte can be tested on a bioassay with reduced risk of matrix interference.

The general procedure of SPE consists of four steps:

1. **Conditioning**: 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.

2.2.2 Protein denaturation

The term denaturation is used to designate defined changes in the molecular structure of proteins, losing their native shape caused by non-physiological conditions (chemical, physical, and biological agents). The weak chemical bonds (hydrogen bonds) and interactions that are responsible for the highly ordered structure of the protein in its natural state are disrupted (Koshland & Haurowitz, 2019). Thereby, the proteins become biologically inactive. Denatured proteins have a looser, unfolded state and irregular structure; most are insoluble (Koshland & Haurowitz, 2019). A protein denaturation step allows the discrimination and removal of proteins in a matrix. In other words, the denaturation procedure can be seen as an attempt to extract the interfering compounds, specifically proteins. For this reason, protein denaturation can be part of the primary separation step that is applied before EDA, in this case, separating the interfering compounds from the toxicants of interest in the matrix.

2.2.3 Bioassay

A biological assay or bioassay is a tool employed to detect biologically active substances, based upon biological responses on a certain type of living matter (Aldana, 2010; Panuganti , 2015). It is essential in monitoring environmental pollutants. Bioassay is of two types: *in-vitro* and *in-vivo* assays. *In-vivo* bioassays are carried out in living organisms such as mice, rat etc.; whereas *in-vitro* bioassay is carried out using a part of cells or tissue derived from living organisms, such as, human, rat, etc. For this study, focus is brought to *in vitro* bioassays.

In the context of EDA, the choice of bioassay studies (endpoint and effect-based testing system) is of utmost importance, as it drives which type of compounds will be identified (Froment, 2017). Different bioassays describe different effects that may be caused by different types of chemicals. Biological activity in EDA is typically measured through, e.g. nuclear receptor binding (e.g. ER, AR, AhR) or the interaction with transport proteins (e.g. TTR protein-binding for thyroid hormone disruption) (Lamoree & Vinggaard, 2017). In the framework of this study, focus is brought to thyroid hormone disruption.

2.2.3.1 Thyroid hormone disrupting compounds (THDCs)

The indoor environment is an important source of attention for human biomonitoring. Toxicants in the indoor environment can be thyroid hormone disrupting compounds (THDCs), such as polycyclic aromatic hydrocarbons (PAHs), poly-chlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), organophosphate flame retardants (OPFRs), poly- and perfluoroalkyl substances (PFASs) and plasticizers, resembling structurally and chemically thyroid hormones (THs). These have been widely used over the past few decades (Plaßmann, Brack, & Krauss, 2014) and as a result, rising levels of these compounds have been found in the bodies of humans.

Thyroid hormones are produced by the thyroid gland. These hormones are polar non-steroidal and are only soluble in blood plasma. The major form of thyroid hormone in the blood is thyroxine (T_4). For it to be transported and reach the location in the body where it has its effect, the hormone weakly binds (non-covalently) (Brack W. , 2011) to proteins such as transthyretin (TTR), also known as prealbumin, in human blood (Froment, 2017). Although TTR is not the most dominant transport protein, TTR is of importance because of its capacity to transport T_4 across the placenta and the blood-brain-barrier (Meerts, et al., 2002). Thyroid hormone disruption can be affected through a variety of pathways. One mode of thyroid hormone interference is the TH transport through the body, by blocking the binding between the thyroid hormone T_4 and the transport protein TTR. Disruption of the binding of T_4 with TTR is caused by the competition between the endogenous thyroid hormone T_4 and the exogenous T_4 (structurally and chemically) resembling contaminants and, thus, replacing the natural T_4 hormone (Ouyang, et al., 2017). By doing so, the T_4 transport from the site of production to the site of conversion is reduced (Froment, 2017). Other interferences created by thyroid hormone disrupting compounds are within the TH metabolism and TH excretion (Lamoree & Vinggaard, 2017).

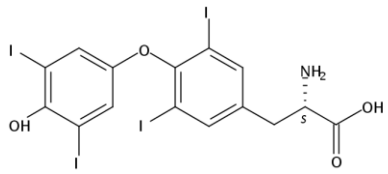


Figure 3. *Structure of T_4 thyroxine hormone (ECHA, 2018)*

Thyroid hormones play an important role in the regulation (development and growth) of an organism and in the maintenance of a normal physiological state, especially in embryos (Weiss, et al., 2015). Thyroid disrupting contaminants may have a critical impact for normal growth and differentiation of many tissues and organs, and may alter metabolic regulations in higher organisms and perinatal development of the central nervous system (Plaßmann, Brack, & Krauss, 2014). The pollution of these chemicals in the indoor environment is believed to be able to contribute to abnormal thyroid function, respiratory diseases, cancer, neuropsychological disorders and neuron-developmental deficits, etc. (Xiao & Liang-Hong, 2012). For this reason, Thyroid hormone (TH) disrupting compounds are potentially important contaminants (Ouyang, et al., 2017) and could have devastating effects on individuals, as well as on whole populations (Weiss, et al., 2015). Therefore, it is important to identify thyroid hormone-disrupting compounds (THDCs) in monitoring programs.

2.2.3.2 TTR-FITC-T_4 assay

The interference of environmental pollutants with the thyroid hormone system is assessed by the competition between the (natural) endogenous hormone T_4 and the T_4-resembling exogenous compounds, i.e. THDCs, for the binding to TTR. Currently, the most successful bio-analytical method to detect and evaluate TH disruptors, which target the blood transport of TH in environmental samples, is the thyroxine-transthyretin (T_4-TTR) binding assay. Recently, a report proposed the utilization of a fluorescent probe in competitive bio-assays (Ren & Guo, 2012) as labels or tracers for visualization and localization of biomolecules (Valeur & Berberan-Santos, 2012).

For the TTR-FITC-T_4 assay, a fluorescence probe, i.e. fluorescein isothiocyanate (FITC), is covalently associated to a T_4 conjugate (Xiao & Liang-Hong, 2012) that will serve as a signal reporter (FITC-T_4). This fluorescent T_4 conjugate (FITC-T_4) is employed to study the binding affinities of THDCs and T_4 to the major thyroid hormone transport protein TTR (Xiao & Liang-Hong, 2012). In the TTR-FITC-T_4 assay, the binding competitiveness to TTR is between the T_4 fluorescent conjugate (FITC-T_4) and the THDCs. It is expected that the T_4 moiety of the conjugate would provide site specificity for proteins (Xiao & Liang-Hong, 2012), whereas the fluorescein probe serves as a signal reporter. A thyroid hormone disrupter able to bind to TTR will decrease the fluorescence intensity, making it possible to measure its impact on TTR (Ren & Guo, 2012). One of the requirements for a fluorescent probe is signal modulation after binding with the target protein (TTR), which can be a change in its fluorescence intensity, emission wavelength, or degree of polarization (Xiao & Liang-Hong, 2012). Fluorescein is composed of two parts of xanthene, the chromophore part, and benzene, and exhibits excitation at 490 nm and emission at 514 nm ($\lambda_{max}/\lambda_{em}$ = 490/514 nm) (Nishi, Isobe, Zhu, & Kiyama, 2015).

To obtain less complex, small volume fractions and a high throughput EDA, multi-well microplate format (96, 384, 1536) may be used in combination with miniaturised *in vitro* bioassays (Lamoree & Vinggaard, 2017). The development of bioassays in microplates has several advantages. Firstly, the format allows testing of a large number of samples with the help of a microplate reader to generate the data. Furthermore, this format can be directly linked with chemical fractionation with the help of a special fraction collector. Finally, the cost of a bioassay can be lowered when the assay is simplified, as it does not require keeping animals in the laboratory, and most of the time when it is downscaled as well (as it requires less solvent use and more samples can be run in a day) (Froment, 2017).

2.2.4 Separation technique for fractionation

A chromatographic separation technique is primarily applied in EDA for fractionation. Separation techniques in EDA are applied to create a simpler matrix to a limited number of major toxicants. Reducing the number of chemicals (discrimination) is primordial in order to facilitate the non-targeted identification of the active compounds, while conserving the responses observed during the bioassays. Additionally, fractionation is also applied to avoid interference of endogenous hormones with bioassays by separating these from the biological samples. Liquid chromatography (LC) has been the most commonly reported fractionation technique used in a context of EDA. This technique allows rapid and efficient fractionation (Froment, 2017). Fractions are tested again in the respective *in vitro* bioassay used prior to fractionation. The application of bioassays inherently facilitates a prioritisation of fractions to be studied for the presence of toxic CECs (Lamoree & Vinggaard, 2017).

Fractions are typically collected by automated fraction-collectors. The fraction- collectors inject, commonly known as 'spot', the eluting compounds from the LC-column in the wells of the bioassay plates at regular time points with steady interval times (e.g. every minute).

2.2.5 Chemical analysis

Effect-directed analysis (EDA) has been developed to identify and confirm major toxicants in complex mixtures (Lamoree & Vinggaard, 2017). The identification and confirmation of non-target analytes is obtained by a mass spectrometer after chromatographic separation. Mass spectrometry is a powerful instrumental method of analysis as it finds the abundance and mass of each isotope in an element, allowing the determination of its relative atomic mass, and finds the relative molecular mass of substances made of molecules (AQA, 2015).

Even after several steps of fractionation, mixtures may be still complex, requiring high resolution separation prior to mass spectrometric analysis (Brack, Ulrich, & Bataineh, 2011). To address this complexity issue,

chromatography coupled to quadruple-time-of-flight mass spectrometry (Q-ToF-MS) shows high sensitivity, resolution and accuracy of complex mixtures (World Health Organization Regional Office for Europe, 2015).

2.2.6 Blood Sample

Human biomonitoring is an analytical approach which focuses directly on measuring the volume of toxic chemical compounds present in the body, such as in bio-fluids. The application of EDA to human samples such as blood has great potential, as it may provide insight into which chemicals are found in the human body (i.e. the internal exposome) that we have not paid attention to so far. Chemicals in blood are in continuous contact with the whole organism and is in equilibrium with the organs and tissues where chemicals are deposited. Therefore, blood is the preferred matrix for human biomonitoring (World Health Organization Regional Office for Europe, 2015). In addition, sampling of biological materials takes a number of factors into account, which can influence the fate of environmental pollutants accumulated in biota, such as metabolism, depuration rates, excretion, stress, viability and condition of the organisms (Vrana, et al., 2005), which can hardly be considered when sampling abiotic material.

3. Methodology

The main objective of the present study is to determine whether the new serum sample preparation method developed by the HBM4EU project, i.e. protein denaturation, is suitable for EDA and how its efficacy compares to an existing (VU) sample preparation method. The VU sample preparation includes protein denaturation and solid-phase extraction. The novel HBM4EU sample preparation method will be deemed suitable and effective if the toxicological activity of spiked compounds in bovine serum is detected in a bioassay spectrum (from bio-testing) and if these latter can be identified in the chromatograms (from chemical analysis). The efficacy will also be determined by yielding a ratio that expresses the difference in recovery of the spiked compounds in the whole extracts of the HBM4EU and in the whole extracts of the VU. The efficacy determination of the HBM4EU sample preparation also involves the comparison of the two EDA products (chromatograms and bioassay spectra) of the HBM4EU extracts with those using the VU sample preparation method.

During this project, two experiments will be conducted before the determination of the suitability and efficacy of the HBM4EU sample preparation method for EDA. Primarily, the selected EDA method that includes LC-Q-ToF-MS, LC-fractionation and a TTR-FITC-T_4 bioassay, will be evaluated for functionality, i.e. Proof-of-Concept, by directly testing the bioassay and the chemical analysis method using a Standard Mixture (MiSSe, 2018) that consists of seven thyroid hormone disrupting compounds. Purposely, the mixture is chosen to exclude an extraction step (sample preparation step), since the it has a very simple matrix containing the thyroid hormone disruptors. This Proof-of-Concept experiment will be performed four times. Secondly, it is of interest if an extraction step is suitable for the selected EDA analysis. Therefore, a solid- phase extraction will be performed on Standard Reference material (SRM) 2585 dust as a sample preparation step, followed by the EDA procedure. In this experiment, a total of seven extracts will be retrieved. If these two experiments give positive results on the functionality of the EDA combined with an extraction method, the main objective of the present study will be performed.

The newly HBM4EU sample preparation proposes protein denaturation as an extraction method of toxicants in serum. For this study, the toxicants of interest to be chemically analysed and bio-tested in EDA are thyroid hormone disrupting compounds (THDCs). Therefore, clear bovine serum samples will be spiked with a selection of TH- disrupting compounds before extraction Comparable samples will be subject to the VU sample preparation method. During the extraction, the spiked TH-disrupting substances will be isolated from the serum matrix, for instance lipids and proteins. In this manner, the extract matrix of the toxicants will be adequate for both bio-testing and chemical analysis in EDA. Half of the extracts (A) will be directly bio-tested in dilution series, to determine the T_4-toxicological equivalence activity of the recovered THDCs in the whole extract. The T_4-equivalence is further explained in *section 3.5.6.2*. The other half of the extracts (B) will be separated using liquid chromatography. This LC-separation step will be performed twice; the first time, the LC-column will be coupled to a fraction collector and the second time the LC-column will be connected to a Q-ToF-MS. The LC-separation coupled to a fraction collector will be used to separate and eliminate (non-polar) compounds in the extracts that do not contribute to the TH-disrupting activity and that could interfere in the bioassay measurements. The (polar) TH-disrupting compounds will elute in the first 18 minutes from a reversed-phase column (C18) and will be injected, commonly known as 'spotted' or 'fractionated', by a fraction collector (FractioMate) into an *in vitro* TTR-FITC-T_4 bioassay plate for bio-testing. Compounds eluting after 18 minutes will be directly transferred to the waste. Furthermore, the LC-separation process coupled to a Q-ToF-MS will be performed with the purpose to set the recovered bioactive fractions from the bio-testing in relation to the identified compounds in chemical analysis, that is to say TH-disruptors. See figure 4 and Appendix II for a representation on the strategy of this research.

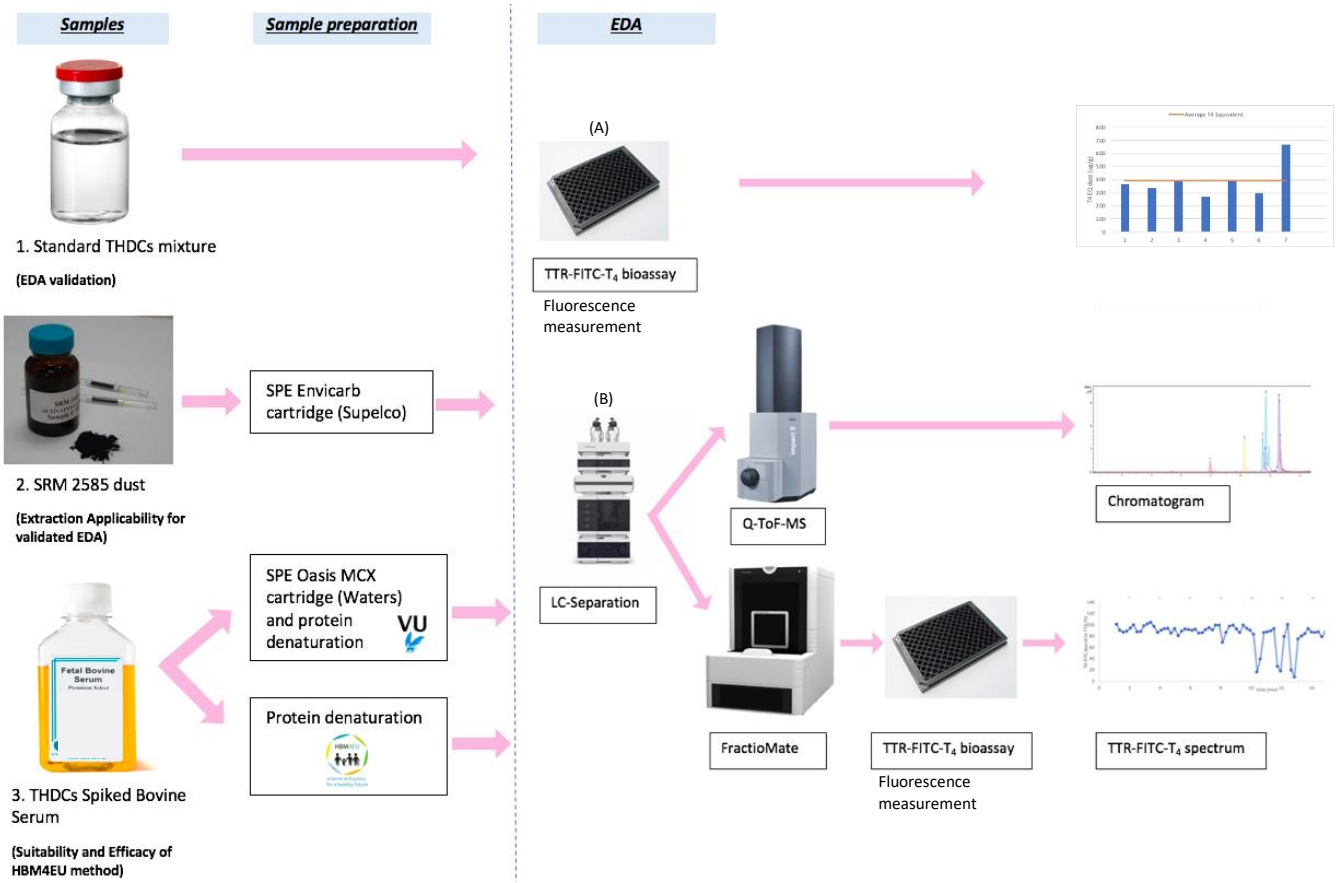


Figure 4. *An overview of the strategy to obtain the objectives of this study. First, a Proof-of-Concept experiment on the EDA method is performed using a Standard mixture that allows the exclusion of a sample preparation step. For this experiment, the sample is only subject to LC-separation (B), followed by chemical analysis and bio-testing. Second, the suitability of an extraction before EDA is evaluated by performing a solid-phase extraction on SRM2585 dust. Half of the SRM2585 extracts is directly bio-tested (A) and the other half undergoes a separation process (B) before chemical analysis and bio-testing. Thirdly, the HBM4EU and VU sample preparation is performed on bovine serum, followed by the whole EDA method; half of the extracts (A) are subject to direct bio-testing, the other half to LC-separation before chemical analysis and bio-testing.*

3.1 Chemicals and Materials

Fluorescein isothiocyanate (FITC, >90%), L-thyroxine (T4, >98%), Transthyretin (TTR), formic acid (HCOOH, 99% v/v) in 2-propanol (4:1, v/v), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and SRM2595 dust were supplied by Sigma (Zwijndrecht, The Netherlands). Methanol (HPLC grade) was purchased by J.T. Baker (Deventer, The Netherlands). Acetonitrile was retrieved from BioSolve (Dieuze, France). Milli-Q Reference A+ purification system (Millipore, Bedford, MA, USA). Furthermore, dimethylsulfoxide (DMSO) and EDTA was obtained from Acros (Geel,Belgium). Bovine serum was provided by Gibco (Netherlands). Sodium chloride and 2, 4, 6- TBP was purchased from Riedel-de Haen (Seelze, Germany). 6-OHBDE47, TBBPA, 5-OHBDE47, 4-OHCB107, 6-OHBDE99 and 4-OHCB187 (1 mg/mL in DMSO) were supplied by the group of Prof. Bergman, ACES, Stockholm University, Sweden. The Envicarb SPE cartridges were purchased from Supelco (Zwijndrecht, Netherlands). The OASIS catridges MCX were procured from Waters (Milford MA, USA). The black polystyrene 96-well microplates 655076 were retrieved from Greiner Bio-One (Frickenhausen, Germany). Reversed Phase (RP) ZORBAX Rapid Resolution High Definition (RRHD) Eclipse plus C18 (2.1 x 50 mm, 1.8µm particle size) column, Infinity 1290 UPLC pump and autosampler were from Agilent (Amstelveen, Netherlands). Daltonics Compact Q-ToF and the software tool Compass Hystar (2019) were from Bruker (Leiderdorp, Netherlands). A Syring Pump model 100 was from kd Scientific (Aarle-Rixel, the Netherlands). The Varioskan Flash multimode plate reader combined with the software SkanitRE for Varioscanflash2.4.5 were from Thermo Fisher Scientific (Waltham, MA, USA). A CentriVap Vacuum Concentration System combined with a coldtrap was from Labconco (Missouri, USA). The FractioMate FRM100 developed by SPARK and the VU Engineering Groups on Electronics and Precision Mechanics was of usage for fractionation.

3.2 EDA Proof-of-Concept: Preparation of the Standard Mixture (MiSSe, 2018)

The selected EDA is first proved for functionality, id est Proof-of-Concept, by directly testing the chosen bioassay and the chemical analysis method using a selected THDC Standard Mixture (MiSSe, 2018). The chemical analysis should identify via mass spectrometry the target THDCs in the mixture, eluting from the LC-column at specific retention times. At the same retention times, the bioassay after fractionation should provide biological responses based on the TTR-binding properties of these target compounds. The chemical are identified in a chromatogram and the bio-active response (bioassay) is displayed in the form of a spectrum. Both spectrum and chromatogram plot retention times on the x-axis. The bioassay spectrum allows the detection of unidentified compounds that have a TTR-binding toxicity and that eluted at specific retention times from the LC-column after separation. The chromatogram allows the identification of these compounds, as they should elute from the column at the same specific retention times. The Proof-of-Concept experiment is performed on four Standard Mixture replicates.

The chosen Standard Mixture (MiSSe, 2018) consists of seven compounds known to have an effect on thyroxine (T_4) transport in blood by binding to transthyretin protein (TTR). These compounds include five brominated flame-retardants and two PCB metabolites, see table 1 and Appendix I. The concentration is of 100x the EC50 (the concentration that gives 50% of the maximum response in the TTR-FITC-T_4 assay). The Standard Mixture is selected since the effect of these compounds on the bioassay is well established (Ouyang, et al., 2017), as well as the most abundant fragment ions detected in MS (see table 3). As the most abundant fragment ions of the compounds are already known, the compounds can easily be identified by looking at the retrieved chromatograms of those ions from the chemical analysis. Consequently, the biological responses in the bioassay can be aligned to the peaks of the chromatograms.

***Table 1**. Concentration of the seven compounds in the standard mixture at 100x EC50, diluted MilliQ: MeOH*

Standard Mixture (100xEC50) (MiSSe, 2018)				
CAS.no	**Compound**	**Molecular weight (mg/mmol)**	**Concentration(µg/ml)**	**Concentration (mM)**
79-94-7	TBBPA	543.875	1.7	0.0032
118-79-6	2,4,6-TBP	330.801	0.8	0.0025
79755-43-4	5-OHBDE47	501.794	0.8	0.0015
297742-10-0	6-OHBDE47	580.687	5.0	0.0086
152969-11-4	6-OHBDE99	342.421	2.3	0.0068
158076-68-7	4-OHCB107	411.305	2.1	0.0050
60348-60-9	4-OHCB187	564.691	0.6	0.0011

3.3 Standard Reference Material (SRM 2585) dust sample

It is of interest if an extraction step is suitable for the approved EDA method. Therefore, a solid- phase extraction is performed on Standard Reference material (SRM) 2585 dust sample as a preparation step, followed by the EDA procedure, see figure 4. The SRM 2585 contains a wide range of substances listed in Appendix XVI.

Before the extraction, approximately 50mg of SRM2585 is added to 5mL methanol in a 15mL polypropylene tube. This is performed seven times for a total of seven SRM2585 replicates. The mixtures are then vortexed for a minute and ultra-sonicated for 15 minutes. Subsequently, the mixtures are centrifuged for 5 minutes at 1500rpm. The supernatant of each SRM2585 replicate containing the compounds of interest is carefully separated and collected. Consequently, the whole procedure, starting with the addition of 5mL of methanol, is repeated another time on the seven residues using 5mL acetonitrile instead of methanol. The supernatant is then collected and combined with the previous supernatant of each replicate, ready to be loaded on the SPE cartridges.

For this study, the Envicarb SPE cartridges are selected for the extraction. The SPE catridges are activated two times with 5mL methanol:acetonitrile (1:1 v/v). As follow, the seven supernatants are loaded on the SPE cartridges. This is then followed by a rinsing step using 0.5 ml methanol:acetonitrile (1:1 v/v) four times to collect the target extracts from the cartridge. The collected extracts are then split 1:1 by half (A:B). A total of fourteen test tubes are set for evaporation to a final volume of 0.2mL, under a gentle nitrogen stream at room temperature. After the extraction, half of extracts (A) is reconstructed in 40µL dimethylsulfoxide. Consequently, each A-extract is diluted in a range of 1x-3x-10x-30x (A_d) and tested directly on the TTR-FITC-T_4 competitive binding bioassay. The other half of the extracts (B) is resuspended in 40µL MilliQ. These B-extracts are kept for LC-separation, followed by chemical analysis and bio-testing.

3.4 Sample preparation of spiked bovine serum

Bovine serum is selected as an example of a liquid biological sample because no or only very low levels of contaminants are expected in this type of serum. In other words, bovine serum is clearer compared to serum from top predators, such as polar bears (Simon, et al., 2011). Bovine serum (1mL) is spiked with 10µL of the Standard Mixture (MiSSe, 2018) mentioned in *section 3.2* (table 1). The spiked serum solution is prepared in triplicate and stored in the refrigerator at 4°C to equilibrate overnight. This spiking procedure is used prior to the HBM4EU and VU sample preparation method. In parallel, three (non-spiked) serum blank (SB) replicates and three MilliQ procedure blank (PB) replicates are prepared and these undergo the same procedural treatments as the spiked serum solutions.

3.4.1 HBM4EU Sample Preparation: Protein denaturation

The newly HBM4EU sample preparation proposes protein denaturation as an extraction method of toxicants from serum samples (see Appendix XII). 3mL of Acetonitrile (ACN) is added to the spiked serum solutions, serum blanks and procedure blanks. As follow, the mixtures are vortexed for 20 seconds and sonicated in an ultrasonic bath for 5 minutes. Then, the mixtures are centrifuged at 3000rpm for 5 minutes to separate and collect approx. 3.8mL liquid layer. The liquid layer of the spiked serum, serum blank and procedure blank replicates (approx. 1.9mL) is then split in half (A:B). Afterwards, all liquid layers (A and B) are set under a gentle nitrogen (N_2) flow for evaporation to a final volume of 0.1mL, at room temperature.

Eventually, half of extracts (A) is reconstructed in 40µL of dimethylsulfoxide for which dilution series are made. These dilutions series (A_d) are tested on a TTR-FITC-T_4 competitive binding assay. The other half (B-extracts) is reconstructed in 40µL MilliQ. These extracts are kept for LC-separation, followed by chemical analysis and bio-testing.

3.4.2 VU Sample Preparation: Protein denaturation and Solid Phase Extraction (SPE)

The extraction method of the Vrije Universiteit Amsterdam (VU) includes a protein denaturation step and SPE procedure. The protocols can be found in Appendix XIII.

3.4.2.1 Protein denaturation

In order to denaturate serum proteins, 1mL formic acid (HCOOH, 99% v/v) in 2-propanol (4:1, v/v) is added in a 1:1 (v/v) ratio to spiked serum (1mL), as well as the serum blank and procedure blank replicates. As follow, the mixtures are vortexed and, consequently, sonicated in an ultrasonic bath for 10 minutes. Then, the the spiked serum solutions, non-spiked serum solutions and procedure blanks are stored in the dark for 50minutes at room temperature. Eventually, 1 mL of water/2-propanol (4:1, v/v) is added to the replicates. The resulting mixtures are again sonicated for another 10minutes. Finally, the mixtures are diluted with approximately 6mL of water until the organic solvent percentage (iso-propanol and solvent of the spiking mixture) is less than 5%. This allows the reduction of the organic solvent on the SPE procedure. In order to obtain clear samples, a centrifugation step at 3000rpm for 5 minutes is required for pellet separation before extraction.

3.4.2.2 Solid Phase Extraction

The Oasis MCX cartridge (150mg, 6mL, Waters) is selected for this study, because highest chemically determined recoveries of TH-disrupting compounds was obtained for this cartridge in a pilot experiment comparing seven different cartridges (Simon E. , 2013). Approximately 1.7mL of the spiked serum samples, serum blanks and procedure blanks are extracted on the cartridge. The cartridge is first conditioned by adding MeOH (3mL) and equilibrated with water (3mL). Subsequently, 3mL of the treated spiked serum samples, serum blanks and procedure blanks is loaded. MeOH, water and the replicates are passed through the cartridges dropwise. The cartridges are washed with 3mL water containing 1.8% HCOOH formic acid. After the washing step, the SPE sorbent material is dried completely before elution by using vacuum. The adsorbed compounds are eluted from the MCX cartridges with 4 x 0.75 mL 100% MeOH into glass tubes. The collected eluates are then split by half (A:B) into other glass tubes. For this reason, a total of eighteen glass test tubes are subject to evaporation under a gentle nitrogen stream at room temperature to a volume of 0.1 mL. After evaporation, half of extracts (A) are reconstructed in 40µL of dimethylsulfoxide for which dilution series (A_d) are prepared for direct bio-testing. The other half of the extracts (B) is resuspended in 40µL of MilliQ for LC-separation, followed by chemical analysis and bio-testing.

3.5 LC separation for Fractionation and Chemical analysis

A Reversed Phase (RP) Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse plus C18 (2.1 x 50 mm, 1.8 µm particle size) column at 45 °C is used for the LC-separation using an Agilent Infinity 1290 UPLC pump and autosampler. 20µl of standard mixture, sample and blank (MeOH/H2O 1:1 (v:v)) is separately injected at a flow rate of 500 µL/min in 90% mobile phase A (100% MilliQ H_2O) and 10% mobile phase B (100% ACN). Over a period of 18 minutes, the solvent gradient increases to 99% mobile phase B. This ratio (99%B:1%A) is kept for another 12 minutes after which the gradient decreases to 10% mobile phase B and 90% mobile phase A again in half a minute. This is kept constant for 4.5 minutes. The total runtime is of 35 minutes. The gradient changes are shown in Table 2. After the LC-separation, the flow is diverted off-line to either the Bruker Compact Q-ToF-MS for chemical analysis or the FractioMate for the collection of fractions to be used in the bioassay.

***Table 2**. LC gradient over total period of 35minutes, separated in three steps. Mobile phase A consists of MilliQ and mobile phase B consists of acetonitrile (ACN)*

Process	*Separation/elution polar analytes*	*Flushing of nonpolar compounds*		*(Re-)acclimatize the column*	
Time (min)	0	18	30	30.5	35
Mobile phase B (%)	10	99	99	10	10
Mobile phase A (%)	90	1	1	90	90

3.5.1 Fractionation

Fractionation is performed using liquid chromatography (LC), in order to reduce complexity of the sample. In the first 18 minutes of the LC run, fractions are collected using the FractioMate well spotter, suitable for 96-wells black polystyrene plates. In each experiment (n=1), the polystyrene 96- well plate is first filled with 10µL of 10% DMSO: 90% MilliQ, respectively, as keeper in order to increase recoveries. Fractions are spotted in the bioassay plate by the FractioMate. Considering a total spotting time of 18 minutes and a flow of 500µL/min, 80 fractions can be collected in 80 wells at 13.5 seconds intervals, resulting in an end volume of approximately 117µL eluate per well. After fractionation, the collected fraction is stored overnight at -4°C. Consequently, the fraction is completely dried in a Centrivap concentrator for 4hours at 25°C and dissolved in 50µL of TRIS buffer. Eventually, the plates are shaken for 30 minutes at 700rpm using a plate-shaker, after which the bioassay test is performed.

3.5.2 Chemical analysis

Time-of-flight detection is performed using a Bruker Compact Q-ToF. The instrument is equipped with an orthogonal electrospray ionization source (ESI) in positive mode and a negative polarized mass spectrometer. For ESI^+ (positive) the capillary voltage is maintained at 4200 V, the gas flow to the nebulizer is set to 1.8 bar, the drying temperature is 220 °C, and the drying gas flow is 9.0 L/min. The spectra are recorded in the range of 112–1472 m/z during the first 18 minutes. For calibration, 20µL of sodium formate (1M NaOH, formic acid, MilliQ, propanol) is injected at the beginning of each chromatographic run at a rate of 35µL/h, using the kd Scientific auto-injector (Kd Scientific, 2016) equipped with a syringe. Data files are calibrated post-run on the average spectrum from this time segment, using the Bruker HPC (high-precision calibration) algorithm. The most abundant M-H monoisotopic ions of the THDCs from the Standard Mixture (MiSSe, 2018) measured on the spectra are listed in table 3.

***Table 3.** M-H monoisotopic (m/z)[1] of the THDCs product ions in the Standard Mixture (MiSSe, 2018)*

Compound	**CAS no.**	**Chemical formula**	**Molecular weight Mw (g/mol)**	**M-H monoisotopic (m/z)**	**Most abundant M-H monoisotopic (m/z)**
TBBPA	79-94-7	$C_{15}H_{12}Br_4O_2$	543.875	538.7487	542.7447
2,4,6-TBP	118-79-6	$C_6H_3Br_3O$	330.801	326.765	328.763
5-OHBDE47	79755-43-4	$C_{12}H_6Br_4O_2$	501.794	496.7018	500.6977
6-OHBDE47	297742-10-0	$C_{12}H_6Br_4O_2$	580.69	496.7018	500.6977
6-OHBDE99	152969-11-4	$C_{12}H_5Br_5O_2$	342.421	574.6122	578.6082
4-OHCB107	158076-68-7	$C_{12}H_5Cl_5O$	411.305	338.8699	340.867
4-OHCB187	60348-60-9	$C_{12}H_3Cl_7O$	564.691	406.7919	408.7892

[1] The (most) abundant M-H monoisotopic (m/z) of TBBPA, 2,4,6-TBP, 5-OHBDE47, 6-OHBDE47, 6-OHBDE99, 4-OHCB107 and 4-OHCB187 were experimentally determined.

3.6 TTR-FITC-T_4 assay for biological analysis

For this experiment, a 96-well plate with a total volume of 200µL per well is used for the for the TTR-FITC-T_4 bioassay to enhance the assay throughput. A black polystyrene (PS) plate with flat bottom wells is selected, due to relatively lower T_4 adsorption to the wall of the wells and good fluorescence measurement property. FITC-T_4 and TTR protein is added in the wells filled with sample. Subsequently, TTR-binding competitiveness is created between the FITC-T_4 and the T_4-ressembling compounds present in the wells.

3.6.1 TTR-FITC-T_4 Goodness-of-Fit

A Goodness-of-Fit test determines if a bioassay experiment works. For this study, it is relevant to establish if the TTR-FITC-T_4 bioassay method is able to provide a good representation regarding the TTR-binding activity of the T_4 in relation to its concentration (calibration curve). Therefore, the TTR-binding activity (y-axis) of the reference compound T_4 is plotted versus its concentration (x-axis) using T_4 calibration standards in multiple dilutions. The y-axis expresses the competitive TTR-binding between T_4 from the standards and the fluorescent FITC-T_4, when these two are set together in presence of the TTR protein. It is expected that at higher T_4 concentrations in the standards, the likelihood that FITC-T_4 binds to TTR is less. Accordingly, the fluorescence intensity (y-axis) is lower. In other words, the calibration curves show that a higher concentration in T_4 leads to a higher potency of TTR binding, as the fluorescence percentage is lower. In this study, seven to eight T_4 calibration standards are diluted in DMSO to concentrations of 0.0002, 0.002, 0.006, 0.02, 0.06, (0.2,) 0.6 and 2.0µM. A calibration curve with a square of the correlation coefficient (R^2) value of 1 (-0.1 SD) indicates a good fit of the TTR-FITC-T_4 bioassay method. Each dilution of the reference compound T_4 is measured in duplicate in each bioassay (see figure 5 and 6).

3.6.2 Bioassay preparation after LC-separation

During the first 18 minutes of the LC-run, 80 fractions (S1-80) are collected by the FractioMate well spotter in a polystyrene 96-well plate that is pre-filled with 10µL of 10% DMSO: 90% MilliQ. Eventually, after fractionation and solvent evaporation, the bioassay plate is prepared by directly dosing 2µL calibration standards (T_4 St. 1-6 and blank with concentrations of 2.0, 0.6, 0.06, 0.02, 0.006, 0.002, 0.0002µM, accordingly), as well as their duplicate (*d*), 50µL TRIS-buffer, 50µL TTR and 100µL FITC-T_4 in the correct wells. The whole plate (+TTR) is tested for the TTR-binding activity. However, two wells include DMSO blank without TTR for the background correction (-TTR), see figure 5. The background correction principle is further elaborated in *section 3.6.5.1*. In these two wells 50µL of TRIS buffer is added to obtain a total volume of 200µL. Subsequently, the plate is shaken for 5minutes at 600rpm without temperature on the plate shaker. As follow, the plate is incubated at 4°C for 2 hours. When incubated with TTR, the fluorescent intensity produced by the bonded FITC-T_4 is enhanced making it possible to calculate the amount of FITC-T_4 bonded with TTR.

		With + TTR									
DMSO blank -TTR	DMSO blank -TTR	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
DMSO blank +TTR	DMSO blank +TTR	S20	S19	S18	S17	S16	S15	S14	S13	S12	S11
T4 St.1 +TTR	T4 St.1_d +TTR	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30
T4 St.2 +TTR	T4 St.2_d +TTR	S40	S39	S38	S37	S36	S35	S34	S33	S32	S31
T4 St.3 +TTR	T4 St.3_d +TTR	S41	S42	S43	S44	S45	S46	S47	S48	S49	S50
T4 St.4 + TTR	T4 St.4_d + TTR	S60	S59	S58	S57	S56	S55	S54	S53	S52	S51
T4 St.5 + TTR	T4 St.5_d + TTR	S61	S62	S63	S64	S65	S66	S67	S68	S69	S70
T4 St.6 + TTR	T4 St.6_d + TTR	S80	S79	S78	S77	S76	S75	S74	S73	S72	S71

***Figure 5**. Representation of the bioassay scheme for the 96 well-TTR-FITC-T_4 binding assay using T_4 standards (T_4 St.), DMSO blanks and sample. The plate is pipetted with TTR protein after fractionation (+TTR), apart from the two DMSO blank (-TTR). 'd' stands for duplicate and 'S' stands for sample spotted in the bioassay after LC-separation.*

3.6.3 Bioassay preparation for the extracts in dilution series

The left half of the 96-well plate is tested for the TTR-binding (+TTR) activity in the diluted extracts, the right half is kept for the background correction (-TTR). The background correction concept is further explained in section *3.6.5.2* of this report. Each extract is first dissolved in the carrier solvent DMSO in dilutions of 1x-3x-10x-30x-100x-300x-1000x-3000x (dilution series may vary between the experiments). 2µL of the extracts (S) and their dilution solutions (x), as well as their duplicate (S_d), is directly dosed into the assay medium, which minimizes concentrations of the applied DMSO solvent to be of 1%. The 96-well plate is also pipetted with 2µL T_4 calibration curve standards (T_4 St. 1-7 and blank with concentrations of 2.0, 0.6, 0.2, 0.06, 0.02, 0.006, 0.002, 0.0002µM, accordingly), as shown in figure 6. 50µL of TRIS-buffer and 50µL of TTR protein is pipetted to the left side (+TTR) and 100µL of TRIS-buffer is pipetted to the right side (-TTR). Finally, 100µL of the FITC-T_4 is pipetted over the whole 96-well plate. It has to be considered that the concentrations in the wells of the

calibration curve standards (blank, St. $_{1\text{-}7}$) and (dilution) extracts, as well as their duplicate (*d*), are a 100 times diluted from their stock concentration. The correct reasoning for this is that only 2µL of their stock solutions is pipetted in a well containing 200µL of other mixtures.

Based on this pipetting scheme, it can be summarized that all wells within the bioassay plate consist of the FITC-T_4 and some TRIS-buffer. The wells are also injected with (diluted) extracts and a range of calibration standards, as well as their duplicates, individually. Half of the (diluted) extracts and calibration standards are set in the presence of the TTR protein (+TTR). The other half of the samples and calibration standards are not mixed with the TTR protein (-TTR). Comparison can be made between the observed activities obtained from the wells in presence of the TTR protein and those without the TTR protein.

With TTR (+TTR)						Without TTR (-TTR)					
DMSO blank	DMSO $blank_d$	S1 (1x)	$S1_d$ (1x)	S2 (1x)	$S2_d$ (1x)	DMSO blank	DMSO $blank_d$	S1 (1x)	$S1_d$ (1x)	S2 (1x)	$S2_d$ (1x)
T4 St.1	T4 $St.1_d$	S1 (3x)	$S1_d$ (3x)	S2 (3x)	$S2_d$ (3x)	T4 St.1	T4 $St.1_d$	S1 (3x)	$S1_d$ (3x)	S2 (3x)	$S2_d$ (3x)
T4 St.2	T4 $St.2_d$	S1 (10x)	$S1_d$ (10x)	S2 (10x)	$S2_d$ (10x)	T4 St.2	T4 $St.2_d$	S1 (10x)	$S1_d$ (10x)	S2 (10x)	$S2_d$ (10x)
T4 St.3	T4 $St.3_d$	S1 (30x)	$S1_d$ (30x)	S2 (30x)	$S2_d$ (30x)	T4 St.3	T4 $St.3_d$	S1 (30x)	$S1_d$ (30x)	S2 (30x)	$S2_d$ (30x)
T4 St.4	T4 $St.4_d$	S1 (100x)	$S1_d$ (100x)	S2 (100x)	$S2_d$ (100x)	T4 St.4	T4 $St.4_d$	S1 (100x)	$S1_d$ (100x)	S2 (100x)	$S2_d$ (100x)
T4 St.5	T4 $St.5_d$	S1 (300x)	$S1_d$ (300x)	S2 (300x)	$S2_d$ (300x)	T4 St.5	T4 $St.5_d$	S1 (300x)	$S1_d$ (300x)	S2 (300x)	$S2_d$ (300x)
T4 St.6	T4 $St.6_d$	S1(1000x)	$S1_d$(1000x)	S2 (1000x)	$S2_d$(1000x)	T4 St.6	T4 $St.6_d$	S1(1000x)	$S1_d$(1000x)	S2 (1000x)	$S2_d$(1000x)
T4 St.7	T4 $St.7_d$	S1(3000x)	$S1_d$(3000x)	S2(3000x)	$S2_d$(3000x)	T4 St.7	T4 $St.7_d$	S1(3000x)	$S1_d$(3000x)	S2(3000x)	$S2_d$(3000x)

Figure 6. *Representation of the pipetting scheme of the extracts (1x) and their dilutions (3x-10x-30x-100x-300x-1000x-3000x) for the 96 well-TTR-FITC-T_4 binding assay using T_4 standards, DMSO blanks and sample extracts (all in duplicate). One half (left) is pipetted with extra TTR protein.*

The plate is shaken for 5 minutes at 600rpm without temperature on the plate shaker. As follow, the plate is incubated at 4°C for 2 hours. When incubated with TTR, the fluorescent intensity produced by the bonded FITC-T_4 is enhanced making it possible to calculate the amount of FITC-T_4 bonded with TTR.

3.6.4 Fluorescence spectrometer

Once the bioassay is fully prepared with the adequate solutions in its wells, the plate is measured as fast as possible due to temperature shifts after incubation at 4°C. The fluorescence intensity is monitored at 490nm for the excitation and 514nm for emission (Ouyang, et al., 2017), using the FluTTR protocol on the Varioskan Flash multimode plate reader. The competitive FITC-T_4 or THDCs binding potency to TTR is estimated by the percentage of fluorescence intensity. The measurement with the highest increase in fluorescence reveals the FITC-T_4 binding to TTR.

3.6.5 Qualitative and Quantitative Data Processing: Suitability and Efficacy determination

In this study, three main experiments will be carried out multiple times: 1) EDA Proof-of-Concept, 2) applicability of an extraction method for the approved EDA method, and 3) determination of the suitability of the HBM4EU sample preparation for EDA analysis and its efficacy compared to an existing sample preparation method. For the first experiment, only *qualitative* data processing is of importance. For the other two experiments, *quantitative* in addition to qualitative data processing is applied. Qualitative testing consists of associating the found compounds in the MS chromatograms with the toxicological response from the bioassay of the sample fractions. Quantitative determination estimates the amount of TTR-binding activity in the whole extracts (in terms of T_4-equivalence).

With regard to the main objective of this study, the *suitability* of the HBM4EU sample preparation for EDA analysis implicates qualitative data processing. For the latter, the sample preparation method is suitable for EDA if the spiked toxicants in the serum are retrieved in the EDA products of the extracts: bioassay spectra and chromatograms. The *efficacy* of the HBM4EU sample preparation addresses quantitative data processing; calculating the amount of T_4 (µg) required in the whole serum extract (mL) to obtain the same measured TTR-binding activity that is from THDC origin. This is known as the T_4-equivalence (T_4-EQ) and is further elucidated in this section. A ratio can be established expressing the recovered THDCs from the VU and HBM4EU method (*VU:HBM4EU*) by comparing the T_4-equivalence values of the two methods. Efficacy evaluation also involves qualitative data processing by verifying if all spiked compounds are recovered in both bioassay spectra and chromatograms and by comparing the two EDA products of the HBM4EU method with those obtained after performing the VU sample preparation method, for instance with regard to peak heights in the HBM4EU and VU chromatograms.

3.6.5.1 Qualitative data processing of the fractionated samples

Qualitative data processing takes advantage of EDA products after LC-separation (B in figure 4): LC-Q-ToF-MS chromatograms and bioassay spectra after fractionation.

After LC-separation of the samples in the C18 column, the eluting compounds are collected in a 96-well plate. The FractioMate spots the eluting solution in one well during 13.5 seconds. After those 13.5 seconds, the injection needle moves to the next well, repeating the same step. In total, 18 minutes are required with intervals of 13.5 seconds (injection time/well), to fill all the 80 wells of the 96-well plate. Since the TTR-binding analytes, i.e. THDC, elute after various retention times, it can be expected that some wells of the bioassay plate contain specific THDCs and other wells only contain mobile phase. The plate is then prepped with FITC-T_4 and TTR-protein. In the wells where there are the eluted TTR-binding analytes, the binding competitiveness to TTR is between the T_4 fluorescent conjugate (FITC-T_4) and the THDCs. During fluorescence analysis, there will be little to no fluorescence intensity where a thyroid hormone disrupter is able to bind to TTR, as it is not associated to a fluorescent probe. The wells in which only mobile phase is spotted by the FractioMate, the fluorescence intensity is high, since the TTR automatically binds to the FITC-T_4.

A background correction is required for the obtained fluorescence measurements. It is considered that DMSO does not affect the measurement signals, as it is present in such low concentrations. Nevertheless, the bioassay plates themselves give a background signal caused by, for instance, negligible contamination. This signal interferes with the signal of interest, the one of the TTR-binding compounds in the samples. The procedure blank and each sample is spotted individually into one plate. Since it is of interest to set the measurements of the fractionated samples relative to those of the procedure blank, it is relevant to get rid of the signal variations of the different plates. Correction is done using the following equation (1) (Ouyang, et al., 2017):

Equation (1):

$$\mathrm{F}_{\text{Corrected Sample}} = \mathrm{F}_{\text{Sample}} - \mathrm{F}_{(\text{Average DMSO}-\text{TTR})}$$

The input value F_{Sample} represents the fluorescence of the samples and a blank. The blank is solely the matrix wherein the other samples are diluted. The $F_{average\ DMSO\ -TTR}$ is the average fluorescence measurement of the two DMSO wells without TTR (see figure 5).

After background correction, the percentage fluorescence intensities or, in other words, the T_4-FITC ability to bind to TTR (y-axis) can be plotted against retention times (x-axis) in a bioassay spectrum. The spectrum displays downwards peaks at retention times where the TTR-protein is bound to a thyroid disrupting compound, when competing with the T_4 associated with the FITC fluorescent probe. That is to say, at retention times where there is little fluorescence, a compound having the same TTR-binding functionality as T_4 is found. Therefore, the fluorescence intensities are an indication to the presence of TTR-binding compounds in the samples. The fluorescence percentage can be calculated by:

Equation (2):

$$\mathrm{F}_{\text{Sample}}(\%) = \frac{\mathrm{F}_{\text{Corrected Sample}}}{\mathrm{F}_{\text{Blank Sample}}} \times 100$$

The input value $F_{Corrected\ sample}$ represents the fluorescence intensities measured by the fluorescence spectrometer for the sample. The $F_{Blank\ Sample}$ expresses the fluorescence intensities measured of the blank sample. The blank is simply the matrix in which the sample is originally diluted.

Considering the fact that the TTR-binding analytes have eluted during fractionation at the same retention times as when the LC-column was connected to the Q-ToF-MS, the peaks from the chromatograms can be set against the peaks from the bioassay spectrum. The peaks at specific retention times in the chromatograms and in the spectrum can be aligned. These can be considered to belong to the same compounds.

3.6.5.2 Quantitative data processing of diluted extracts

Quantitative data processing only considers bioassay measurements of diluted extracts (A in figure 4).

The plate layout of the diluted extracts, as described in figure 6, includes a background correction to determine their biological activities in the absence of any sample matrix. The fluorescence background correction for the extracts is calculated using equation (3) (Ouyang, et al., 2017)). This calculation is also applied for the duplicate DMSO measurements, considering the '*sample*' input in the equation (3) as DMSO. The output is an $F_{Corrected\ DMSO}$ value. Consequently, the $F_{Corrected\ Sample}$ is set relative against the and average of the $F_{Corrected\ DMSO}$ values for the calculation of the fluorescence percentage, F_{Sample} (%) (Equation 4, (Ouyang, et al., 2017)).

Equation (3):

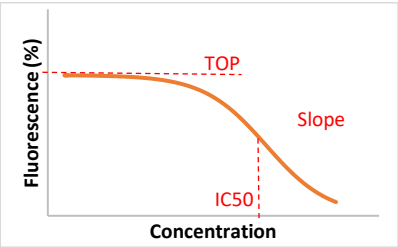
$$F_{Corrected\ Sample} = F_{Sample+TTR} - F_{Average\ Sample-TTR}$$

Equation (4):

$$F_{Sample}(\%) = \frac{F_{Corrected\ Sample}}{F_{Average\ Corrected\ DMSO}} \times 100$$

The major principle in toxicology is to establish a concentration-response relationship between exposure to a toxic substance and an observed effect. It is necessary to calculate the T_4-equivalence (µg/mL or µg/g) in the whole extract; the amount of T_4 (µg) necessary to exhibit the same TTR-binding activity measured from the TTR-binding toxicants, i.e. THDCs, in the extract. T_4 –equivalence is further clarified in this section. In this TTR-competitive binding assay, the measure of the concentration-response relationship is the inhibitory concentration 20% (IC20) (response). IC20 is chosen based on the study '*Miniaturization of a transthyretin binding assay using a fluorescent probe for high throughput screening of thyroid hormone disruption in environmental samples*', published by Ouyang (2017). The inhibitory concentration is a measure of a substance's potency in disrupting a specific biological or biochemical function. In the context of this study, the IC is the concentration of a THDC at which it inhibits the T_4 hormone to bind with the TTR protein.

The IC20 can be found using the equation (5) (IVM, BDS and Education Centre-VU, 2005) of the concentration-response curve. The logarithmical scaled x-axis is the concentration of an inhibitor, i.e. THDC, and the y-axis is the percentage of fluorescence (response). A high percentage of fluorescence suggests a low percentage of inhibition and vice versa. Therefore, at the lowest concentration of an inhibitor, the dose-response curve reaches a maximum fluorescence percentage, the '*Top*', where the inhibition percentage is zero. Thus, one or multiple factors in the test system become typically rate-limiting. In the middle of the curve there is a 'turning point', where the slope of the curve no longer increases with increasing concentration. Given the symmetrical shape of the curve, this turning point is exactly half-way the slope, in other words where the inhibition or the fluorescence is 50% of the maximum. The concentration that describes the location of this point is called the "50% inhibition concentration" or "half maximal inhibitory concentration (IC_{50})". This quantitative measure indicates how much of an inhibitor is needed to impede the biological TTR-binding with T_4 by half. Considering the fluorescence-inhibition pattern (IC(%)=1-F(%)), the inhibition of 20% (IC20) is at a fluorescence of 80%. The concentration-fluorescence response curve is shown in figure 7 together with its corresponding equation (5). Such a concentration-fluorescence response curve is to be obtained for each sample and T_4 solution that has been tested in various concentrations; a sample concentration-fluorescence response curve and a T_4 concentration-fluorescence response curve. For these concentration-fluorescence response curves, the *Top, IC50* and *Slope* parameters differ.



Equation (5):

$$y = \frac{Top}{1 + \left(\frac{x}{IC50}\right)^{Slope}}$$

Figure 7. *A concentration-fluorescence relationship presented in a sigmoidal curve. In the equation, 'y' is the relative fluorescence (%), the 'Top' describes the maximum fluorescence response of a sample, 'x' is the concentration of a sample, 'IC50' is the concentration where there is 50% inhibition, and the 'Slope' describes the change in the percentage inhibition responding as the concentration increases (IVM, BDS and Education Centre-VU, 2005).*

In order to obtain the correct Top, Slope and IC50 values, a SOLVER- algorithm is used in Excel. The SOLVER-algorithm uses least square regression to determine the parameter values that are of best fit. Briefly, random values are inserted for the Top, slope and IC50 input parameters; for the Top a value of approx. 100, for the slope a value of 1, and for the IC50 a concentration value at which the previously calculated $F_{sample}(\%)$ was around 50%. The x-value input is the concentration of a sample. Consequently, a *y* value is retrieved, considered as the 'predicted' fluorescence percentage, $F_{Predicted\ Sample}$ (%). Then, the positive Sum of Squares (SOS) value is calculated using the following equation (6) (CFI Education Inc, 2019).

Equation (6):
$$\mathrm{SOS} = [\mathrm{F_{Sample}}(\%) - \mathrm{F_{Predicted\ Sample}}(\%)]^2$$

An SOS value is retrieved for each *x* input. The SOS values are summed up to a total value 'SUM SOS'. Finally, the SOLVER- algorithm is used, instructed to generate the smallest SUM SOS value by adapting the Top, IC50 and Slope input values. As a result, the adequate fix parameters (Top, Slope and IC50) of equation (5) are found. For various concentration values (*x*-input), the inhibition percentage (*y*-input) can be calculated. In this manner, a sample concentration-fluorescence response curve and a T_4 concentration-fluorescence response curve is retrieved.

From these curves, **interpolation** allows the finding of the $IC20_{sample}$ and $IC20_{T4}$. Usually, when constructing a calibration curve, the y-axis is plotted corresponding to the known x-axis values. Interpolation is going the other way around, plotting from the y-axis to the unknown x-axis. For each sample, the fluorescence (%) measurements are filled out on the y-axis. In order to determine the corresponding concentrations (x-axis), equation (5) is rewritten as in equation (7) (IVM, BDS and Education Centre-VU, 2005). The concentration (x) at which the fluorescence intensity is of 80% (y= 80), the inhibition percentage is 20.

Equation (7):
$$\mathbf{x = [(\frac{Top}{y} - 1)^{\frac{1}{Slope}}] \times IC50}$$

The retrieved $IC20_{sample}$ and $IC20_{T4}$ values can be used to calculate the **T_4 – Equivalence (T_4-EQ)** in a sample. The T_4-equivalence gives information on the amount of toxicity present in the sample. The T_4-resembling compounds, i.e. THDCs, have the same ability to bind to TTR protein as the thyroxine hormone. T_4-Equivalence (T_4-EQ) describes the total amount of T_4 (µg) required in a sample for the thyroxine hormone to give off the equivalent TTR-binding activity measured from the THDCs in that same sample. For instance, 1mM of a T_4-resembling compound has same TTR- binding activity as 1mM of the T_4 hormone. The T_4- Equivalence, expressed in µg/mL or µg/g, is calculated as such (IVM, BDS and Education Centre-VU, 2005):

Equation (8):
$$\mathrm{EQ_{T4}} = \frac{IC20_{sample} \times IC20_{T4} \times F_{sample\ dilution} \times M_{T4} \times V_{extract\ sample\ in\ DMSO}}{Z_{total\ sample}} \times 100$$

In the equation, the *IC20* is the concentration value at which there is 20% inhibition, the $F_{sample\ dilution}$ is the dilution factor of the sample when pipetted in the bioassay (in this case the $F_{sample\ dilution}$ is equal to 100 since 2µL is pipetted into 200µL wells), the M_{T4} represents the molecular mass of the T_4 hormone (M_{T4} =776,87 g/mol), $V_{extract\ sample\ in\ DMSO}$ is the volume of the sample after transfer in DMSO after the extraction step, and the $Z_{total\ sample}$ is the initial total mass or volume of the sample.

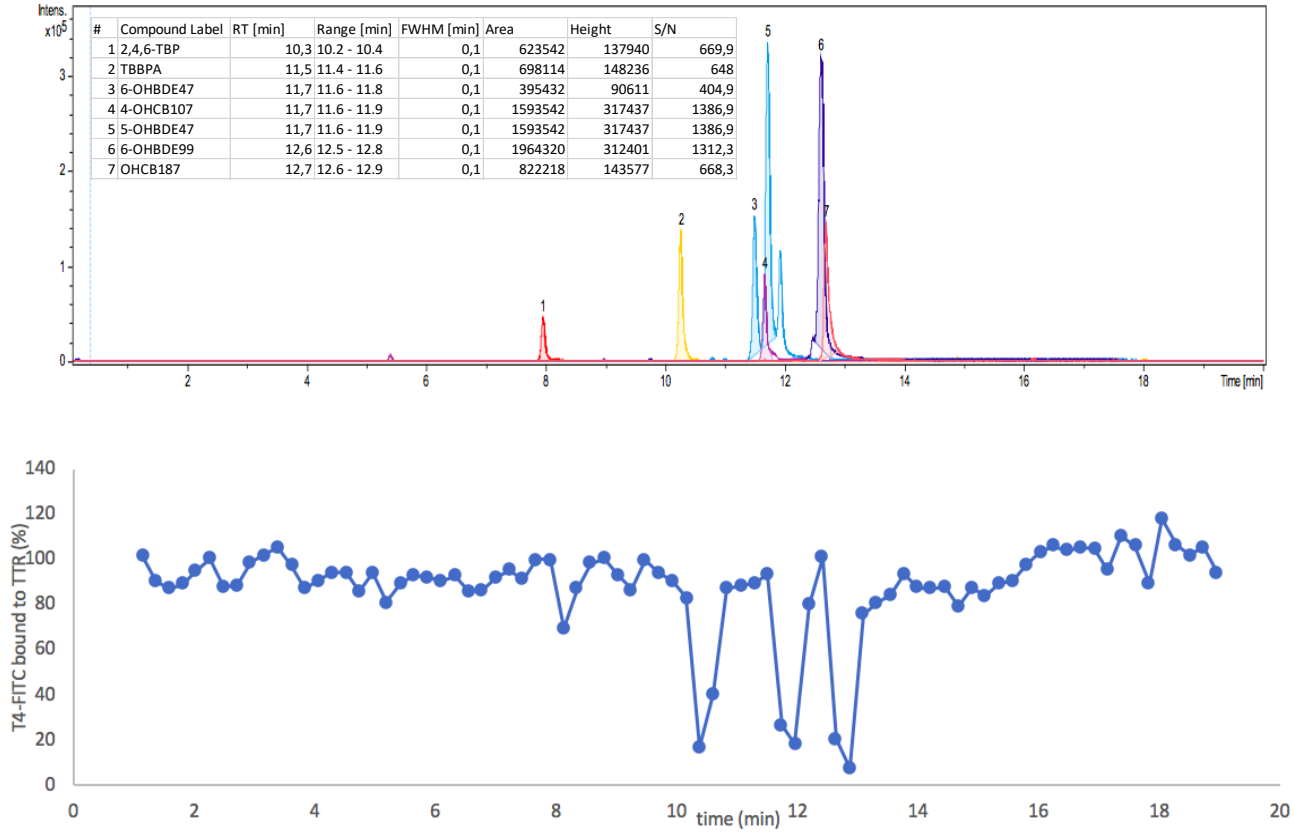
4. Results

The main objective of the present study is to determine whether the new blood sample preparation method, i.e. protein denaturation, developed by the HBM4EU project is suitable for EDA and how its efficacy compares to an existing (VU) sample preparation method that includes protein denaturation and SPE. Before performing the main objective, two experiments were conducted; an EDA Proof-of-Concept experiment using a THDC Standard Mixture (MiSSe, 2018) and an experiment on the suitability of an extraction procedure for the EDA method, applying SPE on an SRM2585 dust sample.

4.1 Proof-of-Concept: Effect-Directed analysis (EDA) method

The major components of the EDA method set to proof includes (1) liquid chromatography as a chemical tool for fractionation and for compound identification using mass spectrometry (Froment, 2017; HBM4EU, s.d.), (2) together with the chosen TTR-FITC-T_4 *in vitro* bio-assay in which the fractions are spotted. The result of interest in the EDA Proof-of-Concept experiment is the relationship between the chemical identification and the bio-active response (after fractionation) of the compounds from the chosen Standard Mixture (MiSSe, 2018). For the approval of the EDA procedure (Proof-of-Concept), this simple matrix mixture is directly injected in the LC-column for chemical analysis (LC-Q-ToF-MS) and fractionation in the TTR-FITC-T_4 bioassay plate, excluding a sample preparation step.

Since the most abundant fragment ions of the compounds are already known, the compounds were easily identified by looking at the retrieved chromatograms of those ions, using the Bruker software tool Compass Hystar (Bruker, 2019). The bio-assay response was then aligned to the retention times from the chemical analysis. The TTR-FITC-T_4 assay used was of good fit with a R^2 value of 0.9937 (see Appendix III, figure 24). In the TTR-FITC-T_4 assay, all compounds showed competitive TTR-binding activity at the same retention time intervals as the compounds found in the chromatograms. The chromatogram-bioassay relationship is shown in figure 8. This experiment was carried out four times. All four experiments provided results with the same chromatogram-bioassay relationship pattern (see Appendix III).



#	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	2,4,6-TBP	10,3	10.2 - 10.4	0,1	623542	137940	669,9
2	TBBPA	11,5	11.4 - 11.6	0,1	698114	148236	648
3	6-OHBDE47	11,7	11.6 - 11.8	0,1	395432	90611	404,9
4	4-OHCB107	11,7	11.6 - 11.9	0,1	1593542	317437	1386,9
5	5-OHBDE47	11,7	11.6 - 11.9	0,1	1593542	317437	1386,9
6	6-OHBDE99	12,6	12.5 - 12.8	0,1	1964320	312401	1312,3
7	OHCB187	12,7	12.6 - 12.9	0,1	822218	143577	668,3

Figure 8. *Chromatogram (compound peaks) and bioassay (compound biological activity) products of an SRM2585 extract. The peaks from both product can be aligned and determined to belong to the same THDCs in the Standard Mixture.*

4.2 Suitability of an extraction for the selected EDA method

In order to determine if any extraction method is suitable for the selected EDA method, solid-phase extration using Envicarb SPE cartridges was performed on a Standard Reference Material (SRM) 2585 dust sample before chemical analysis and bio-testing of the EDA procedure.

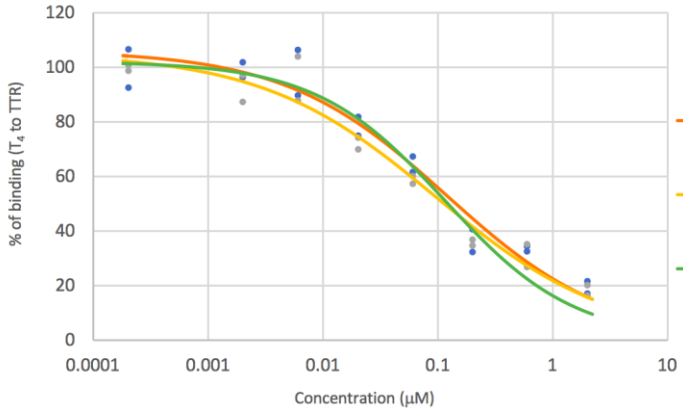
A total of eight extracts was obtained, i.e. seven SRM2585 extracts and one Procedure Blank (PB) extract. For each extract, the half reconstructed in DMSO (A) was directly subject to bio-testing in various dilutions (1x-3x-10x-30x). The other part resuspended in MilliQ (B) was injected in the LC-column for both chemical analysis and fractionation. The fractions were then collected, subject to bio analysis on the TTR-FITC-T_4 bioassay.

4.2.1 Toxicological recovery for Quantitative analysis

The seven extracts of the SRM2585 dust samples diluted in DMSO (A) were tested each on the bioassay in dilution series (1x-3x-10x-30x). A total of three bioassay plates were prepared for the seven SRM2585 dust extracts. Each plate also included T_4 standard solutions to provide a fluorescence-concentration relationship graph of the reference compound T_4.

A fluorescence-concentration relationship graph of the reference compound T_4 was plotted in Figure 8. Measurements on the T_4 calibration standards were performed three times, providing three calibration curves. The R^2 values obtained for all three calibration lines were 0.9567 (Cal 1), 0.9629 (Cal 2) and 0.9791 (Cal 3), close to a value of 1.0. Therefore, all three plates were of good fit.

The T_4 calibration curves are used to find the 20% inhibition concentration (IC20) of the T_4 calibrations standards. The IC20 is found at a FITC-T_4 TTR-binding potency of 80%. The three calibration curves gave IC20 values of 0.020µM, 0.013µM and 0.022µM, see figure 9.

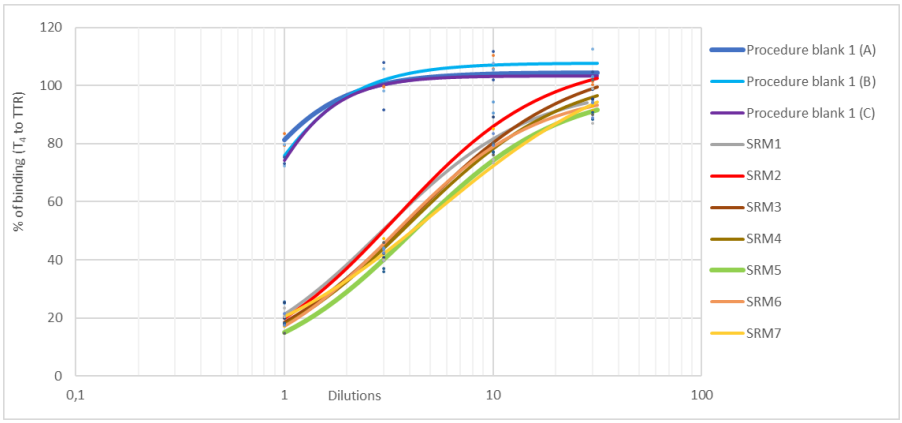


20% Inhibition of T_4

T_4 Calibration	IC20 T_4 (µM)
1	0.020
2	0.013
3	0.022

***Figure 9**. Fluorescence-concentration calibration curves of T_4. The logarithmically scaled x-axis shows the relative concentration (µM) of T_4. On the y-axis, the percentage of FITC-T_4 binding to TTR is indicated. At a fluorescence of 80% (y-axis) the IC20 (x-axis) of T_4 can be found. The 20% inhibition of T_4 from the three calibration curves is at concentrations of 0.020, 0.013 and 0.022µM. The calibration curves are also of good fit with squared correlation coefficients (R^2) of 0.9567, 0.9629 and 0.9791.*

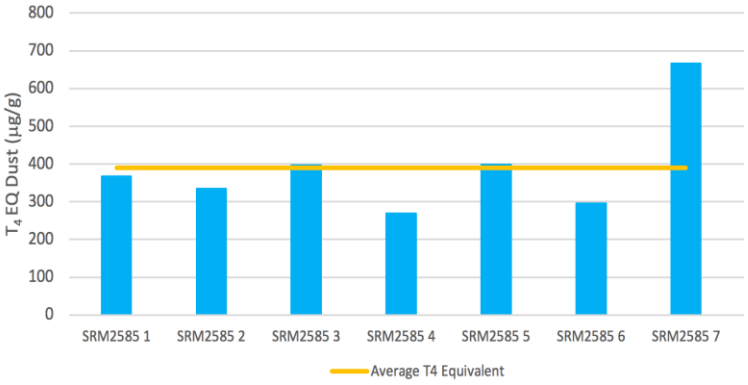
Furthermore, it was calculated that 20% inhibition (IC20) in the extracts SRM2585 1, SRM2585 2, SRM2585 3, SRM2585 4, SRM2585 5, SRM2585 6 and SRM2585 7 is obtained at dilutions of 10.25, 7.86, 9.87, 11.32, 13.97, 11.12 and 19.88, respectively (see figure 10). The toxicity is the same as a concentration of 0.020µM T_4 hormone in the SRM2585 1-2-3 samples, 0.013µM T_4 hormone in SRM2585 4-5-6 samples and 0.022µM T_4 hormone in SRM2585 7 sample.



20% Inhibition of the diluted SRM2585 samples	
Sample	IC20 (dilution)
SRM1	10.25
SRM2	7.86
SRM3	9.87
SRM4	11.32
SRM5	13.97
SRM6	11.12
SRM7	19.88

Figure 10. *Fluorescence-dilution calibration curves of SRM2585 samples and Procedure blank 1. The logarithmically scaled x-axis shows the relative dilutions. On the y-axis of the figure, the percentage of FITC-T_4 binding to TTR is indicated. At a fluorescence of 80% (y-axis) the IC20 (x-axis) of TTR-binding compounds in the extracts can be found. The 20% inhibition of the TTR-binding compounds is at dilutions of 10.25 (extract 1), 7.86 (extract 2), 9.87 (extract 3), 11.32 (extract 4), 13.97 (extract 5), 11.12 (extract 6) and 19.88 (extract 7).*

The IC20 values of the T_4 standards and the IC20 of the extracts are needed to estimate the T_4-equivalence (µg/g) in the SRM2585 dust extracts. From the interpolation results, it is calculated (equation (8) *section 3.6.5.2*) that the measured TTR-binding activity of unknown compounds per gram SRM2585 dust extract is equivalent to approximately 389.87µg T_4 hormone binding to TTR protein in a gram of that same dust extract. All seven SRM2585 extracts have comparable T_4- equivalence values (see figure 11). This indicates that the recoveries of the extracted TTR-binding compounds from the SRM2585 replicates are consistent.



Sample	T_4 -EQ Dust (µg/g)	Average T_4 -EQ of dust (µg/g)
SRM 1	368.19	
SRM 2	334.09	
SRM 3	396.50	
SRM 4	268.66	398.87
SRM 5	398.98	
SRM 6	295.55	
SRM 7	667.11	

Figure 11. *T_4 -Equivalence of the SRM2585 dust in the seven extracts (µg/g). The T_4-Equivalence average is of 398.87µg/g.*

4.2.2 Qualitative analysis

The seven SRM2585 dust sample extracts reconstructed in MilliQ water (B) were separated by the LC-column twice. During the first LC-separation, the eluting compounds from the SRM2585 sample extracts were first collected into a 96-well bioassay plate using a FractioMate. The plate was then bio-tested from which a TTR-binding potency spectrum of the fractions was obtained. The TTR-FITIC-T_4 bioassay was of good fit with a R^2 value of 0.9975 (see Appendix IV, figure 27). The SRM2585 sample extracts were also chemically analysed with mass spectrometry during the second LC-separation. The chromatogram from the chemical analysis can be then set against the bioassay spectrum. The recovery screening revealed that the SRM2585 dust sample includes compounds with a T_4 toxicological activity. Despite the finding of TTR binding compounds in the SRM2585 dust samples, the compounds could not be further identified. Nevertheless, the results obtained from both chemical analysis and bio-testing suggest that compounds that bind to the TTR can be extracted and found within the chromatograms and bioassay spectra. Around the retention times 7.44min and 15.8min, there are unknown compounds in the SRM2585 sample that have a TTR-binding potency. Such results could also be retrieved from the other six SRM2585 dust extracts, see Appendix IV.

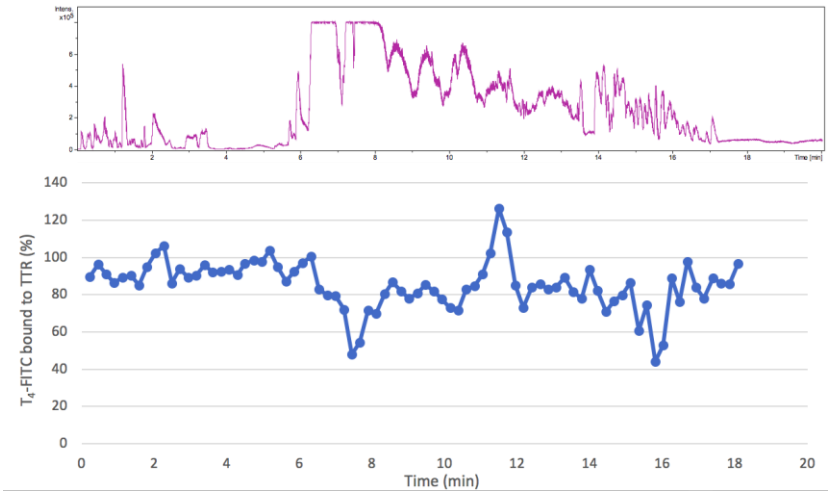


***Figure 12**. Chromatogram of a SRM2585 extracts with its corresponding biological activity spectrum, set over time (min).*

4.3 HBM4EU sample preparation

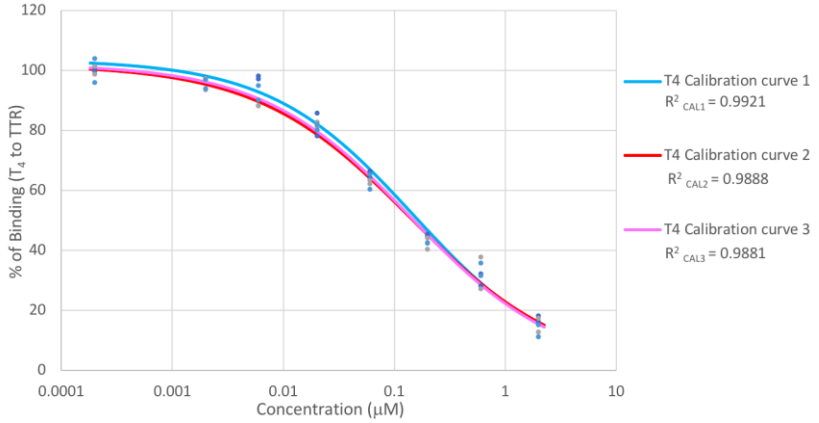
The extraction method consisted of a protein denaturation procedure of bovine serum. Before the HBM4EU procedure was applied, three samples were prepared in triplicate; a Procedure Blank MilliQ (PB1, PB2, PB3), a Serum Blank (non-spiked) (SB1, SB2, SB3), and a Spiked Serum solution (SS1, SS2, SS3) for which the Standard Mixture (MiSSe, 2018) was pipetted into the serum sample. The Procedure Blanks are only used as controls. Therefore, the results obtained on these solutions can be found in Appendix V and VI. The results obtained on the Spiked Serum and Serum Blanks are found within this section.

4.3.1 Quantitative analysis

The A-extract samples of the Spiked Serum (SS1, SS2, SS3) and Serum Blank (SB1, SB2, SB3) reconstructed in DMSO were subject to direct bio-testing after the extraction procedure. These were tested in multiple dilutions (1x-3x-10x-30x-60x-100x-300x-1000x-3000x), due to their expected high activity. Each Spiked Serum sample was tested in an individual bioassay plate with a Serum Blank; SS1 together with SB1 etc. (see Appendix XII). Therefore, three plates were prepared, wherein T_4 standards in various concentrations were also included, in order to retrieve a fluorescence-concentration graph of T_4.

A fluorescence-concentration relationship graph of the reference compound T_4 was plotted in figure 13. Measurements on the T_4 calibration standards were performed three times, providing three calibration curves. The R^2 values obtained for all three calibration lines were 0.9921 (Cal 1), 0.9888 (Cal 2) and 0.9881 (Cal 3). The bioassay is estimated of good fit.

The calibration curves of the reference compound T_4 were fitted to find the 20% inhibition concentration (IC20) of the T_4 calibrations standards. The IC20 is found at a fluorescence of 80%. The three calibration curves gave IC20 values of 0.024µM, 0.018µM, 0.020 and 0.019µM, see figure 13.

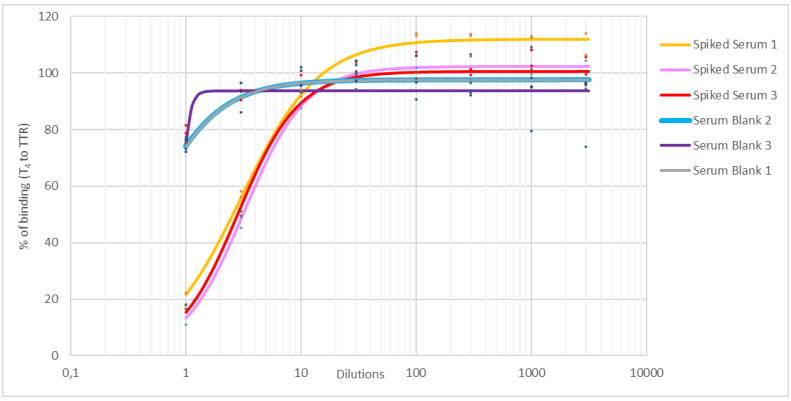


20% Inhibition of T_4

T_4 Calibration	IC20 T_4 (µM)
1	0.024
2	0.018
3	0.019

***Figure 13.** Fluorescence-concentration calibration curves of T_4. The logarithmically scaled x-axis shows the relative concentration (µM) of T_4. On the y-axis of the figure, the percentage of binding of FITC-T4 to TTR is indicated.*

Interpolation allowed the finding of the IC20 of the Spiked Serum and Serum Blank samples. The more diluted sample caused a response in the 20% inhibition window, see figure 14. 20% inhibition (IC20) of T_4-equivalent toxic activity in the three spiked serum samples is obtained at dilutions of 6.16, 6.56 and 6.46. The toxicity is the same as in concentrations of 0.024μM, 0.018μM and 0.019μM T_4 hormone in the spiked serum 1-2-3, respectively. The activity of the Serum Blanks is considered too low to make a reliable fit of the fluorescence-concentration curve. However, some activity is observed at the 1x dilution.

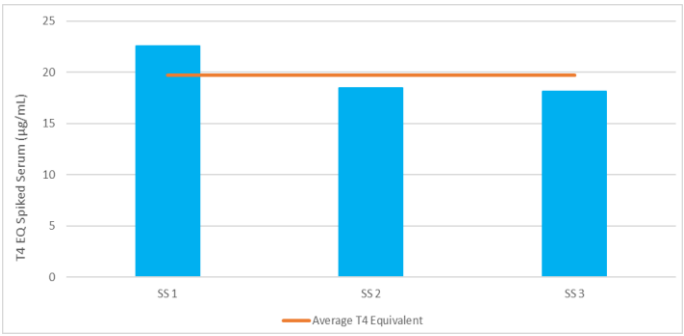


20% Inhibition of the diluted serum extracts

Sample	IC20 (dilution)
SS 1	6.16
SS 2	6.56
SS 3	6.46

***Figure 14**. Fluorescence-concentration graph relationship of the Spiked Serum (SS) and Serum Blank (SB) samples. IC20 was found to be at 6.16, 6.56 and 6.46 dilutions of SS1, SS2, SS3, respectively.*

After interpolation, the IC20 values of the T_4 standards and the IC20 of the extracts are required to calculate with equation (8) of *section 3.6.5.2* the T_4 equivalence (μg/mL) in the Spiked Serum extracts. The TTR- binding potencies of the Spiked Serum were determined by taking the dilution of the extract and the volume of the serum extracted into account. It is calculated that the TTR-binding activity of the spiked THDCs per millilitre serum extract is equivalent to approximately 19.72μg T_4 hormone (binding to TTR) in a millilitre serum extract. All Spiked Serum extracts have analogous T_4- equivalence (see figure 15). In other words, the spiked THDCs in the serum samples were recovered steadily between the triplicates, after the extraction.



Spiked serum Sample	T_4-Eq spiked serum (μg/mL)	Average T_4-EQ of spiked serum(μg/mL)
SS 1	22.60	
SS 2	18.47	19.72
SS 3	18.11	

***Figure 15.** T_4-equivalence in the Spiked Serum from the HBM4EU extracts. The T_4-equivalent average is of 19.72μg/mL.*

4.3.2 Qualitative analysis

The Spiked Serum extracts (B) diluted in MilliQ were separated by the LC-column twice. During the first LC-separation, the eluting compounds from the extracts were collected into a 96-well bioassay plate using a FractioMate. The plate was then bio-tested from which a TTR-binding potency spectrum of the fractions was obtained. Additionally, the bioassay was estimated a good fit with an R^2 value of 0.9945 (see Appendix VI, figure 39). During the second LC-separation, the Spiked Serum extracts were chemically analysed with mass spectrometry. The chromatogram (Compass Hystar Bruker Software) from the chemical analysis was then aligned to the bioassay spectrum. The protein denaturation method successfully extracted the seven spiked THDCs in the serum samples as their activities could be measured in the TTR-FITC-T_4 competitive binding assay and these could be identified in the chromatograms. Figure 16 shows the spectrum and chromatogram of an SS sample. Comparable bioassay spectra and chromatograms were also retrieved for the other two Spiked Serum extracts (see Appendix VI). The recovery screening in the chromatograms reveal that the spiked compounds eluted mainly in the retention time interval of 7 to 14 minutes.

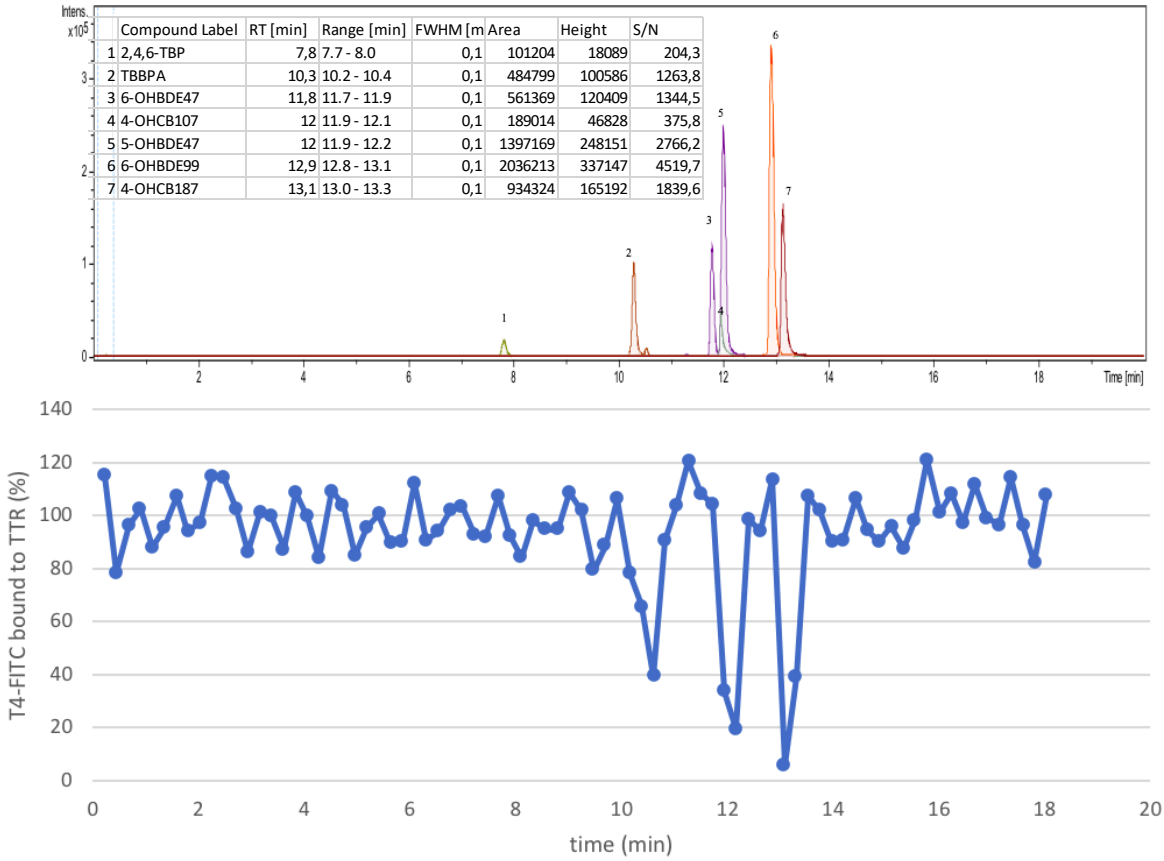


Figure 16. *Chromatogram of the Spike Serum extracts (HBM4EU sample preparation) with its corresponding biological activity spectrum, set over time (min).*

4.4 VU sample preparation

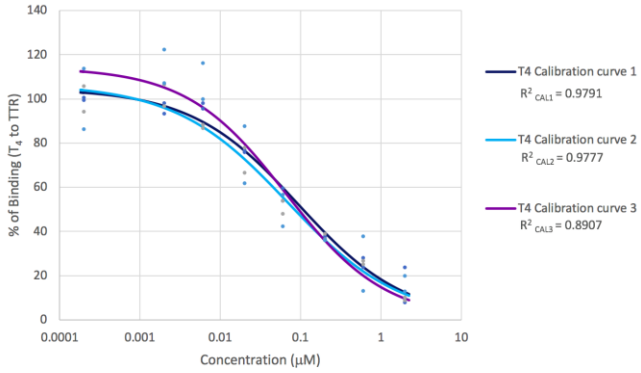
A Sample preparation was performed according to the method developed by the Vrije Universiteit Amsterdam (VU). The method consisted of a protein denaturation procedure and a solid-phase extraction of toxicants from bovine serum. Before the VU method was carried, three samples were prepared in triplicate as in the HBM4EU method; a Procedure Blank MilliQ (PB1, PB2, PB3), a Serum Blank (non-spiked) (SB1, SB2, SB3), and a Spiked Serum solution (SS1, SS2, SS3) for which the Standard Mixture (MiSSe, 2018) was pipetted into the serum sample. The Procedure Blanks are only used as controls. Therefore, the results obtained on these solutions can be found in Appendix VII and VIII. The results retrieved on the Spiked Serum and Serum Blanks are found within this section.

4.4.1 Quantitative analysis

After the application of the VU extraction method, the Spiked Serum and Serum Blank extracts (A) were subject to direct bio-testing. These were tested in more dilutions (1x-3x-10x-30x-60x-100x-300x-1000x-3000x), due to their expected high activity. Each Spiked Serum sample was tested in an individual bioassay plate with a Serum Blank; SS1 together with SB1 etc. (see Appendix XII). Therefore, three plates were prepared, wherein T_4 standards in various concentrations were also included, in order to retrieve a fluorescence-concentration graph of T_4.

A fluorescence-concentration relationship graph of the reference compound T_4 was plotted in Figure 10. Measurements on the T_4 calibration standards were performed three times, providing three calibration curves. The R^2 values obtained for all four calibration lines were 0.9791 (Cal 1), 0.9777 (Cal 2) and 0.8907 (Cal 3). Considering the irregular experimental conditions of the bioassay plates, the R^2 values are considered of good fit.

The T_4 calibration curves were used to find the 20% inhibition concentration (IC20) of the T_4 calibrations standards. The IC20 is found at a TTR-binding potency of 80%. The four calibration curves gave IC20 values of 0.015µM, 0.012µM, and 0.020µM, see figure 17.

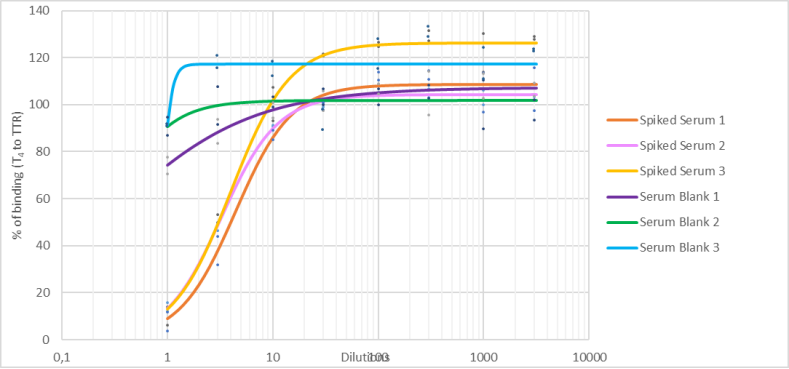


20% Inhibition of T_4

T_4 Calibration	IC20 T_4 (µM)
1	0.015
2	0.012
3	0.020

***Figure 17**. Fluorescence-concentration calibration curves of T_4, with its corresponding table. The logarithmically scaled x-axis shows the relative concentration (μM) of T_4. On the y-axis of the figure, the percentage of binding of FITC-T_4 to TTR is indicated.*

Interpolation allowed the finding of the IC20 of the Spiked Serum and Serum Blank samples. The more diluted sample caused a response in the 20% inhibition window, see figure 18. 20% inhibition (IC20) of T_4-equivalent toxic activity in the three spiked serum samples is obtained at dilutions of 8.30, 6.74 and 5.66. The toxicity is the same as in concentrations of 0.015µM, 0.012µM and 0.020µM T_4 hormone in the Spiked Serum 1-2-3, respectively. The activity of the Serum Blanks is considered too low to make a reliable fit of the fluorescence-concentration curve. However, some activity is observed at the 1x dilution.

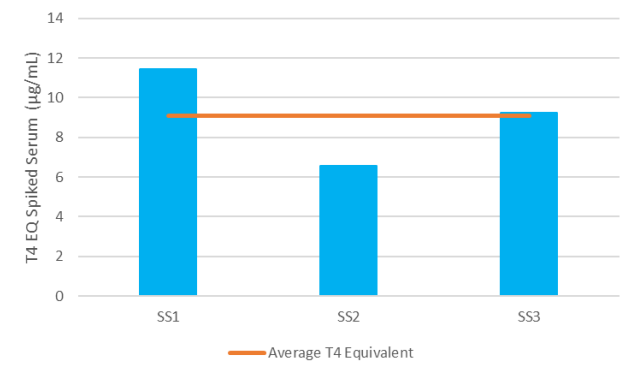


20% Inhibition of the diluted serum samples

Sample	IC20 (dilution)
SS 1	8.30
SS 2	6.74
SS 3	5.66

***Figure 18.** Fluorescence-concentration graph relationship of the Spiked Serum (SS) together with Serum Blank (SB) samples, tested by the miniaturized TTR-FITC-T_4 binding assay. IC20 was found to be at 8.30, 6.74 and 5.66 dilutions of SS1, SS2, SS3, respectively.*

The IC20 values of the T_4 standards and of the extracts are used to calculate the T_4 equivalence (µg/g) in the Spiked Serum extracts, by adopting the equation (8) of *section 3.6.5.2*. The TTR- binding potencies of the Spiked Serum were determined by taking the dilution of the extract and the volume of the serum extracted into account. It is calculated approximately 9.08µg T_4 hormone (binding to TTR) per millilitre serum extract is necessary to emit the same TTR-binding activity of spiked THDCs in a millilitre serum. All spiked serum samples have similar T_4- equivalence (see figure 19).

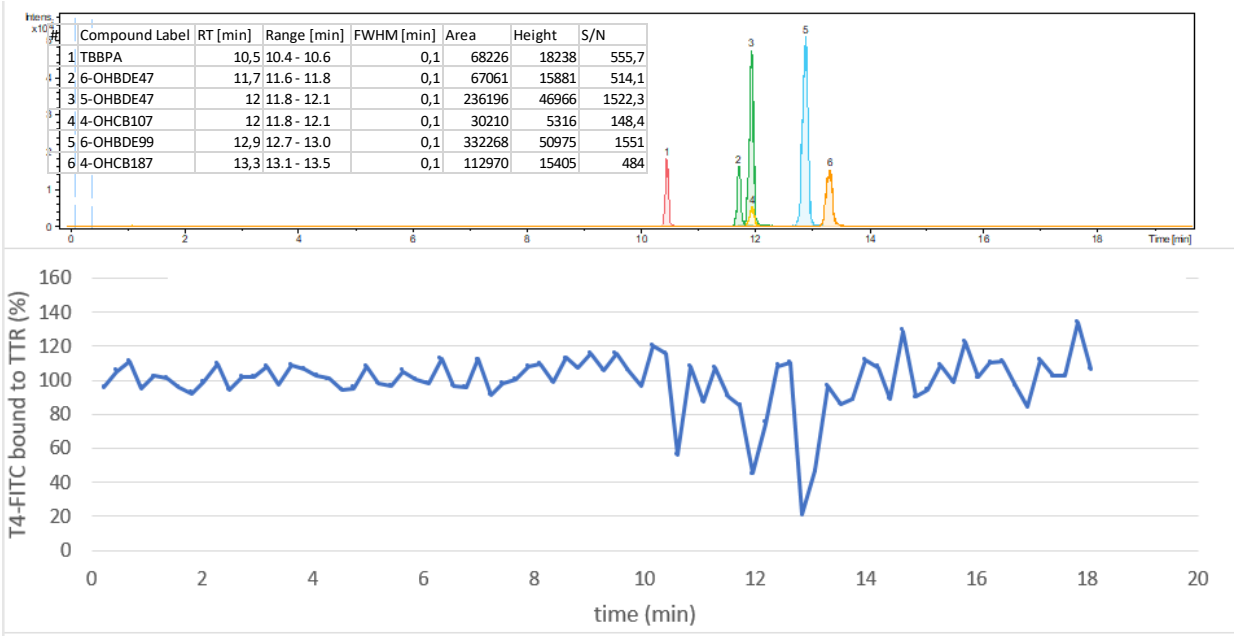


Spiked serum Sample	T_4-EQ spiked serum (µg/mL)	Average T_4-EQ of spiked serum(µg/mL)
SS 1	11.45	
SS 2	6.55	9.08
SS 3	9.25	

***Figure 19.** T_4 -equivalence in the Spiked Serum from the VU extracts. The T_4 equivalent average is of 9.08 µg/mL.*

4.4.2 Qualitative analysis

The B-extracts (Spiked Serum extracts) transferred in MilliQ were separated by the LC-column twice. During the first LC-separation, the eluting compounds from the extracts were first collected into a 96-well bioassay plate using a fraction collector. The TTR-FITC-T_4 bioassay was tested to be of good fit (R^2 of 0.9867 Appendix VIII, figure 49). A TTR-binding activity spectrum of the fractions in the plate was obtained. The biological activities of the spiked THDCs in the serum samples could successfully be assessed in the TTR-FITC-T_4 competitive binding assay. During the second LC-separation, the seven THDCs in the Spiked Serum extracts were tentatively identified with mass spectrometry. The identification strategy was established by looking for compound matches from compiled mass libraries. The chromatograms retrieved from the Bruker Compass Hystar Software were then set aligned to the bioassay spectrum. Figure 20 shows the spectra and chromatogram of one Spiked Serum sample. Such bioassay and chemical recoveries were also obtained for the other two Spiked Serum extracts (see Appendix VIII). The recovery screening in the chromatograms reveal that the spiked compounds eluted mainly in the retention time range of 10 to 14 minutes. The protein denaturation and solid- phase extraction method could successfully extract the biologically active compounds present in the Spiked Serum samples.



#	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	TBBPA	10,5	10.4 - 10.6	0,1	68226	18238	555,7
2	6-OHBDE47	11,7	11.6 - 11.8	0,1	67061	15881	514,1
3	5-OHBDE47	12	11.8 - 12.1	0,1	236196	46966	1522,3
4	4-OHCB107	12	11.8 - 12.1	0,1	30210	5316	148,4
5	6-OHBDE99	12,9	12.7 - 13.0	0,1	332268	50975	1551
6	4-OHCB187	13,3	13.1 - 13.5	0,1	112970	15405	484

Figure 20. *Chromatogram of a spiked serum extract (VU sample preparation) with its corresponding biological activity spectrum, set over time (min).*

4.5 T_4- equivalence in the Spiked Serum

The average T_4-equivalence in the Spiked Serum was 19.72µg/mL (±2.49 SD) after a protein denaturation (HBM4EU). The protein denaturation combined with a solid-phase extraction from the VU method suggested that 9.08µg/mL (±1.28 SD) T_4 hormone is required to mirror the TTR-binding activity of the recovered THDCs in the Spiked Serum extracts. The difference of in T_4-equivalence is of approximately one half. This means that the recovered THDCs ratio from the two methods is of approximately one half, see figure 21.

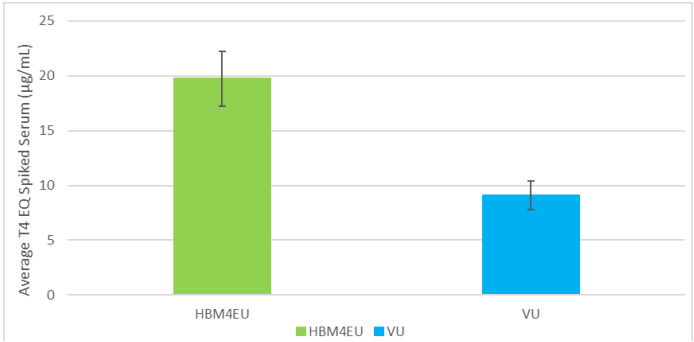


Figure 21. *Average amount (µg) of T_4 hormone required per mL Spiked Serum extract to exhibit the same TTR-binding activity as the THDCs in the SS extracts. The extracts were obtained from the HBM4EU and the VU sample preparation method.*

5. Discussion

The aim of this research is to determine whether the new HBM4EU serum sample preparation method is suitable for EDA and how its efficacy compares to an existing (VU) sample preparation method. The first step was to perform a Proof-of-Concept experiment of the selected EDA method that includes LC-Q-ToF-MS, LC-fractionation and TTR-FITC-T_4 bioassay. This experiment took advantage of a Standard Mixture (MiSSe, 2018) of seven Thyroid Hormone Disrupting Compounds (THDCs). The second experiment was to determine if an extraction step was suitable for the EDA method. Therefore, a Solid Phase Extraction (SPE) method was carried on Standard Reference material (SRM) 2585 dust sample for the preparation step, followed by the EDA procedure. Once these two experiments gave positive results on the functionality of the EDA combined with an extraction method, the main objective of the present study was performed; two complete EDA procedures on serum samples was performed to identify the spiked biologically active Thyroid Hormone Disrupting Compounds (THDCs). The HBM4EU sample preparation method was applied for one EDA procedure and the VU sample preparation method for the other. The two serum sample preparation methods were then evaluated against each other and for EDA suitability and efficacy by a (TTR-FITC-T_4) bioassay and LC-Q-ToF-MS.

5.1 EDA Proof-of-Concept: Standard Mixture (MiSSe, 2018)

The Proof-of-Concept experiment indicated that the selected EDA method is functional for the target toxicants, i.e. THDCs. EDA analysis was performed on a Standard Mixture (MiSSe, 2018) containing the seven THDCs, i.e. TBBPA, 2,4,6-TBP, 5-OHBDE47, 6-OHBDE47, 6-OHBDE99, 4-OHCB107 and 4-OHCB187. All seven compounds were found within the bioassay spectra and identified in chemical analysis. The bioassay recoveries of the THDCs in the Standard Mixture correlate with the chemical recoveries from the mass spectrometry analysis. The EDA method applied is also determined to be reproducible, as the experiment was performed four times and similar chromatograms, as well as bioassay spectra were retrieved.

Nonetheless, two individual peaks were expected to be found in the chromatograms for the compounds that have a most abundant M-H monoisotopic mass of 500.6977 (m/z), i.e. 5-OHBDE47 and 6-OHBDE47. Despite, it is noticeable that three peaks were retrieved for this specific M-H monoisotopic mass. An explanation for this extra peak is based on a certain amount of DMSO solvent present in the Standard Mixture (MiSSe, 2018). Briefly, the Standard Mixture is prepared from individual stock solutions of the seven THDCs. The THDCs are diluted in DMSO in their stock solution. Therefore, when pipetting the compounds into the Standard Mixture matrix, i.e. MilliQ:MeOH, the stock solution's DMSO solvent is also simultaneously added. Accordingly, it is expected that the Standard Mixture has a matrix of approximately 50%MilliQ, 10%MeOH and 40%DMSO. When starting the LC-run, the gradient should be relatively the same as the samples' matrix. In this study, the initial gradient in the LC-column is of 90%MilliQ, which is far from being similar to the solvent matrix of the Standard Mixture. Such deviance can be the cause for a compound to have multiple peaks. In this study, it was the case for 5-OHBDE47.

With regard to the chemical analysis, the chromatograms retrieved show a good resolution and separation of the peaks. Nevertheless, it is apparent that 6-OHBDE99 (peak 6) and OHCB187 (peak 7) co-elute in the bioassay spectrum. A solution for this is the fractionation with a higher resolution. In other words, the spotting time interval into one bioassay well should have a shorter duration than 13.5 seconds. Thus, a better separation of the eluting compounds is obtained in the bioassay plate.

5.2 Extraction suitability for EDA using Standard Reference Material 2585 Dust

The second experiment was to determine if an extraction step was suitable for the selected EDA method. A solid-phase extraction method was carried out on Standard Reference Material (SRM) 2585 dust sample. Dust was selected as it is a relevant indoor exposure matrix. The indoor environment exposure to contaminants can be as much as 1000 times higher compared to the outdoor environment, by reason of a relatively longer residence time, poorer ventilation and slower degradation of contaminants (Ouyang, et al., 2017). Additionally, babies are 100-fold more vulnerable to health risks from contaminants in house dust than adults (JW, et al., 2009). Dust in households may allow uptake of toxicants for humans, notably via dermal route, ingestion, inhalation, and the aggregate exposure, and may reach human tissues such as blood (Plaßmann, Brack, & Krauss, 2014). Therefore, performing EDA studies on dust is of great relevance for human biomonitoring.

The SRM 2585 dust includes various toxic compounds, namely33 PAHs, 30 PCB congeners, 4 chlorinated pesticides, and 15 PBDE congeners (see Appendix XVI). The applied extraction method on SRM2585 was already validated in another research (Ouyang, et al., 2017) and is commonly used. The results from this study show that there is a clear TTR-binding effect in the seven SRM2585 dust replicates. Overall, the method is robust and reproducible for EDA since there is not much difference between the biological testing outcomes of the SRM2585 replicates, see figure 11 and Appendix IV. Compounds from the SRM2585 dust extracts could also be separated using the LC-Q-ToF-MS. However, identification of the compound peaks was not performed, as it fell outside the scope of this study.

Nevertheless, it has to be considered that there may be an effect in the direct bioassay measurements of the diluted (1x-3x-10x) procedure blank and the seven SRM2585 whole extracts. An interpretation to this hypothesis is related to the possibility that the organic solvent ACN did not completely evaporate under the nitrogen flow after the extraction. Organic solvent precipitates proteins (Arakawaa, Kitab, Shirakic, & Ohtaked, 2011) and, therefore, also TTR. Correspondingly, there may be less TTR protein in the bioassay wells, leading to less potency of TTR-binding activity with T_4. As a result, a lower signal is retrieved from the TTR-FITC-T_4 bioassay measurements of the whole extracts. Such an effect, i.e. an ACN solvent influence, does not appear in the bio-active response after fractionation, as the fractions in the wells are thenceforth set to complete evaporation using a CentriVap concentrator (see *section 3.5.1*). A potential solution to the ACN effect in the bioassay of the diluted extracts is to do an additional evaporation step after pipetting 0.4mL DMSO into the 0.2mL extract (diluted in ACN). The 0.6mL extract mixture can be evaporated back to 0.4mL. After this evaporation step, the certainty that only DMSO is the residual in the extract is higher, as ACN evaporates faster than DMSO.

On the other hand, upward peaks were found in the bioassay spectra of the SRM2585 extracts after fractionation (see Appendix IV). These peaks are artefacts, as in this case the measured fluorescence intensity of the blank is smaller than the fluorescence value of the extracts. These peaks appear mostly towards the end of the bioassay spectra, which indicates that the fluorescence measurements from the wells in the last row of the bioassay plate gave this peak effect. An explanation may be with regard to the gradient mobile phase spotted in the bioassay plate. Over the fractionation period, the solvent gradient increased to 99% mobile phase B (100%ACN). Therefore, the last row of the bioassay plate consists mostly of ACN solvent. After fractionation, plates were stored overnight before the evaporation step. It is possible that the solvent dissolved the polystyrene plate, pulling particles out that could interfere with the bio-active response.

Another phenomenon was visible in the bioassay spectra of the procedure blank after fractionation. That is to say, a regular pattern of downward peaks was perceived (see Appendix IV, figure 34). An interpretation to the outcome of these peaks may be with regard to the manner of pipetting TTR into the bioassay wells, when preparing the plate for fluorescence measurement. The TTR protein working solution (TTR diluted in Tris-buffer) was pipetted in the wells per row (from right to left) using an automatic pipette. After one row, the automatic pipette was refilled with the adequate amount of TTR working solution to fill the next row of wells. Before reloading the automatic pipette, the TTR working solution was whirl-mixed for homogenization. However, it can be speculated that the TTR protein was heavy enough to accumulate at the tip of the automatic pipette. As a result, TTR was pipetted in the first wells of the row, yet, Tris-buffer was predominantly pipetted towards the end of the row. As a result, there may be less TTR protein in the bioassay wells in the last columns of the plate, leading to less TTR-binding activity in this plate segment. This is visible with a lower fluorescence signal. Such a pattern was perceived less after a change in the pipetting pattern (see Appendix IV, figure 35).

Looking at the chromatograms of the SRM2585 extracts, there is plateau forming between retention times 6 and 8 minutes (see figure 12). This is the cause of overloading the detector with the analytes of interest. As a result, there are missing peaks. Plateau forming prohibits identification and quantification of the analytes. In order to avoid such outcome, the extract should be further diluted.

Furthermore, identification of the compounds was not performed in this study. In spite of that, identification can be carried out using the Metaboscape Software (MetaboScape Bruker , 2019). The software executes automatic deconvolution based on a regression and correlation algorithm. This allows the suspect screening of compounds at very close retention times based on the monoisotopic (m/z) and isotopic distribution. In order to verify, an LC- run and TTR-FITC-T_4 bioassay should be performed for the individual compounds.

Moreover, performing an EDA experiment on the metabolites of the compounds in SRM2585 dust would be of interest. The reasoning is based on the fact that most compounds are metabolized within the human body and phase 1 metabolites have a higher TTR-binding activity (Gutleb, et al., 2010). An S9 fraction experiment (Jia & Liu, 2007) could be performed to metabolize compounds, in order to evaluate if there is indeed a stronger toxic activity in the bioassay and, also, to investigate if there are more peaks found in the chromatograms. This would show a good representation of toxicity realistic conditions.

5.3 Suitability of HBM4EU sample preparation method for EDA analysis

The HBM4EU sample preparation methods could successfully extract the biologically active compounds present in the Spiked Serum samples. However, the THDCs could not be recovered simultaneously in both EDA products: bioassay spectra and chromarograms. With regard to the EDA products after performing the HBM4EU sample preparation method, all compound peaks were found in the chromatograms. However, the TTR-binding activity of 2,4,6-TBP could not be recovered in the bioassay. Therefore, the chromatogram peak of 2,4,6-TBP could not be correlated to the bioassay spectrum. Nevertheless, the chemical recoveries in combination with the bioassay recoveries of the various TH- disruptors from the extracts, demonstrate the suitability of the developed HBM4EU serum sample preparation method for an EDA study.

5.4 Efficacy evaluation: comparison HBM4EU and VU sample preparation method

Both HBM4EU and VU sample preparation methods are robust since the fluorescence-concentration curves and the T4-EQ values are precise and akin between replicates.

Yet, it is noticeable from both HBM4EU and VU sample preparation methods that the fluorescence-concentration curves of the Serum Blank tail down-wards at lower dilutions levels, indicating an effect (see figures 14 and 18). Matrix effect, i.e. lipids and endogenous hormones, may be a justification for such a pattern. From a paper in the Chemosphere Journal (Ouyang, et al., 2017), it is determined that lipids have a solid effect on signal in the bioassay. This matrix effect is almost inevitable at high serum concentrations. This effect pattern was more expected when performing the HBM4EU sample preparation, since the method does not include a clean-up step as in the SPE procedure from the VU method. Nevertheless, both methods have a lower efficacy for high concentrations of serum samples. There is a higher chance of ion suppression in chemical analysis, impeding quantification. The limit of detection (LOD) also becomes higher, which is not favourable.

Despite, all spiked compounds were recovered and identified within the EDA products, i.e. chromatograms and bioassay spectra, after applying the HBM4EU sample preparation method. In contrast, this was not the case when performing the VU sample preparation method. With regard to the chemical analysis of the extracts, a compound was lost, namely 2,4,6-TBP, after the SPE extraction of the VU sample preparation method. This is perceived from the bioassay spectra and chromatograms. Contrary to the HBM4EU sample preparation method, it has to be considered that the VU sample preparation method includes a clean-up step, part of the SPE procedure, which introduces additional sample loss, resulting to compound loss. The loss of 2,4,6-TBP may also be the consequence of a turbid solution obtained after the solid-phase extraction. Therefore, an extra filtration step was required by centrifuging the extracts in filtration-vials. These filtration vials separated the cloudy material from the extracts. Nevertheless, such an additional step increased the likelihood of loss of compounds. Therefore, the HBM4EU has a higher efficacy than the VU method with regard to compound-loss probability.

Additionally, peak heights in the chromatograms of the HBM4EU extracts vary between values of approximately 2×10^4 and 3×10^5. Regarding the chemical analysis of the VU extracts, peak heights fell in the range of approximately 3×10^3 to 5×10^4. The peak height difference between the two methods is of a factor 10. Therefore, it can be determined that the HBM4EU has a higher efficacy in terms of chemical recovery.

Furthermore, the sample preparation method developed by HBM4EU has a higher efficacy than the method from the VU with regard to the recovered toxicological compounds from the extracts, expressed in T_4-equivalence. The resulting Spiked Serum (SS) extracts from the HBM4EU method have an average T_4-equivalence of 19.72μg/mL. This value is almost double as much as the average T_4-equivalence 9.08μg/mL in the (VU) SS extracts. Since the T_4-EQ represents the amount of T_4 hormone in a sample (μg/mL), it can be

elaborated that the HBM4EU SS-extracts would include twice as much T_4 hormones as found in the VU SS-extracts, suggesting twice as much TTR-binding activity (in the HBM4EU SS-extracts). This TTR-binding activity of the T_4 hormones reflects the concrete TTR-binding activity in the SS-extracts, which is the activity of the THDCs. Therefore, it can be determined that the HBM4EU extraction recovered approximately twice as much THDCs in the SS-extracts than the VU method (ratio 2:1) (see figure 21). In other words, the protein denaturation of the HBM4EU sample preparation method extracted more spiked THDCs from the serum samples than the VU sample preparation did, combining protein denaturation with a solid-phase extraction method. In this regard, the HBM4EU sample preparation method is twofold more effective than the VU sample preparation method.

5.5 Prioritization

There is no sufficient experimental proof to provide a solid statistical conclusion on the suitability and efficacy level of the HBM4EU sample preparation method for Effect-direct Analysis. This study provides only an *indication* for these two peculiarities. Despite, this study provides the option of prioritization in the selection of the sample preparation method for EDA studies; it gives an indication that the HBM4EU sample preparation is suitable and more effective than the VU sample preparation method. Consequently, this study can be used to prioritize the HBM4EU sample preparation method to be further tested statistically for reliability (only three spiked serum samples were tested in this study) and reproducibility, to be compared to other sample preparation methods and, perhaps, to be applied in EDA studies for human biomonitoring purposes.

Conclusion

It can be concluded that the HBM4EU serum sample preparation method is suitable for Effect-Directed Analysis. The method allows a recovery of all bioactive compounds of interest from serum in the EDA products, with little loss of compounds: bioassay spectra and chromatograms. Nevertheless, the HBM4EU sample preparation method is suitable to the extent of using low serum concentrations, especially when quantification is part of the chemical analysis. Thus, to avoid ion suppression and a high limit of detection (LOD), which are unfavourable. In parallel, the VU sample preparation method also requires low serum concentrations to avoid a matrix effect.

In comparison to an existing sample preparation method of the Vrije Universiteit Amsterdam, it can be concluded that the HBM4EU method has an overall higher efficacy. The HBM4EU method is two times more effective than the VU method with regard to THDC recoveries in the whole extracts, in terms of T_4-equivalence. In this study, the average T_4-equivalence using the new sample preparation method was calculated to be of 19.72μg/g. In contrast, the average T_4-equivalence obtained from the VU sample preparation method was 9.08μg/g. The ratio of the recovered THDCs is 2:1 between the methods (HBM4EU:VU). Concerning peak height in chemical analysis, the efficacy of the HBM4EU method could also be qualitatively determined superior than the VU method, with a difference factor of 10. After applying the HBM4EU method, peak heights in the chromatograms vary between values of approximately 2×10^4 and 3×10^5. The chromatograms from the chemical analysis after performing the VU sample preparation method gave peak heights in the range of approximately 3×10^3 to 5×10^4. Furthermore, the HBM4EU method is more effective than the VU method with regard to the compound-loss. The VU sample preparation is more vulnerable to loss of compounds, since the method involves more steps. This susceptibility was observed in this study. The HBM4EU sample preparation method recovered all THDCs in the chemical analysis. In other words, all compounds were identified in the chromatograms. Contrary to the HBM4EU sample preparation method, 2,4,6-TBP was lost in the VU sample preparation, as it was not identified in the chromatograms.

Finally, this study provides an indication on the suitability and efficacy of the newly sample preparation method developed by the European HBM4EU initiative for Effect-directed Analysis. Therefore, the method should be further tested for statistical reliability and reproducibility, in order to ascertain its efficacy with regard to other sample preparation methods. Consequently, EDA studies could be performed applying the HBM4EU sample preparation method for human biomonitoring purposes.

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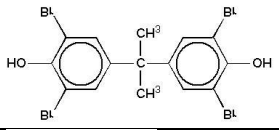
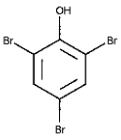
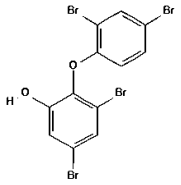
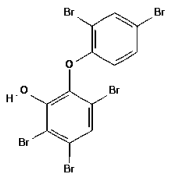
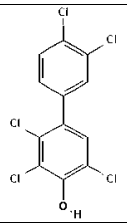
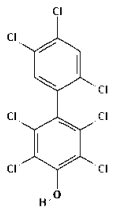
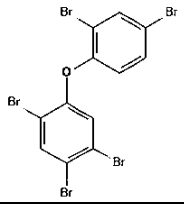
Appendices

Appendix I

Structure of 5 brominated flame retardants and 2 PCB metabolites.

CAS.no	Compound	IUPAC	Structure	Source (ECHA and PubChem)
79-94-7	TBBPA	2,2',6,6',-tetrabromo-4,4'-isopropylidenediphenol		https://echa.europa.eu/registration-dossier/-/registered-dossier/14760
118-79-6	2,4,6-TBP	2,4,6-Tribromophenol		https://echa.europa.eu/substance-information/-/substanceinfo/100.003.890
79755-43-4	5-OHBDE47	3,5-dibromo-2-(2,4-dibromophenoxy)phenol		https://pubchem.ncbi.nlm.nih.gov/compound/3086109#section=Top
297742-10-0	6-OHBDE47	2,3,5-tribromo-6-(2,4-dibromophenoxy)phenol		https://pubchem.ncbi.nlm.nih.gov/compound/21576164#section=Top
152969-11-4	6-OHBDE99	2,3,6-trichloro-4-(3,4-dichlorophenyl)phenol		https://pubchem.ncbi.nlm.nih.gov/compound/177947#section=Top
158076-68-7	4-OHCB107	2,3,5,6-tetrachloro-4-(2,4,5-trichlorophenyl)phenol		https://pubchem.ncbi.nlm.nih.gov/compound/178007#section=Top
60348-60-9	4-OHCB187	1,2,4-tribromo-5-(2,4-dibromophenoxy)benzene		https://pubchem.ncbi.nlm.nih.gov/compound/36159#section=Names-and-Identifiers

Appendix II

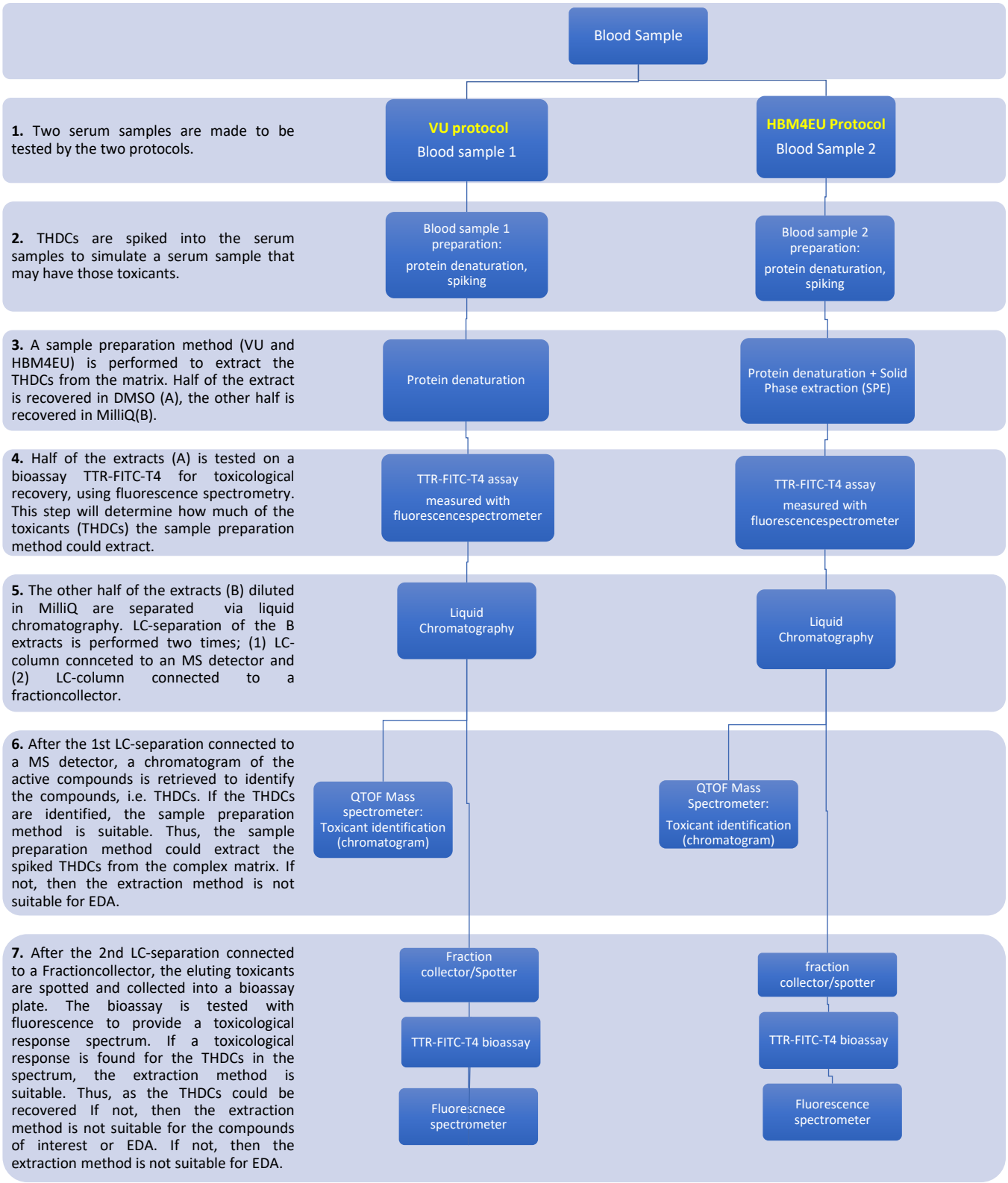


Figure 22. *The workflow of this research using an Effect-Directed Analysis (EDA) to evaluate the suitability of the sample preparation to EDA and their efficacy.*

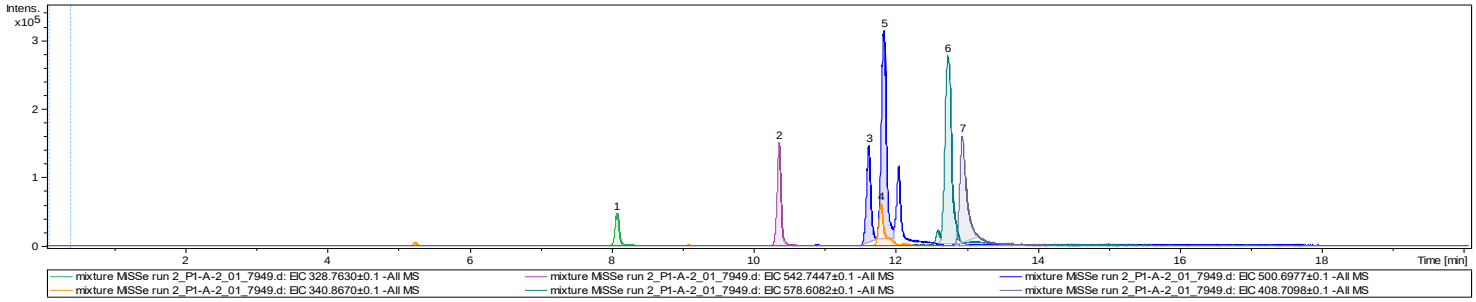
Appendix III

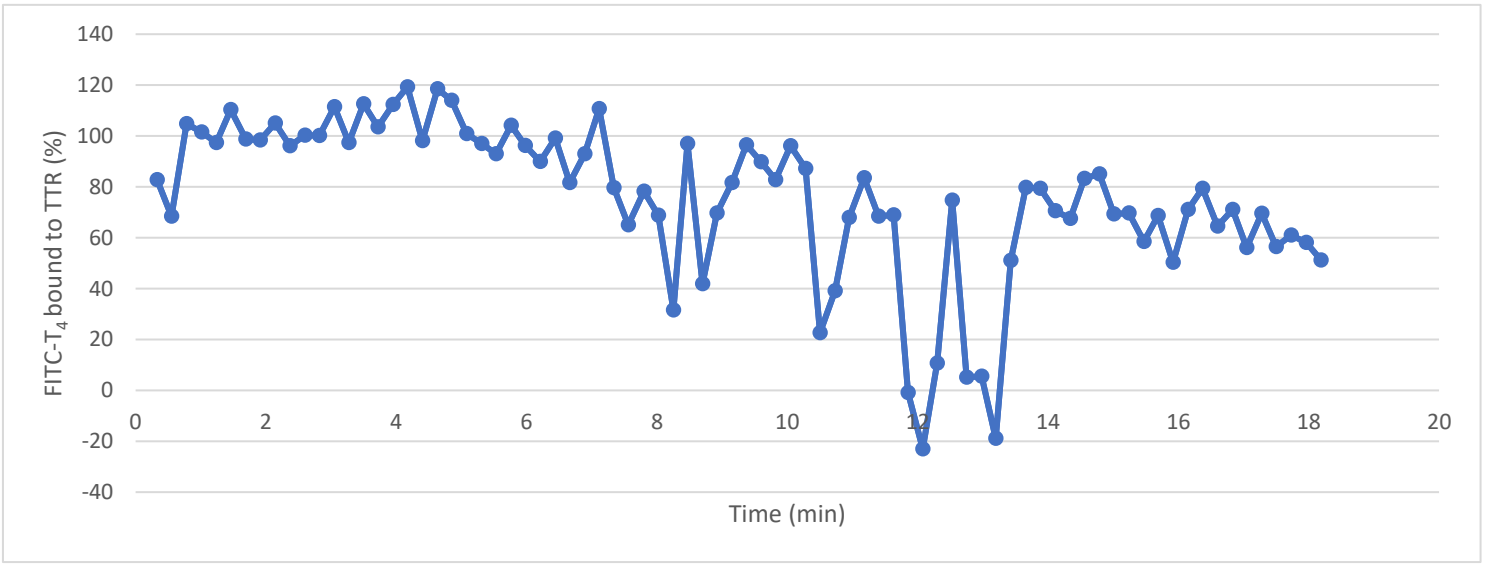
Standard Mixture EDA products

All seven compounds were identified in the chromatograms and could be correlated to the bioassay spectra.

Standard Mixture EDA Experiment 1:

#	Compound Label	RT [min]	Range [min]	FWHM [min]	Height	Area	S/N
1	2,4,6-TBP	8,1	8.0 - 8.2	0.1	47242	182157	379.8
2	TBBPA	10,4	10.3 - 10.5	0.1	151391	570402	1323
3	6-OHBDE47	11,6	11.5 - 11.7	0.1	141367	592181	856.2
4	4-OHCB107	11,8	11.7 - 12.0	0.1	61275	315434	393.6
5	5-OHBDE47	11,9	11.7 - 12.0	0.1	304888	1559588	1845.7
6	6-OHBDE99	12,7	12.6 - 12.9	0.1	273469	1785478	2178.8
7	4-OHCB187	12,9	12.8 - 13.2	0.1	157640	1026568	1581.2





$R^2 = 0.9632$

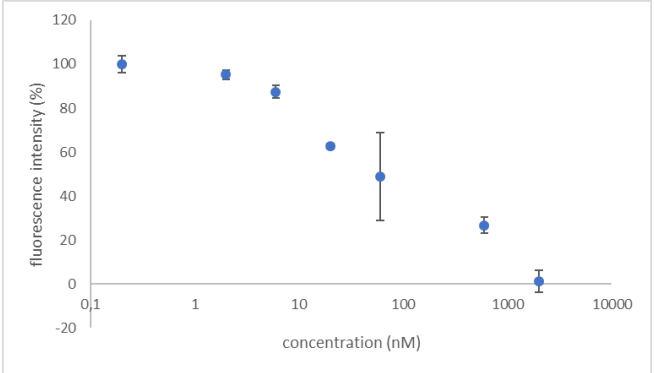
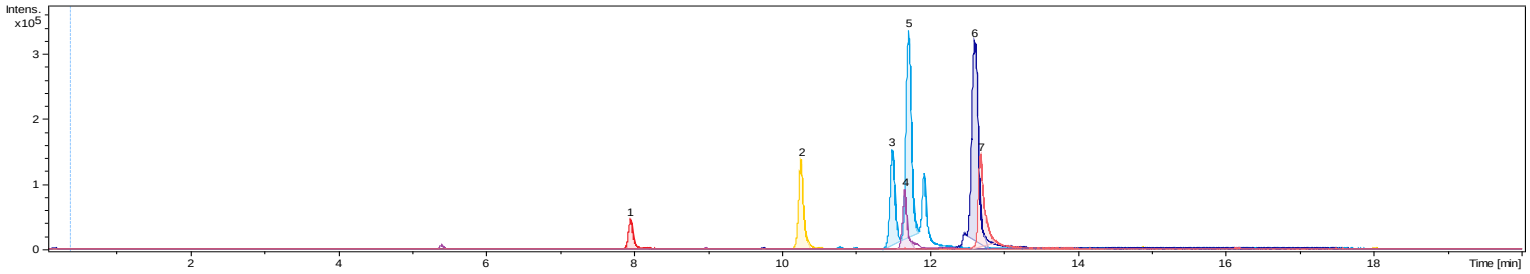
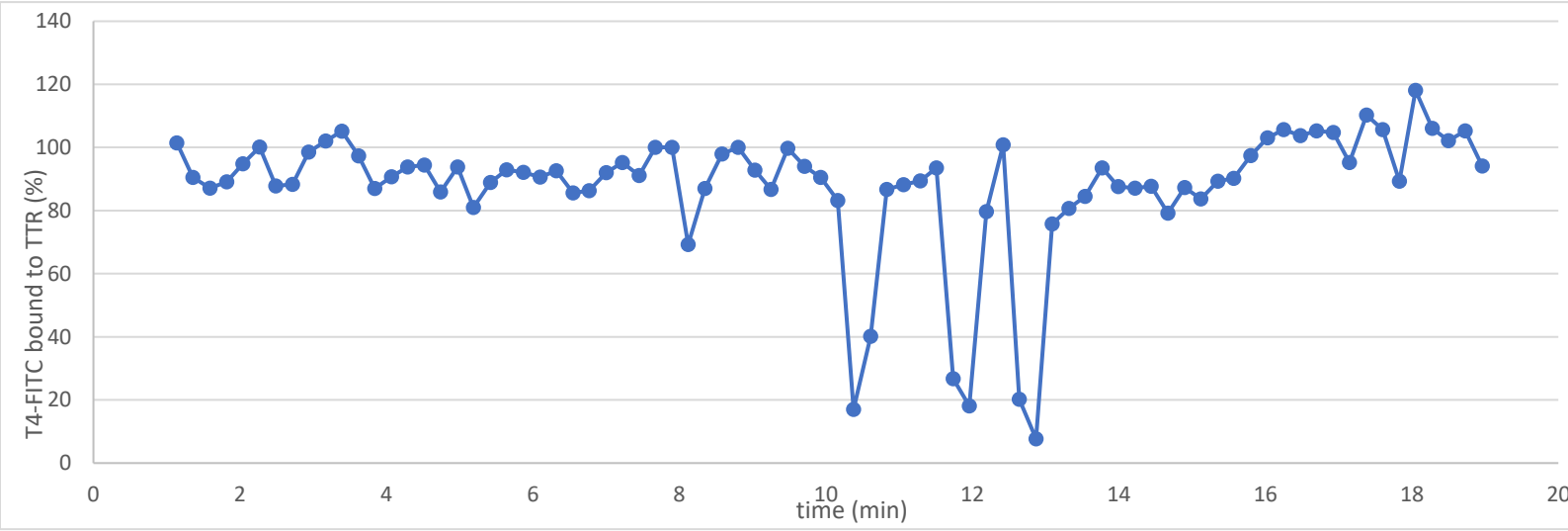


Figure 23. *Chromatogram with its adjacent information and TTR-FITC-T_4 bioassay spectrum with its corresponding calibration curve of Standard Mixture sample1.*

Standard Mixture EDA Experiment 2:

#	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	2,4,6-TBP	10.3	10.2 - 10.4	0.1	623542	137940	669.9
2	TBBPA	11.5	11.4 - 11.6	0.1	698114	148236	648
3	6-OHBDE47	11.7	11.6 - 11.8	0.1	395432	90611	404.9
4	4-OHCB107	11.7	11.6 - 11.9	0.1	1593542	317437	1386.9
5	5-OHBDE47	11.7	11.6 - 11.9	0.1	1593542	317437	1386.9
6	6-OHBDE99	12.6	12.5 - 12.8	0.1	1964320	312401	1312.3
7	4-OHCB187	12.7	12.6 - 12.9	0.1	822218	143577	668.3





$R^2 = 0.9937$

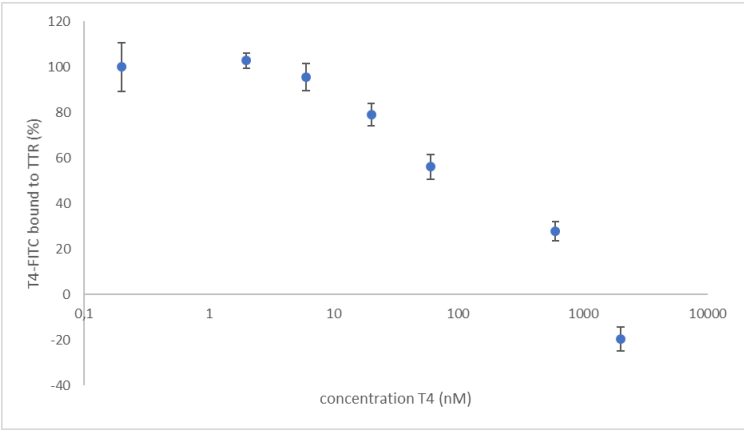
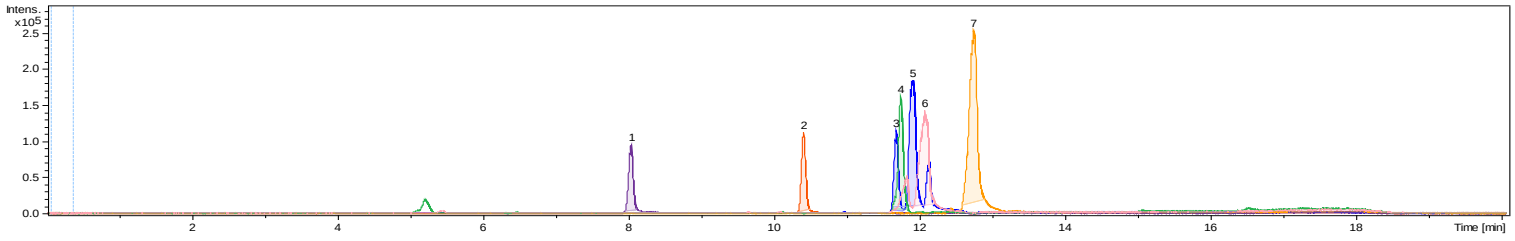
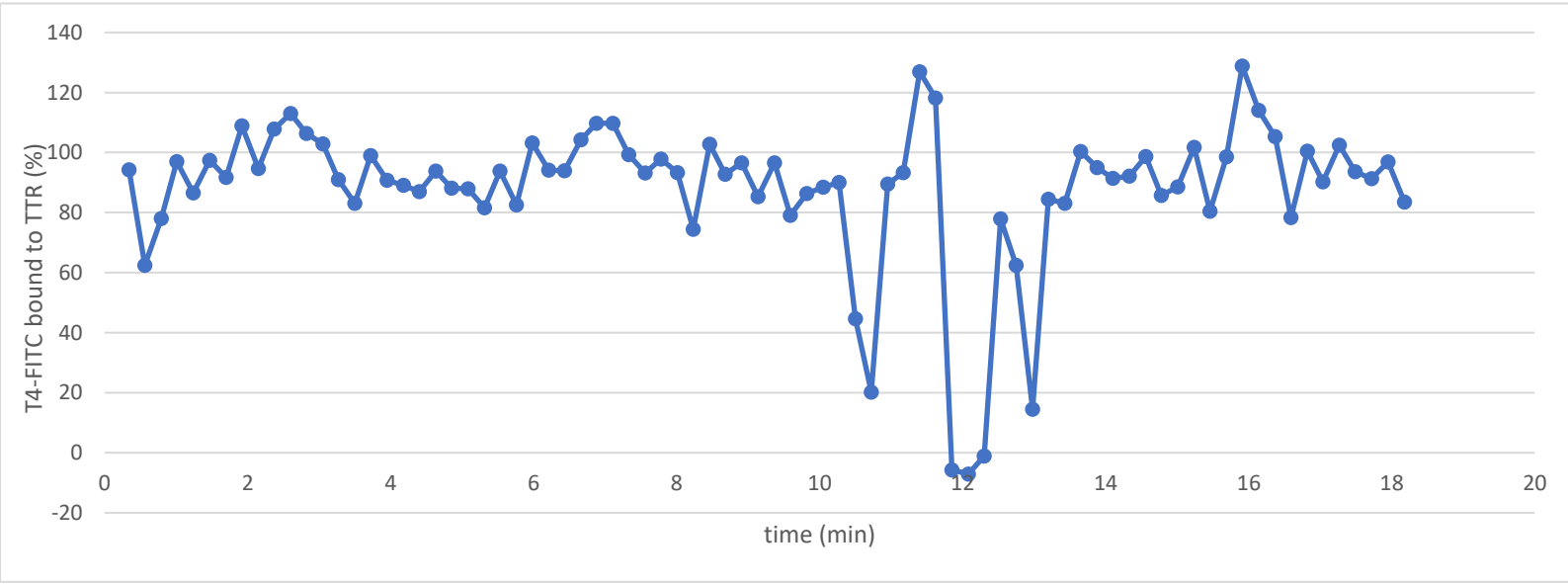


Figure 24. *Chromatogram with its adjacent information and TTR-FITC-T_4 bioassay spectrum with its corresponding calibration curve of Standard Mixture sample2.*

Standard Mixture EDA Experiment 3:

#	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	2,4,6 -TBP	8.1	8.0 - 8.1	0.1	361170	90493	827.2
2	TBBPA	10.4	10.4 - 10.5	0.1	418835	107859	474.6
3	6-OHBDE47	11.7	11.6 - 11.8	0.1	469758	111298	391.3
4	4-OHCB107	11.8	11.6 - 11.8	0.1	708458	156573	482.9
5	5-OHBDE47	11.9	11.6 - 12.0	0.1	1471663	172908	607.9
6	4-OHCB187	12.1	11.9 - 12.2	0.1	1058986	131695	403.7
7	6-OHBDE99	12.8	12.6 - 12.9	0.1	1762535	237432	2170.3





$R^2 = 0.9865$

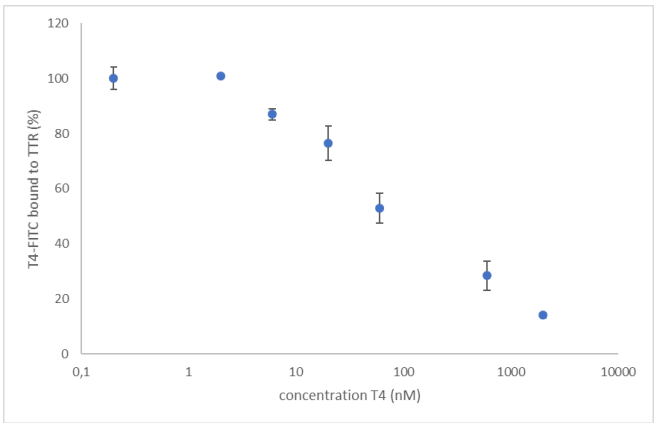
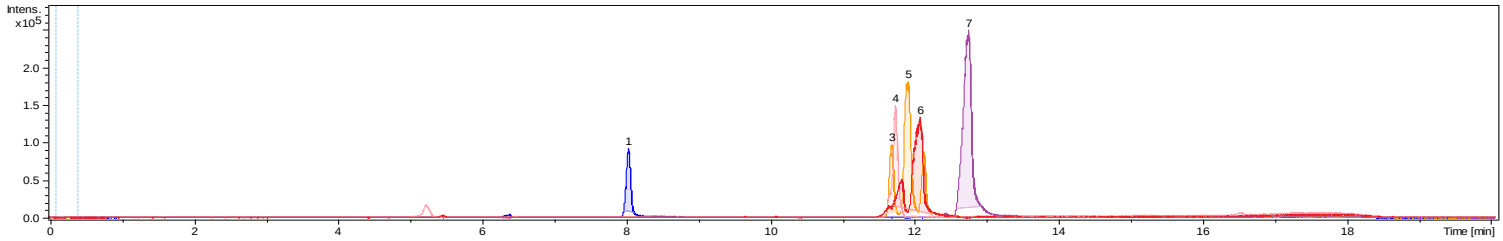
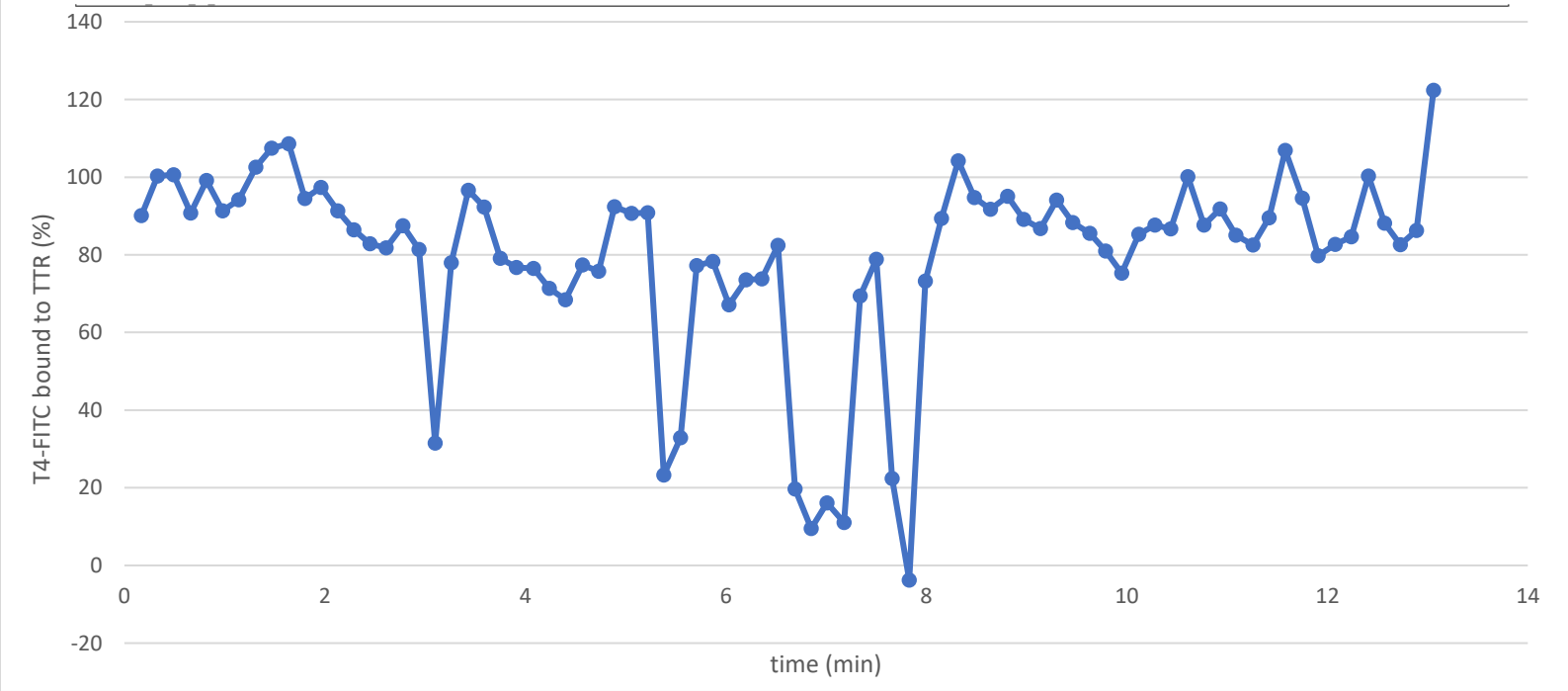


Figure 25. *Chromatogram with its adjacent information and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of Standard Mixture sample3.*

Standard Mixture EDA Experiment 4:

#	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	2,4,6-TBP	8.1	8.0 - 8.1	0.1	316655	84032	336.6
2	TBBPA	10.4	10.4 - 10.5	0.1	393854	103993	456.6
3	6-OHBDE47	11.7	11.6 - 11.8	0.1	348980	87476	304.3
4	4-OHCB107	11.8	11.6 - 11.8	0.1	661443	141800	434.7
5	5-OHBDE47	11.9	11.8 - 12.0	0.1	1023480	171806	597.7
6	4-OHCB187	12.1	11.7 - 12.3	0.1	1192435	124967	378.5
7	6-OHBDE99	12.8	12.6 - 12.9	0.1	1812114	235753	871.5





$R^2 = 0.9725$

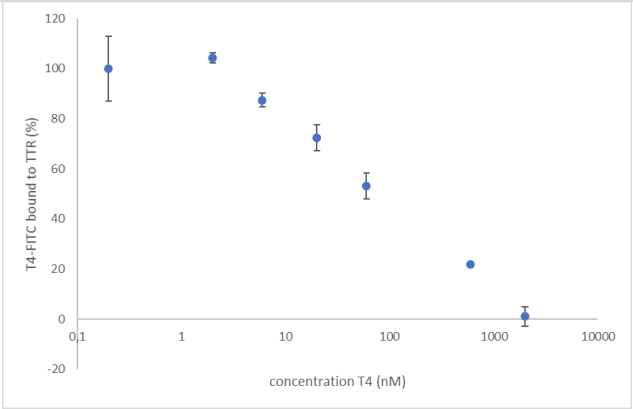
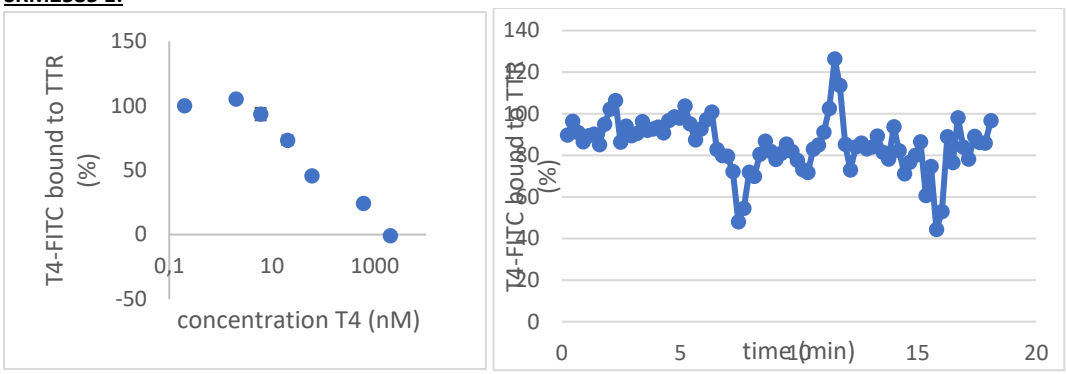


Figure 26. *Chromatogram with its adjacent information and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of Standard Mixture sample4.*

Appendix IV

SRM2585 EDA products: TTR-FITC-T4 bioassay and Chromatograms after LC-separation (fractionation)

SRM2585 1:



R^2 = 0.9975

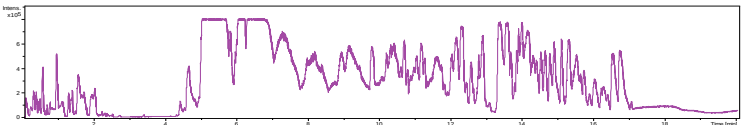
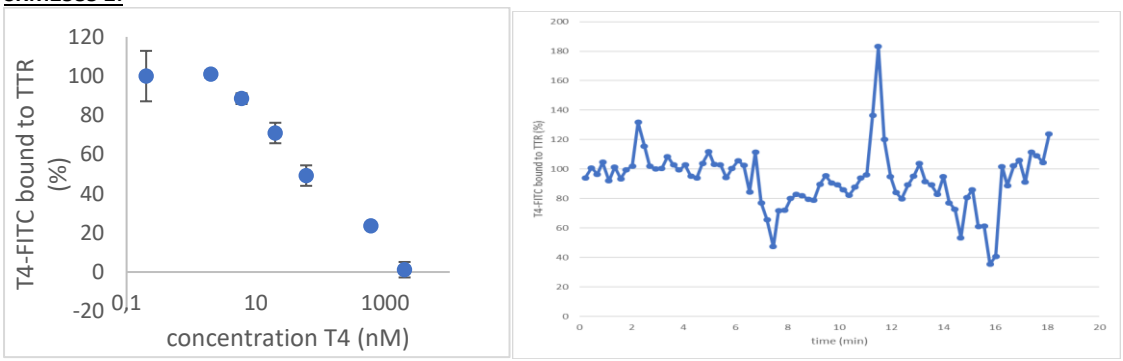


Figure 27*.Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of SRM2585 1.*

SRM2585 2:



R^2= 0.9946

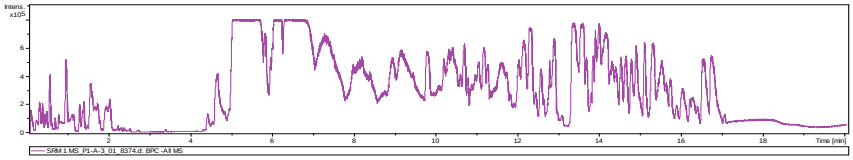


Figure 28. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of SRM2585 2.*

SRM2585 3:

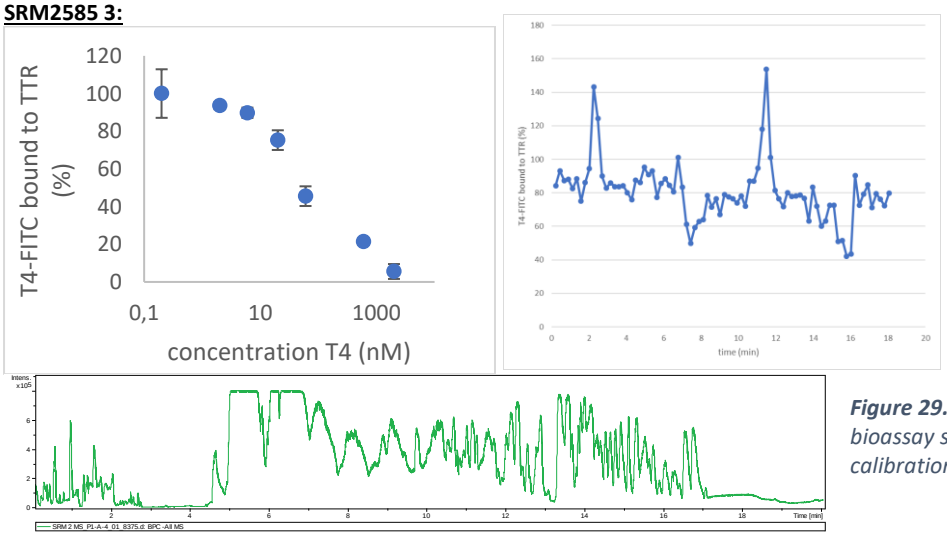
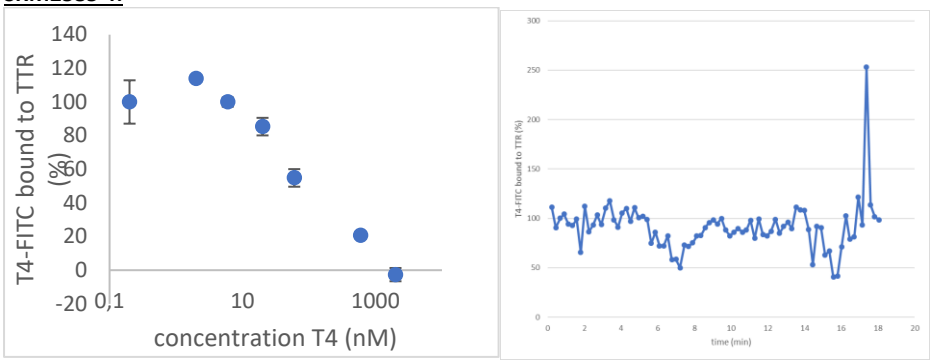


Figure 29. . *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of SRM2585 3.*

SRM2585 4:



R^2 = 0.9895

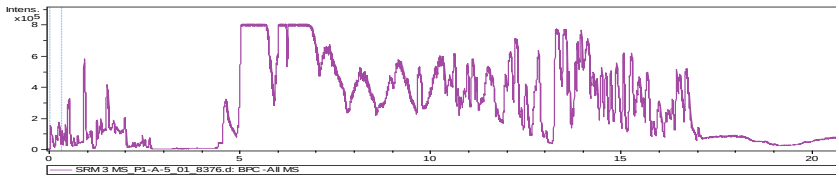
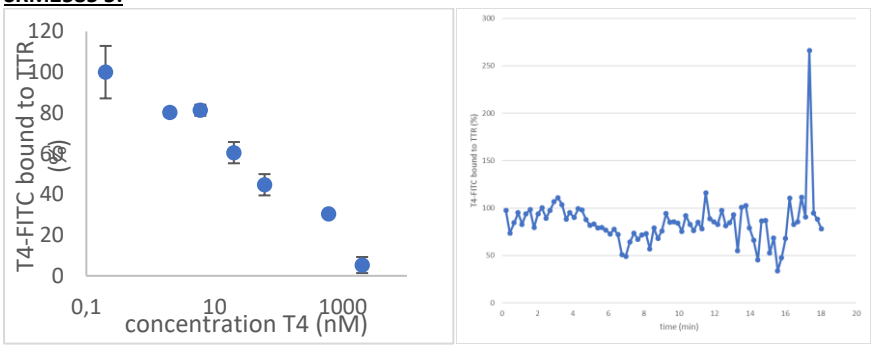


Figure 30. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of SRM2585 4.*

SRM2585 5:



R^2 = 0.9872

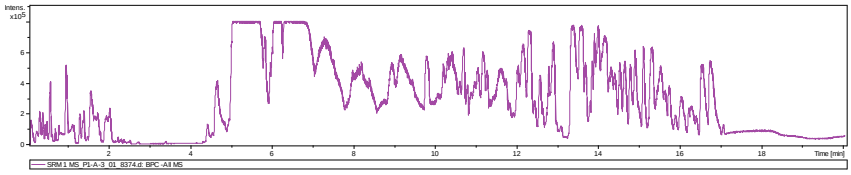
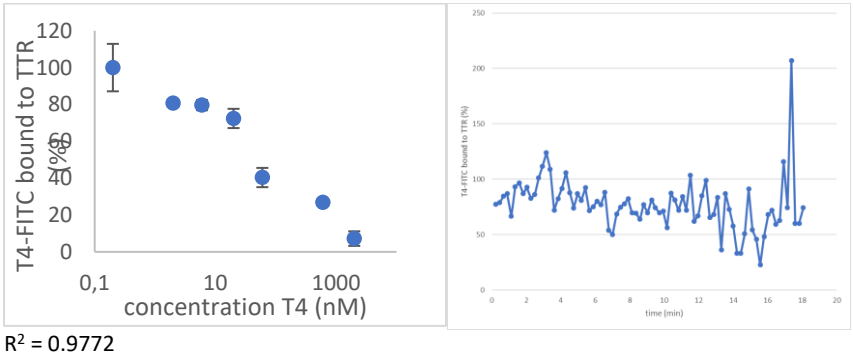


Figure 31. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of SRM2585 5.*

SRM2585 6:



R^2 = 0.9772

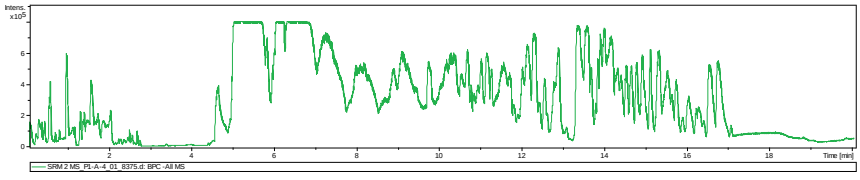
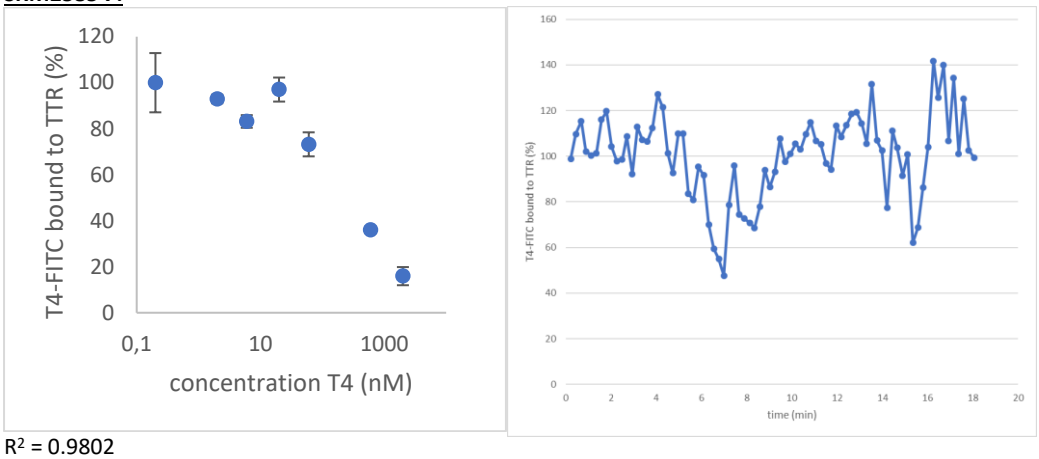


Figure 32. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of SRM2585 6.*

SRM2585 7:



R^2 = 0.9802

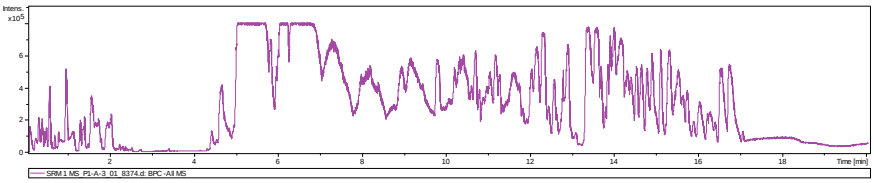
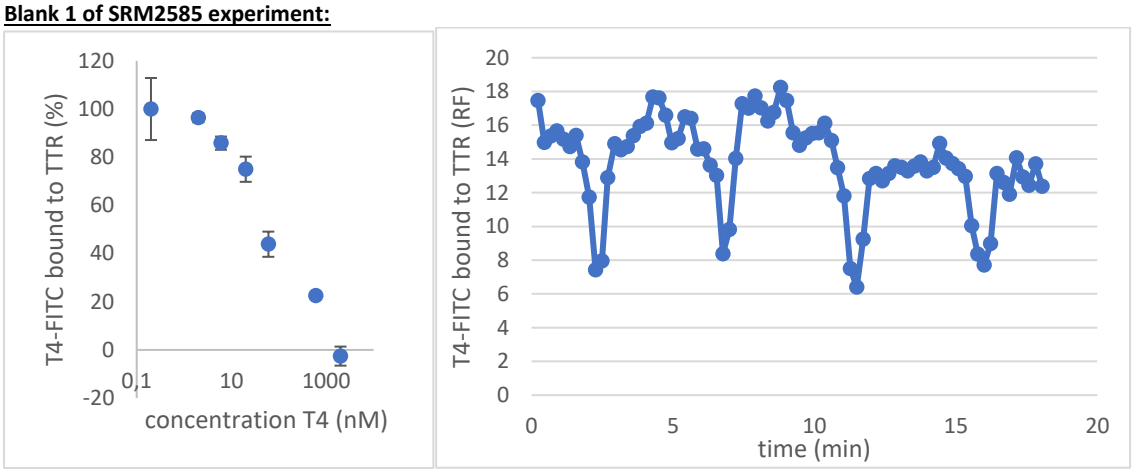


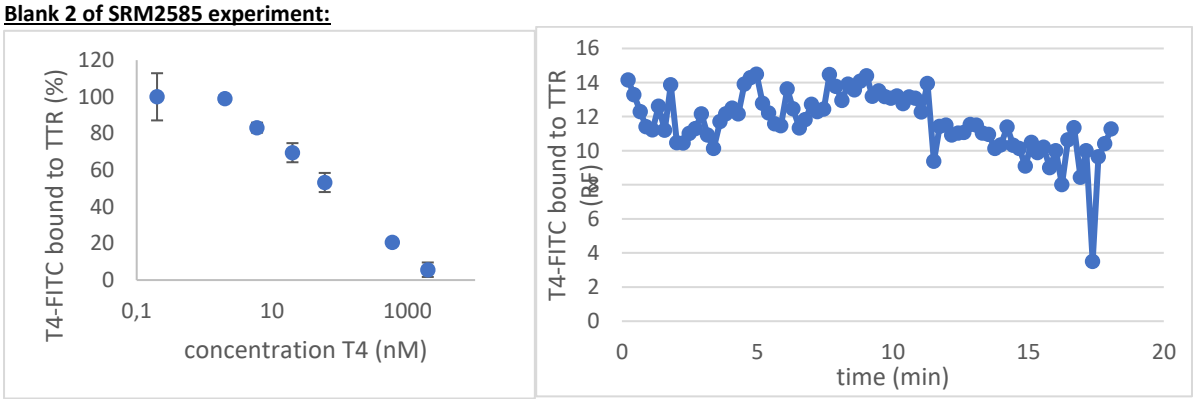
Figure 33. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of SRM2585 7.*

Blank 1 of SRM2585 experiment:



R^2 =0.9633 ***Figure 34.*** *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of Blank 1.*

Blank 2 of SRM2585 experiment:

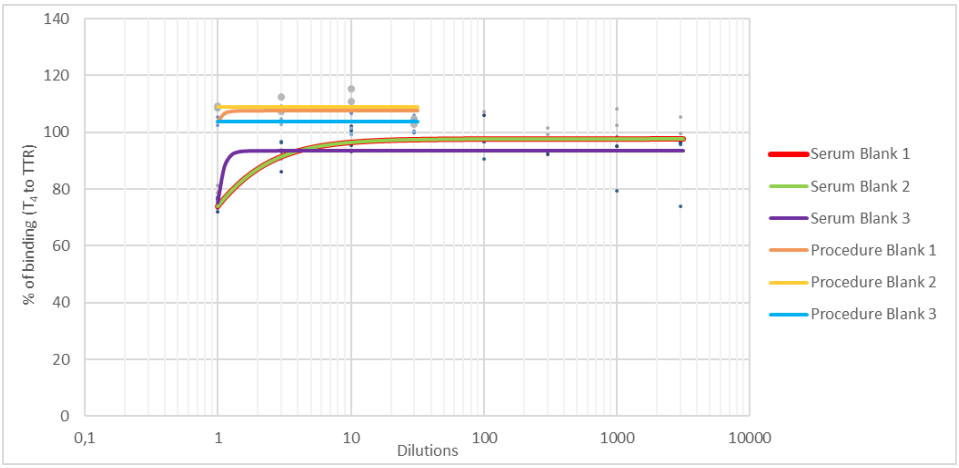


R^2 = 0.9948 ***Figure 35.*** *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of Blank 2.*

Appendix V

Quantitative data processing of the Procedure blanks and Serum Blanks of HBM4EU method

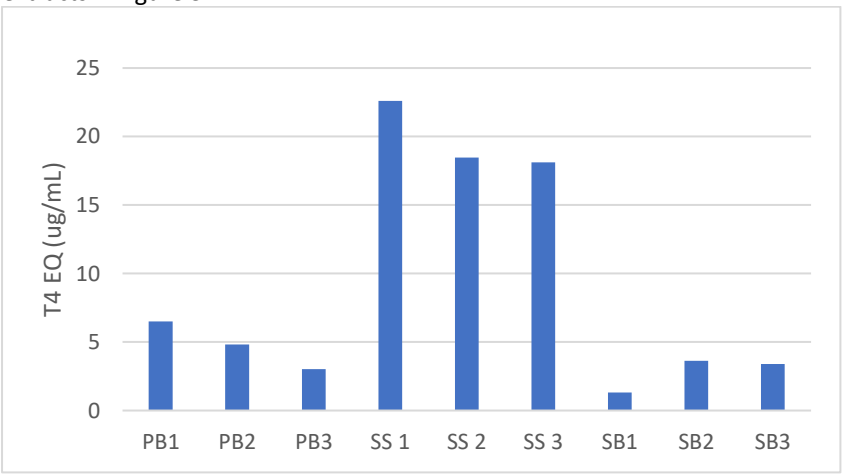
The extracts of the procedure blanks (PB1, PB2, PB3) were tested on a bioassay plate in dilution series (1x-3x-10x-30x) and the Serum Blanks (SB1, SB2, SB3) were tested each on the bioassay in dilution series (1x-3x-10x-30x-60x-100x-300x-1000x-3000x). Interpolation allowed the finding of the IC20 of the Serum Blanks and Procedure Blanks. The 20% inhibition concentration of these solutions can be found in the figure 36 here below:



Sample	IC20 (dilution)
PB1	0.85
PB2	0.63
PB3	0.43
SB1	0.35
SB2	1.17
SB3	1.04

Figure 36. *Fluorescence-concentration graph relationship of the Procedure Blanks and the Serum Blanks, tested by the miniaturized TTR-FITC-T4 binding assay.*

The T_4-EQ of the Procedure Blanks and the Serum blanks are set against the T4-EQ of the Spiked Serum extracts in figure 37:



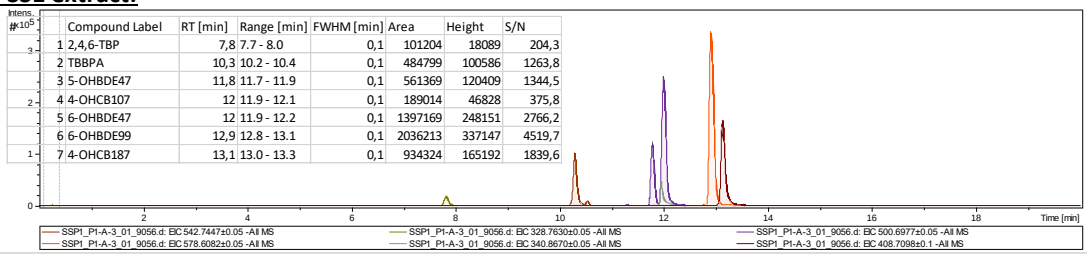
sample	T_4 EQ blanks (ug/mL)
PB1	6.50
PB2	4.81
PB3	3.03
SB1	1.31
SB2	3.63
SB3	3.40

Figure 37. *T_4-EQ of the Procedure blanks and the Serum Blanks in relation to the T_4-EQ of the Spiked Serum extracts.*

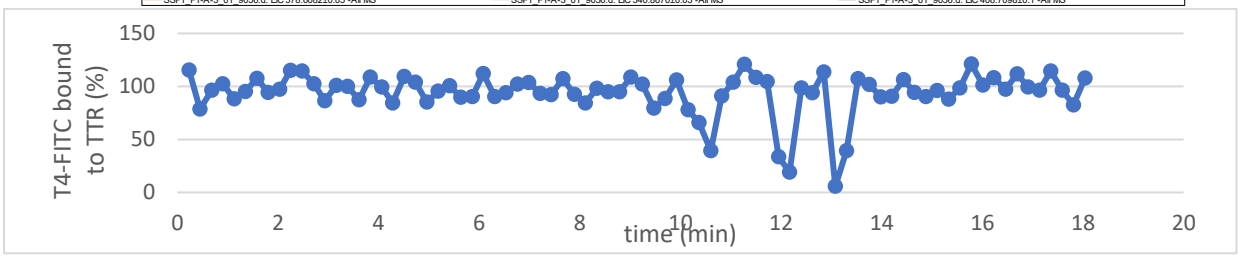
Appendix VI

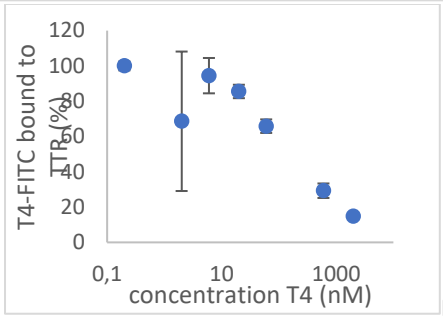
Qualitative data processing of the HBM4EU method extracts

HBM4EU SS1 extract:



	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	2,4,6-TBP	7,8	7.7 - 8.0	0,1	101204	18089	204,3
2	TBBPA	10,3	10.2 - 10.4	0,1	484799	100586	1263,8
3	5-OHBDE47	11,8	11.7 - 11.9	0,1	561369	120409	1344,5
4	4-OHCB107	12	11.9 - 12.1	0,1	189014	46828	375,8
5	6-OHBDE47	12	11.9 - 12.2	0,1	1397169	248151	2766,2
6	6-OHBDE99	12,9	12.8 - 13.1	0,1	2036213	337147	4519,7
7	4-OHCB187	13,1	13.0 - 13.3	0,1	934324	165192	1839,6

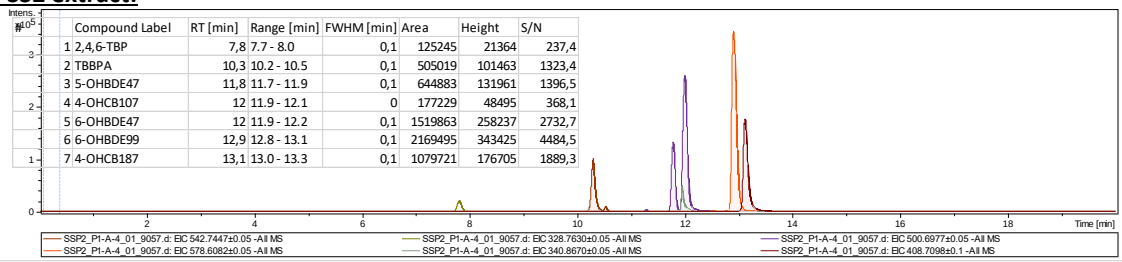




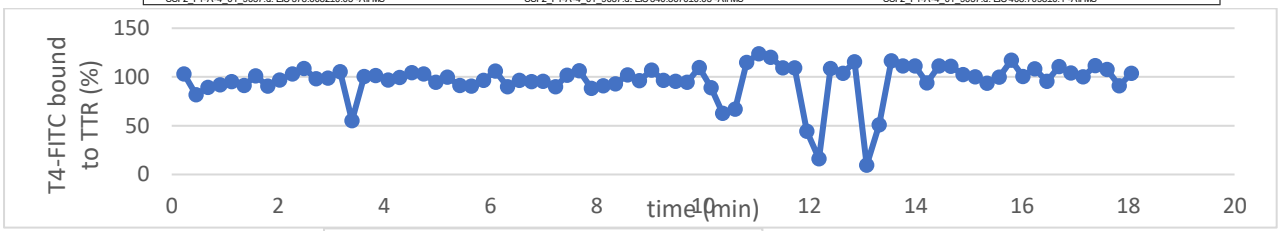
R^2 = 0.8380

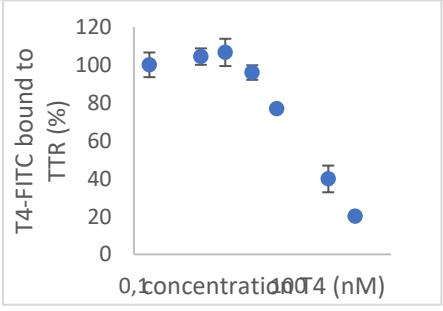
Figure 38. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of HBM4EU extract SS1.*

HBM4EU SS2 extract:



	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	2,4,6-TBP	7,8	7.7 - 8.0	0,1	125245	21364	237,4
2	TBBPA	10,3	10.2 - 10.5	0,1	505019	101463	1323,4
3	5-OHBDE47	11,8	11.7 - 11.9	0,1	644883	131961	1396,5
4	4-OHCB107	12	11.9 - 12.1	0	177229	48495	368,1
5	6-OHBDE47	12	11.9 - 12.2	0,1	1519863	258237	2732,7
6	6-OHBDE99	12,9	12.8 - 13.1	0,1	2169495	343425	4484,5
7	4-OHCB187	13,1	13.0 - 13.3	0,1	1079721	176705	1889,3

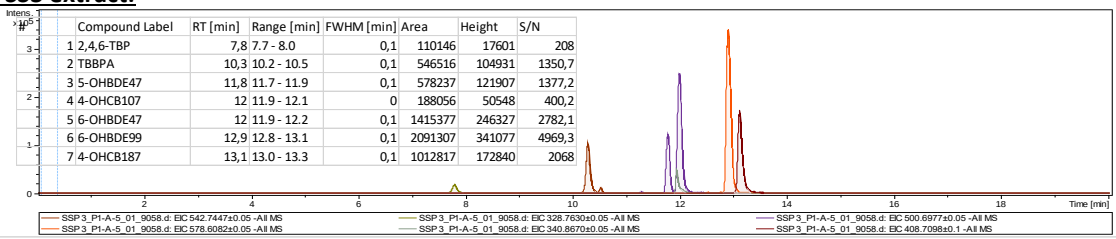




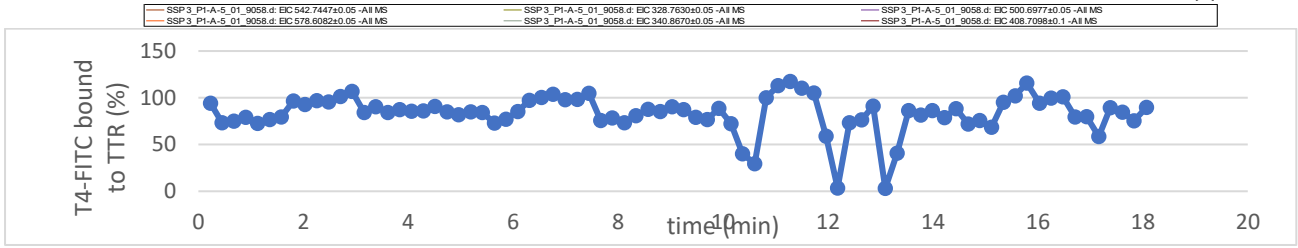
R^2 = 0.9945

Figure 39. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of HBM4EU extract SS2.*

HBM4EU SS3 extract:



#	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	2,4,6-TBP	7,8	7.7 - 8.0	0,1	110146	17601	208
2	TBBPA	10,3	10.2 - 10.5	0,1	546516	104931	1350,7
3	5-OHBDE47	11,8	11.7 - 11.9	0,1	578237	121907	1377,2
4	4-OHCB107	12	11.9 - 12.1	0	188056	50548	400,2
5	6-OHBDE47	12	11.9 - 12.2	0,1	1415377	246327	2782,1
6	6-OHBDE99	12,9	12.8 - 13.1	0,1	2091307	341077	4969,3
7	4-OHCB187	13,1	13.0 - 13.3	0,1	1012817	172840	2068



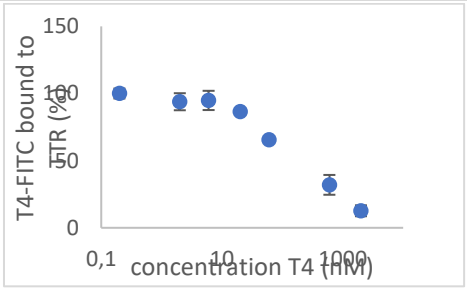
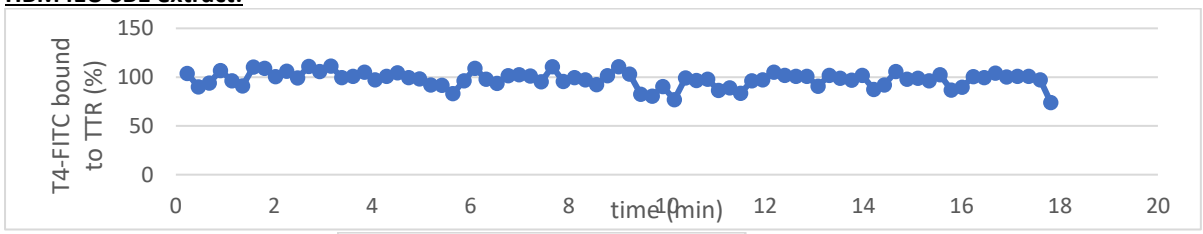


Figure 40. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of HBM4EU extract SS3.*

$R^2 = 0.9883$

HBM4EU SB1 extract:



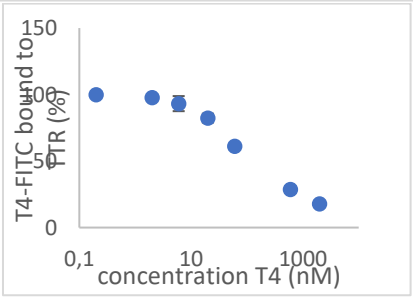
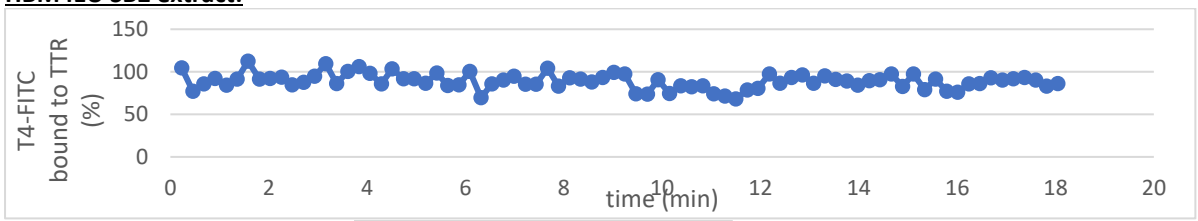


Figure 41. *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of HBM4EU extract SB1.*

$R^2 = 0.9959$

HBM4EU SB2 extract:



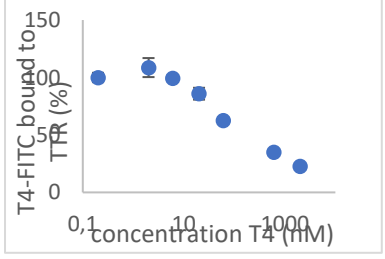
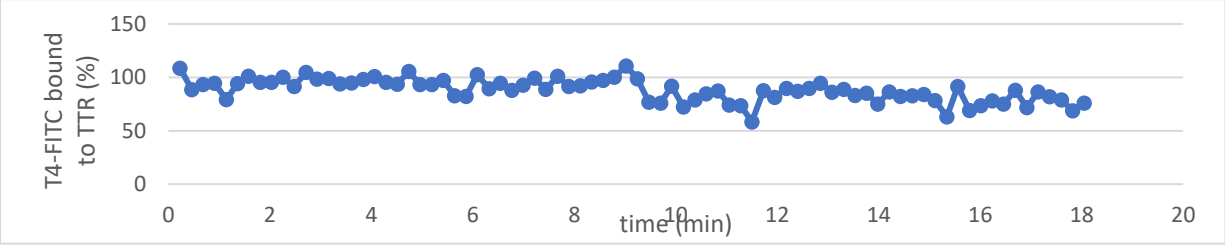
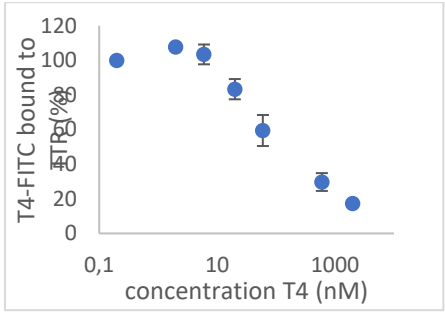


Figure 42*. TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of HBM4EU extract SB2.*

$R^2 = 0.9970$

HBM4EU SB3 extract:

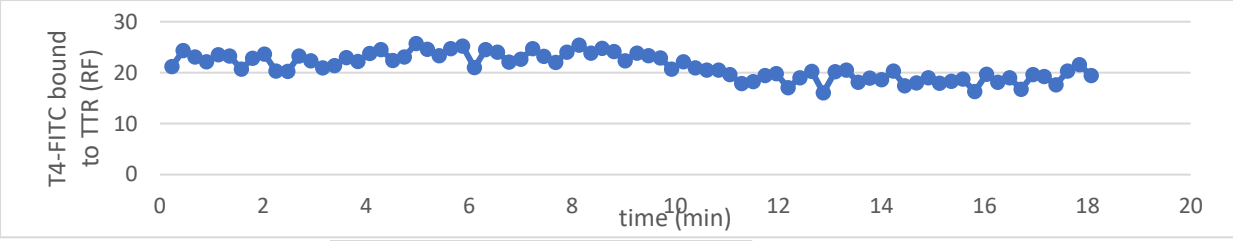


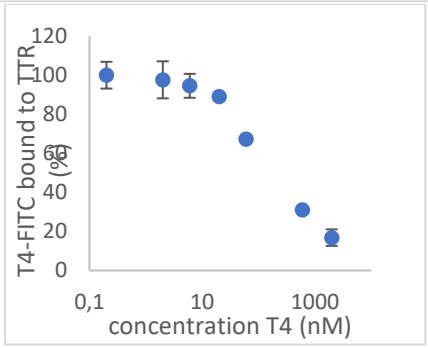


R^2 = 0.9838

Figure 43. *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of HBM4EU extract SB3.*

HBM4EU PB 1 extract:

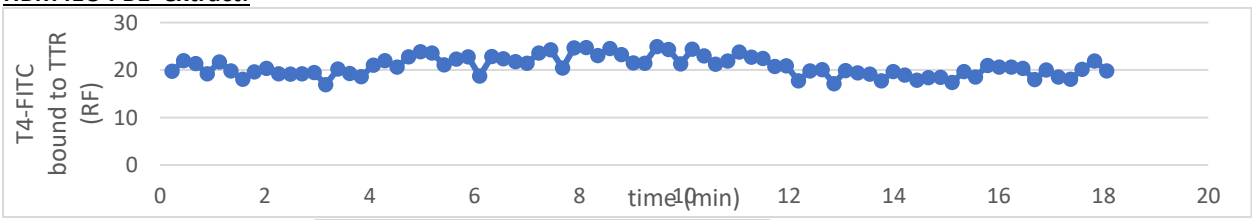


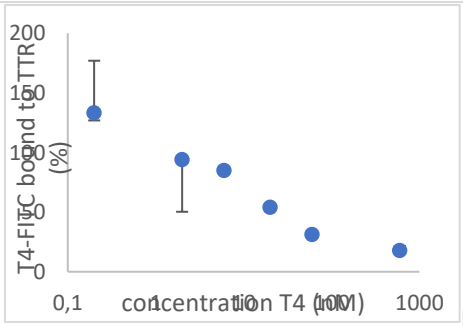


R^2 =0.9759

Figure 44. *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of HBM4EU extract PB1*

HBM4EU PB1' extract:





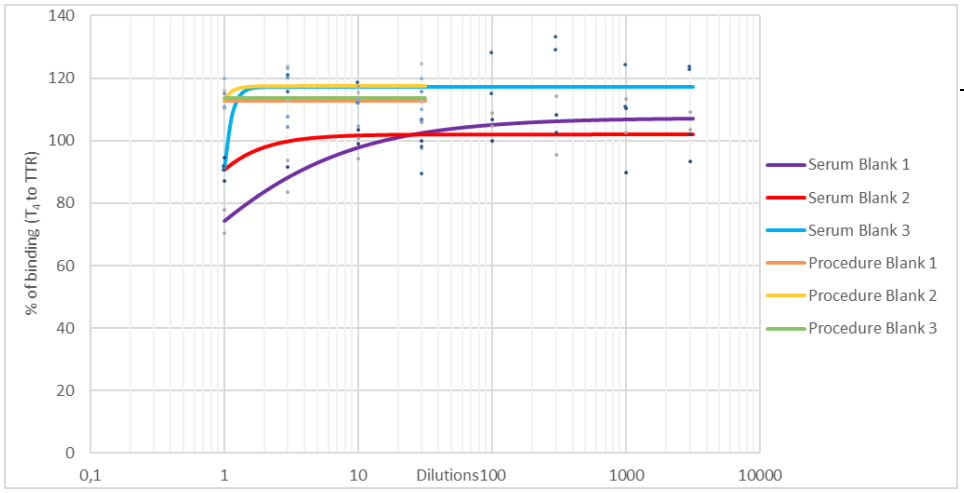
R^2 = 0.9160

Figure 45. *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of HBM4EU extract PB1'.*

Appendix VII

Quantitative data processing of the Procedure blanks and Serum Blanks of VU method

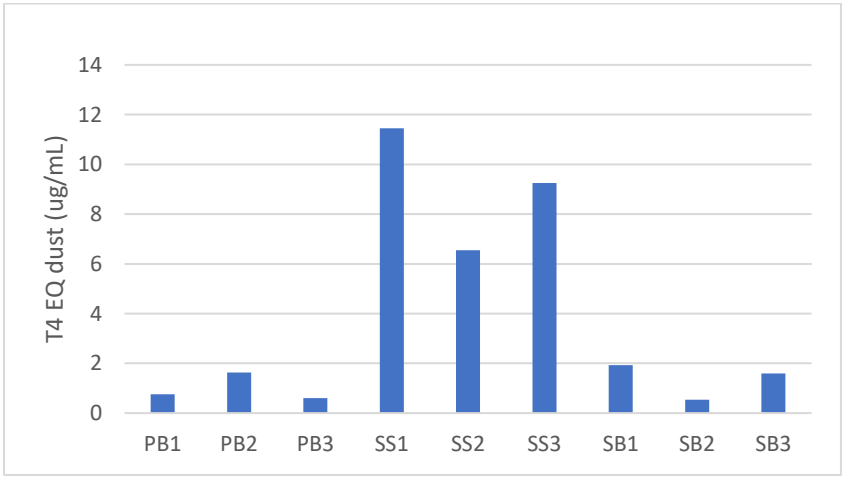
The extracts of the procedure blanks (PB1, PB2, PB3) were tested on a bioassay plate in dilution series (1x-3x-10x-30x) and the Serum Blanks (SB1, SB2, SB3) were tested each on the bioassay in dilution series (1x-3x-10x-30x-60x-100x-300x-1000x-3000x). Interpolation allowed the finding of the IC20 of the Serum Blanks and Procedure Blanks. The 20% inhibition concentration of these solutions can be found in the figure 46 here below:



Sample	IC20 (dilution)
PB1	0.293496013
PB2	0.705045705
PB3	0.264946253
SB1	1.496780673
SB2	0.603086551
SB3	0.948282048

***Figure 46**. Fluorescence-concentration graph relationship of the Procedure Blanks and the Serum Blanks, tested by the miniaturized TTR-FITC-T4 binding assay.*

The T_4-EQ of the Procedure Blanks and the Serum blanks are set against the T4-EQ of the Spiked Serum extracts in figure 47:



sample	T_4 EQ blanks (ug/mL)
PB1	0.75
PB2	1.63
PB3	0.60
SB1	1.93
SB2	0.53
SB3	1.59

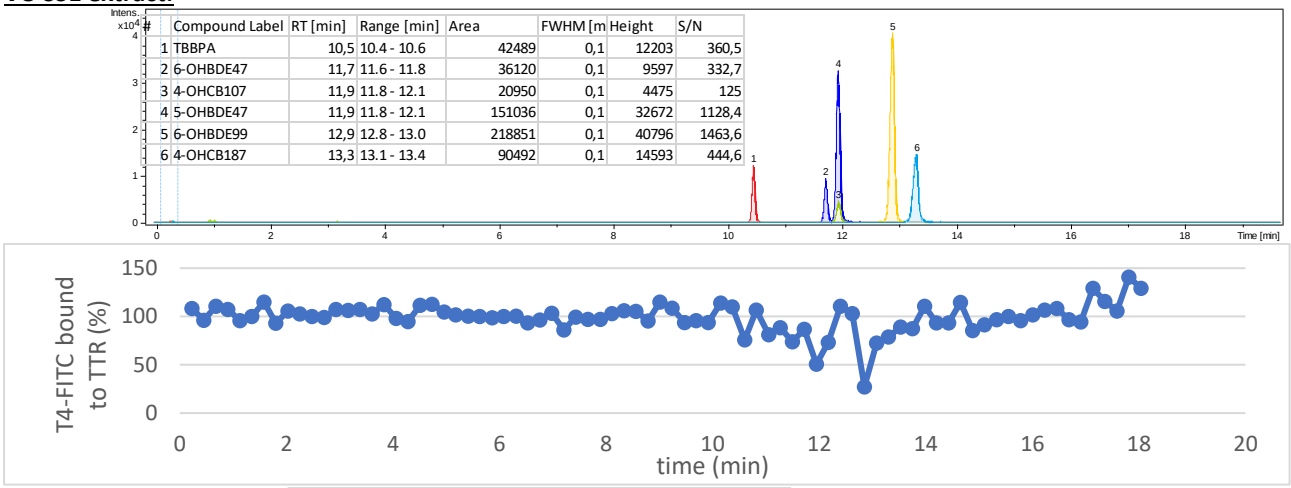
***Figure 47.** T4-EQ of the Procedure blanks and the Serum Blanks in relation to the T4-EQ of the Spiked Serum extracts.*

Appendix VIII

Qualitative data processing of the VU method extracts.

VU SS1 extract:

#	Compound Label	RT [min]	Range [min]	Area	FWHM [m	Height	S/N
1	TBBPA	10,5	10.4 - 10.6	42489	0,1	12203	360,5
2	6-OHBDE47	11,7	11.6 - 11.8	36120	0,1	9597	332,7
3	4-OHCB107	11,9	11.8 - 12.1	20950	0,1	4475	125
4	5-OHBDE47	11,9	11.8 - 12.1	151036	0,1	32672	1128,4
5	6-OHBDE99	12,9	12.8 - 13.0	218851	0,1	40796	1463,6
6	4-OHCB187	13,3	13.1 - 13.4	90492	0,1	14593	444,6



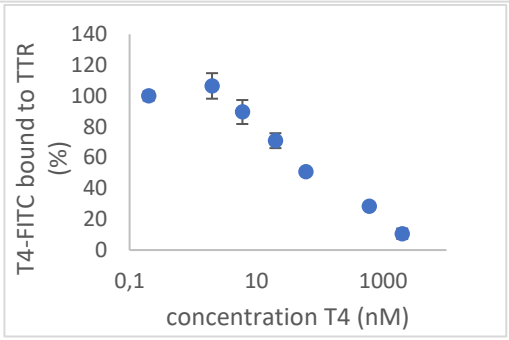
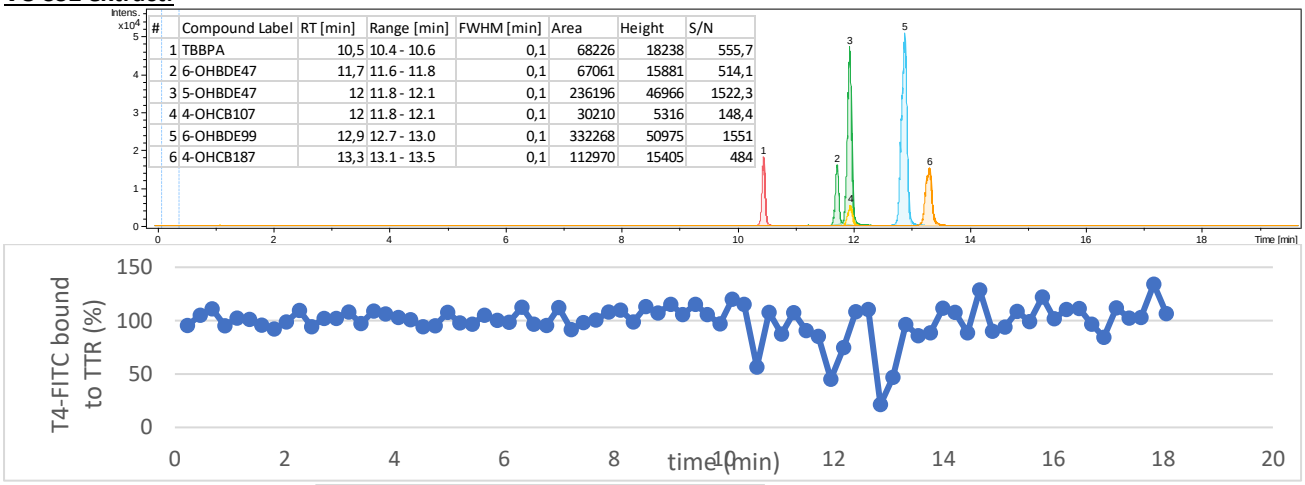


Figure 48. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of VU extract SS1.*

$R^2 = 0.9865$

VU SS2 extract:

#	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	TBBPA	10,5	10.4 - 10.6	0,1	68226	18238	555,7
2	6-OHBDE47	11,7	11.6 - 11.8	0,1	67061	15881	514,1
3	5-OHBDE47	12	11.8 - 12.1	0,1	236196	46966	1522,3
4	4-OHCB107	12	11.8 - 12.1	0,1	30210	5316	148,4
5	6-OHBDE99	12,9	12.7 - 13.0	0,1	332268	50975	1551
6	4-OHCB187	13,3	13.1 - 13.5	0,1	112970	15405	484



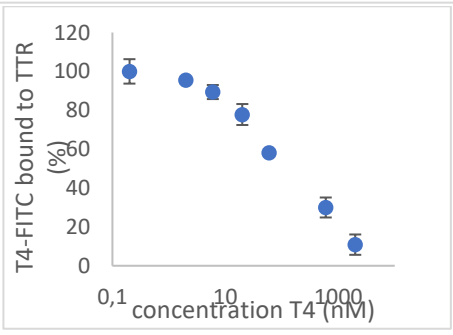
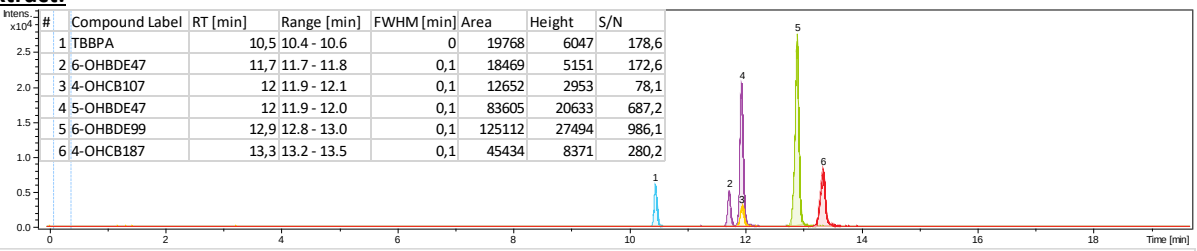


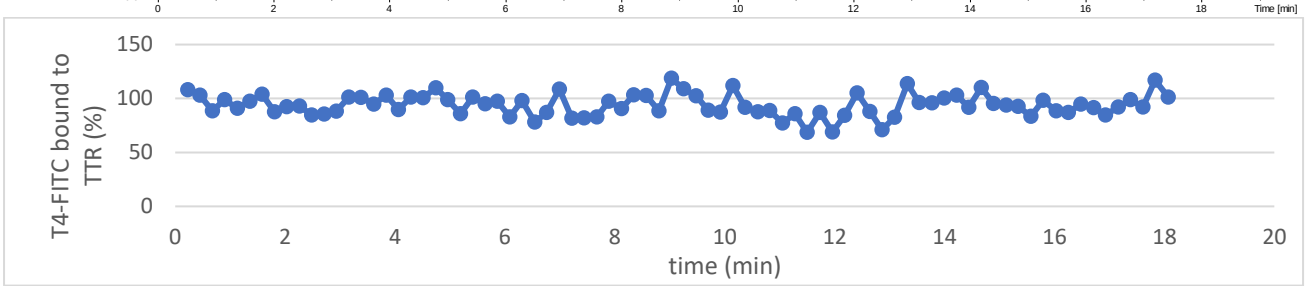
Figure 49. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of VU extract SS2.*

$R^2 = 0.9867$

VU SS3 extract:



#	Compound Label	RT [min]	Range [min]	FWHM [min]	Area	Height	S/N
1	TBBPA	10,5	10.4 - 10.6	0	19768	6047	178,6
2	6-OHBDE47	11,7	11.7 - 11.8	0,1	18469	5151	172,6
3	4-OHCB107	12	11.9 - 12.1	0,1	12652	2953	78,1
4	5-OHBDE47	12	11.9 - 12.0	0,1	83605	20633	687,2
5	6-OHBDE99	12,9	12.8 - 13.0	0,1	125112	27494	986,1
6	4-OHCB187	13,3	13.2 - 13.5	0,1	45434	8371	280,2



(the THDCs were lost during the bio-testing)

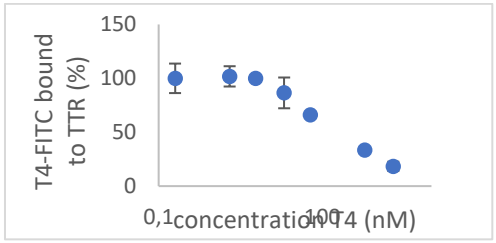
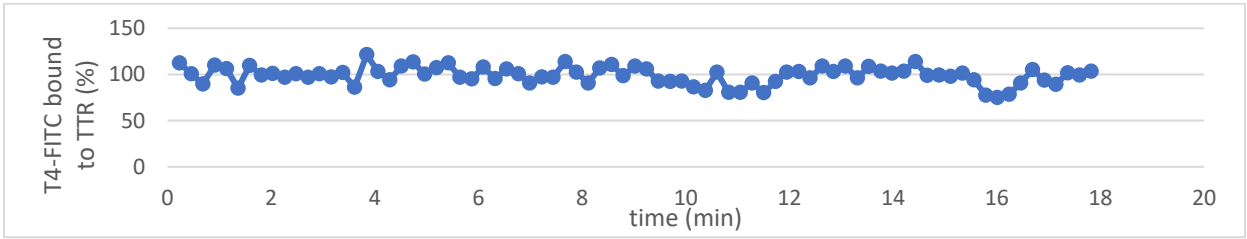


Figure 50. *Chromatogram and TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of VU extract SS3.*

R^2 = 0.9823

VU SB1 extract:



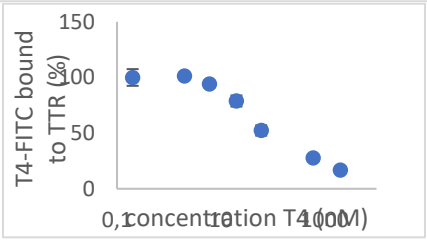
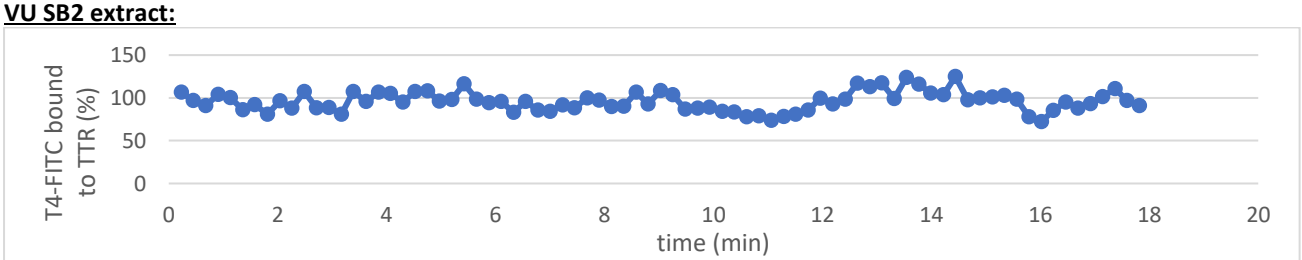


Figure 51. *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of VU extract SB1.*

R^2 = 0.9914

VU SB2 extract:



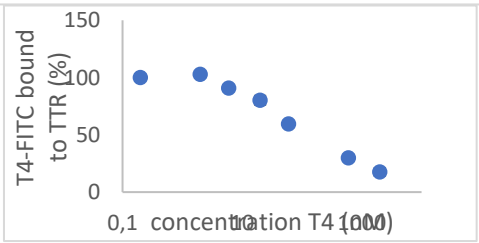
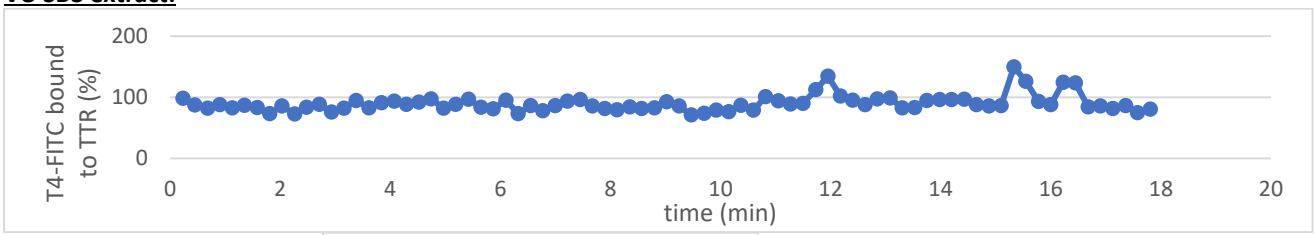


Figure 52. *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of VU extract SB2.*

R^2 = 0.9953

VU SB3 extract:



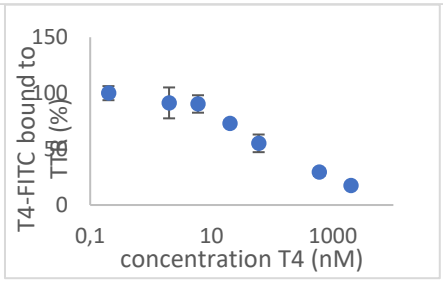
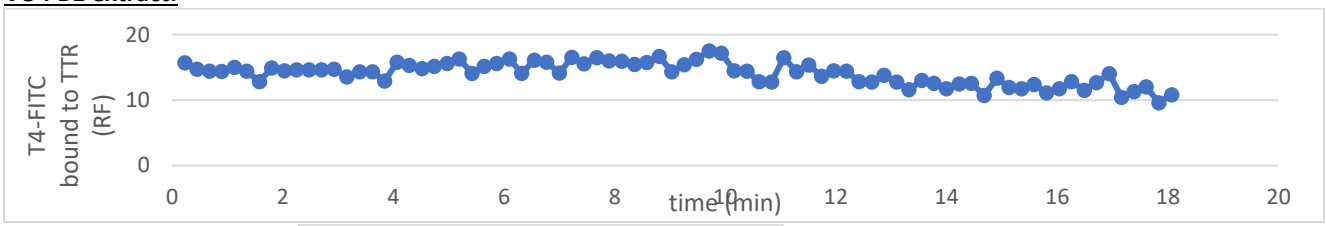


Figure 53. *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of VU extract SB3.*

$R^2 = 0.9794$

VU PB1 extract:



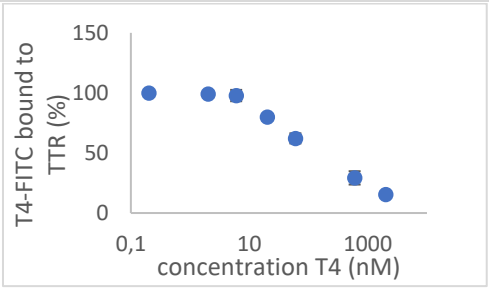
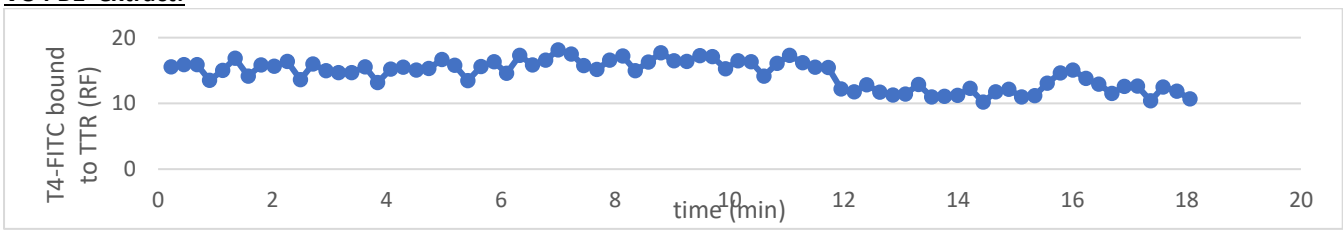


Figure 54. *TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of VU extract PB1..*

$R^2 = 0.9917$

VU PB1' extract:



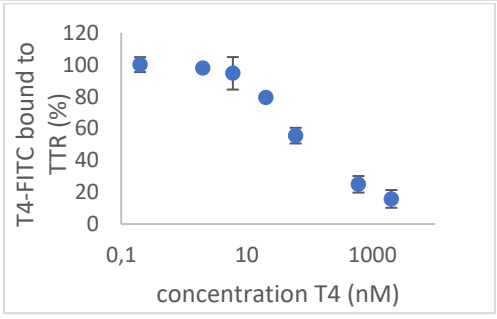


Figure 55*. TTR-FITC-T4 bioassay spectrum with its corresponding calibration curve of VU extract PB1'.*

$R^2 = 0.9889$

Appendix IX

Bioassay Protocols[2]

F-T4 protocol - JH Kamstra

Generation of FITC-T4

Reagents:
Pyridine
Trimethylamine
FITC fluorescein 5-isothiocyanate (Sigma: F-7250)
T4
LH20 sephadex (overnight in milliQ)
0.2 M NH_4Acetate (pH=4.0, with acidic acid)
0.05 M NH_4HCO_3
0.05 M $NaHCO_3$ (pH=8.5)

Procedure

- Make a pyridine/water/trimethylamine (PWT) mixture:
  - 9 mL pyridine
  - 1.5 mL milliQ
  - 0.1 mL trimethylamine
- Weigh 10 mg FITC and dissolve in 0.5 mL PWT (51.4 mM) in amber glass vial
- Weigh 10 mg T4 and dissolve in 1 mL PWT (25.7 mM) in amber glass vial
- Mix both vials and incubate 1 hr at 37ºC.
- Precipitate mixture with 20 mL 0.2 M NH_4Acetate (pH=4) in a 50 mL greiner tube
- Centrifuge 10 min at 1000g.
- Discard supernatant
- Add 20 mL of MilliQ water and mix vigorously.
- Centrifuge 10 min at 1000g.
- Discard the supernatant
- Dissolve the pellet in 5 mL 0.05 M NH_4HCO_3
- Mix thoroughly until the FITC-T4 is dissolved
- Optionally incubate at 37ºC for a few minutes.
- Make a column of LH20 in a burette (0.9 cm diameter) of about 4.5 cm
- Equilibrate the LH20 by passing 3 column volumes of 0.05 M $NaHCO_3$
- Add 0.5 mL of the FITC-T4 to the LH20 column
- Rinse with 10 column volume of 0.05 M $NaHCO_3$
- Elute with milliQ water by collecting 10 mL fractions until the yellow color is eluted from the column
- Test in each fraction the TTR binding with the same parameters as the T4-TTR procedure.
  - Add 2 µL fraction in a well
  - Add 150 µL TRIS buffer
  - Add 50 µL TTR working solution
  - ALSO PERFORM BACKGROUND MEASUREMENT WITHOUT TTR
- TTR-T4FITC should enhance the fluorescence. The fraction with the highest increase in fluorescence is considered the FITC-T4 fraction.
- Concentration is determined by measuring the absorbance at 490 nm with the molar extinction coefficient of 7.8×10^4 M/cm^{-1}.

[2] This document is property of the Vrije Universiteit Amsterdam.

TTR-FITC-T4 assay

Reagents
EDTA (EDTA.2H2O) (Acros organics, M = 372,23 g/mol, [6381-92-6], cat. no. 147855000)
2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), Sigma, M=121.1 g/mol, [77-86-1], cat. no. T1503
Demi water
Sodium chloride (NaCl), Riedel de Haën, M=58.45 g/mol, [7647-14-5], cat. no. 31434
Thyroxine (T4), Sigma, M=776.87 g/mol, [51-48-9], cat. no. T2376
Prealbumine (TTR), Sigma, M ca. 55000 g/mol, [87090-18-4], cat. no.P-1742
Dimethylsulfoxide (DMSO), Acros, M=78,13 g/mol, [67-68-5], cat. no. 167852500

Materials

Refrigerator 4°C.
Vortex
2-20 µl pipette
20-200 µl pipette
200-1000 µl pipette
Multi pipette
Trays
Tappered vials, 1-2 ml, 12x32mm, screw
Screw caps 8 mm with PTFE-liner
Reaction tubes (1.5 ml)
200 µl Pipette tips with filter
Low binding 96 wells plates – black

Methods

Preparations

TRIS buffer:

Substance	Concentration	Mw.	Weight (gr)
TRIS-Base (4.1.2)	0.1 M	121.1	12.11
NaCl (4.1.5)	0.1 M	58.44	5.84
EDTA (4.1.1)	1 mM	372.23	0.372

Dissolve all of the above substances in 800 ml milli Q water. Adjust the pH to 8.0 with 1 M HCl. Bring the volume to a total volume of 1 liter with milliQ water.
Storage life: 2 month

TTR stock solution in Tris-buffer (3.64 µM)
Pay attention: TTR is a labile substance. Careful handling is required to prevent denaturation of the protein. Do not vortex!

Substance	Concentration	Mw.	Gewicht	Volume

TTR (prealbumine)	3.636 µM	55000	1 mg	5 ml

Divide the stock solution in portions of 40 µl in 1.5 ml reaction tubes. Store at -20 ºC.

Storage life: 1 year

TTR working solution (0.12 µM)

For 48 wells (1 96 well plate):
Add 2655 µl TRIS buffer to 95 µl of TTR stock solution.
Storage life: make new each experiment

T4 stock solution

Substance	Concentration	Mw.	Gewicht per DMSO
T4	1 mM	776.9	0.777 mg/ml

Weigh some T4 and add DMSO until a concentration of 1 mM. Divide into portions of 1 mL and store at -20ºC.
Storage life: 3 years

T4 calibration curve:

Standard	concentration T4 (µM)	Use standard for dilution	V standard (µl)	µl DMSO
1	200	T4 stock	200	800
2	60	T4 stock	60	940
3	20	1	100	900
4	6	2	100	900
5	2	3	100	900
6	0.6	4	100	900
7	0.2	5	100	900
0	0	n.v.t.	n.v.t.	200

Storage life: 1 year –20 °C.

Procedure:

- Dilute the FITC-T4 (75.2 µM stock in -80°C) to a concentration of 220 nM in 10.5 mL TRIS buffer. (Keep in dark, always make fresh, this is enough for 1x96 well plate).
- Pipette the 96 well plate with 2 µL of DMSO, T4 calibration or samples according to figure 1.
- The left half is for the TTR binding, the right half is for the background correction (-TTR).
- Add to the left half 50 µL of buffer and to the right half 100 µL of buffer.
- Add 50 µL of TTR working solution to the left half of the plate.
- Add 100 µL of T4-FITC to the entire plate.
- Shake for 5 minutes at 600 rpm on the plate shaker (no TEMP).
- Incubate 2 hrs at 4 ºC.
- Measure the plate using the FluTTR protocol on the varioscan.
- Make sure to turn on the machine, load the protocol and measure as fast as possible due to temperature shifts!
- Save the report and process data.

Figure 1: Layout 96 well plate

With TTR						Without TTR					
T4 0	T4 0	S1	S1	S9	S9	T4 0	T4 0	S1	S1	S9	S9
T4 2	T4 2	S2	S2	S10	S10	T4 2	T4 2	S2	S2	S10	S10
T4 6	T4 6	S3	S3	S11	S11	T4 6	T4 6	S3	S3	S11	S11
T4 20	T4 20	S4	S4	S12	S12	T4 20	T4 20	S4	S4	S12	S12
T4 60	T4 60	S5	S5	S13	S13	T4 60	T4 60	S5	S5	S13	S13
T4 200	T4 200	S6	S6	S14	S14	T4 200	T4 200	S6	S6	S14	S14
T4 600	T4 600	S7	S7	S15	S15	T4 600	T4 600	S7	S7	S15	S15
T4 2000	T4 2000	S8	S8	S16	S16	T4 2000	T4 2000	S8	S8	S16	S16

Data processing:

- Subtract the +TTR measurement from the –TTR measurement (e.g. A1-A7, B1-B7 etc etc)
- Process data like old T4 measurements.

Appendix X

SRM2585 Extraction Method (EDA)

This protocol contains the steps necessary for extraction of dust samples to be used in the T4-FITC-TTR assay and chemicals analysis using HPLC-Q-ToF-MS.

Extraction

1 Weight approximately 50mg of sample (House dust, dryer lint or SRM) in a 15mL polypropylene tube.

2 Add 5 mL of methanol.

3 Vortex the tube for 1 minute.

4 Ultra-sonicate for 15 minutes.

5 Centrifuge for 5 minutes at 1500 rpm (make sure a counter weight is added in the centrifuge (blank or another sample).

6 Transfer the supernatant to another polypropylene tube.

7 Perform steps 2-6 on the residue using 5 mL acetonitrile instead of methanol

8 Combine the supernatants

Clean-up

9 Activate the SPE cartridge 2 times with 5 ml methanol:acetonitrile (1:1 v/v)

10 Load sample extract on the SPE cartridge

11 Rinse SPE cartridge 4 times with 0.5 ml methanol:acetonitrile (1:1 v/v)

12 Evaporate collected solution to 0.2 ml under a gentle nitrogen stream at room temperature

13 Add 0.4 ml MilliQ

(Ouyang, et al., 2017)

Appendix XI

SRM 2585 Bioassay plate layout protocol of dilution extracts

(Make the SRM2585/blank dilutions in 100µL pellets)

SAMPLE 1	**SAMPLE 2**	**SAMPLE 3**	**SAMPLE 4**
3X Sample 1 (30µL sample 1 +60µL DMSO)	3X Sample 2 (30µL sample 2 +60µL DMSO)	3X Sample 3 (30µL sample 3 +60µL DMSO)	3X Sample 4 (30µL Sample 4+60µL DMSO)
10X Sample 1 (10µL sample 1 +90µL DMSO)	10X Sample 2 (10µL sample 2 +90µL DMSO)	10X Sample 3 (10µL sample 3 +90µL DMSO)	10X Sample 4 (10µL Sample 4+90µL DMSO)
30X Sample 1 (3µL sample 1 +87 µL DMSO)	30X Sample 2 (3µL sample 2 +87 µL DMSO)	30X Sample 3 (3µL sample 3 +87 µL DMSO)	30x Sample 4 (3µL Sample 4+87 µL DMSO)
SAMPLE 5	**SAMPLE 6**	**SAMPLE 7**	**BLANK 1**
3X Sample 5 (30µL sample 5 +60µL DMSO)	3X Sample 6 (30µL sample 6 +60µL DMSO)	3X Sample 7 (30µL sample 7 +60µL DMSO)	3X Blank 1 (30µL blank+60µL DMSO)
10X Sample 5 (10µL sample 5 +90µL DMSO)	10X Sample 6 (10µL sample 6 +90µL DMSO)	10X Sample 7 (10µL sample 7 +90µL DMSO)	10X Blank 1 (10µL blank+90µL DMSO)
30X Sample 5 (3µL sample 5 +87 µL DMSO)	30X Sample 6 (3µL sample 6 +87 µL DMSO)	30X Sample 7 (3µL sample 7 +87 µL DMSO)	30x Blank 1 (3µL blank+87 µL DMSO)
BLANK 2	**BLANK 3**		
3X Blank 2 (30µL Blank 2 +60µL DMSO)	3X Blank 3 (30µL Blank 3 +60µL DMSO)		
10X Blank 2 (10µL Blank 2 +90µL DMSO)	10X Blank 3 (10µL Blank 2 +90µL DMSO)		
30x Blank 2 (3µL Blank 2 +87 µL DMSO)	30x Blank 3 (3µL Blank 3 +87 µL DMSO)		

- **Bioassay:**

	With TTR						Without TTR					
	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO Blank	DMSO Blank	Procedure blank 1	Procedure blank 1	SRM 1	SRM 1	DMSO Blank	DMSO Blank	Procedure blank	Procedure blank	Procedure blank 2	Procedure blank 2
B	T4 7	T4 7	Procedure blank 1 3x	Procedure blank 1 3x	SRM 1 3x	SRM 1 3x	T4 7	T4 7	Procedure blank 1 3x	Procedure blank 1 3x	SRM 1 3x	SRM 1 3x
C	T4 6	T4 6	Procedure blank 1 10x	Procedure blank 1 10x	SRM 1 10x	SRM 1 10x	T4 6	T4 6	Procedure blank 1 10x	Procedure blank 1 10x	SRM 1 10x	SRM 1 10x
D	T4 5	T4 5	Procedure blank 1 30x	Procedure blank 1 30x	SRM 1 30x	SRM 1 30x	T4 5	T4 5	Procedure blank 1 30x	Procedure blank 1 30x	SRM 1 30x	SRM 1 30x
E	T4 4	T4 4	SRM 2	SRM 2	SRM 3	SRM 3	T4 4	T4 4	SRM 2	SRM 2	SRM 3	SRM 3
F	T4 3	T4 3	SRM 2 3x	SRM 2 3x	SRM 3 3x	SRM 3 3x	T4 3	T4 3	SRM 2 3x	SRM 2 3x	SRM 3 3x	SRM 3 3x
G	T4 2	T4 2	SRM 2 10x	SRM 2 10x	SRM 3 10x	SRM 3 10x	T4 2	T4 2	SRM 2 10x	SRM 2 10x	SRM 3 10x	SRM 3 10x
H	T4 1	T4 1	SRM 2 30x	SRM 2 30x	SRM 3 30x	SRM 3 30x	T4 1	T4 1	SRM 2 30x	SRM 2 30x	SRM 3 30x	SRM 3 30x

	With TTR						Without TTR					
	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO Blank	DMSO Blank	Procedure blank 2	Procedure blank 2	SRM 4	SRM 4	DMSO Blank	DMSO Blank	Procedure blank 2	Procedure blank 2	SRM 4	SRM 4
B	T4 7	T4 7	Procedure blank 2 3x	Procedure blank 2 3x	SRM 4 3x	SRM 4 3x	T4 7	T4 7	Procedure blank 2 3x	Procedure blank 2 3x	SRM 4 3x	SRM 4 3x
C	T4 6	T4 6	Procedure blank 2 10x	Procedure blank 2 10x	SRM 4 10x	SRM 4 10x	T4 6	T4 6	Procedure blank 2 10x	Procedure blank 2 10x	SRM 4 10x	SRM 4 10x
D	T4 5	T4 5	Procedure blank 2 30x	Procedure blank 2 30x	SRM 4 30x	SRM 4 30x	T4 5	T4 5	Procedure blank 2 30x	Procedure blank 2 30x	SRM 4 30x	SRM 4 30x
E	T4 4	T4 4	SRM 5	SRM 5	SRM 6	SRM 6	T4 4	T4 4	SRM 5	SRM 5	SRM 6	SRM 6
F	T4 3	T4 3	SRM 5 3x	SRM 5 3x	SRM 6 3x	SRM 6 3x	T4 3	T4 3	SRM 5 3x	SRM 5 3x	SRM 6 3x	SRM 6 3x
G	T4 2	T4 2	SRM 5 10x	SRM 5 10x	SRM 6 10x	SRM 6 10x	T4 2	T4 2	SRM 5 10x	SRM 5 10x	SRM 6 10x	SRM 6 10x
H	T4 1	T4 1	SRM 5 30x	SRM 5 30x	SRM 6 30x	SRM 6 30x	T4 1	T4 1	SRM 5 30x	SRM 5 30x	SRM 6 30x	SRM 6 30x

	With TTR						Without TTR					
	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO Blank	DMSO Blank	Procedure blank 1	Procedure blank 1	SRM 7	SRM 7	DMSO Blank	DMSO Blank	Procedure blank 1	Procedure blank 1	SRM 7	SRM 7
B	T4 7	T4 7	Procedure blank 3 3x	Procedure blank 3 3x	SRM 7 3x	SRM 7 3x	T4 7	T4 7	Procedure blank 3 3x	Procedure blank 3 3x	SRM 7 3x	SRM 7 3x
C	T4 6	T4 6	Procedure blank 3 10x	Procedure blank 3 10x	SRM 7 10x	SRM 7 10x	T4 6	T4 6	Procedure blank 3 10x	Procedure blank 3 10x	SRM 7 10x	SRM 7 10x
D	T4 5	T4 5	Procedure blank 3 30x	Procedure blank 3 30x	SRM 7 30x	SRM 7 30x	T4 5	T4 5	Procedure blank 3 30x	Procedure blank 3 30x	SRM 7 30x	SRM 7 30x
E	T4 4	T4 4					T4 4	T4 4				
F	T4 3	T4 3					T4 3	T4 3				
G	T4 2	T4 2					T4 2	T4 2				
H	T4 1	T4 1					T4 1	T4 1				

- Pipette 2µL of the calibration standards
- Pipette 2µL of the (SRM) + (procedure blank) samples after vortexing;
- Pipette 2µL of the (SRM) + (procedure blank) dilutions (x) and after vortexing;
- Add in 96 wells 50µL of TRIS buffer `
- Measure fluorescence + save data

+ TTR half: Add 50µL of TTR in the wells (48 wells)
→ *so far, total amount per well : 2µL X, 50µL of TRIS, 50µL of TTR in all the wells*

-**TTR half:** Add 50µL of TRIS buffer in the (48 wells)
→ *so far, total amount per well: 2µL X, 100µL of TRIS in all the wells*

- Measure fluorescence + save data
- Add 100µL of FITC-T4 in all the 96 wells of the plate (total volume/well: 202µL)
- Shake for 5min (600rpm)
- Incubate for 2hours
- Measure fluorescence + save data

SOLUTION PREPARATION

4 plates:

TTR working solution (-20°C fridge stock):

- 4 X 95µL TTR stock solution (from 2 vials) (using a 100µL pipette)
- 10 000µL + 1x620µL TRIS BUFFER

→ **DON'T VORTEX!**

FITC-T4 solution (-80°C fridge stock, C5-4-2):

- 4x 23.46µL FITC T4 (from 4 vials) (using 100µL pipette)
- 42mL TRIS buffer (2x 21mL)

→ **VORTEX**
→ **ALUMINIUM COVERAGE**

Appendix XII

Blood extraction HBM4EU

Spiking:

1: Pipet 3mL of serum or MilliQ in a 15ml polypropylene tube(weigh).

2: Add 30uL of spiking solution to the 3mL of serum (weigh).

3: Whirl mix the solution using the vortex mixer.

4: Distribute the 3ml of spiked serum, non-spiked serum or MilliQ in portion of 1ml in 3 15ml polypropylene tubes (weigh).

5: Place the tubes in the refrigerator at 4°C and equilibrate overnight.

Extraction:

1: Add 3ml of acetonitrile to the spiked serum, non-spiked serum or mille for protein precipitation (weigh).

2: Vortex the tubes for 20 seconds.

3: Ultra sonicate the tubes for 5 minutes.

4: Centrifuge for 5 minutes at 3000 rpm.

5: Separate and collect 3.8ml of the liquid layer (weigh).

6: Transfer half the volume (1.9ml) to a new 15mL polypropylene tube (weigh).

7: Evaporate at room temperature under a gentle nitrogen flow till a volume of approximately 0.1mL (weigh).

8: Resuspend one tube in 0.4mL DMSO and the other in 0.4mL 25% MeOH in MilliQ (weigh).

(HBM4EU, s.d.)

Appendix XIII

Blood extraction VU :

Spiking:

1: Pipet 1mL of serum or MilliQ in a 15ml polypropylene tube (weigh).

2: Add 10uL of spiking solution to the 1mL of serum (weigh).

3: Whirl mix the solution.

4: Place the tubes in the refrigerator at 4°C and equilibrate overnight.

Protein denaturation:

1: Add 1 mL (1:1 ratio!) formic acid (99%) in 2-propanol (4:1, v/v) (weigh)

2: Vortex the tubes for 20 seconds.

3: Ultra sonicate the tubes for 10 minutes.

4: Store in the dark at room temperature for 50 minutes.

5: Add 1 mL water/2-propanol (4:1, v/v) (weigh).

6: Sonication for 10 minutes

7: Dilute with water until organic solvent (2-propanol+spiking mix) is less than 5% (add 6ml of water) (weigh).

8: Centrifuge for 5 minutes at 3000 rpm and collect supernatant in new 15ml polypropylene tubes (weigh). If samples are clear, no need for centrifugation.

SPE Extraction (on Oasis MCX cartridges, 150 mg, 6 mL, Waters)

1: Condition cartridges with 3 mL MeOH

2: Equilibrate cartridges with 3 mL water

3: Loading samples

4: Wash with 3 mL water (containing 1.8% formic acid)

5: Dry SPE sorbent (vacuum)

6: Elute 4 x 0.75 mL MeOH (no vacuum)

7: Split eluate 1:1 into another glass test tube (weigh).

8: Evaporation under a gentle nitrogen stream at room temperature until 0.1 mL (weigh).

9: Resuspend one tube in 0.4mL DMSO and the other in 0.4mL MilliQ (weigh).

(Simon, et al., 2011)

Appendix XIV

Serum Bioassay plate layout protocol (dilution series of extracts)

SSP 1	**SSP2**	**SSP3**
3X SSP1 (30µL sample +60µL DMSO)	3X SSP2 (30µL sample+60µL DMSO)	3X SSP3 (30µL sample +60µL DMSO)
10X SSP 1 (10µL sample +90µL DMSO)	10X SSP2 (10µL sample+90µL DMSO)	10X SSP3 (10µL sample +90µL DMSO)
30X SSP 1 (10µL sample + 90µL DMSO from 3X)	30X SSP2 (10µL sample + 90µL DMSO from 3X)	30X SSP3 (10µL sample + 90µL DMSO from 3X)
100X SSP 1 (10µL sample + 90µL DMSO from 10X)	100X SSP2 (10µL sample + 90µL DMSO from 10X)	100X SSP3 (10µL sample + 90µL DMSO from 10X)
300X SSP 1 (10µL sample + 90µL DMSO from 30X)	300X SSP2 (10µL sample + 90µL DMSO from 30X)	300X SSP3 (10µL sample + 90µL DMSO from 30X)
1000X SSP 1 (10µL sample + 90µL DMSO from 100X)	1000X SSP2 (10µL sample + 90µL DMSO from 100X)	1000X SSP3 (10µL sample + 90µL DMSO from 100X)
3000X SSP 1 (10µL sample + 90µL DMSO from 300X)	3000X SSP2 (10µL sample + 90µL DMSO from 300X)	3000X SSP3 (10µL sample + 90µL DMSO from 300X)
SB 1	**SB2**	**SB3**
3X SB1 (30µL Sample +60µL DMSO)	3X SB2 (30µL sample +60µL DMSO)	3X SB3 (30µL sample +60µL DMSO)
10X SB1 (10µL Sample +90µL DMSO)	10X SB2 (10µL sample +90µL DMSO)	10X SB3 (10µL sample +90µL DMSO)
30x SB1 (10µL sample + 90µL DMSO from 3X)	30X SB2 (10µL sample + 90µL DMSO from 3X)	30X SB3 (10µL sample + 90µL DMSO from 3X)
100X SB1 (10µL sample + 90µL DMSO from 10X)	100X SB2 (10µL sample + 90µL DMSO from 10X)	100X SB3 (10µL sample + 90µL DMSO from 10X)
300X SB1 (10µL sample + 90µL DMSO from 30X)	300X SB2 (10µL sample + 90µL DMSO from 30X)	300X SB3 (10µL sample + 90µL DMSO from 30X)
1000X SB1 (10µL sample + 90µL DMSO from 100X)	1000X SB2 (10µL sample + 90µL DMSO from 100X)	1000X SB3 (10µL sample + 90µL DMSO from 100X)
3000X SB1 (10µL sample + 90µL DMSO from 300X)	3000X SB2 (10µL sample + 90µL DMSO from 300X)	3000X SB3 (10µL sample + 90µL DMSO from 300X)
PB 1	**PB 2**	**PB 3**
3X PB1 (30µL sample +60µL DMSO)	3X PB2 (30µL sample +60µL DMSO)	3X PB3 (30µL sample +60µL DMSO)
10X PB1 (10µL sample +90µL DMSO)	10X PB2 (10µL sample +90µL DMSO)	10X PB3 (10µL sample +90µL DMSO)
30X PB1 (3µL sample +87 µL DMSO)	30x PB2 (3µL sample +87 µL DMSO)	30x PB3 (3µL sample +87 µL DMSO)

- **Bioassay:**

	With TTR						Without TTR					
	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO Blank	DMSO Blank	Procedure SB**1** 1X	Procedure SB**1** 1X	Procedure SSP**1** 1X	Procedure SSP**2** 1X	DMSO Blank	DMSO Blank	Procedure SB**1** 1X	Procedure SB**1** 1X	Procedure SSP**1** 1X	Procedure SSP**2** 1X
B	T4 7	T4 7	SB1 3x	SB1 3x	SSP1 3x	SSP1 3x	T4 7	T4 7	SB1 3x	SB1 3x	SSP1 3x	SSP1 3x
C	T4 6	T4 6	SB1 10x	SB1 10x	SSP1 10x	SSP1 10x	T4 6	T4 6	SB1 10x	SB1 10x	SSP1 10x	SSP1 10x
D	T4 5	T4 5	SB1 30x	SB1 30x	SSP1 30x	SSP1 30x	T4 5	T4 5	SB1 30x	SB1 30x	SSP1 30x	SSP1 30x
E	T4 4	T4 4	SB1 100x	SB1 100x	SSP1 100x	SSP1 100x	T4 4	T4 4	SB1 100x	SB1 100x	SSP1 100x	SSP1 100x
F	T4 3	T4 3	SB1 300x	SB1 300x	SSP1 300x	SSP1 300x	T4 3	T4 3	SB1 300x	SB1 300x	SSP1 300x	SSP1 300x
G	T4 2	T4 2	SB1 1000x	SB1 1000x	SSP1 1000x	SSP1 1000x	T4 2	T4 2	SB1 1000x	SB1 1000x	SSP1 1000x	SSP1 1000x
H	T4 1	T4 1	SB1 3000x	SB1 3000x	SSP1 3000x	SSP1 3000x	T4 1	T4 1	SB1 3000x	SB1 3000x	SSP1 3000x	SSP1 3000x

	With TTR						Without TTR					
	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO Blank	DMSO Blank	Procedure SB**2** 1X	Procedure SB**2** 1X	Procedure SSP**2** 1X	Procedure SSP**2** 1X	DMSO Blank	DMSO Blank	Procedure SB**2** 1X	Procedure SB**2** 1X	Procedure SSP**2** 1X	Procedure SSP**2** 1X
B	T4 7	T4 7	SB2 3x	SB2 3x	SSP2 3x	SSP2 3x	T4 7	T4 7	SB2 3x	SB2 3x	SSP2 3x	SSP2 3x
C	T4 6	T4 6	SB2 10x	SB2 10x	SSP2 10x	SSP2 10x	T4 6	T4 6	SB2 10x	SB2 10x	SSP2 10x	SSP2 10x
D	T4 5	T4 5	SB2 30x	SB2 30x	SSP2 30x	SSP2 30x	T4 5	T4 5	SB2 30x	SB2 30x	SSP2 30x	SSP2 30x
E	T4 4	T4 4	SB2 100x	SB2 100x	SSP2 100x	SSP2 100x	T4 4	T4 4	SB2 100x	SB2 100x	SSP2 100x	SSP2 100x
F	T4 3	T4 3	SB2 300x	SB2 300x	SSP2 300x	SSP2 300x	T4 3	T4 3	SB2 300x	SB2 300x	SSP2 300x	SSP2 300x
G	T4 2	T4 2	SB2 1000x	SB2 1000x	SSP2 1000x	SSP2 1000x	T4 2	T4 2	SB2 1000x	SB2 1000x	SSP2 1000x	SSP2 1000x
H	T4 1	T4 1	SB2 3000x	SB2 3000x	SSP2 3000x	SSP2 3000x	T4 1	T4 1	SB2 3000x	SB2 3000x	SSP2 3000x	SSP2 3000x

	With TTR						Without TTR					
	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO Blank	DMSO Blank	Procedure SB**3** 1X	Procedure SB**3** 1X	Procedure SSP**3** 1X	Procedure SSP**3** 1X	DMSO Blank	DMSO Blank	Procedure SB**3** 1X	Procedure SB**3** 1X	Procedure SSP**3** 1X	Procedure SSP**3** 1X
B	T4 7	T4 7	SB3 3x	SB3 3x	SSP3 3x	SSP3 3x	T4 7	T4 7	SB3 3x	SB3 3x	SSP3 3x	SSP3 3x
C	T4 6	T4 6	SB3 10x	SB3 10x	SSP3 10x	SSP3 10x	T4 6	T4 6	SB3 10x	SB3 10x	SSP3 10x	SSP3 10x
D	T4 5	T4 5	SB3 30x	SB3 30x	SSP3 30x	SSP3 30x	T4 5	T4 5	SB3 30x	SB3 30x	SSP3 30x	SSP3 30x
E	T4 4	T4 4	SB3 100x	SB3 100x	SSP3 100x	SSP3 100x	T4 4	T4 4	SB3 100x	SB3 100x	SSP3 100x	SSP3 100x
F	T4 3	T4 3	SB3 300x	SB3 300x	SSP3 300x	SSP3 300x	T4 3	T4 3	SB3 300x	SB3 300x	SSP3 300x	SSP3 300x
G	T4 2	T4 2	SB3 1000x	SB3 1000x	SSP3 1000x	SSP3 1000x	T4 2	T4 2	SB3 1000x	SB3 1000x	SSP3 1000x	SSP3 1000x
H	T4 1	T4 1	SB3 3000x	SB3 3000x	SSP3 3000x	SSP3 3000x	T4 1	T4 1	SB3 3000x	SB3 3000x	SSP3 3000x	SSP3 3000x

	With TTR						Without TTR					
	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO Blank	DMSO Blank	Procedure PB1 1X	Procedure PB1 1X	Procedure PB2 1X	Procedure PB2 1X	DMSO Blank	DMSO Blank	Procedure PB1 1X	Procedure PB1 1X	Procedure PB2 1X	Procedure PB2 1X
B	T4 7	T4 7	PB1 3x	PB1 3x	PB2 3x	PB2 3x	T4 7	T4 7	PB1 3x	PB1 3x	PB2 3x	PB2 3x
C	T4 6	T4 6	PB1 10x	PB1 10x	PB2 10x	PB2 10x	T4 6	T4 6	PB1 10x	PB1 10x	PB2 10x	PB2 10x
D	T4 5	T4 5	PB1 30x	PB1 30x	PB2 30x	PB2 30x	T4 5	T4 5	PB1 30x	PB1 30x	PB2 30x	PB2 30x
E	T4 4	T4 4	Procedure PB3 1X	Procedure PB3 1X			T4 4	T4 4	Procedure PB3 1X	Procedure PB3 1X		
F	T4 3	T4 3	PB3 3x	PB3 3x			T4 3	T4 3	PB3 3x	PB3 3x		
G	T4 2	T4 2	PB3 10x	PB3 10x			T4 2	T4 2	PB3 10x	PB3 10x		
H	T4 1	T4 1	PB3 30x	PB3 30x			T4 1	T4 1	PB3 30x	PB3 30x		

- Pipette 2µL of the calibration standards
- Pipette 2µL of the samples+ sample dilutions after vortexing;
- Add in 96 wells 50µL of TRIS buffer `
- Measure fluorescence + save data

+ TTR half: Add 50µL of TTR in the wells (48 wells)
→ *so far, total amount per well : 2µL X, 50µL of TRIS, 50µL of TTR in all the wells*

-**TTR half:** Add 50µL of TRIS buffer in the (48 wells)
→ *so far, total amount per well: 2µL X, 100µL of TRIS in all the wells*

- Measure fluorescence + save data
- Add 100µL of FITC-T4 in all the 96 wells of the plate (total volume/well: 202µL)
- Shake for 5min (600rpm)
- Incubate for 2hours
- Measure fluorescence + save data

SOLUTION PREPARATION

4 plates

TTR working solution (-20°C fridge stock):

- 4 x 95µL TTR stock solution (from 4 vials) (using a 100µL pipette)
- 10 620 uL TRIS Buffer (10mL + 620uL)

→ **DON'T VORTEX!**

FITC-T4 solution (-80°C fridge stock, C5-4-2):

- 4 x 23.46µL FITC T4 (using 100µL pipette)
- 42 mL TRIS buffer (use 2 x 21mL)

→ **VORTEX**
→ **ALUMINIUM COVERAGE**

Appendix XX

Liquid chromatography (coupled to Q-ToF-MS or FractioMate) laboratory experiment protocol

Day 1

1. LC preparation			**Total time**
Step	Machine	Computer	1h12
	• Take out previous column • Solvent A_1: MilliQ • Solvent B_1: ACN	• Open sample table in LC software • Open a xml file • Select row; click acquisition	15 min
Pump purging (5min)	• Fix column	• PRIME	7 min
Auto-sampler purging (3min)		• PURGE	10 min
Acclimatising (15 min + 15 min)		• Pump: A:50/B:50 (15min) • Pump: A:90/B:10 (15min)	40 min

2. FractionMate			**Total time**
Step	Prepare/Machine	Computer	6h
Pre-fractionation	• Add 10µL DMSO (10%) in 80 wells per plate (0.2mL DMSO and 1.8mL milliQ)		20 min (10:40- 11:00)
Fractionation		• Action: select 96 tray; choose plate position (**backtray** or front-tray) • Adjust rows/columns • Activation setting: select start delay • Total spot time: 18min • Click Operate MS software (purple color) • Start run LC software → press start Fractionmate (turn on) (35 min) (X4) • After spotting: save list well spotted @time • STORE plate: **close the plates with tape**	2h40 (11:15-13:55)

3. Q-ToF-MS			**Total time**
Step	Machine	Computer	1h50
Direct injection (day 1)	• Connect syringe tube to **NEBULIZER** • Kd Scientific: 180µL/h (rate) • Speed injection up (right arrow; RUN button together) • Press run (Kd Scientific) (**BEFORE** press operate MS software) → arrow blinking	• Press operate (MS software) (**AFTER** press run Kd Scientific) • MS Software check: - NEGATIVE polarity - Na Formate - HPC Press zooming 1% + calibrate → check score: >99% re-calibrate/change zooming % to less (0.5%) → **CLICK ACCEPT!** • turn pump on (right click; control; pump ON; ok) → Check leaks (if yes, clean sensors)	20 min (14:00 -14:20)
Sample analysis	• Kd Scientific: 35µL/h (**BEFORE** LC start run) • **PUT TUBE BACK TO MS!**	• LC software: start run (**AFTER** Kd scientific run) (35min) (X2) (check if Line: 1, blank)	3hmin (14h30-17h30)

Day 2

Evaporation	• Put the plates in centrifuge (between the bumps) • Press on program 1 of centrifuge centrivap		8h20 (8:00-16:20)
Incubation	• put 50µL TRIS buffer in all wells with fractions (80 wells) • **close the plates with tape** • incubate the plates overnight at 4°C		overnight

Day 3

		Total time:
		3h10
preparation	• take plates out of the fridge • mix the plate using a plate mixer (700rpm) (30min)	35min (8:55-9:30)
Fluorescence analysis	• software: SkanitRE for Varioscanflash 2.4.5 • open method • open tray; • insert plate • close tray • execute	25min (9:40-10:05)

1. Pipetting Start 10h15 End 12h00	**Total time:**
	45 min (10:15-12:00)

Plate 1, 2, 3, 4:

		With TTR									
DMSO blank -TTR	**DMSO blank -TTR**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
DMSO blank +TTR	**DMSO blank +TTR**	**S20**	**S19**	**S18**	**S17**	**S16**	**S15**	**S14**	**S13**	**S12**	**S11**
T4 St.7 +TTR	**T4 St.7 +TTR**	**S21**	**S22**	**S23**	**S24**	**S25**	**S26**	**S27**	**S28**	**S29**	**S30**
T4 St.6 +TTR	**T4 St.6 +TTR**	**S40**	**S39**	**S38**	**S37**	**S36**	**S35**	**S34**	**S33**	**S32**	**S31**
T4 St.5 +TTR	**T4 St.5 +TTR**	**S41**	**S42**	**S43**	**S44**	**S45**	**S46**	**S47**	**S48**	**S49**	**S50**
T4 St.4 + TTR	**T4 St.4 + TTR**	**S60**	**S59**	**S58**	**S57**	**S56**	**S55**	**S54**	**S53**	**S52**	**S51**
T4 St.2 + TTR	**T4 St.2 + TTR**	**S61**	**S62**	**S63**	**S64**	**S65**	**S66**	**S67**	**S68**	**S69**	**S70**
T4 St.1 + TTR	**T4 St.1 + TTR**	**S80**	**S79**	**S78**	**S77**	**S76**	**S75**	**S74**	**S73**	**S72**	**S71**

- Add 2µL calibration curve standards {7,6,5,4,2,1}
- Add in DMSO –TTR blank 50µL TRIS Buffer
- Add in +TTR wells 50µL TTR working solution
- Add 100µL FITC-T_4 solution

Appendix XXI

Compounds in SRM2585

Table 1. Certified Concentrations for Selected PAHs in SRM 2585

	Mass Fraction (dry-mass basis)[a] (µg/kg)
Naphthalene[d,e,f]	266 ± 8[b]
Dibenzothiophene[d,e,f,g]	109 ± 8[c]
Phenanthrene[d,e,f,g,h,i]	1920 ± 20[c]
Anthracene[d,e,f,g,h,i]	96.0 ± 5.2[c]
4H-cyclopenta[*def*]phenanthrene[d,e,f,g]	117 ± 10[c]
3-Methylphenanthrene[e,f,g,j]	293 ± 36[c]
2-Methylphenathrene[e,f,g]	352 ± 40[b]
9-Methylphenanthrene[e,f,g,j]	205 ± 16[c]
1-Methylphenanthrene[e,f,g]	197 ± 29[b]
Fluoranthene[d,e,f,g,h,i]	4380 ± 100[c]
Pyrene[d,e,f,g,h,i]	3290 ± 30[c]
Benzo[*ghi*]fluoranthene[d,e,f,g,h,i]	317 ± 11[c]
Benzo[c]phenanthrene[d,e,f,g,h,i]	288 ± 10[c]
Benz[*a*]anthracene[d,e,f,g,h,i]	1160 ± 54[c]
Chrysene[e,h,i]	2260 ± 60[b]
Triphenylene[e,h,i]	589 ± 17[b]
Benzo[*b*]fluoranthene[d,f,g,h,i,j]	2700 ± 90[c]
Benzo[*j*]fluoranthene[d,f,g,j]	1320 ± 110[c]
Benzo[*k*]fluoranthene[d,f,g,h,j]	1330 ± 70[c]
Benzo[*a*]fluoranthene[d,f,g,j]	74.5 ± 8.1[c]
Benzo[*e*]pyrene[d,f,g,h,i,j]	2160 ± 80[c]
Benzo[*a*]pyrene[d,f,g,h,i,j]	1140 ± 10[c]
Perylene[d,f,g,h,i,j]	387 ± 23[c]
Benzo[*ghi*]perylene[d,f,g,h,i,j]	2280 ± 40[c]
Indeno[1,2,3-*cd*]pyrene[d,f,g,h,i,j]	2080 ± 100[c]
Dibenz[*a,j*]anthracene[d,f,g,h,i,j]	267 ± 9[c]
Dibenz[*a,c*]anthracene[d,f,g,h,i,j]	183 ± 25[c]
Dibenz[*a,h*]anthracene[d,f,g,h,i,j]	301 ± 50[c]
Benzo[*b*]chrysene[d,f,g,h,i]	182 ± 6[c]
Picene[d,f,g,h,j]	413 ± 15[c]
Coronene[d,f,g,h,i,j]	603 ± 38[c]
Dibenzo[*b,k*]fluoranthene[d,f,g,h,i,j]	596 ± 22[c]
Dibenzo[*a,e*]pyrene[d,f,g,h,i,j]	477 ± 67[c]

[a] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture.
[b] Certified values are unweighted means of the results from three analytical methods. The uncertainty listed with the value is an expanded uncertainty about the mean, with coverage factor 2, calculated by combining a between-method variance [13] with a pooled, within-method variance following the ISO Guides [2].
[c] Certified values are weighted means of the results from four to six analytical methods [14]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), except for phenanthrene and benzo[*a*]pyrene for which a coverage factor of 4 was used, calculated by combining a between-method variance incorporating inter-method bias with a pooled within-source variance following the ISO Guide [2].
[d] GC/MS (Ia) on a 50 % phenyl-substituted methylpolysiloxane phase after PFE with DCM.
[e] GC/MS (IIIa) on a relatively non-polar proprietary phase after Soxhlet extraction with 50 % hexane/50 % acetone mixture.
[f] GC/MS (IV) on a 50 % phenyl-substituted methylpolysiloxane phase after PFE with DCM.
[g] GC/MS (IIa) on a 50 % phenyl-substituted methylpolysiloxane phase after PFE with DCM.
[h] GC/MS (Ib) on a liquid crystalline phase after PFE with DCM.
[i] GC/MS (IIb) on a liquid crystalline phase after PFE with DCM.
[j] GC/MS (IIIb) on a 50 % phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with 50 % hexane/50 % acetone mixture.

(National Institute of Standards & Technology, 2011)

Table 2. Certified Concentrations for Selected PCB Congeners in SRM 2585

PCB Congener[a]		Mass Fraction (dry-mass basis)[b] (µg/kg)		
PCB 18	(2,2',5-Trichlorobiphenyl)[e,f,g,h]	12.8	±	1.0[c]
PCB 28	(2,4,4'-Trichlorobiphenyl)[f,g]	13.4	±	0.5[d]
PCB 31	(2,4',5-Trichlorobiphenyl)[f,g]	14.0	±	0.5[d]
PCB 44	(2,2'3,5'-Tetrachlorobiphenyl)[e,f,g,h]	18.1	±	1.9[c]
PCB 52	(2,2',5,5'-Tetrachlorobiphenyl)[e,f,g,h]	21.8	±	1.9[c]
PCB 56	(2,3,3',4-Tetrachlorobiphenyl)[e,f,g]	4.42	±	0.28[d]
PCB 70	(2,3',4',5-Tetrachlorobiphenyl)[e,f,g,h]	13.1	±	1.2[c]
PCB 74	(2,4,4',5-Tetrachlorobiphenyl)[e,f,g,h]	5.22	±	0.51[c]
PCB 87	(2,2',3,4,5'-Pentachlorobiphenyl)[e,f,g,h]	16.6	±	0.8[c]
PCB 92	(2,2',3,5,5'-Pentachlorobiphenyl)[f,g,h]	5.48	±	0.72[d]
PCB 95	(2,2',3,5',6-Pentachlorobiphenyl)[f,g,h]	22.7	±	2.6[d]
PCB 99	(2,2',4,4',5-Pentachlorobiphenyl)[,f,g,h]	11.6	±	0.4[c]
PCB 101	(2,2',4,5,5'-Pentachlorobiphenyl)[f,g,h]	29.8	±	2.3[d]
PCB 105	(2,3,3',4,4'-Pentachlorobiphenyl)[f,g,h]	13.2	±	1.4[d]
PCB 107	(2,3,3',4,5'-Pentachlorobiphenyl)[e,f,g]	4.14	±	0.47[d]
PCB 110	(2,3,3',4',6-Pentachlorobiphenyl)[e,f,g,h]	28.1	±	3.7[c]
PCB 118	(2,3',4,4',5-Pentachlorobiphenyl)[e,f,g,h]	26.3	±	1.7[c]
PCB 138	(2,2',3,4,4',5'-Hexachlorobiphenyl)[e,f,g,h]	27.6	±	2.1[c]
PCB 146	(2,2',3,4',5,5'-Hexachlorobiphenyl)[e,f,g,h]	4.89	±	0.38[c]
PCB 149	(2,2',3,4',5',6-Hexachlorobiphenyl)[e,f,g,h]	24.4	±	1.9[c]
PCB 151	(2,2',3,5,5',6-Hexachlorobiphenyl)[f,g,h]	6.92	±	0.64[d]
PCB 153	(2,2',4,4',5,5'-Hexachlorobiphenyl)[e,f,g,h]	40.2	±	1.8[c]
132	(2,2',3,3',4,6'-Hexachlorobiphenyl)[i]			
PCB 158	(2,3,3',4,4',6-Hexachlorobiphenyl)[e,f,g,h,i]	4.50	±	0.43[c]
PCB 163	(2,3,3',4',5,6-Hexachlorobiphenyl)[e,f,g,h]	7.2	±	1.2[c]
PCB 170	(2,2',3,3',4,4',5-Heptachlorobiphenyl)[e,f,g,h]	8.8	±	1.0[c]
PCB 174	(2,2',3,3',4,5,6'-Heptachlorobiphenyl)[e,f,g,h]	8.83	±	0.47[c]
PCB 180	(2,2',3,4,4',5,5'-Heptachlorobiphenyl)[e,f,h]	18.4	±	3.2[d]
PCB 183	(2,2',3,4,4',5',6-Heptachlorobiphenyl)[e,f,g,h]	5.27	±	0.39[c]
PCB 187	(2,2',3,4',5,5',6-Heptachlorobiphenyl)[e,f,g,h]	11.3	±	1.4[c]
PCB 206	(2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl)[e,h]	3.81	±	0.13[d]

[a] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [15] and later revised by Schulte and Malisch [16] to conform with IUPAC rules; for the specific congeners mentioned in this SRM, PCB 107 is different in the numbering systems. Under the Ballschmiter and Zell numbering system, the IUPAC PCB 107 is listed as PCB 108.

[b] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture.

[c] Certified values are weighted means of the results from four analytical methods [14]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), calculated by combining a between-method variance incorporating inter-method bias with a pooled within-source variance following the ISO Guide [2].

[d] Certified values are unweighted means of the results from two or three analytical methods. The uncertainty listed with the value is an expanded uncertainty about the mean, with coverage factor 2, calculated by combining a between-method variance [13] with a pooled, within-method variance following the ISO Guide [2].

[e] GC-ECD (I) on a relatively non-polar proprietary phase after PFE extraction with DCM.

[f] GC/MS (I) on a relatively non-polar proprietary phase; same extracts analyzed as in GC-ECD (I).

[g] GC/MS (II) on a 50 % phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with 50 % hexane/50 % acetone mixture.

[h] GC/MS (III) on a 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM.

[i] PCB 153 is the primary component with PCB 132 contributing at most 10 % to the concentration based on the data from method GC/MS (II).

(National Institute of Standards & Technology, 2011)

Table 3. Certified Concentrations for Selected Chlorinated Pesticides in SRM 2585

	Mass Fraction (dry-mass basis)[a] (µg/kg)		
4,4'-DDE[d,e,f,g]	261	±	2[b]
4,4'-DDD[d,f]	27.3	±	0.8[c]
2,4'-DDT[d,f]	44.5	±	3.9[c]
4,4'-DDT[d,f,g]	111	±	23[c]

[a] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture.
[b] Certified values are weighted means of the results from four analytical methods [14]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence) except for 4,4'-DDE with a coverage factor of 10, calculated by combining a between-method variance incorporating inter-method bias with a pooled within-source variance following the ISO Guide [2].
[c] Certified values are unweighted means of the results from two or three analytical methods. The uncertainty listed with the value is an expanded uncertainty about the mean, with coverage factor 2, calculated by combining a between-method variance [13] with a pooled, within-method variance following the ISO Guide [2].
[d] GC-ECD (I) on a relatively non-polar proprietary phase after PFE extraction with DCM.
[e] GC/MS (I) on a relatively non-polar proprietary phase; same extracts analyzed as in GC-ECD (I).
[f] GC/MS (II) on a 50 % phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with 50 % hexane/50 % acetone mixture.
[g] GC/MS (III) on a 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM.

Table 4. Certified Concentrations for Selected PBDE Congeners in SRM 2585

PBDE Congener[a]		Mass Fraction (dry-mass basis) (µg/kg)		
PBDE 17	(2,2',4-Tribromodiphenyl Ether)[c,d,e,f]	11.5	±	1.2[b]
PBDE 28	(2,4,4'-Tribromodiphenyl Ether)[c,d,e,f]	46.9	±	4.4[b]
33	(2',3,4-Tribromodiphenyl Ether)[g]			
PBDE 47	(2,2',4,4'-Tetrabromodiphenyl Ether)[c,d,e,f]	497	±	46[b]
PBDE 49	(2,2',4,5'-Tetrabromodiphenyl Ether)[c,d,e]	53.5	±	4.2[b]
PBDE 85	(2,2',3,4,4'-Pentabromodiphenyl Ether)[c,d,e,f]	43.8	±	1.6[b]
PBDE 99	(2,2',4,4',5-Pentabromodiphenyl Ether)[c,d,e,f]	892	±	53[b]
PBDE 100	(2,2',4,4',6-Pentabromodiphenyl Ether)[c,d,e,f]	145	±	11[b]
PBDE 138	(2,2',3,4,4',5'-Hexabromodiphenyl Ether)[c,d,e,f]	15.2	±	2.0[b]
PBDE 153	(2,2',4,4',5,5'-Hexabromodiphenyl Ether)[c,d,e,f]	119	±	1[b]
PBDE 154	(2,2',4,4',5,6'-Hexabromodiphenyl Ether)[d,e]	83.5	±	2.0[h]
PBDE 155	(2,2',4,4',6,6'-Hexabromodiphenyl Ether)[d,e]	3.94	±	0.34[h]
PBDE 183	(2,2',3,4,4',5',6-Heptabromodiphenyl Ether)[c,d,e,f]	43.0	±	3.5[b]
PBDE 203	(2,2',3,4,4',5,6',6-Octabromodiphenyl Ether)[c,e]	36.7	±	6.4[h]
PBDE 206	(2,2',3,3',4,4',5,5',6-Nonabromodiphenyl Ether)[c,e]	271	±	42[h]
PBDE 209	(Decabromodiphenyl Ether)[c,e,f]	2510	±	190[h]

[a] PBDE congeners are numbered according to IUPAC rules.
[b] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture. Certified values are weighted means of the results from four analytical methods [14]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence) except for PBDE 153 with a coverage factor of 10, calculated by combining a between-source variance incorporating inter-method bias with a pooled within-source variance following the ISO [2].
[c] GC/NCI-MS (I) on a 15 m 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM.
[d] GC/EI-MS (I) on a 15 m 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM (same extracts as GC/NCI-MS (I)).
[e] GC/EI-MS (II) on a 60 m 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM.
[f] GC/NCI-MS (II) on a 15 m 5 % phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with DCM.
[g] Using the 15 m column, PBDE 28 and PBDE 33 coelute; however, using the 60 m column, the two isomers are separated. Based on the data from the 60 m column, the concentration of PBDE 33 is less than the detection limit of 2.2 µg/kg.
[h] Certified values are unweighted means of the results from two or three analytical methods. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2, calculated by combining a between-method variance [23] with a pooled, within-method variance following the ISO Guide [2].

(National Institute of Standards & Technology, 2011)

Table 5. Reference Concentrations for Selected PAHs in SRM 2585

	Mass Fraction (dry-mass basis)[a] (µg/kg)		
1-Methylnaphthalene[d,e,f]	150	±	37[b]
2-Methylnaphthalene[d,e,f]	227	±	20[b]
Biphenyl[e,f,g]	88	±	21[b]
Retene[d]	588	±	34[c]
1,7-Dimethylphenanthrene[f,g]	219	±	19[b]
1-Methylfluoranthene[d,f,h]	94	±	11[b]
3-Methylfluoranthene[d,f,h]	235	±	67[b]
8-Methylfluoranthene[d]	132	±	7[c]
4-Methylpyrene[d,f,h]	235	±	27[b]
2-Methylpyrene[d]	345	±	10[c]
1-Methylpyrene[d,f,h]	209	±	69[b]
3-Methylchrysene[d,f,h]	146	±	18[b]
2-Methylchrysene[d]	181	±	4[c]
6-Methylchrysene[d,f]	88	±	14[b]
4-Methyl- and 1-Methylchrysene[d]	94.8	±	4.8[c]
9-Methyl- and 3-Methylbenz[*a*]anthracene[d]	92.3	±	2.5[c]
6-Methyl- and 1-Methylbenz[*a*]anthracene[d]	155	±	5[c]
Anthanthrene[f,g,i]	91	±	27[b]
Dibenzo[*b*,*e*]fluoranthene[f,g,j]	59.6	±	7.5[b]
Naphtho[1,2-*b*]fluoranthene[g,i]	312	±	10[b]
Naphtho[1,2-*k*] and naphtho[2,3-*j*]fluoranthene[f,g,i]	382	±	18[b]
Naphtho[2,3-*b*]fluoranthene[f,g,i]	93	±	30[b]
Naphtho[2,3-*k*]fluoranthene[i]	24.7	±	1.2[c]
Dibenzo[*a*,*k*]fluoranthene[f,j]	14.3	±	3.4[b]
Dibenzo[*j*,*l*]fluoranthene[f,g,i,j]	260	±	26[k]
Dibenzo[*a*,*l*]pyrene[f,g,j]	42.3	±	3.1[b]
Naphtho[2,3-*k*]fluoranthene and Naphtho[1,2-*a*]pyrene[f,g,j]	44.3	±	1.9[b]
Naphtho[2,3-*e*]pyrene[f,g,j]	145	±	29[b]
Naphtho[2,1-*a*]pyrene[f,g,i,j]	379	±	47[k]
Dibenzo[*e*,*l*]pyrene[f,g,i,j]	208	±	14[k]
Benzo[*b*]perylene[g,i]	103	±	24[b]
Dibenzo[*a*,*i*]pyrene[g,i]	105	±	11[b]
Dibenzo[*a*,*h*]pyrene[f]	20.9	±	0.7[c]

[a] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture.
[b] Reference values are unweighted means of the results from two or three analytical methods. The uncertainty listed with the value is an expanded uncertainty about the mean, with coverage factor 2, calculated by combining a between-method variance [13] with a pooled, within-method variance following the ISO Guide [2].
[c] Reference values are the means of results obtained by NIST using one analytical technique. The expanded uncertainty, U, is calculated as $U = ku_c$, where u_c is one standard deviation of the analyte mean, and the coverage factor, k, is determined from the Student's t-distribution corresponding to the associated degrees of freedom and 95 % confidence level for each analyte.
[d] GC/MS (IIa) on a 50 % phenyl-substituted methylpolysiloxane phase after PFE with DCM.
[e] GC/MS (IIIa) on a relatively non-polar proprietary phase after Soxhlet extraction with 50 % hexane/50 % acetone mixture.
[f] GC/MS (IV) on a 50 % phenyl-substituted methylpolysiloxane phase after PFE with DCM.
[g] GC/MS (Ia) on a 50 % phenyl-substituted methylpolysiloxane phase after PFE with DCM.
[h] GC/MS (IIb) on a liquid crystalline phase after PFE with DCM.
[i] GC/MS (Ib) on a liquid crystalline phase after PFE with DCM.
[j] GC/MS (IIIb) on a 50 % phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with 50 % hexane/50 % acetone mixture.
[k] Reference values are weighted means of the results from four analytical methods [14]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), calculated by combining a between-method variance incorporating inter-method bias with a pooled within-source variance following the ISO Guide [2].

(National Institute of Standards & Technology, 2011)

Table 6. Reference Concentrations for Selected PCB Congeners in SRM 2585

PCB Congener[a]		Mass Fraction (dry-mass basis)[b] (µg/kg)		
PCB 49	(2,2'4,5'-Tetrachlorobiphenyl)[e,f,g,h]	16.4	±	3.3[c]
PCB 63	(2,3,4',5-Tetrachlorobiphenyl)[e]	6.69	±	0.26[d]
PCB 66	(2,3',4,4'-Tetrachlorobiphenyl)[f,g,h]	8.5	±	1.9[e]
PCB 121	(2,3',4,5',6-Pentachlorobiphenyl)[h]	18.7	±	0.4[d]
PCB 128	(2,2',3,3',4,4'-Hexachlorobiphenyl)[g,h]	8.1	±	1.6[e]
PCB 177	(2,2',3,3',4,5,6-Heptachlorobiphenyl)[h]	5.50	±	0.44[d]
PCB 178	(2,2',3,3',5,5',6'-Heptachlorobiphenyl)[h]	2.17	±	0.16[d]
PCB 185	(2,2',3,4,5,5',6-Heptachlorobiphenyl)[h]	5.32	±	0.39[d]
PCB 193	(2,3,3',4',5,5',6-Heptachlorobiphenyl)[e]	1.23	±	0.070[d]
PCB 194	(2,2',3,3',4,4',5,5'-Octachlorobiphenyl)[e,h]	4.47	±	0.76[e]
PCB 199	(2,2',3,3',4,5,6,6'-Octachlorobiphenyl)[h]	5.81	±	0.38[d]
PCB 209	Decachlorobiphenyl[e]	2.14	±	0.11[d]

[a] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [15] and later revised by Schulte and Malisch [16] to conform with IUPAC rules; for the specific congeners mentioned in this SRM.
[b] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture.
[c] Reference values are weighted means of the results from four analytical methods [14]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), calculated by combining a between-method variance incorporating inter-method bias with a pooled within-source variance following the ISO Guide [2].
[d] Reference values are the means of results obtained by NIST using one analytical technique. The expanded uncertainty, U, is calculated as $U = ku_c$, where u_c is one standard deviation of the analyte mean, and the coverage factor, k, is determined from the Student's t-distribution corresponding to the associated degrees of freedom and 95 % confidence level for each analyte.
[e] Reference values are unweighted means of the results from two to three analytical methods. The uncertainty listed with the value is an expanded uncertainty about the mean, with coverage factor 2, calculated by combining a between-method variance [13] with a pooled, within-method variance following the ISO Guide [2].
[f] GC-ECD (I) on a relatively non-polar proprietary phase after PFE extraction with DCM.
[g] GC/MS (I) on a relatively non-polar proprietary phase; same extracts analyzed as in GC-ECD (I).
[h] GC/MS (II) on a 50 % phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with 50 % hexane/50 % acetone mixture.
[i] GC/MS (III) on a 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM.

(National Institute of Standards & Technology, 2011)

Table 7. Reference Concentrations for Selected Chlorinated Pesticides in SRM 2585

	Mass Fraction (dry-mass basis)[a] (µg/kg)		
cis-Chlordane[d,e,f]	174	±	45[b]
trans-Chlordane[d,e,f]	277	±	96[b]
cis-Nonachlor[f]	28.0	±	0.6[c]
trans-Nonachlor[d,e,f]	130	±	38[b]
Heptachlor[d,f]	166	±	34[b]
Heptachlor Epoxide[f]	11.3	±	0.6[c]
Dieldrin[d,f]	88	±	21[b]
gamma-hexachlorocyclohexane (HCH)[d]	4.06	±	0.55[c]
Mirex[d]	6.89	±	0.25[c]
Pentachlorobenzene[f]	20.9	±	1.6[c]

[a] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture.
[b] Reference values are unweighted means of the results from two to three analytical methods. The uncertainty listed with the value is an expanded uncertainty about the mean, with coverage factor 2, calculated by combining a between-method variance [13] with a pooled, within-method variance following the ISO Guide [2].
[c] Reference values are the means of results obtained by NIST using one analytical technique. The expanded uncertainty, U, is calculated as $U = ku_c$, where u_c is one standard deviation of the analyte mean, and the coverage factor, k, is determined from the Student's t-distribution corresponding to the associated degrees of freedom and 95 % confidence level for each analyte.
[d] GC-ECD (I) on a relatively non-polar proprietary phase after PFE extraction with DCM.
[e] GC/MS (II) on a 50 % phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with 50 % hexane/50 % acetone mixture.
[f] GC/MS (III) on a 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM.

Table 8. Reference Concentrations for Selected PBDE Congeners in SRM 2585

PBDE Congener[a]		Mass Fraction (dry-mass basis)[b] (µg/kg)		
PBDE 66	(2,3',4,4'-Tetrabromodiphenyl Ether)[d,e,f,g]	29.5	±	6.2[c]
PBDE 75	(2,4,4',6-Tetrabromodiphenyl Ether)[e,f]	4.5	±	1.2[h]
PBDE 190	(2,3,3',4,4',5,6-Heptabromodiphenyl Ether)[d,g]	5.1	±	2.9[h]

[a] PBDE congeners are numbered according to the IUPAC rules.
[b] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture.
[c] Reference values are weighted means of the results from four analytical methods [14]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), calculated by combining a between-source variance incorporating inter-method bias with a pooled within-source variance following the ISO Guide [2].
[d] GC/NCI-MS (I) on a 15 m 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM.
[e] GC/EI-MS (I) on a 15 m 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM (same extracts as GC/NCI-MS (I)).
[f] GC/EI-MS (II) on a 60 m 5 % phenyl-substituted methylpolysiloxane phase after PFE extraction with DCM.
[g] GC/NCI-MS (II) on a 15 m 5 % phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with DCM.
[h] Reference values are unweighted means of the results from two analytical methods. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2, calculated by combining a between-method variance [13] with a pooled, within-method variance following the ISO Guide [2].

Table 9. Information Concentrations for Selected PBDE Congeners in SRM 2585[a]

PBDE Congener[b]		Mass Fraction (dry-mass basis)[c] (µg/kg)
PBDE 25	(2,3',4-Tribromodiphenyl Ether)	<0.2
PBDE 30	(2,4,6-Tribromodiphenyl Ether)	<0.2
PBDE 71	(2,3',4',6-Tetrabromodiphenyl Ether)	<0.2
PBDE 116	(2,3,4,5,6-Pentabromodiphenyl Ether)	<0.2
PBDE 119	(2,3',4,4',6-Pentabromodiphenyl Ether)	<0.2
PBDE 156	(2,3,3',4,4',5-Hexabromodiphenyl Ether)	<0.2
PBDE 181	(2,2',3,4,4',5,6-Heptabromodiphenyl Ether)	<0.3
PBDE 191	(2,3,3',4,4'5',6-Heptabromodiphenyl Ether)	<0.3
PBDE 205	(2,3,3',4,4',5,5',6-Octabromodiphenyl Ether)	<0.5

[a] Concentrations reported on dry-mass basis; material as received contains approximately 2.1 % moisture.
[b] PBDE congeners are numbered according to the IUPAC rules.
[c] The information values are from the method detection limits using a signal to noise value = 100 for method GC/NCI-MS (I).

(National Institute of Standards & Technology, 2011)