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Method development for the extraction of six bisphenols in serum by LC-MS/MS

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Preface

This master thesis was written at the Faculty of Chemistry, Biotechnology and Food Science (KBM) at the Norwegian University of Life Sciences (NMBU) in Ås, Norway. The laboratory work and sample preparation were carried out at the Toxicology unit, Department of Paraclinical Sciences, the Faculty of Veterinary Medicine (VET) at NMBU. All the instrumental analysis was also performed at the Faculty of Veterinary Medicine (VET) at NMBU, during the period from October 2023 to May 2024.

This project has been funded by The Partnership for the Assessment of Risks from Chemicals (PARC). This initiative aims to evaluate and manage the risks associated with chemical substances.

Professor Roland Kallenborn at KBM, NMBU has been the chief supervisor during this period. Professor Jan Ludvig Lyche and PhD candidate Selma Tofte Granerud have been co-supervisors at NMBU.

Keywords: Bisphenols (BPs), Bisphenol A (BPA), method development, solid-phase extraction, method validation, LC-MS/MS

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Partnership for the Assessment of Risks from Chemicals

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Abstract

Bisphenols are a class of chemicals characterized by two phenol groups bonded to a carbon chain or other chemical groups. These chemical substances are applied in everyday products such as plastics, canned goods, thermal paper, and medical devices. Bisphenol A (BPA) is the most studied compound out of the bisphenol family, and exposure to BPA at low concentrations can induce endocrine-disrupting processes affecting the endocrine system and human reproductive capabilities. When two phenol groups react with acetone (CH_3COCH_3), Bisphenol A (-cetone) is formed. By reacting two phenol groups with other functional groups, such as formaldehyde (CH_2O) and sulfonyl (SO_2), other bisphenols like Bisphenol F (-ormaldehyde) and Bisphenol S (-ulfonyl) are synthesized. The regulation of BPA has led to its replacement with other bisphenol analogs, such as BPF and BPS, which can have similar negative impacts on human health. Therefore, it is interesting to develop a method for quantifying low concentrations of various bisphenols in biological samples.

In this study, a preparation method was developed for the quantification of six bisphenols (BPs) in serum samples by LC-MS/MS. The six BPs of interest are: Bisphenol A (BPA; 4,4′-(propane-2,2-diyl)diphenol), Bisphenol AF (BPAF; 4,4′-(1,1,1,3,3,3-hexafluoropropane-2,2-diyl)diphenol), Bisphenol E (BPE; 4,4'-ethylidenebisphenol), Bisphenol F (BPF; 4-[(4-hydroxyphenyl)methyl]phenol), Bisphenol S (BPS; 4-(4-hydroxyphenyl)sulfonylphenol) and Bisphenol Z (BPZ; 4-[1-(4-hydroxyphenyl)cyclohexyl]phenol). The sample preparation method was developed to obtain optimal sensitivity and selectivity of the BPs. The sample preparation included solid-phase extraction (SPE) to improve separation, enhance sensitivity, and reduce potential interferences and matrix effects.

The limit of quantification (LOQ) was found to be 2.1ng/mL for BPA, 0.1ng/mL for BPAF, 0.5ng/mL for BPE, 0.03ng/mL for BPF, 0.2ng/mL for BPS, and 0.2ng/mL for BPZ. Recovery testing was performed to evaluate the accuracy and correction of analyte loss during the extraction method. The robustness was evaluated by analyzing a pre-spiked serum sample with a fixed concentration of 500ng/mL multiple times (n=6) to evaluate the precision and variation in the measurements of the same sample. Lastly, the method was tested on real serum samples from Argentina. The conclusion is that further optimization is needed as the method is not sufficiently sensitive enough to quantify low concentrations of BPs in serum samples.

Sammendrag

Bisfenoler er en gruppe kjemikalier som består av to fenolgrupper bundet til en karbon-kjede eller andre funksjonelle grupper. Disse kjemiske stoffene finnes i hverdagslige produkter som plastikk, hermetikk, termisk papir og medisinsk utsyr. Bisfenol A (BPA) er den mest omtalte i bisfenol-familien og eksponering av BPA i lave konsentrasjoner kan ha hormonforstyrrende egenskaper i forhold til det endokrine systemet og evnen til reproduksjon i mennesker. Når to fenolgrupper reagerer med aceton (CH_3COCH_3), dannes Bisfenol A(-ceton). Ved at to fenolgrupper reagerer med andre funksjonelle grupper, slik som formaldehyd (CH_2O) og sulfonyl (SO_2) dannes andre bisfenoler som Bisfenol F(-ormaldehyd) og Bisfenol S(-ulfonyl). Reguleringen av BPA har ført til at stoffet blir erstattet med andre analoger, slik som BPF og BPS. Disse analogene har potensialet til å ha de samme helseskadelige virkningene hos mennesker som BPA. Det er derfor interessant å utvikle en opparbeidelsesmetode for kvantifisering av lave konsentrasjoner av ulike bisfenoler i biologiske prøver.

I denne oppgaven ble det utviklet en opparbeidelsesmetode for kvantifisering av seks bisfenoler i serum-prøver ved LC-MS/MS. De seks bisfenolene er: Bisfenol A (BPA; 4,4'-(propan-2,2-diyl)difenol), Bisfenol AF (BPAF; 4,4′-(1,1,1,3,3,3-hexafluorpropan-2,2-diyl)difenol), Bisfenol E (BPE; 4,4'-etylidenbisfenol), Bisfenol F (BPF; 4-[(4-hydroksyfenyl) metyl]fenol), Bisfenol S (BPS; 4-(4-hydroksyfenyl)sulfonylfenol) og Bisfenol Z (BPZ; 4-[1-(4-hydroksyfenyl)sykloheksyl]fenol). En opparbeidelsesmetode ble utviklet og optimalisert med hensyn på best mulig sensitivitet og selektivitet av de ulike bisfenolene. Fast-fase ekstraksjon ble benyttet som prøvebehandling for bedre separasjon, oppkonsentrering og reduksjon av mulige interferenser og matrikseffekter.

Kvantifiseringsgrensen ble funnet til å være 2,1ng/mL for BPA, 0,1ng/mL for BPAF, 0,5ng/mL for BPE, 0,03ng/mL for BPF, 0,2ng/mL for BPS og 0,2ng/mL for BPZ. Gjenvinningen ble testet for å evaluere nøyaktighet og korreksjon av tap av analytter under prøveopparbeidelsen. Robusthet ble evaluert ved å analysere en serum-prøve med en fikset konsentrasjon på 500ng/mL gjentatte ganger (n=6) for å evaluere presisjon og variasjon i målingene av samme prøve. Avslutningsvis ble ekte serum prøver fra Argentina testet med den endelige metoden. Konklusjonen er at metoden trenger videre optimalisering, da metoden ikke er tilstrekkelig sensitiv nok til å kvantifisere lave konsentrasjoner av bisfenoler i serum-prøver.

Abbreviations

NMBU	Norwegian University of Life Sciences
VET	The Faculty of Veterinary Medicine
KBM	The Faculty of Chemistry, Biotechnology and Food Science
PARC	The Partnership for the Assessment of Risks from Chemicals
EU	European Union
BPs	Bisphenols
BPA	Bisphenol A ($C_{15}H_{16}O_2$: 4,4'-(propane-2,2-diyl) diphenol)
BPAF	Bisphenol AF ($C_{15}H_{10}F_6O_2$: 4,4′-(1,1,1,3,3,3-hexafluoropropane-2,2-diyl) diphenol)
BPE	Bisphenol E ($C_{14}H_{14}O_2$: 4,4'-ethylidenebisphenol)
BPF	Bisphenol F ($C_{13}H_{12}O_2$: 4-[(4-hydroxyphenyl) methyl] phenol)
BPS	Bisphenol S ($C_{12}H_{10}O_4S$: 4-(4-hydroxyphenyl) sulfonylphenol)
BPZ	Bisphenol Z ($C_{18}H_{20}O_2$: 4-[1-(4-hydroxyphenyl) cyclohexyl] phenol)
PE	Polyethylene ($(C_2H_4)_n$)
PVC	Polyvinyl chloride (C_2H_3Cl)
PP	Polypropylene ($(C_3H_6)_n$)
PS	Polystyrene ($(C_8H_8)_n$)
PC	Polycarbonate ($C_{16}H_{18}O_5$)
PCB	Polychlorinated biphenyl
PAH	Polycyclic aromatic hydrocarbon
EDC	Endocrine-disrupting chemical
EDTA	Ethylenediaminetetraacetic acid
CPD	Citrate-phosphate-dextrose
ESI	Electrospray ionization
SPE	Solid-phase extraction
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LLOQ	Lower limit of quantification
ULOQ	Upper limit of quantification
API	Atmospheric pressure ionization
LC	Liquid chromatography

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
RF	Radio frequency
CID	Collision-induced dissociation
SRM	Selected reaction monitoring
MRM	Multiple reaction monitoring
FDA	U.S. Food and Drug Administration
EMA	European Medicines Agency
IUPAC	International Union of Pure and Applied Chemistry
AOAC	The Association of Official Agricultural Chemists
ME	Matrix effect
R	Correlation coefficient
R^2	Squared correlation coefficient
WLSLR	Weighted least squares linear regression
SD	Standard deviation
RSD	Relative standard deviation
S/N	Signal-to-noise ratio
CRM	Certified reference material
SIL	Stable isotope labelled
IDMS	Isotope dilution mass spectrometry
Milli-Q	Purified water (Type 1)
ISTD	Internal standard
CE	Collision energy

Table of contents

List of figures

List of tables

1 Introduction

Recent concerns surrounding Bisphenol A (BPA) and other bisphenol analogs, are primarily due to its potential estrogenic impact and associated human health risks [1]. These concerns threaten the production of plastics and commercial use of BPA and require investigation of suitable replacements with sufficient thermal and mechanical properties [2].

Bisphenols (BPs) are a class of chemical compounds characterized by two phenol groups connected by bridging carbon atoms or other chemical groups [3]. When two phenol groups react with acetone (CH_3COCH_3), Bisphenol A (-cetone) is formed [4]. By reacting two phenol groups with other functional groups, such as formaldehyde (CH_2O) and sulfonyl (SO_2), other bisphenols like Bisphenol F (-ormaldehyde) and Bisphenol S (-ulfonyl) are formed [5]. BPs are applied in polycarbonates (PC), polyesters, epoxies, and polyimides [3].

In the synthesis of PC, the polymerization of BPs forms long chains of repeating carbonate units, providing the backbone structure for the polycarbonate polymer. BPs serve as building blocks and provide desirable properties to polycarbonate plastics, such as impact and heat resistance, stability, and optical clarity. In epoxy resins, BP is used as an additive for cross-linking functionalities and durability in thermoset polymers. In polyesters and polyimides, adding BPs contributes to mechanical strength and thermal stability in the polymers [6, 7].

1.1 Plastics

Plastics are a class of synthetic organic polymers composed of a long chain or network of repeating units of smaller molecules called monomers. Synthetic polymers are created by chemical reactions, such as polymerization. There are numerous possibilities for building different polymers. Today, they are globally ubiquitous and found in a variety of everyday commercial products and engineering applications. They provide flexibility in design and manufacture, giving products with a desirable combination of properties [6, 8].

Plastic pollution today remains a persistent challenge, with documented impacts on both living and non-living aspects of the environment dating back over half a century [9]. The pollution can arise from natural sources, the environment or it can be man-made, through anthropogenic activities. Anthropogenic activities as the plastic production industry contribute to most of the

global environmental challenges today. The increasing awareness of the immediate and long-term consequences of the use of plastics in commercial products is important, particularly in the light of sustainability across food systems, water resources, environmental conservation, and human health [9, 10].

The origin of plastics can be traced back to the early 20th century, marked by Leo Baekeland's invention of Bakelite in 1907, which is considered the first synthetic plastic [11, 12]. Prior to this milestone, biobased plastics obtained from natural sources were early precursors to the concept of plastics [13, 14]. The plastic industry has since had an exponential increase and an annual growth rate of 4-5% between the years of 1996 and 1999 [15].

Plastics are high molecular weight organic polymers made of various elements such as carbon, hydrogen, oxygen, nitrogen, sulphur and chlorine [16]. A polymer is defined as a large molecule composed of a long chain of small, simple chemical units of methylene (CH_2). Polymers as plastics are categorized based on their chemical composition and their behavior when heat is applied. Thermoplastics are polymers containing repeating aliphatic (linear) carbon atoms in their backbone chains. These are often commodity plastics with chain lengths that can vary between 1000 to 10 000 methylene groups. These polymers usually soften or melt on heating and harden reversibly on cooling, making them easy to be shaped and reshaped. Polyethylene (PE) is a common thermoplastic polymer, and the structure contains a long chain of repeating -(CH_2)- methylene groups.

$$-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$$

Figure 1: Chemical structure of polyethylene (PE), a polymer synthesized through the catalytic polymerization of ethylene.

Polyethylene (PE) is the most emerging polymer and has the largest application in the plastic industry [17]. PE is synthesized by the catalytic polymerization of ethylene. Other emerging thermoplastics are polyvinyl chloride (PVC), polypropylene (PP), polystyrene (PS) and polycarbonate (PC) [7, 18].

The second category of plastics known as thermosets, consists of heterochain polymers. These compounds typically incorporate atoms like oxygen, nitrogen, or sulphur into their backbone chains, alongside carbon. Thermosets are characterized by their cross-linked polymer structure, which makes them durable and resistant to heat, as the covalent bonds are not broken. The application of these resins is often in molded and laminated plastics, such as phenolic and epoxy resins [7, 19].

1.2 Bisphenols

Bisphenols (BPs) are a class of compounds characterized by two hydroxyphenyl groups connected by bridging carbon atoms or other chemical groups. These chemical compounds are used to synthesize polycarbonate (PC) plastics and epoxy resins that are added to a variety of consumer products, such as food and beverage containers, medical devices, thermal paper, and household items [3, 20].

Bisphenol A (BPA; 4,4'-(propane-2,2-diyl) diphenol) is the most widely studied out of the bisphenol family and one of the highest volume chemicals produced worldwide [21]. It is classified as an endocrine-disruption chemical (EDC) and can interfere with the body´s endocrine system [22]. The exposure occurs when commercial products applied with BPA leaches and BPA is released into the environment, but can also come from dust, thermal paper, and medical equipment. Dust is one of the major sources, and products that pollute dust include epoxy-based floor coverings, glue, paint, electronic equipment, and printed circuit boards. Exposure through thermal paper has also been discussed in recent years, where BPA can be absorbed in the body by dermal contact [23, 24]. In addition to this, trace levels of BPA can also be found in water, sediment, and soil [25].

In the later years, there have been an emerging number of studies regarding BPA and its adverse health effects on the endocrine system and carcinogenesis. The application of alternative bisphenols analogs to BPA in BPA-free commercial products has emerged. However, many of these analogs have the potential to induce similar adverse health effects on humans. There is a total of 16 bisphenol analogs that are documented for industrial use, including BPF (4-[(4-hydroxyphenyl)methyl]phenol), BPS (4-(4-hydroxyphenyl)sulfonylphenol) and BPAF (4,4′-(1,1,1,3,3,3-hexafluoropropane-2,2-diyl) as some of the main substitutes for BPA [3, 26].

BPA, BPF and BPS are often present in environmental samples, including soil, sediments, water sewage effluents and sludge. BPA has been the most prevalent bisphenol compound in the previous years, but BPF and BPS have in the more recent years frequently been detected in environmental samples [27, 28].

1.2.1 Endocrine-disrupting chemicals

In all living organisms, hormones regulate various biological processes like metabolism, cell development, and the reproductive system. Similarly, biocatalysts and endocrine biochemical processes occur within microorganisms, including viral processes where there is no classical "body" present. Hormones are essential for life and can be affected by an endocrine disrupting chemical (EDC) [29]. EDCs are chemicals, or a mixture of chemicals, that interfere with any aspect of normal hormone action. These chemicals serve as endocrine disruptors by mimicking or blocking natural hormones and are associated with a wide range of adverse health effects [30-32].

Persistent endocrine disruptors have low water solubility and extremely high lipid solubility, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and bisphenols (BPs). This leads to bioaccumulation in tissue and organs as they travel through the bloodstream [30, 33].

EDCs are found in many consumer products and human exposure to endocrine disruptors can occur through diet, air, dermal contact, and water. The group of compounds identified as EDCs is highly heterogeneous and includes synthetic chemicals used as solvents and lubricants in the plastic industry. Known byproducts that tend to leach from plastic products are compounds such as polychlorinated biphenyls (PCBs), dioxins, bisphenols (BPs), plasticizers (phthalates) and toxic metals [22, 30, 34].

1.3 The aim of this study

The aim of this study is to develop an optimal preparation method for the quantification of bisphenols in serum samples by LC-MS/MS. With the widespread use of bisphenol-containing products, it is important to be able to qualify and quantify low concentration levels of bisphenols in blood and other biological samples to assess the extent of accumulation, exposure, and further risk assessment. This knowledge is essential for developing targeted strategies to reduce or eliminate exposure from plastics to the environment.

1.4 Blood

Blood is a vital fluid medium that transports oxygen and essential nutrients to the body's tissues and cells, and eliminates carbon dioxide (CO_2) and other waste products from the body's systems. The circulation of blood is driven by the heart or other equivalent structures in the body. Blood is considered both a tissue and a fluid. As a tissue, it consists of a collection of specialized cells with specific functions in a suspension of a liquid matrix called plasma. Whole blood contains four principal components; plasma, red blood cells, white blood cells and platelets [35, 36].

1.4.1 Serum and plasma

Whole blood samples intended for laboratory analysis and clinical studies undergo centrifugation-based extraction to yield either plasma or serum as blood derivates. Plasma and serum are prepared differently and are the most analyzed samples in metabolomic studies, and some analyses show difference in analyte concentrations between the two of them [36].

The fundamental difference between these two matrices lies in the pre-centrifugation treatment. Plasma is obtained by the addition of anticoagulants to whole blood, thereby preventing coagulation [37]. This leads to isolation of the liquid plasma from cellular components during centrifugation. Serum is extracted from whole blood when natural clotting occurs spontaneously, with no addition of anticoagulant. Extraction allows removal of fibrin clots, blood cells and other related coagulation factors. The most commonly used anticoagulants for whole blood are ethylenediaminetetraacetic acid (EDTA), citrate-phosphate-dextrose (CPD), and heparin [36, 38].

1.4.2 LC-MS analysis for bisphenols in blood

Analysis of different bisphenols (BPs) in blood samples by liquid chromatography coupled with mass spectrometry (LC-MS) has been developed in multiple studies. Triple quadrupole with electrospray ionization technique (ESI-MS/MS) is also often used in these methods. Common to most methods is that the sample preparation includes a step for solid-phase extraction (SPE) or liquid-liquid extraction (LLE) with a suitable solvent for the matrix of choice [39-44]. In a previous report, HPLC-MS/MS were used as a method for determining the trace levels of eleven bisphenol A analogues in human blood serum. Limit of detection (LOD) in this method was from 0.008-0.039ng/mL and the limit of quantification (LOQ) was from 0.024-0.12ng/mL for all BPs [45]. Another study used a similar method for determining three BPs: BPA, BPF and BPS in cord blood. In this method, LOD was from 0.023-0.038ng/mL and the LOQ was from 0.046-0.052ng/mL for all BPs [46].

1.5 Analysis

1.5.1 Sample preparation

Prior to analysis, samples usually undergo a clean-up in terms of sample treatment or extraction to remove interfering substances or increase the concentration of analyte. Sample preparation also contributes to minimizing matrix effects and protection for the analytical instrument. Analyzing samples with low concentrations of analyte might require a concentration step. The sample preparation is carried out differently depending on the matrix of choice, which could be from biological, pharmaceutical, environmental or food matrices [47].

It is a crucial step of a bioanalytical method to get analytical sample matrices suited and purified for the separation and detection technique of choice. Bioanalysis is a common term for biological matrices, including whole blood, serum, plasma, urine, saliva, and tissues in the body. In most biological samples, many endogenous components such as carbohydrates, proteins, lipids, and salts must be removed from the matrix. In biological samples, chemical derivatization has been widely used to increase the concentration of analytes. Thorough validation of the technique used for the bioanalytical sample preparation needs to be done before employed in a real sample analysis [47-49].

There are several factors that need to be considered prior to quantitative analysis. A suitable preparation method is achieved by (i) selectively isolating the analyte of interest from the matrix and/or simplifying the matrix, (ii) eliminating interfering endogenous components while still optimizing the recovery, and (iii) having a concentration step of the target analyte to enhance the detectability to a level surpassing the limit of detection (LOD) of the analytical instrument [48, 49].

An ideal sample preparation method for quantitative analysis should be simple, efficient, selective and use a small amount of organic solvent. For bioanalytical methods, the sample preparation often includes steps such as protein precipitation, liquid-liquid extraction (LLE) or solid-phase extraction (SPE) [48-50].

1.5.2 Solid-phase extraction (SPE)

Solid-phase extraction (SPE) is an extraction technique used to isolate, purify, and up-concentrate analytes from liquid matrices and can be applied to treat both environmental and biological samples [51].

The extraction process comprises four steps: i) conditioning/equilibrate, ii) sample application, iii) washing, and iv) elution [52]. The underlying principle of this type of extraction method lies in the analyte's ability to distribute itself between the surface of a solid material (sorbent) and a liquid. The solid material usually consisting of silica, is often modified with selected functional groups, facilitating the capture or retention of the analyte. If the interaction between the analyte and the surface surpasses the interaction between the analyte and the liquid, the substance will isolate on the surface. Such solid materials are referred to as sorbents and are selected based on their interaction with the chosen analyte [51, 53].

In sample solutions, sorbents for normal-phase, reverse-phase or ion-exchange extraction are chosen, depending on the interactions and the polarity of the analytes. For normal-phase, the stationary phase will be polar, while the mobile phase is non-polar, and it is reverse for reverse-phase extraction. In ion-exchange extraction, the stationary phase contains charged functional groups (either positive or negative charged) that interact with analytes of the opposite charge. When dealing with samples dissolved in organic solvents, sorbents for reverse-phase extraction are selected. While most sorbents are silica-based, there are also polymeric sorbents available for use in extraction processes [51, 54].

1.5.3 Liquid Chromatography (LC)

Liquid chromatography (LC) is a separation technique that relies on the interactions between a sample with a solid stationary phase and a liquid mobile phase within a column. The approach often involves a solid stationary phase, characterized by different polar groups, and a liquid organic solvent as the mobile phase (often a gradient between organic solvent and water) [55]. A sample is introduced into the column and transported with the mobile phase by flow from a pump that facilitates high pressure. The mechanism of separation relies on adsorption with the stationary phase based on different physicochemical interactions, where various adsorption forces such as dispersion, dipolar, acid-base and complexation come into play. The duration between sample injection into the system and elution from the column is defined as the retention time [56]. Separation by LC is carried out by using normal-phase or reverse-phase silica columns. In reverse-phase columns, there are non-polar-bonded phases eluting compounds with polar solvents [57]. Water (H_2O), methanol (CH_3OH) and acetonitrile (CH_3CN) are common polar solvents for reverse-phase liquid chromatography [58].

1.5.4 Mass Spectrometry (MS)

Mass spectrometry (MS) is an analytical technique used to identify and quantify chemical compounds. It is based on the ionization and separation of analytes by their mass-to-charge ratio (m/z) [59]. MS contributes to high sensitivity and low detection limits, making it a versatile and important analytical tool in many areas such as analytical chemistry, biochemistry, medicine, and environmental analysis. A mass spectrometer consists of an ion source, a m/z separation unit (mass analyzer) and an ion detector, operated under high vacuum conditions. The ions can be single ionized atoms, clusters, fragments, or molecules, and are separated by static or dynamic electrical or magnetic fields. A mass spectrum is a graphical representation of the detected signal intensity of an analyte (abundance) as a function of the m/z. Each peak corresponds to an ion with a specific m/z and the fragmentation patterns can provide information about the structure of the original molecule [60, 61].

1.5.5 Ion source: electrospray ionization (ESI)

Electrospray ionization (ESI) is an atmospheric-pressure ionization (API) technique where the analytes in a sample are ionized and transferred to the gaseous phase prior to mass spectrometric analysis. This technique removes the solvent without losing any analyte and is a soft ionization technique causing minimal fragmentation of the molecules and multiple charged ions. This allows for accurate measurements of masses as the molecular ions remain intact. However, one limitation of this technique is that the reduction of fragments provides less information about the structure of the analytes. This can be surpassed by coupling ESI with tandem mass spectrometry (ESI-MS/MS) [60, 62].

For LC, ESI is the preferred ionization technique and is used to analyze non-volatile and moderately polar to polar compounds. It can be used in either positive or negative mode and can analyze a wide range of small and larger organic compounds. The solution is first introduced to the system by nebulization, as droplets into the source as ions are produced under atmospheric pressure and focused into the mass spectrometer through high vacuum pumping [60].

1.5.6 Mass analyzer: Tandem Mass Spectrometry (MS/MS)

The ions movement through the electrical or magnetic system is affected by their m/z ratio and the ions are separated in the mass analyzer. In a quadrupole mass analyzer, the separation is based on the ion's different trajectories in an oscillating electric field [63]. The system consists of four parallel rods kept at equal distance and arranged where the opposite rods form a pair which is applied with the same amount of voltage. The two pairs are applied with a specific currency and radio frequency (RF) and are the opposite of one another, creating an oscillating electrical field that the ions travel through before detection. The voltage used in the system is selective, where only ions with a certain m/z ratio will reach the detector. Other ions with smaller or greater m/z will collide in the metal rods, get neutralized and fail to reach the detector [61, 63].

An analysis with tandem mass spectrometry (MS/MS) uses multiple mass analyzers, as the triple quadrupole consists of three quadrupoles set up linearly. The quadrupoles are often denoted as Q1, Q2 and Q3. The analyte ion of interest (precursor ion) is selected based on m/z in the first quadrupole (Q1) and collision-induced dissociation (CID) occurs in the second quadrupole (Q2). In Q2, the precursor ions are activated by collision and undergo further

fragmentation. The smaller ion fragments (product ions) resulting from CID are monitored by the third quadrupole (Q3) and provide information about the fragmentation patterns of the molecule [64, 65].

MS/MS can be used in different scan modes, depending on the desired information. Selected reaction monitoring (SRM), also referred to as multiple reaction monitoring (MRM), is the most common mode used for quantitative analysis in complex matrices [66]. In SRM, both Q1 and Q3 selectively filter ions based on the m/z, detecting the precursor ion that generates fragments with the specific m/z [67]. The precursor ion with the highest abundance and signal intensity is selected as the quantifier, ensuring precise and accurate quantification [68].

1.6 Method validation

The validation of an analytical method is to ensure that future measurements in routine analysis are close to the true value content of the analyte in a sample [69]. This ensures that the analytical method is suited for its intended purpose, by producing consistent and reproducible results of high quality [70]. There are regulatory international guidelines for validation of analytical methods, including the U.S. Food and Drug Administration (FDA), Eurachem, European Medicines Agency (EMA), International Union of the Pure and Applied Chemistry (IUPAC) and The Association of Official Agricultural Chemists (AOAC) [71, 72]. There are several parameters that contribute to the validation and increase of the quality of an analytical method. This includes accuracy, linearity, precision, recovery, and robustness [73, 74].

1.6.1 Regression analysis

Regression analysis is a statistical technique that is used to predict the values and model the relationship between a dependent value (y) and when one or more independent variables (x) are known [75]. The experimental data will not always align with the mathematical model and the deviations between the observed and predicted values by the model are called residuals. The goal in regression analysis is to minimize the sum of square residuals, to achieve the best estimation of the model parameters. The simplest form of regression is a linear model and can be used as part of the validation of a method [76].

1.6.2 Linearity

Linearity is the proportional relationship between the analyte response in the instrument and the known concentration of the analyte and is usually assessed by a calibration curve. The linearity of a method is desired to be fully linear, where the data points from the calibration curve form a straight line [77, 78]. The regression line is expressed as shown in Equation 1.1:

$$y = a + bx \tag{1.1}$$

where y is the measured response, a is the y-intercept and b is the slope of the regression line. The b-term is determined by x, which is an independent variable. The regression line is obtained by minimizing the sum of squared vertical deviations (residuals) of the data points from the line [78, 79]. This is achieved by using the "method of least squares", ensuring that the line fits the response as closely as possible. With the regression line, many software packages also provide the correlation coefficient (R) to quantify the strength and direction of the relationship between two variables. The squared correlation coefficient (R^2) explains the proportion of variance in the dependent variable (y) that is explained by the independent variable (x) [80-82]. R is calculated as shown in Equation 1.2:

$$R = \frac{\sum_{i=1}^{n}(x_i-\bar{x})(y_i-\bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2 \sum_{i=1}^{n}(y_i-\bar{y})^2}} \tag{1.2}$$

where $\bar{x}$ and $\bar{y}$ are the means of x and y, respectively.

The value of the coefficient ranges from -1 and 1, where 1 indicates a perfect positive correlation, -1 indicates a perfect negative correlation, and 0 indicates no correlation [82, 83].

The "method of least squares" regards all y-values from the data points as equally important, regardless of the magnitude and potential variability [84]. Dealing with concentration data, large deviations at higher concentrations can influence the regression line more than smaller deviations at lower concentrations. This can lead to inaccuracies in the lower end of the calibration range but can be balanced out when using weighted least squares linear regression (WLSLR) [85, 86].

WLSLR is a method used to fit a linear regression model to data with uneven variations in the data. It is able to reduce the lower limit of quantification (LLOQ) and extend the linear calibration range, leading to higher accuracy and precision [86]. Forcing the intercept of the calibration line through the origin (0,0) can be done when there is evidence that the true intercept does not significantly differentiate from zero (0,0). This will not always be appropriate and can lead to biased results [68].

1.6.3 Limit of detection (LOD) and quantification (LOQ)

The limit of detection (LOD) is defined as the minimum level of an analyte that can be reliably detected in a sample with known concentration by the analytical method. The most common approaches to determine LOD are by the signal-to-noise ratio (S/N) or by the standard deviation (SD) of the response [87]. The S/N method assesses the difference between the analyte response and the background noise in the blank samples. Limit of quantification (LOQ) is often defined as the lowest concentration of analyte in a sample that can be quantified with an acceptable accuracy and precision under the experimental conditions. The upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) is the highest and lowest concentration level of an analyte that can be quantified with an acceptable accuracy and precision in the analytical method.

The LOD and LOQ can be determined by multiplying the standard deviation (SD) of the response or noise of a blank sample by 3 and 10 times, respectively [86, 87]. Both LOD and LOQ provide a measurement of the sensitivity and reliability of an analytical method and can be estimated by both statistical approaches and visual definition [88].

1.6.4 Recovery

Throughout an analytical process, from sample preparation to analysis, the potential of analyte loss can occur, making the recovery an important aspect of the validation of all analytical methods. Recovery is the ratio between measured concentration and the amount of analyte theoretically present or added in the sample. There are different procedures for assessing recovery, by adding certified reference material (CRM) or a surrogate compound with similar chemical and physical properties as the analyte [89].

Results from the test materials of the same matrix can be corrected and adjusted based on the recovery measured for the CRM used. There are potentially several problems that can occur when using reference materials, namely: (i) the validity of any recovery estimate relies on the assumption that the analytical method used is unbiased; (ii) the selection of appropriate reference materials is slightly limited; (iii) available reference materials cannot always match the matrix of the test materials [90]. The optimal surrogate is an isotopic-modified variant of the analyte which is often used in isotope dilution approaches. This procedure allows the correction of analyte loss and an unbiased estimation of the concentration of the native analyte in the original matrix sample. The specific acceptance criteria for the recovery percentages for most analytical methods are 70-120% [91].

A simple and efficient applied method to determine the recovery is by spiking. A spiked sample is created by adding a fixed concentration of the analyte to a matrix blank sample as the first step of the sample preparation. The recovery can be determined after applying the chosen analytical procedure, by the amount of analyte loss and the ratio between the theoretical spiked concentration and the measured concentration in samples at the same level. However, a disadvantage of this approach is the lack of sample specificity. It assumes identical recoveries for all samples, which may not always be the case [92].

1.6.5 Accuracy

Accuracy is defined as the deviation in the measured analyte concentration from the true value. The accuracy of a measurement can be calculated from the deviation between the analyte concentration response and theoretical concentration in the sample, expressed as shown in Equation 1.3:

$$E(\%) = \frac{Measured\ conc.}{Theoretical\ conc.} \times 100\% \quad (1.3)$$

where the measured concentration is the analyte response, and the theoretical concentration is the known concentration in the spiked sample in the first step of sample preparation [93, 94].

1.6.6 Precision

Precision is defined as the repeatability of a series of analyte measurements (n) of the same sample and the reproducibility of a measurement. It is affected by random errors that can occur in all measurements in analytical methods [74]. The standard deviation (SD) and the relative

standard deviation (RSD) are the most common statistical terms for estimating the precision of a data set. SD indicates how the dispersion of data is from the mean and a measure of the precision of the data. SD is obtained as shown in Equation 1.4:

$$SD = \sqrt{\sum_i \frac{(x_i - x)^2}{n-1}} \quad (1.4)$$

where x_i is the individual measurements and n refers to the number of measurements. The mean of all measurements is represented as x and is obtained as shown in Equation 1.5.

$$RSD = \frac{1}{n}\sum_{i=1}^{n} x_i \quad (1.5)$$

The size of the measurement of SD is dependent on the size of the data set used, which makes it difficult to compare the SD to other data sets. Therefore RSD % is obtained, calculated as shown in Equation 1.6.

$$RSD(\%) = \left(\frac{SD}{x}\right) \times 100\% \quad (1.6)$$

In statistics, RSD is used to calculate a standardized measure of the ratio between the standard deviation and the mean. This measurement reflects the precision of the average of the results. RSD is often stated as percentage % RSD for the uncertainty among the variety of the measurements. A higher RSD % indicates that the data is more spread from the mean and a lower RSD % suggests less spreading from the mean, indicating better precision. RSD <15% is often used in laboratory assays for trace analysis [95-97].

1.6.7 Carry-over

The term carry-over is used when parts of a previous sample injection are present in the system and affect the analysis of the subsequent samples [98]. This can occur from various sources, including incomplete elution of analytes, adsorption of analytes of surfaces in the analytical system or incomplete flushing of the system. Carry-over can be estimated by injecting blank samples containing only solvent (instrument blank) after a sample run. This can identify if there is any residue contamination present in the system [68, 99, 100].

1.6.8 Method uncertainty

One critical aspect in analytical chemistry is determining and calculating a method's uncertainty that can arise from various sources. This includes incomplete definitions of measurand, sampling, matrix effects and interferences [101]. Method uncertainty provides a quantitative measure of the reliability and reproducibility of analytical results [102].

The uncertainty of measurements is the doubt that exists about the result of any measurement and is not a fixed value. The interval of uncertainty can either deviate upwards or downwards from the expected value. Specifically, measurement uncertainty plays a significant role in method uncertainty, impacting various aspects of method validation. It is essential for evaluating the performance of analytical methods, particularly in areas such as quality assurance and method validation through considering factors such as precision and recovery [103, 104].

1.7 Quality control

Method validation and quality control are important aspects to consider when ensuring the accuracy, reliability, and consistency of analytical measurements. Method validation refers to the process of confirming that a particular method is suitable for its intended use by evaluating its performance characteristics through necessary criteria for accuracy, linearity, precision, and other parameters [105].

Quality control (QC) focuses on monitoring and maintaining the quality of processes and products to ensure consistency and reliability. Quality control aims to identify and correct errors, deviations, or variations that can affect the validity of the analytical results [106].

1.7.1 Applicability

The applicability of a method consists of a set of features that provides information about the identity of the analyte of interest (e.g., nature and speciation), the concentration range to operate efficiently, identification of the type of matrix considered for the method validation, a comprehensive protocol outlining (describing equipment, reagents, analytical and quality control procedures, including calibration and safety precautions) and the intended method application with the following critical requirements.

(1) the analytical procedure must be validated as a whole, including sample treatment prior to analysis; (2) the analytical procedure must be validated covering the full range of analyte concentrations specified in the method scope; and (3) the analytical procedure must be validated for each kind of matrix where it will be applied [107, 108].

1.7.2 Selectivity and specificity

Selectivity refers to the degree to which a method can accurately quantify and differentiate the analyte of interest and expecting interfering compounds present under stated conditions for the sample matrix. It is impractical to assess every potential interference, so it is advisable to focus on the interferences that are likely to occur. By eliminating interfering compounds, selectivity provides accuracy and reliability of the analytical measurements. The absence of interfering effects can translate to specificity, where specificity = 100% selectivity [109, 110].

Selectivity can be checked by implementing chromatographic solvent blanks (instrument blanks) containing only mobile phase solvents [110].

1.7.3 Retention

The retention time (RT) in a chromatographic method is dependent on its partition coefficient, also known as the distribution constant (K_d). The K_d is the equilibrium constant for an analyte in between the stationary phase and the mobile phase, described by the following Equation 1.7:

$$K_d = \frac{c_s}{c_m} \tag{1.7}$$

with c_s being the analyte concentration in the solid stationary phase and c_m being the analyte concentration in the mobile phase [111]. Compounds with a higher K_d exhibit more affinity towards the stationary phase and move at a slower rate through the column. This results in longer RT for these compounds than for compounds with lower K_d.

The identification of compounds can be done by retention indices (RIs). By comparing a sample's retention time to an external standard analyzed under identical conditions, deviations from the expected value can be identified. A too rapid elution can affect the retention time, as it may not be consistent and can lead to co-elution of other compounds. Too much retention can result in broad peaks in the chromatogram [112, 113].

Retention shift in chromatography refers to the change in the time it takes for a compound to travel through a chromatographic column compared to the RT of an external reference standard [114]. If the peak of interest shifts significantly, it may overlap with other peaks or interfere with baseline noise, resulting in incorrect quantification and affecting the accuracy and robustness of the method [114, 115].

1.7.4 Standards

Standards represent the pure form of the compounds of interest and are applied to calibrate analytical instruments and methods. Primary standards are characterized by their high purity, stability, and traceability to international standards, making them ideal for establishing reference points in analytical chemistry [116]. They are expected to have extremely high levels of purity, often exceeding 99.9% with accurately known concentrations [117]. Secondary standards are prepared from primary standards and are used for routine calibration, validation, and quality control purposes. While secondary standards are not required to have the same level of purity as primary standards, they still need to meet certain purity criteria to ensure accurate and reliable results [116].

To quantify analyte concentrations accurately, a calibration curve with external standard solutions is constructed, containing standard, internal standard (ISTD) and solvent used in the method. A range of concentrations, usually a selection of high, medium, and low, are prepared to generate the calibration curve. It is recommended to use at least 5 levels of concentration points that are spread across the concentration range of 50-150% of the expected working range, including a blank sample. The calibrants should be injected at the beginning and end of every chromatographic run. The standard can also identify the compound of interest in a sample by comparing the retention time of the peak when analyzed under the identical conditions [54, 118].

Certified reference materials (CRM) are often used as external standards in the validation of analytical methods and assessing accuracy, traceability, and reproducible results among different laboratories over time. CRM are reference materials with known properties and have been established to fit for its indended use. CRMs are a subset of standards that have been certified for specific properties with a known level of uncertainty in the measurements [119].

The internal standard (ISTD) holds similar physiochemical properties as the analyte of interest and shows similar behavior when extracted or detected in an analytical system. Given that they are almost chemically identical to the analyte, they can be distinguished by using a mass spectrometer (MS). It is important that the concentration of ISTD is identical in the test samples as for the prepared standard solutions used to create the calibration curve. ISTD is added in the first step when preparing the samples, before further sample preparation. This is to control for any loss, variations, and ionization, and correct for matrix effects. Control samples are analyzed more regularly to obtain precision. The concentration of ISTD is known, making any changes in the concentration after detection to be adjusted for. Two common types of ISTD are stable isotope-labelled (SIL) and isotope dilution mass spectrometry (IDMS). SILs are often incorporated in IDMS, as it utilizes the addition of known amounts of SILs to the sample to determine the concentration of the analyte [120-122].

1.7.5 Calibration study

The response function or calibration curve of an analytical method is defined by the relationship between an analytical signal (response) and the concentration of the analyte of interest [110]. The response function for chromatographic methods can be linear or non-linear and is obtained by using samples containing standard solutions with known concentrations prepared in a matrix sample or solvent. To obtain the best adapted calibration function, there are several statistical models that can be investigated and tested based on the linearity of the regression line. Linear regression is the most common model used, but if the relationship is non-linear or more complex, exponential, or logarithmic regression can be more appropriate [123].

The linear range should cover the expected concentrations of the analyte in the samples being analyzed. It is typically determined during method development and validation and may vary depending on the sensitivity of the analytical method and the detection limits of the instrument. The linear range should extend from the lower limit of quantification (LLOQ) to a higher concentration where the linearity begins to deviate significantly [124].

In the calibration of curves, the weighting of the data points plays a crucial role in ensuring accuracy and reliability. The choice of weighing factors, such as $1/x$ or $1/x^2$, can impact the slope of the calibration curve in different ways [125].

1.7.6 Matrix

Matrix-matching is preferred in quantitative analysis, to ensure consistency and that the blank samples, calibration curve, quality control samples and test samples have the same matrix. This can eliminate or reduce matrix effects or keep it at a constant level [126].

Ion suppression is a matrix effect often present in chromatographic analysis by LC-MS and affects the slope of the calibration curve. This issue occurs when endogenous compounds present in the matrix sample interfere with the ionization process. Ion suppression can affect the peak shape in the chromatogram and the ionization efficiency of analytes to either be reduced or enhanced, which affects the signal intensity and can lead to non-linear responses in the calibration curve. This will influence the detection capability as the analyte signal changes, affecting the limit of detection (LOD), signal-to-noise (S/N) ratio and the range of calculated concentrations [127].

The saturation of detector response at high concentrations can also affect the slope of the calibration curve, where the concentrations do not increase proportionally with the signal intensity [128]. Matrix-analyte interactions can also contribute to non-linear effects, where the co-elution of analytes with matrix components can lead to distorted and overlapping peaks in the chromatogram. The sum of non-linear effects leads to inaccuracies in the quantification and can affect the accuracy, precision, and sensitivity of the analytical method [129, 130].

1.7.7 Precursor and product ion

In mass spectrometry (MS) techniques for quantification, often tandem mass spectrometry (MS/MS), mass-selected ions (precursor ions) are induced to dissociate into smaller fragments called product ions. The result is a mass spectrum containing signals for all the smaller product ions that originate from one precursor ion [131].

Product ion scanning can be used to identify fragment patterns with great certainty by their mass-to-charge ratio (m/z) and acts like a fingerprint. By using product ions, one can determine the molecular composition and structural characteristics of the original compound. Selecting the most optimal product ions can be challenging at times, as the most abundant fragment is not always the best choice. Other significant fragments that provide cleaner chromatograms can occur, with an excellent signal-to-noise ratio (S/N) [132, 133].

1.7.8 Single reaction monitoring (SRM) and multiple reaction monitoring (MRM)

Single reaction monitoring (SRM) and Multiple reaction monitoring (MRM) are targeted mass spectrometry techniques that enable the detection and quantification of specific molecules within complex mixtures.

In single reaction monitoring (SRM), specific precursor and product ion pairs are monitored by MS. This method provides high sensitivity, reproducibility, and the ability to analyze multiple compounds. In SRM, one precursor-product ion transition is monitored at a time and is a subset of multiple reaction monitoring (MRM) [134]. In MRM, only specific precursor-product ion transitions are tracked using tandem mass spectrometry (MS/MS). Unlike scanning all product ions from precursor fragmentation as in a product ion scan, MRM focuses on predefined pairs specific to the target analyte [135, 136].

MRM is also a valuable tool in qualitative (Qual) and quantitative (Quant) analysis and determination of concentration levels of the compound of interest. By integrating and determining the area ratio between the quantifying and qualifying peaks, Quant can be used for accurate and reliable quantification [137].

1.7.9 Neutral loss scan

Neutral loss scanning is a MS technique used for the detection and identification of specific molecules based on the loss of a particular fragment during the fragmentation process. This type of scan involves monitoring the loss of a neutral fragment from precursor ions to identify the compounds of interest [138]. It is often used to identify specific functional groups or chemical modifications within molecules. By monitoring the loss of a particular neutral fragment, the identification of certain structural features and chemical composition of the analyte of interest can be done [138, 139].

1.7.10 Errors

Errors in analytical chemistry can arise from various sources, including contamination of samples or reagents, calculation errors, operator mistakes and instrument malfunction or other equipment. The term error is defined as the difference between the measured value from an experimental observation and the true value. By minimizing error, accuracy, precision and

reliability can be obtained, however, there are different types of experimental errors that can occur [68].

A systematic error is a source specific error that is constant or proportionally different from the true value, usually caused by a chemical or an instrumental problem. This type of error can occur by using wrongly calibrated analytical instruments or errors in the sample preparation. Systematic errors are problematic and can lead to false conclusions, whether it be a false positive or negative conclusion. Random errors are unpredictable variations within the measurements and can be high or low. This gives variation in the repeated observation of the measurand. This type of error cannot be compensated for but by increasing the number of observations it can be reduced [68, 140].

One can avoid errors by conducting daily instrument checks prior to the analysis and visually inspect chromatograms before or during a run to ensure acceptable separation, peak shape, and other parameters [141].

1.7.11 General quality assurance

The purity of reference compounds and contamination control are crucial factors when working with trace analysis. The reference compounds and solvents used in the method need to have a high purity level (≥ 98%) to be able to exceed acceptable levels of certainty [142].

Establishing a robust standard operating procedure (SOP) and good laboratory practices can minimize the need for unnecessary work or redoing. These practices, combined with laboratory skills, can enhance the likelihood of successful outcomes during method development or performing a validated method significantly [143].

1.7.12 Contamination control and control samples

Maintaining good cleaning protocols and lab routines is essential to prevent contamination of equipment and the environmental surroundings. All equipment used from sampling, sample preparation to chromatographic analysis can affect and interfere with the results, leading to unwanted matrix effects and possible false conclusions [144].

An effective and thorough cleaning routine is optimal to get rid of possible interferences from the equipment used in the analytical method. Soaking the equipment in soap overnight in a

separate container than other equipment intended for use, for extra caution. Thereafter rinse all equipment thoroughly for soap residue with Type 2 and Type 1 water (milli-Q) or in a dishwasher without soap. All clean equipment intended to be used should be flushed with the solvent used in the analytical method to remove contaminants or soap residue.

Injection of an instrumental blank sample (pure water, solvent, or a mix of them) after running highly concentrated samples and every tenth injection should be included for monitoring the carry-over and other contaminations from the system. A matrix blank sample containing "zero sampling material" is prepared and stored the same way as the other prepared samples until analysis. The matrix blank sample should in theory not contain any of the analytes of interest but can be refined and calculated within the measuring results if it is not accessible with a total clean matrix blank sample. A reagent blank (blind sample) is prepared by substituting the matrix with the solvent used in the method or water and preparing it as a real sample. This can detect if any of the reagents are contaminated [145, 146].

1.7.13 Data processing

Data processing is a crucial step in generating quantitative data from raw data in analytical results. While automated algorithms for peak integration are available in most modern software packages, it is still important to inspect each chromatogram. When inspecting a chromatogram, it is important to ensure that accurate baselines are drawn and that the correct peaks have been selected. The integration of the peaks should be consistent, and one should be critical when adjusting. Following this process for standards, the ratio between the analytes and associated ISTD is used to establish the calibration line and quantify the quality control (QC) samples and samples with unknown concentrations [68].

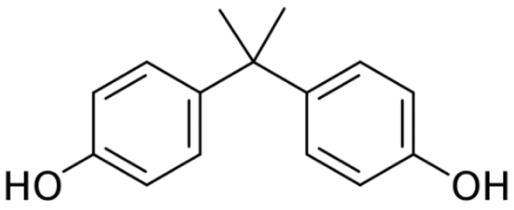
2 Experimental methods

2.1 Choice of analytes

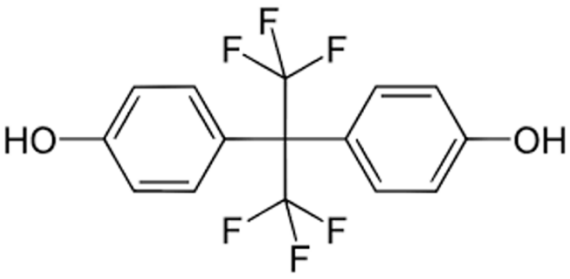
In this study, the focus was on BPA (4,4'-(propane-2,2-diyl) diphenol) along with five analogues that have garnered significant attention and replaced BPA in various commercial products. The five analogues include BPAF (4,4′-(1,1,1,3,3,3-hexafluoropropane-2,2-diyl), BPE (4,4'-ethylidenebisphenol), BPF (4-[(4-hydroxyphenyl) methyl] phenol), BPS (4-(4-hydroxyphenyl) sulfonyl phenol) and BPZ (4-[1-(4-hydroxyphenyl) cyclohexyl] phenol).

Table 1. BPs used in the study, including IUPAC name, chemical formula, Log P and CAS-number.

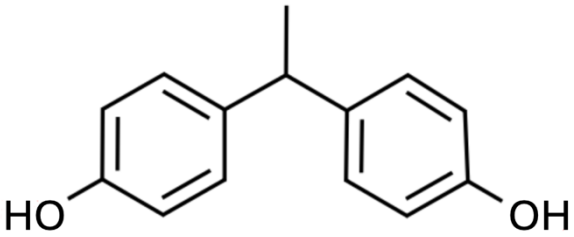
Chemical	IUPAC name	Chemical formula	Log P (K_{ow})	CAS-number
Bisphenol A	4,4'-(propane-2,2-diyl) diphenol	$C_{15}H_{16}O_2$	3,4	80-05-7
Bisphenol AF	4,4′-(1,1,1,3,3,3-hexafluoropropane-2,2-diyl)	$C_{15}H_{10}F_6O_2$	4,5	1478-61-1
Bisphenol E	4,4'-ethylidenebisphenol	$C_{14}H_{14}O_2$	3,2/3,9	2081-08-5
Bisphenol F	4-[(4-hydroxyphenyl) methyl] phenol	$C_{13}H_{12}O_2$	2,9	620-92-8
Bisphenol S	4-(4-hydroxyphenyl) sulfonyl phenol	$C_{12}H_{10}O_4S$	1,9	80-09-1
Bisphenol Z	4-[1-(4-hydroxyphenyl) cyclohexyl] phenol	$C_{18}H_{20}O_2$	5,0/5,4	843-55-0



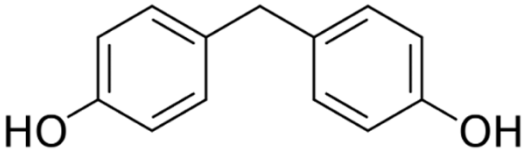
Bisphenol A



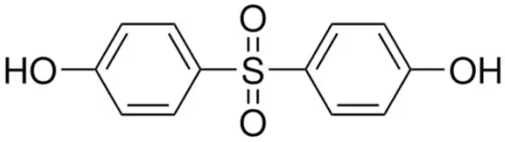
Bisphenol AF



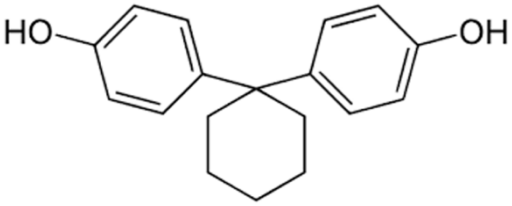
Bisphenol E



Bisphenol F



Bisphenol S



Bisphenol Z

Figure 2: The molecular structure of the six targeted BPs of interest in this study; Bisphenol A (BPA), Bisphenol AF (BPAF), Bisphenol E (BPE), Bisphenol F (BPF), Bisphenol S (BPS) and Bisphenol Z (BPZ).

2.2 Materials and chemicals

2.2.1 List of chemicals

An overview of all chemicals used in the analytical method is attached in the Appendix in Tables 13 and 14. The six bisphenols with associated CAS numbers and IUPAC nomenclature are listed in Table 1 and their molecular structure is illustrated in Figure 2.

2.2.2 List of equipment

The analysis was conducted using Agilent Technologies 1290 LC system coupled with a 6495 mass selective detector (triple quadrupole, QQQ) from Agilent Technologies. The LC-MS system operates in triple quadrupole with electrospray ionization (ESI-MS/MS) in negative mode. For a comprehensive overview of the equipment and software utilized in the analysis, it is listed in the Appendix in Table 15.

2.3 Method

2.2.1 Preparation of standard stock solution

Table 2. Standard solutions and concentration of the standards obtained from AccuStandard. The initial concentrations for dilutions are used in subsequent analysis.

Standard solution	Concentration (mg/mL)
BPA (4,4'-(propane-2,2-diyl) diphenol)	10
BPAF (4,4′-(1,1,1,3,3,3-hexafluoropropane-2,2-diyl))	10
BPE (4,4'-ethylidenebisphenol)	10
BPF (4-[(4-hydroxyphenyl) methyl] phenol)	10
BPS (4-(4-hydroxyphenyl) sulfonyl phenol)	10
BPZ (4-[1-(4-hydroxyphenyl) cyclohexyl] phenol)	10

A stock solution was prepared by dissolving all standards to a concentration of 10µg/mL in methanol (MeOH). This stock solution was used for spiking samples and for constructing the calibration curve.

2.2.2 Preparation of internal standard solution

Table 3. Internal standard solutions applied in all samples for qualification and quantification confirmation purposes in the analysis.

Internal standard	Concentration (µg/mL)
BPA$^{13}C_{12}$	100
BPAF$^{13}C_{12}$	100
BPF$^{13}C_{12}$	100
BPS$^{13}C_{12}$	100

BPE$^{13}C_6$ and BPZ$^{13}C_{12}$ were obtained in solid form, each internal standard in 1mg. A stock solution containing a mix of the internal standards (ISTD) for the BPs was prepared to achieve a final concentration of 1µg/mL for each ISTD, dissolved in methanol (MeOH).

2.2.3 Preparation of mobile phases

Mobile phase A consisted of a solution containing 1mM ammonium acetate in milli-Q water. The preparation involved the addition of 200µL of 5mol/L ammonium acetate to 1.0L of milli-Q water. Mobile phase B was MS-grade/ LC-grade methanol (MeOH).

2.2.4 Calibration curve

A series of standard solutions with known concentrations were prepared to correlate the response of the analytical instrument with the concentration of the analyte. There were prepared eight dilutions of the standard solution in methanol (MeOH) with the concentrations of 0.1, 1, 5, 10, 50, 100, 200 and 500ng/mL. The ISTD was included by adding 20µL in each point at a fixed concentration of ISTD with 1µg/mL. The internal standard mix consisted of BPA$^{13}C_{12}$, BPAF$^{13}C_{12}$, BPE$^{13}C_6$, BPF$^{13}C_{12}$, BPS$^{13}C_{12}$ and BPZ$^{13}C_{12}$. The final concentration of the ISTD in the samples after sample preparation was 100ng/mL.

The algorithm used for the calibration curve is a linear regression algorithm. The method determines the best-fitting straight line through a set of data points and the calibration curve relates the response of the detector (peak area) to the concentration of the analyte. The slope of the calibration was set to ignore the origin and the weighing of the regression line was set to 1/x.

2.2.5 Preparation of test serum samples

The serum samples were from the Faculty of Veterinary Medicine (VET) at NMBU. The samples originate from the Central Laboratory and are fetal calf serum. They serve as standard serum material often employed in cell culture experiments and are utilized as blind material for spiking recovery samples, method development and validation purposes. They were kept frozen at -20°C until sample preparation. When ready, the serum samples were put at room temperature for about 2 hours prior to the sample preparation to defrost.

The samples were prepared as shown in the Appendix in Table 18, containing the following:

- A blank sample with only water.
- Three replicas of serum sample with only ISTD (matrix blank).
- Five pre-spiked serum samples with ISTD and std with levels of known concentrations: 1, 5, 10, 100, and 200ng/mL.
- Five un-spiked serum samples without ISTD (blind sample).

In method validation, a blank sample often consists of the solvent or matrix used in the analysis along with an internal standard (ISTD). Solvent blanks are used to evaluate the background levels of impurities or interferences within the analytical system and can be used for identification of potential contamination from reagents, equipment, or the environment. Matrix blanks are used for determining LOD, LOQ and assessing method sensitivity.

The blind samples include the solvent or matrix used in the analysis but omit the internal standard (ISTD) and pre-spiking. These samples undergo identical sample preparation steps as the other serum samples. By post-spiking the blind samples with standard and internal standard (ISTD) after sample preparation and before analysis, one can evaluate the recovery and accuracy of the analytical method. Comparing different spiked samples allows for the assessment of matrix effects and analyte loss during sample preparation. Blind samples were spiked with the same known concentrations after sample preparation as for the pre-spiked samples. Additionally, samples containing only methanol (MeOH) were spiked with the same five known concentration levels. The full composition of the prepared samples is shown in the Appendix in Table 18, and consisted of 17 samples in total.

2.2.6 Solid-Phase Extraction (SPE)

The serum samples were extracted and eluted at The Faculty of Veterinary Medicine at NMBU in Ås, Norway.

The samples were prepared as shown in the Appendix in Table 18. The extraction process was carried out on a SPE vacuum manifold at a consistently steady flow rate of 1-3 drops per second. The cartridges used to conduct the separation were Oasis® HLB 6cc Vac Cartridge 150mg, 30µm from Waters™. The sample preparation was conducted after "Oasis HLB Cartridges and 96-Well Plates Care and Use Manual" from Waters™. SPE and HLB cartridges are often used in the sample preparation of biological samples and were carried out following the recommended steps in Waters' manual.

The cartridges were first washed with 3mL methanol (MeOH) and equilibrated with 3mL milli-Q water. It was important to not let the cartridges run dry and let there be some droplets left in the cartridge before loading new material. The prepared samples were loaded onto the cartridge and washed with 4mL 5% methanol in milli-Q water. The elution was carried out by applying 4mL methanol (MeOH) to the cartridge. The eluate was evaporated until dryness at 40°C under flow of nitrogen gas (N_2). The dry residue was reconstituted in 200µL of 40% methanol in milli-Q water. The same as for the mobile phase gradient (60/40) at the start of the instrumental analysis. The prepared samples were kept frozen at -20°C until analysis.

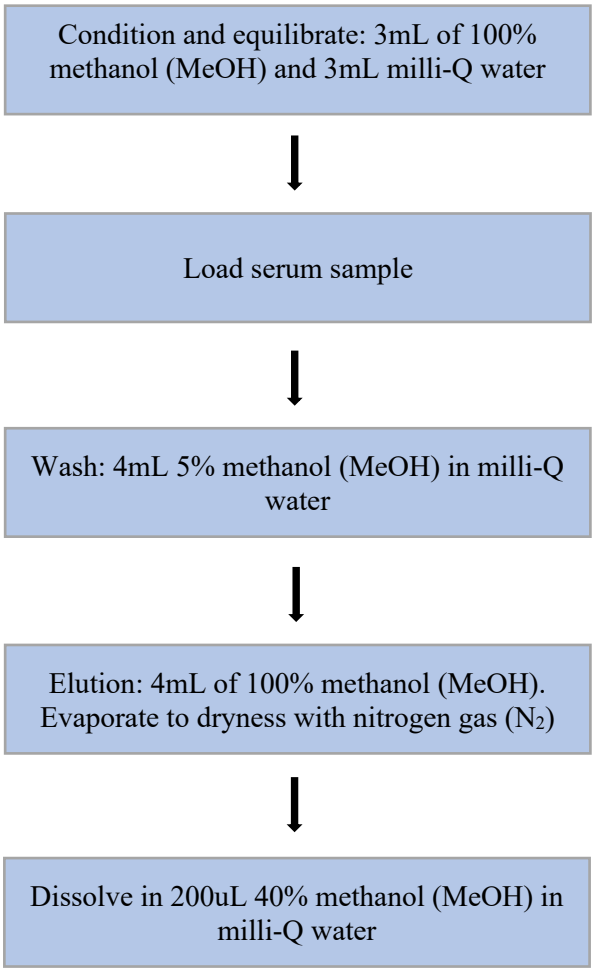


Figure 3: The different stages and volumes used in the solid-phase extraction (SPE) method.

2.2.7 Calibration curve with serum samples

A series of standard solutions with known concentrations including serum samples as matrix were prepared to correlate the response of the analytical instrument with the concentration of the analyte. The composition of the serum samples is shown in the Appendix in Table 17. The extraction process was performed as described in subchapter 2.2.6.

There were prepared eight dilutions of the standard solution with concentrations of 0.1, 1, 5, 10, 50, 100, 200 and 500ng/mL. The ISTD was included by adding 20µL in each point at a fixed concentration of ISTD with 1µg/mL. The internal standard mix consisted of BPA$^{13}C_{12}$, BPAF$^{13}C_{12}$, BPE$^{13}C_{6}$, BPF$^{13}C_{12}$, BPS$^{13}C_{12}$ and BPZ$^{13}C_{12}$. The final concentration of the ISTD in the samples after sample preparation was 100 ng/mL.

2.3 Analysis

2.3.1 LC conditions

The mobile phase A consisted of 1 mM ammonium acetate in milli-Q water, while mobile phase B was methanol (MeOH). The analysis was conducted with a mobile phase flow rate of 0.45mL/min and an injection volume of 10µL was introduced into the LC-MS instrument. The whole analysis spanned a total time of 11 minutes for each sample. The chromatographic separation utilized a Raptor Biphenyl LC column with a diameter of 2.1mm and length of 50mm from Waters™. The particle size of the column packing was 1.8µm. The column temperature was maintained at 30°C throughout the analysis.

The ratio of the solvents in the mobile phase was adjusted to find the gradient with the most optimal separation of the BPs in the samples. Three gradients were tested: 50/50 (standard), 60/40 and 70/30. Based on the physiochemical properties of the BPs it was determined that the gradient containing 60% mobile phase A and 40% mobile phase B were the most efficient. There were also tested multiple hold times; 1-6.5 (standard hold time), 1-7, 1-7.5, 2-7 and 2-7.5 min for when the mobile phase gradient shifted from 60/40 to 10% A and 90% B. The most optimal hold time, which was used for the analysis, was 2-7.5 minutes.

Table 4. Hold times and mobile phase gradients throughout analysis of one sample, developed by me.

Time (min)	A (%)	B (%)
0.0	60	40
1.0	50	50
2.0	50	50
7.5	10	90
8.5	10	90
9.0	60	40
11.0	60	40

The gradient of the mobile phase throughout the analysis are shown in Table 4 and consists of 60% of A in 1 min, then adjusting to 50% in 1 min. Thereafter lowering to 10% A and hold for 5.5 min. Then end with 60% A in 0.5 min.

2.3.2 MS conditions

Under MS conditions, electrospray ionization (ESI) was selected as the ionization technique and the analytes were analyzed in negative mode. The collision energy (CE) was already optimized prior to analysis. Tables 5 and 6 illustrate the mass transitions and collision energy for each individual BPs compound.

Table 5. The precursor ion, transition, collision energy (CE), retention time (RT), and ion polarity for the standards of the six target analytes.

Analyte	Precursor ion (m/z)	Transition (m/z)	Collision energy (V)	Retention time (min)	Ion polarity
BPA	227.1	227.1 → 212.1	20.0	3.508	Negative
BPAF	335.1	335.1 → 197.1	24.0	3.818	Negative
BPE	213.1	213.1 → 198.1	16.0	3.048	Negative
BPF	199.1	199.1 → 199.1	0	2.526	Negative
BPS	249.0	249.0 → 108.0	32.0	1.179	Negative
BPZ	267.1	267.1 → 173.1	28.0	5.109	Negative

Table 6. The precursor ion, transition, collision energy (CE), retention time (RT), and ion polarity for the internal standards of the six target analytes.

Analyte	Precursor ion (m/z)	Transition (m/z)	Collision energy (V)	Retention time (min)	Ion polarity
BPA13C12	239.2	239.2 → 224.2	12.0	3.508	Negative
BPAF13C12	347.2	347.2 → 277.2	25.0	3.818	Negative
BPE13C6	219.1	219.1 → 204.2	16.0	3.048	Negative
BPF13C12	211.2	211.2 → 99.1	23.0	2.526	Negative
BPS13C12	257.2	257.2 → 112.1	32.0	1.179	Negative
BPZ13C12	279.1	279.1 → 179.2	32.0	5.109	Negative

2.3.3 Data processing and quantification

The MRM chromatograms were processed by using Agilent MassHunter Qualitative Analysis version 10.0 and Agilent MassHunter QQQ Quantitative Analysis version 10.2. The chromatograms were integrated automatically and visually inspected. The peaks were manually integrated if necessary. The identification of the different BPs were done by comparing the RT of the MRM visually in the calibration without matrix and the final calibration curve with serum as the matrix. The matched samples with added standard solution were compared and the quantification of the BPs were done by using stable isotope labelled (SIL) internal standard.

2.4 Method validation

The final method underwent a simplified validation process to assess its suitability for quantitative measurements. This validation was not complete, but a simplified validation was performed to test the potential for using the method in quantitative analyses. The following parameters were evaluated: limit of detection (LOD), limit of quantification (LOQ), linearity, precision, robustness and recovery.

The LOD and LOQ were determined based on the calibration curve and the matrix blank samples. The upper and lower limits of quantification were established to accurately represent the methods sensitivity across the linear range of the calibration curve.

The recovery was calculated by Equation 1.3 mentioned in subchapter 1.6.5 and the precision and robustness were calculated by analyzing the same matrix sample over multiple chromatogram runs (n=6).

3 Results and discussion

3.1 Identification of compounds of interest

The identification of the six BPs was performed by LC-MS/MS and is illustrated in Figure 4. For BPF, BPS and BPZ, the lower qualifiers were selected for better visualization.

Table 7. MRM Transitions for identifying all the six bisphenols

Analyte	MRM transitions
BPA$^{13}C_{12}$	239.2 → 224.2
BPAF$^{13}C_{12}$	347.2 → 327.3
BPE$^{13}C_{6}$	219.1 → 204.2
BPF$^{13}C_{12}$	211.2 → 99.1
BPS$^{13}C_{12}$	257.2 → 96.1
BPZ$^{13}C_{12}$	279.1 → 179.2

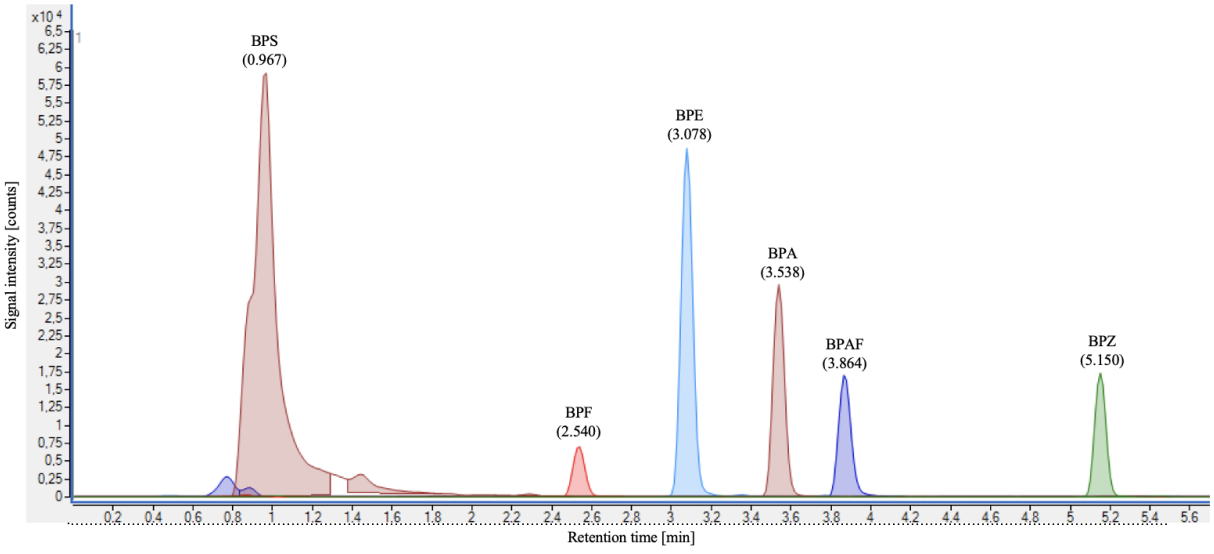


Figure 4: The chromatogram showing the identification of the six targeted BPs in one of the high spiked samples (500ng/mL) using Total Ion Chromatogram (TIC) and Multiple Reaction Monitoring (MRM) Chromatogram.

Figure 4 shows the chromatographic peaks of BPA, BPAF, BPE, BPF, BPS and BPZ with the analytical method and the transitions used are listed in Table 7. BPF had a low signal intensity

compared to the other chromatographic peaks, which could be caused by the presence of matrix effects and ion suppression. The other BPs had better peak resolution with fewer interfering compounds in the chromatogram. The serum sample matrix could contain many interfering compounds due to its complexity, which makes the analysis more challenging, as the retention time (RT) tended to shift for some samples. While the peak shape may appear satisfactory, any retention shifts can impact the accuracy of automated integration. Retention shift can lead to inaccuracies in peak integration, making manual integration necessary for ensuring precise quantification.

As for BPF, the automated integration was not optimal as the compound was difficult to quantify for the lower concentration levels in the calibration curve. By manually integrating the chromatographic peaks in Agilent Masshunter QQQ Quantitative, the precise areas under the peaks can be accurately determined. When manually integrating the peaks, it is crucial to maintain consistency in the integration process across all chromatographic peaks for the same compound. This ensures accuracy and reliability in the determination of peak areas. Additionally, manual integration can also induce human errors, as the integration is subjective.

The separation of BPS show some tailing in the peak shape, indicating inaccurate peak area measurements and interactions between the analyte and the stationary phase of the column. Tailing can affect the reliability and sensitivity of the quantitative results, particularly for the lower concentrations where the peak resolution may be compromised. In cases of tailing, such as with BPS, there is not a definitive approach to manually integrate the peak. When the peak shape is not optimal, one must decide where to cut the peak. It is essential to ensure capture of the total sufficient area, as this can impact the concentration values in the calibration curve. Therefore, it is crucial to integrate consistently to match the integration area for all peaks of the same compound. Tailing is a common issue when analyzing BPS, which may be due to the compound's high polarity as described in different studies [147, 148]. The polarity makes it challenging to retain the compound in the column, despite using a column designed for biphenyls. As for the other five BPs, the peaks are well-defined, symmetrical and indicate accurate integration and quantification of the analytes. Peak shape will be optimal when the analyte of interest is eluted from the column simultaneously. If the peak shape is not optimal, it can be due to incomplete elution of the substance from the column. With optimal peak shape, automated integration will typically perform well, but minor manual adjustments may still be necessary.

There have been challenges associated with the quantification of BPF at lower concentrations. One of the primary difficulties in analyzing BPF lies in its chromatographic behavior, where its peaks in chromatograms often appear messy or exhibit poor peak shape. At lower concentrations, the signal intensity generated by BPF may approach or fall below the detection limit of the analytical instrument. The quantification of BPF can be affected by matrix effects in a complex sample matrix as serum. Matrix interfering components can cause ion suppression or enhancement, leading to inaccuracies in the quantification of BPF.

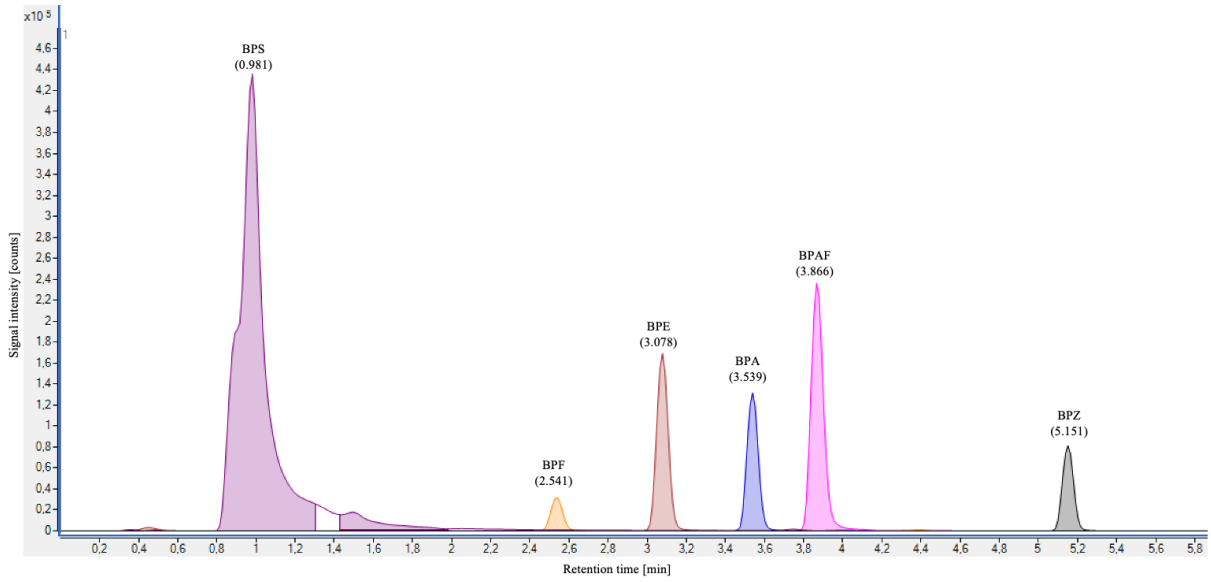


Figure 5: The chromatogram showing the quantification of the six targeted BPs in one of the high spiked samples (500ng/mL) using Total Ion Chromatogram (TIC) and Multiple Reaction Monitoring (MRM) Chromatogram.

The abundance of the BPs in serum samples was assessed, revealing distinct patterns in their relative concentrations. BPS emerged as the predominant compound, exhibiting the highest abundance among the BPs analyzed. Following the next highest abundance was observed for BPAF, indicating its considerable presence in the sample. The lower quantifier (335.1 → 197.1) was chosen for BPAF, as the higher peak abundance from the quantifier at (335.1 → 265.1) would have exceeded BPS and the other compounds as illustrated in Figure 6. As for BPF, the lower qualifier 199.1 → 93.1 was chosen for better visualization as the higher qualifier peak included background noise and interference as shown in Figure 7.

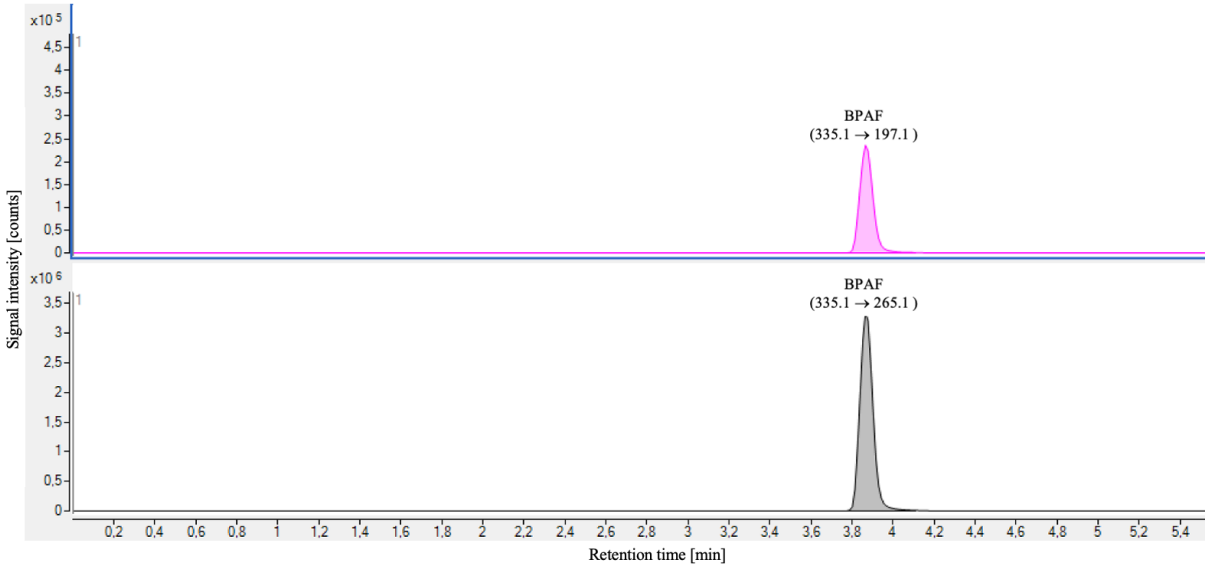


Figure 6: The chromatogram showing the identification of BPAF in one of the high spiked samples (500ng/mL) using Total Ion Chromatogram (TIC) and Multiple Reaction Monitoring (MRM). Chromatogram. The lower qualifier (335.1 → 197.1) has been chosen for better visualization.

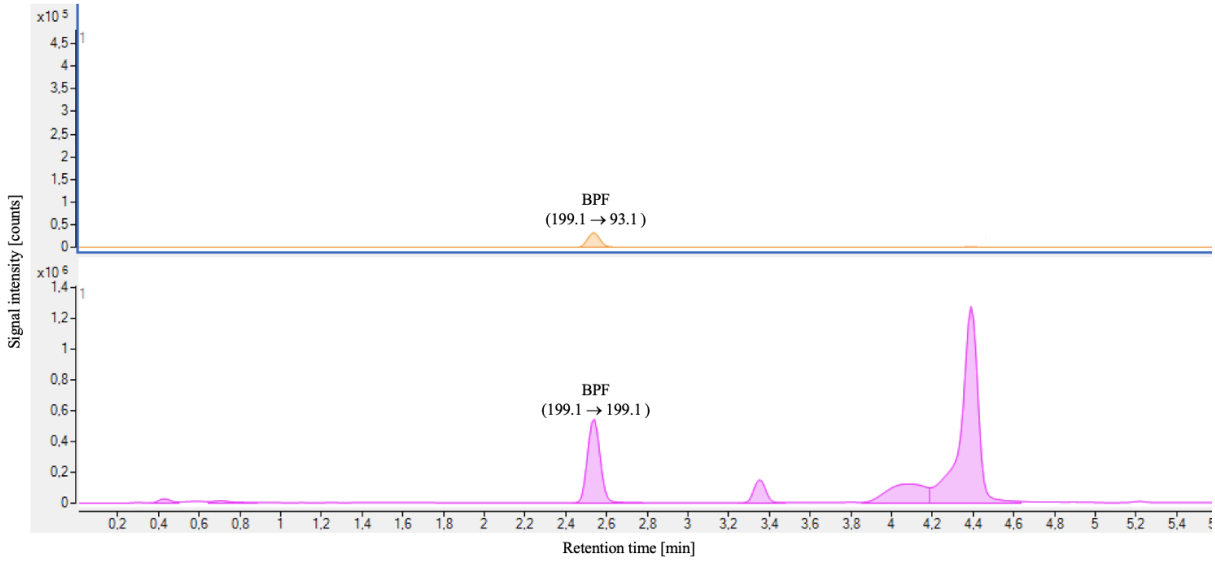


Figure 7: The chromatogram showing the identification of BPF in one of the high spiked samples (500ng/mL) using Total Ion Chromatogram (TIC) and Multiple Reaction Monitoring (MRM) Chromatogram. The lower qualifier (199.1 → 93.1) has been chosen for better visualization.

The chromatographic peaks for the solvent blank (instrument blank) and the matrix blank had levels of background noise, but were found to be below LOD and LOQ. The signal levels of BPA and in the blank samples were higher than for the other BPs throughout the validation

process, but below LOD as illustrated in Figure 8. The levels of contamination of BPA were expected in the analysis as BPA is ubiquitous in the environment and can easily contaminate low concentration samples. There were taken some preliminary measures to minimize contamination from equipment and the surrounding environment at the laboratory. Equipment was thoroughly washed and rinsed according to instructions, and plastic equipment such as test tubes, vials, and pipette tips were replaced with glass wherever feasible.

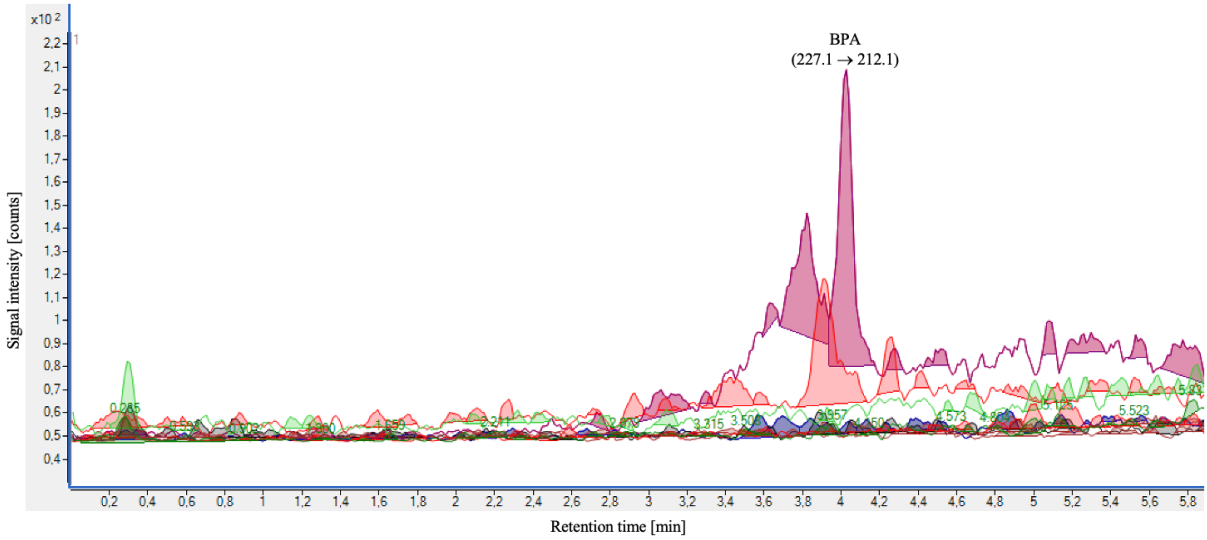


Figure 8: The chromatogram showing the identification of BPs in one of the solvent blank samples using Total Ion Chromatogram (TIC) and Multiple Reaction Monitoring (MRM). The most abundant qualifier (227.1 → 212.1) for BPA is visualized at the highest peak.

A calibration curve with ISTD, and different concentrations of standard solutions in methanol (MeOH) were generated as described in subchapter 2.2.4. and used for assessing the sample preparation method and the first tests with serum samples. After successful testing of the prepared serum samples, a new calibration curve including serum as matrix underwent the same sample preparation method as for the test serum samples.

3.2 Method validation

3.2.1 Linearity

A total of 8 calibrators were used in this method validation, to form a calibration curve within the range of 0.1-500ng/mL. The linear ranges were based on the measurement of the peak area of the compounds versus the peak area of the corresponding deuterated ISTD analog. Validation standards were analyzed to investigate the linear range from ULOD at 0.1ng/mL to the ULOD at 500ng/mL. Increased residuals were observed at the lowest concentration range. When deciding on a calibration model, a weighted (1/x) line and ignoring the origin, was found to give the best fit to the data points for all BPs based on inspection of residuals and calibration curve fit. With this weighting, the variations in the lower and higher concentrations levels influenced the slope of the curve more equally. That is desired as the lower range of concentrations is of focus in this study.

The weighting options of force, include, and ignore origin were considered in the analysis, with a preference of weighing the lower concentrations levels equal to the higher concentrations. Variations in concentrations, particularly at higher levels, will naturally influence the slope for the calibration curve more than the lower levels. Deviations in the measurements of the data point with concentration at 500ng/mL will have a greater impact on the slope compared to the lower values. Weighting by $1/x^2$ would accentuate the influence of lower concentrations more than for 1/x but lead to more forcing of the curve's slope. It is ideal to minimize the changes of weighing to achieve the desired results but it can be done to improve the curve if needed.

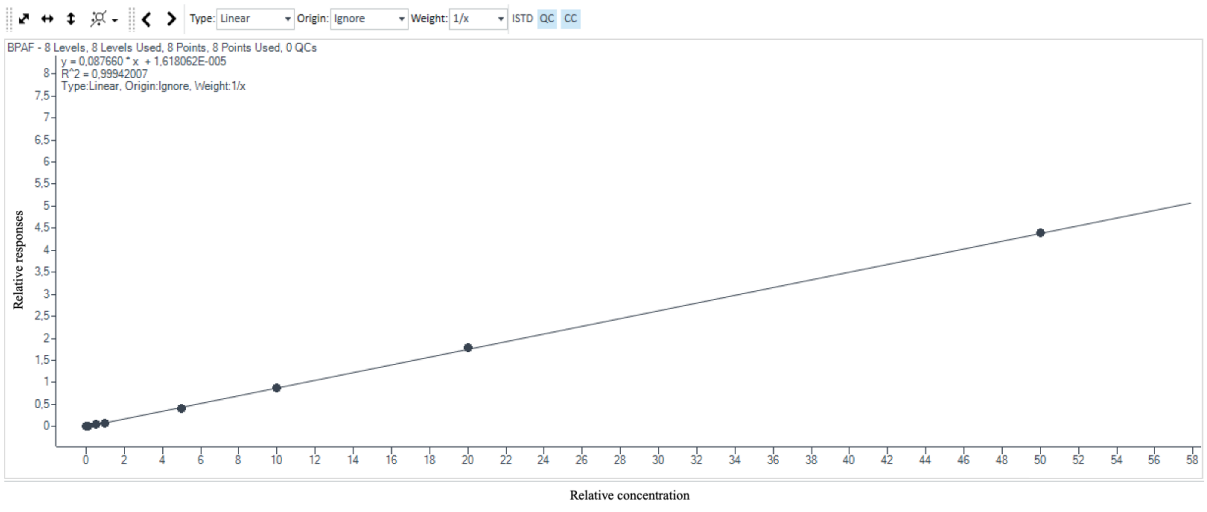


Figure 9: Calibration curve of BPAF from 0.1ng/mL to 500ng/mL.

Five of the BPs compounds were found to be quantified having an $R^2 \geq 0.999$. BPF had a slightly lower R^2 value of 0.993. The calibration curve for BPAF is illustrated in Figure 9. As for BPA, BPE, BPF, BPS and BPZ, the calibration curves are illustrated in the Appendix in Figures 11-15.

Table 8. The squared correlation coefficient for all six bisphenols.

Analyte	Squared correlation coefficient (R^2)
BPA	0.999
BPAF	0.999
BPE	0.999
BPF	0.993
BPS	0.999
BPZ	0.999

3.2.2 LOD and LOQ

The detection and quantification limits were calculated to assess if the total of amount native concentration of the standards in the serum samples was quantifiable. For this method, 4 blank samples were picked where the response was used to calculate the standard deviation. Based on this, the concentration of the blank samples was calculated using the mean of each BPs before determining the detection and quantification limits. These limits were obtained by multiplying the standard deviation by 3 (for LOD) and 10 (for LOQ). LLOQ and ULOQ were established at 0.1ng/mL and 500ng/mL, respectively.

The LOD and LOQ for BPF are 0.07ng/mL and 0.2ng/mL, respectively. It is lower than for any of the other BP compounds, indicating that BPF is easier to detect and quantify. However, it was challenging to detect BPF in the blank samples, leading to difficulties in establishing its LOD and LOQ. BPA was determined with the highest LOD and LOQ at 0.8ng/mL and 2.1ng/mL, respectively. It is expected that the LOD and LOQ are highest for BPA, given their presence in low concentration levels in many of the analyses conducted in this study. BPA is widely used in various applications, making it the most common BP component today. Although efforts have been made to reduce plastic equipment use and contamination control, eliminating all contamination remains difficult. Many researchers dealing with BPA in

analytical methods describe problems with contaminations in analyses of the compound [149, 150].

Table 9. LOD and LOQ determined for the six BPs in ng/mL.

	BPS	BPF	BPE	BPA	BPAF	BPZ
<LOD	0,07	0,01	0,4	0,8	0,04	0,06
<LOQ	0,2	0,03	0,5	2,1	0,1	0,2

3.2.3 Recovery

To evaluate the clean-up methods by sample preparation, the recovery of the six BPs of interest was monitored. The recovery of the spiked serum samples is shown in Table 10 for the:

a) Pre-spiked serum samples before sample preparation in 5 concentration levels.
b) Post-spiked serum blind samples after sample preparation with std and ISTD in 5 concentration levels.
c) Post-spiked methanol (MeOH) samples with std and ISTD in 5 concentration levels.

The recovery for all three sample types was within (70-110%), whereas the pre-spiked serum samples had an average recovery of 105%. The post-spiked serum samples had an average recovery of 101% and the post-spiked methanol (MeOH) samples had an average of 106%.

The recoveries obtained in solid-phase extraction (SPE) are found above 100%, which might indicate the presence of matrix effects in this sample preparation method that may cause an overestimation of analyte concentrations in the samples. While SPE can be helpful for cleaning and concentrating samples, its vulnerability to matrix effects highlights the need to further validate and optimize the analytical method.

Table 10. Recovery (%) in pre-spiked samples, post-spiked serum samples and post-spiked methanol (MeOH) samples (1-200 ng/mL) compared with theoretical known concentration. The post-spiked serum samples were prepared as blind samples under sample preparation and spiked with std and ISTD after sample preparation and before analysis.

	BPS Results	BPF Results	BPE Results	BPA Results	BPAF Results	BPZ Results
Name	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**
Pre-spiked serum sample 1ng/mL	100,0	n.a	102,1	168,7	89,2	99,0
Pre-spiked serum sample 5ng/mL	100,7	115,9	99,5	116,5	100,6	108,5
Pre-spiked serum sample 10ng/mL	109,4	139,9	117,2	113,9	108,0	116,2
Pre-spiked serum sample 100ng/mL	102,1	111,1	108,4	104,7	104,4	112,4
Pre-spiked serum sample 200ng/mL	100,2	108,87	104,1	101,2	100,8	107,6
Post-spiked serum sample 1ng/mL	106,5	n.a	96,8	133,8	97,0	116,3
Post-spiked MeOH sample 1ng/mL	99,6	115,8	106,7	100,9	101,1	106,3
Post-spiked serum sample 5ng/mL	100,2	103,1	101,1	108,8	100,0	107,8
Post-spiked MeOH sample 5ng/mL	89,3	101,5	92,0	99,2	99,1	95,6
Post-spiked serum sample 10ng/mL	98,7	103,2	98,2	100,2	90,5	103,2
Post-spiked MeOH sample 10ng/mL	98,1	101,1	100,1	100,3	97,4	100,8
Post-spiked serum sample 100ng/mL	100,3	102,4	100,0	101,5	100,3	103,6
Post-spiked MeOH sample 100ng/mL	100,0	101,4	101,0	101,7	100,7	102,3
Post-spiked serum sample 200ng/mL	101,3	107,8	108,4	109,6	107,3	115,1
Post-spiked MeOH sample 200ng/mL	120,7	132,6	130,8	128,4	127,0	134,7

* Accepted recovery (%) ranges from 70-110.
* The measurements highlighted in red are outliers for the acceptable range for recovery rate.
* n.a = not found any detectable peaks for the compound.

3.2.4 Precision and robustness

Precision and robustness are vital parameters that need to be assessed in the validation of a method. It is important to evaluate the spread of the data and determine the validity of a single test result. Precision was evaluated by calculating the relative standard deviation (RSD) from the analyte peak area of six runs of the same serum spiked sample with a concentration of 500ng/mL over several chromatogram runs. The RSD % was calculated from Equation 1.6 discussed in subchapter 1.6.6.

The precision ranges between 1 % and 4 % for the different BP compounds as shown in Table 11. The precision ranges observed (1% to 4% RSD) for the different BPs indicate low variations among replicate measurements. A low RSD% indicates low variability among replicate measurements, demonstrating the method's ability to produce consistent and reproducible results. The criteria of <15% RSD ensures that the method meets the precision requirements for each analyte transition, enhancing confidence in the accuracy and reliability of quantitative measurements.

Table 11. Precision rates of the quantifying ions.

Analytes	MRM	R (%)	RSD (%)
BPA	227.1 → 212.1	97	2
BPAF	335.1 → 265.1	106	3
BPE	213.1 → 198.1	99	1
BPF	199.1 → 199.1	97	4
BPS	249.0 → 108.0	93	4
BPZ	267.1 → 173.1	100	2

BPF and BPS show higher RSD % values compared to the other BPs. While still being within an acceptable range, the slightly higher variability may indicate a lower level of precision compared to BPA, BPAF, BPE, and BPZ. BPF and BPS may be more susceptible to matrix effects compared to the other BPs, including ion suppression. Ion suppression can affect the concentration values in the chromatographic analysis and contribute to a higher RSD% as the residuals deviate more from the data points in the calibration curve. For the lower concentration levels, the deviations from the expected response of the calibration curve are high and compromise the precision of BPF. BPF exhibits a lower and poorer peak shape compared to

other BP components, which can be a contributing factor to RSD%. For BPS, the peak shape and the integration of these peaks may contribute to variations in the measurements and concentration values. Peak shape is not consistently uniform, and manually integrating each peak can lead to errors and inconsistencies in the integration.

Multiple reaction monitoring (MRM) in Qual and Quant are important tools to determine the concentration levels of the compound of interest. Qual is used to identify the different analytes in a sample and Quant can be used to ensure that the area ratio between quantifying and qualifying peaks falls within a certain range. A quality control factor is that the quantifying and qualifying peaks resemble each other. Differences within the peaks can lead to incorrect results and false positives. When working with bisphenols, it can be tricky because there are not always good qualifiers available for BP compounds.

3.2.5 Blanks samples

The chromatographic peaks in the blank samples (solvent and matrix) are illustrated in Figure 10. For better visualization, the qualifiers for BPS and BPAF were chosen. The contribution of signals of BPS in the matrix blank is of unknown origin. Signals of the compounds in solvent and matrix blank indicate that contamination sources are present in the analytical process, somewhere in between sample preparation and analysis. Due to some difference in the RT in the matrix blank and the solvent blank for the different BPs, this indicates that the are some contributions of matrix effects. Matrix effects can influence the analyte retention and ionization, leading to variations in peak shape, signal intensity, and RT.

BPS has a short RT (<1min), which contributes to a high uncertainty when identifying and quantifying the analyte because of possible signals from the dead volume of the column. The dead volume represents the portion of the column where analytes do not undergo separation, potentially leading to spurious peaks and inaccuracies in quantification. Signals of BPS were produced throughout the analytical process, which strengthened the possibility of problems with the short RT. BPS can be challenging to quantify without a stable isotope-labelled standard (SIL) because it can introduce significant interference. It is not difficult to identify the compound, but when working with more extensive methods, using a stable isotope-labelled standard might be advisable due to its polarity and short retention time.

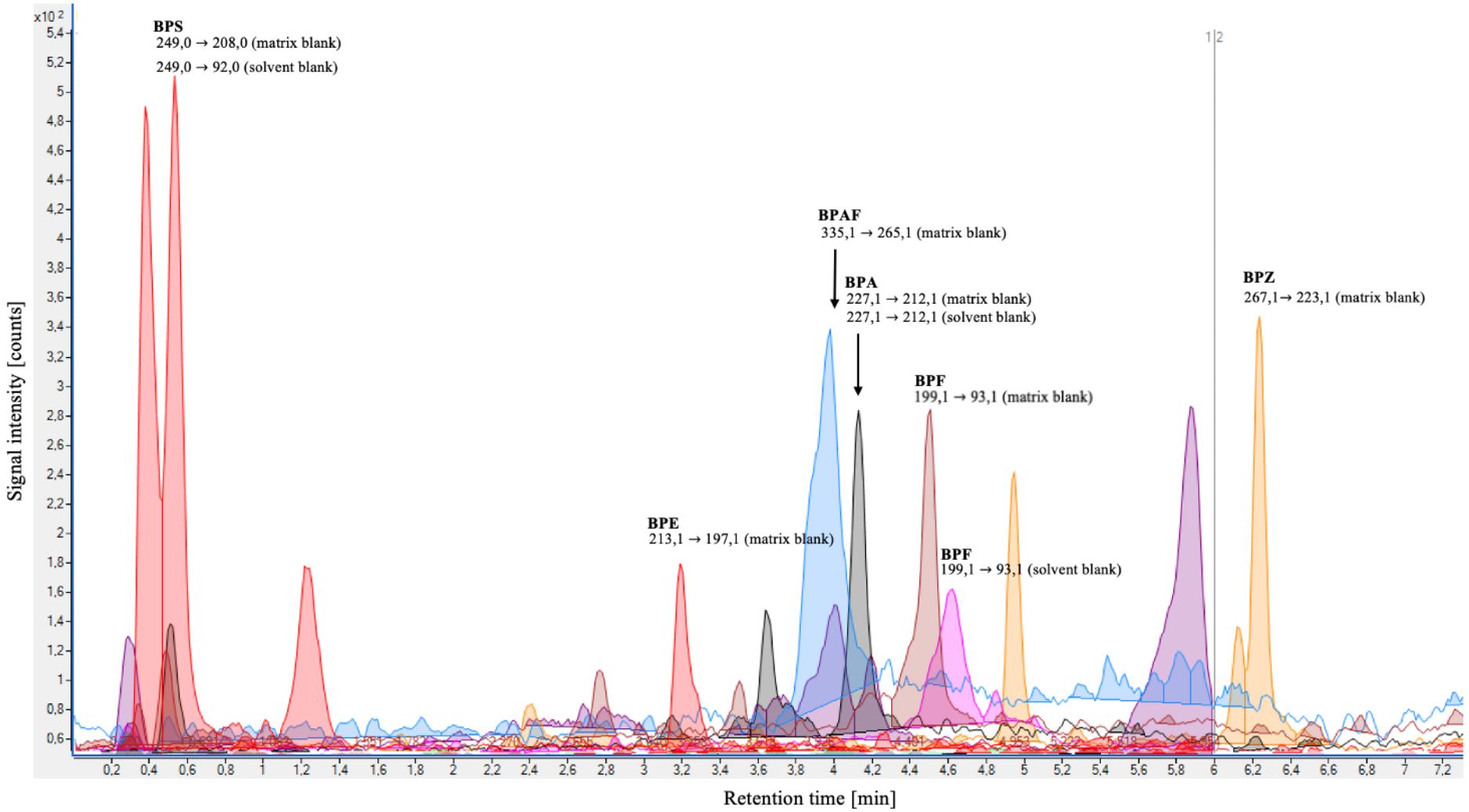


Figure 10: Chromatogram of solvent blanks and matrix blanks BPF (199,1), BPE (213,1), BPA (227,1), BPS (249,0), BPZ (267,1) and BPAF (335,1).

3.3 Serum sample analysis

After optimizing and developing the preparation method for serum samples, the method was tested on human blood samples from two districts in Argentina, South America to check whether the method was sufficiently sensitive to detect bisphenols in the real samples. The blood samples have been used in an earlier study to determine levels of different environmental contaminants in pregnant women and infants. It was not specified whether these samples were serum or plasma. However, it is presumed that they are plasma samples as they usually are easier to analyze as more of the impurities and interferences have been eliminated than for serum samples. The developed method was tested on 18 different samples at the Faculty of Veterinary Medicine (VET).

The samples went through the same sample preparation as for the other samples that had been tested earlier and the calibration curve including the matrix. The concentration levels in the different samples before sample preparation, including SPE are shown in the Appendix in Table 25. LOD and LOQ used are listed in Table 9. There were detected levels of BPS over LOQ in one of the 18 samples but none of the other BPs were detected above LOQ in any of the samples, as shown in Table 12. Sample 17 exceeded the LOQ with a concentration of 0.3ng/mL. For BPAF, BPE, BPS and BPZ, some of the concentrations of the analytes were detected above LOD, but not LOQ. Given that the compounds can be detected and identified in some of the serum samples, there is a need for optimization of the method to better quantify these bisphenols.

Table 12. LOD and LOQ for the 18 real human samples from Argentina with ISTD in ng/mL.

Name	Start volume (mL)	BPS results Final Conc. (ng/mL)	BPF results Final Conc. (ng/mL)	BPE results Final Conc. (ng/mL)	BPA results Final Conc. (ng/mL)	BPAF results Final Conc. (ng/mL)	BPZ results Final Conc. (ng/mL)	End volume (mL)
Serum sample 1	0,5	0,09	n.a	0,3	0,04	0,06	0,03	0,2
Serum sample 2	0,5	0,04	n.a	0,2	0,3	0,05	0,03	0,2
Serum sample 3	0,5	0,04	n.a	0,4	0,2	0,06	0,05	0,2
Serum sample 4	0,5	0,03	n.a	0,3	0,1	0,05	0,01	0,2
Serum sample 5	0,5	0,05	n.a	0,2	0,1	0,03	0,07	0,2
Serum sample 6	0,5	0,05	n.a	0,3	0,3	0,04	0,06	0,2
Serum sample 7	0,5	0,04	n.a	0,3	0,2	0,04	0,07	0,2
Serum sample 8	0,5	0,05	n.a	0,3	0,3	0,02	0,04	0,2
Serum sample 9	0,5	0,06	n.a	0,4	0,2	0,03	0,08	0,2
Serum sample 10	0,5	0,3	n.a	0,4	0,3	0,04	0,04	0,2
Serum sample 11	0,5	0,2	n.a	0,4	0,1	0,04	0,05	0,2
Serum sample 12	0,5	0,2	n.a	0,3	0,1	0,04	0,05	0,2
Serum sample 13	0,5	0,2	n.a	0,3	0,5	0,05	0,02	0,2
Serum sample 14	0,5	0,2	n.a	0,3	0,2	0,06	0,08	0,2
Serum sample 15	0,5	0,2	n.a	0,3	0,2	0,05	0,02	0,2
Serum sample 16	0,5	0,2	n.a	0,3	0,1	0,04	0,03	0,2
Serum sample 17	0,5	0,2	n.a	0,3	0,3	0,04	0,04	0,2
Serum sample 18	0,5	0,2	n.a	0,3	0,3	0,03	0,02	0,2

4 Conclusion and future perspectives

4.1 Conclusion

In conclusion, the study aimed to develop a robust method for the quantification of six BPs in serum samples using LC-MS/MS. The method developed implemented solid-phase extraction (SPE) in the sample preparation to enhance sensitivity, selectivity, and reduce potential interferences and matrix effects. LOD ranged from 0.01-0.8ng/mL and LOQ ranged from 0.03-2.1ng/mL for all BPs. However, further optimization is necessary to improve the sensitivity, particularly for detecting low concentrations of BPF in biological samples. RSD% for all the six bisphenols ranged from 1-4%. Recovery testing and robustness evaluation highlighted the method's accuracy and precision, but additional optimalization of the method is required to meet the desired sensitivity levels.

18 serum samples from Argentina were analyzed to assess the method's performance in a practical setting. One sample (17) exceeded the LOQ for BPS with a concentration of 0.3ng/mL. None of the other BPs were detected in the samples above LOQ. For some of the samples, the concentration of the analytes was above the LOD for BPAF, BPE, BPS and BPZ, but not for LOQ. Given that the compounds can be detected and identified in the serum samples, further optimization of the method is needed to quantify these BPs better. The findings suggest that while the method shows promise, it requires further optimization to reliably quantify low concentrations of bisphenols in serum samples. Continued refinement of the method parameters, such as sample preparation techniques and instrument settings, is essential to enhance the sensitivity and ensure accurate quantification of BPs in biological matrices. Addressing the challenges associated with quantifying low concentrations of BPs in biological samples is crucial for assessing their potential health impacts and implementing effective regulatory measures to mitigate risks associated with these compounds.

4.2 Future perspectives

The method developed in this study is not yet complete in terms of validation and needs further refinement to achieve better sensitivity, accuracy, and precision, enabling analysis of the serum samples and inter-laboratory samples. To improve the accuracy and precision of the method, additional sample preparation steps can be introduced to clean up the samples and concentrate the analytes better. Laboratories interested in further research on bisphenols (BPs) should test potential sources of environmental contamination from the surroundings in the laboratory, including the equipment used. There were observed some contamination in this study and the origin is unknown. When working with bisphenols, it is essential to detect these sources of contamination to exclude further contamination.

Also, the sample preparation method used in this study may not be the most optimal for BPs in serum samples. It is desirable to achieve a lower limit of detection (LOD) and limit of quantification (LOQ) for serum matrix samples. It is desired to test if smaller volumes of serum can be used with the developed method, for animal testing purposes as it is not as accessible as other matrices. The aim is that this method can be further optimized and used for research and assessment purposes of risks from chemicals by PARC.

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5 Appendix

5.1 Materials and chemicals

The tables present the different chemicals, bisphenol standards and instruments used in this study.

Table 13. Chemicals used in the study.

Chemical	Purity/gradient	CAS-number	Manufacturer
Methanol	LC-MS grade	67-56-1	VWR, Radner, PA, USA
2-propanol	LC-MS grade	67-63-0	VWR, Radner, PA, USA
Ammonium acetate	≥99.99%	631-61-8	VWR, Radner, PA, USA

Table 14. BPs standards used in the study.

Chemical	Purity/gradient	CAS-number	Manufacturer
Bisphenol A	99.6	80-05-7	AccuStandard, New Haven, CT, USA
Bisphenol AF	100.0	1478-61-1	AccuStandard, New Haven, CT, USA
Bisphenol E	100.0	2081-08-5	AccuStandard, New Haven, CT, USA
Bisphenol F	100.0	620-92-8	AccuStandard, New Haven, CT, USA
Bisphenol S	98.0	80-09-1	AccuStandard, New Haven, CT, USA
Bisphenol Z	100.0	843-55-0	AccuStandard, New Haven, CT, USA

Table 15. Instruments, equipment, and software used in the study.

Product	Specifications/model	Manufacturer
LC system	1290 Infinity II LC system	Agilent Technologies, Santa Clara, CA, USA
MS	6495 triple quadrupole LC/MS system (6495 LC/TQ)	Agilent Technologies, Santa Clara, CA, USA
LC column	Raptor Biphenyl 1.8µm 90A 50mm length x 2.1mm ID	Waters Corporation, Tauton, MA, USA
Software	Agilent Masshunter QQQ Quantitative Analysis version 10.2 Agilent Masshunter Qualitative Analysis version 10.0	Agilent Technologies, Santa Clara, CA, USA
SPE column	Oasis® HLB 6cc Vac Cartridge 150mg	Waters Corporation, Tauton, MA, USA
SPE manifold	VacMaster™ 20 Sample Processing Station	Biotage®, Uppsala, Sweden
Nitrogen evaporator	REACTI-THERM 3 HEAT #TS-18824	Thermo Fisher Scientific, Waltham, MA, USA
Shaker	MS2 Minishaker	IKA, Wilmington, NC, USA
Automated pipettor	1. mLINE Adjustable Volume, Single Channel Pipette, 20 - 200uL 2. mLINE Adjustable Volume, Single Channel Pipette, 100 - 1000uL	Sartorius (Biohit), Helsinki, Finland

5.2 Sample contents before sample preparation

Table 16. The composition of the different samples for the calibration curve (0.1-500ng/mL).

Calibration curve					
Conc. std mix	**Std mix (µL)**	**MeOH (µL)**	**Volume ISTD (µL)**	**Std curve conc. (ng/mL)**	**End volume (µL)**
10 µg/mL	50	850	100	500	1000
10 µg/mL	20	880	100	200	1000
10 µg/mL	10	890	100	100	1000
200 ng/mL	250	650	100	50	1000
200 ng/mL	50	850	100	10	1000
200 ng/mL	25	875	100	5	1000
5 ng/mL	200	700	100	1	1000
5 ng/mL	20	880	100	0.1	1000

*ISTD 1µg/mL corresponds to a final concentration of 100ng/mL ISTD in the standard curve.

Table 17. The composition of the different matrix samples for the calibration curve (0.1-500ng/mL) before further sample preparation.

Calibration curve with matrix							
Serum (µL)	**Conc. std mix**	**Std mix (µL)**	**MeOH (µL)**	**Volume ISTD (µL)**	**Water (µL)**	**Std curve conc. (ng/mL)**	**End volume (µL)**
500	1µg/mL	100	30	20	2300	500	200
500	1µg/mL	40	90	20	2300	200	200
500	1µg/mL	20	110	20	2300	100	200
500	100ng/mL	100	30	20	2300	50	200
500	100ng/mL	20	110	20	2300	10	200
500	50ng/mL	20	110	20	2300	5	200
500	5ng/mL	40	90	20	2300	1	200
500	1ng/mL	20	110	20	2300	0.1	200

*ISTD 1µg/mL corresponds to a final concentration of 100ng/mL ISTD in the standard curve.

Table 18. The composition of the different samples for the method development tests prior to further sample preparation..

	Serum (µL)	Conc. std mix	Std mix (µL)	MeOH (µL)	Volume ISTD (µL)	Water (µL)	Std curve conc. (ng/mL)	End volume (µL)
Sample	500		0	130	20	2300		200
	500	5ng/mL	40	90	20	2300	1	200
	500	50ng/mL	20	110	20	2300	5	200
	500	100ng/mL	20	110	20	2300	10	200
	500	1µg/mL	20	110	20	2300	100	200
	500	1µg/mL	40	90	20	2300	200	200
Blind	500		0	150	0	2300		200
Blank			0	150	0	2800		200
Rep	500	10µg/mL	50	0	100	2300	500	1000

*ISTD 1µg/mL corresponds to a final concentration of 100ng/mL ISTD in the samples.

*There were 5 blind samples prepared which were post-spiked after sample preparation and before analysis.

*Rep = sample was prepared to calculate and determine the robustness of the analytical method.

Table 19. The composition of the different blind samples that were spiked after sample preparation and before analysis.

	Sample (µL)	**Conc. std mix**	**Std mix (µL)**	**MeOH (µL)**	**Volume ISTD (µL)**	**Std curve conc. (ng/mL)**
Serum	140	5ng/mL	40	0	20	1
Serum	140	50ng/mL	20	20	20	5
Serum	140	100ng/mL	20	20	20	10
Serum	140	1µg/mL	20	20	20	100
Serum	140	1µg/mL	40	0	20	200
Methanol	140	5ng/mL	40	0	20	1
Methanol	140	50ng/mL	20	20	20	5
Methanol	140	100ng/mL	20	20	20	10
Methanol	140	1µg/mL	20	20	20	100
Methanol	140	1µg/mL	40	0	20	200

*ISTD 1µg/mL corresponds to a final concentration of 100ng/mL ISTD in the samples.

5.3 Calibration curves

Calibration curves including matrix of the six BPs from subchapter 3.2.1. The figures show linearity, slope, and R^2 for BPA, BPE, BPF, BPS and BPZ.

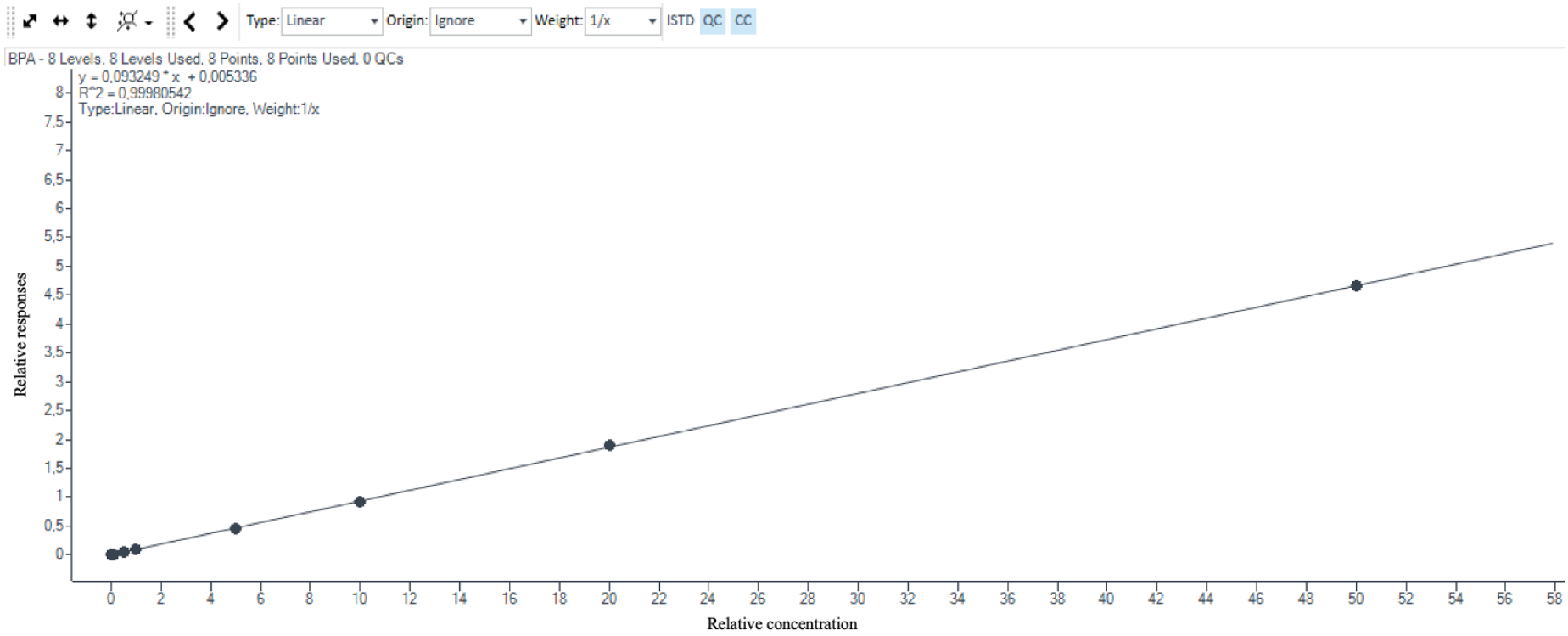


Figure 11: Calibration curve of BPA from 0.1ng/mL to 500ng/mL.

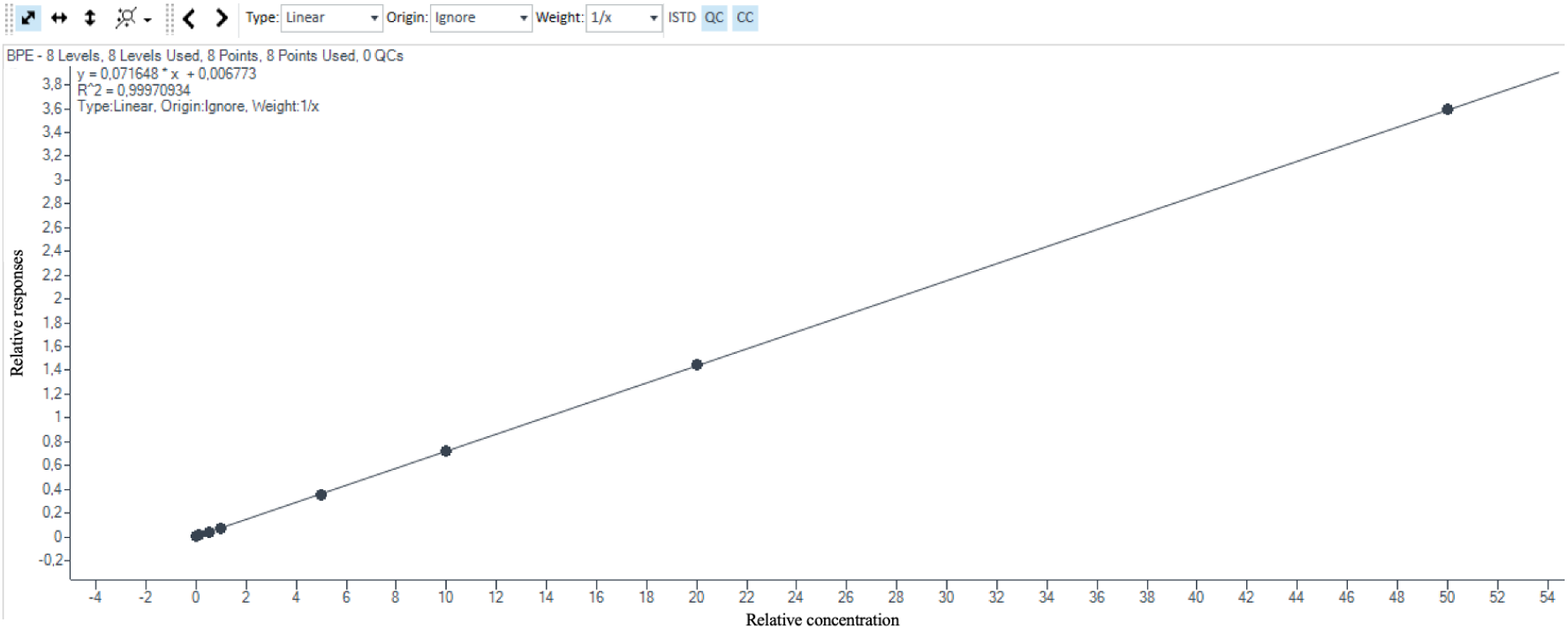


Figure 12: Calibration curve of BPE from 0.1ng/mL to 500ng/mL.

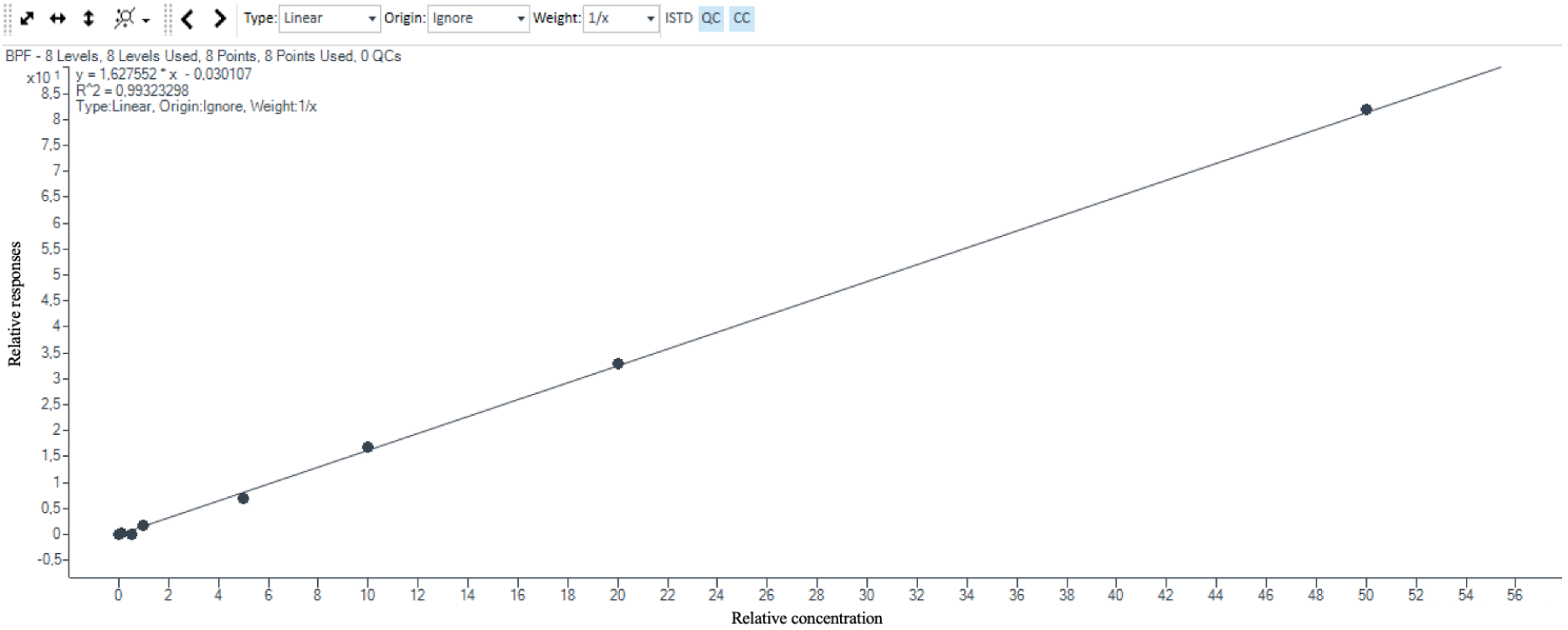


Figure 13: Calibration curve of BPF from 0.1ng/mL to 500ng/mL.

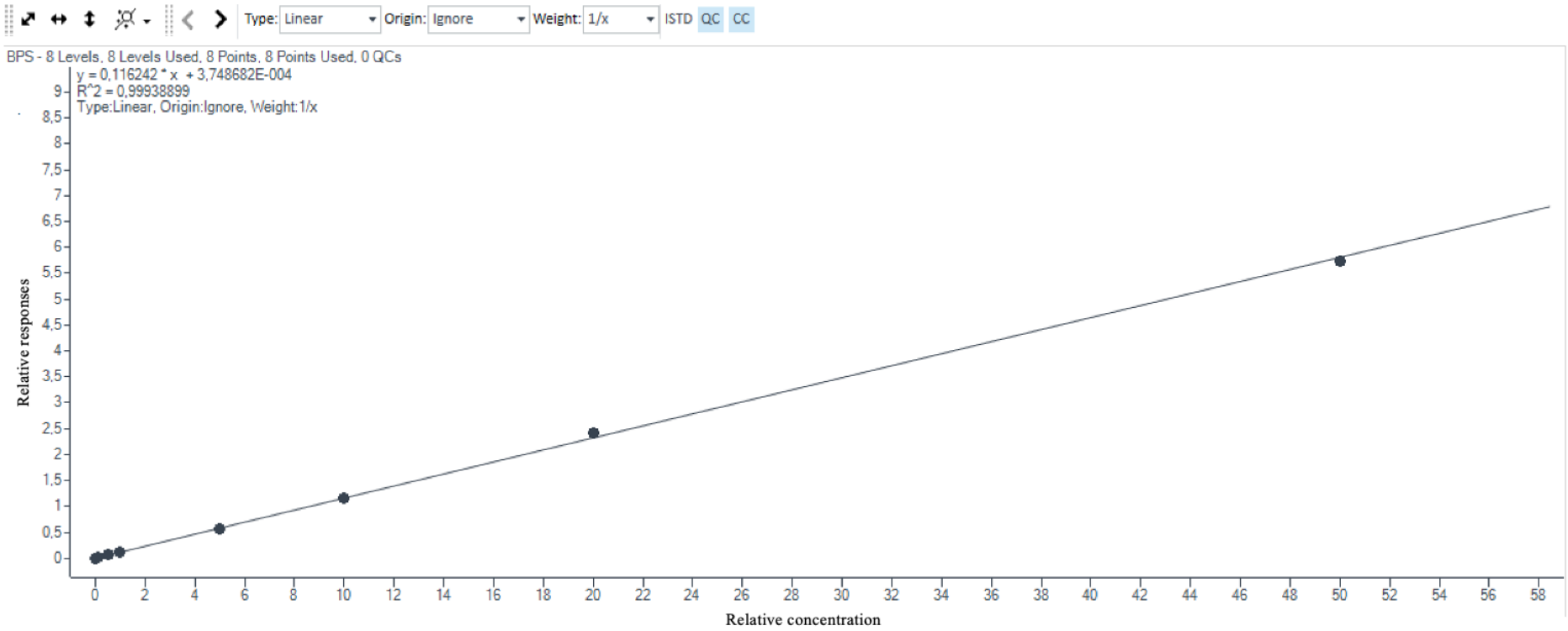


Figure 14: Calibration curve of BPS from 0.1ng/mL to 500ng/mL.

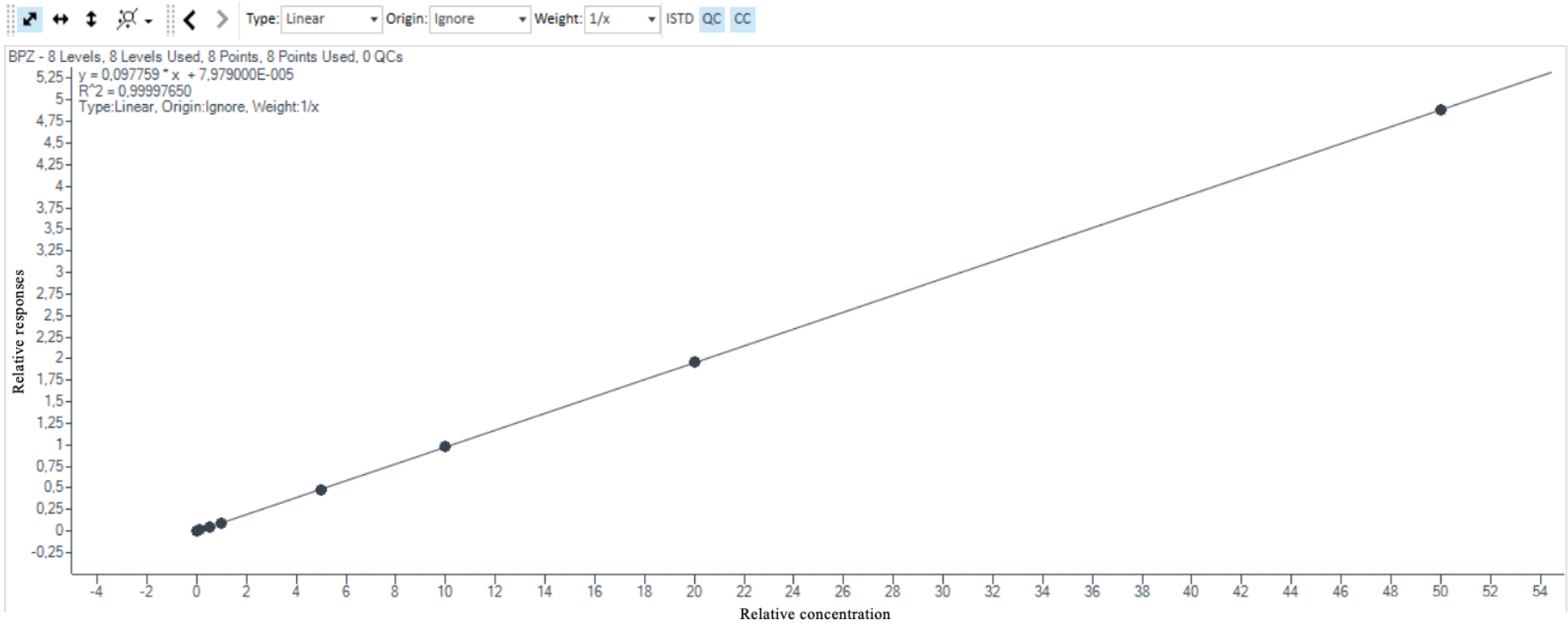


Figure 15: Calibration curve of BPZ from 0.1ng/mL to 500ng/mL.

5.4 Raw data

Table 20. Results of the measurement for the pre-spiked serum samples and post-spiked blind serum samples and methanol (MeOH) with 5 concentration levels (1-200ng/mL).

	BPS Results	BPF Results	BPE Results	BPA Results	BPAF Results	BPZ Results
Name	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**
Pre-spiked serum sample 1ng/mL	1,0	n.a	1,0	1,7	0,9	1,0
Pre-spiked serum sample 5ng/mL	5,0	5,8	5,0	5,8	5,0	5,4
Pre-spiked serum sample 10ng/mL	10,9	14,0	11,7	11,4	10,8	11,6
Pre-spiked serum sample 100ng/mL	102,1	111,1	108,4	104,7	104,4	112,4
Pre-spiked serum sample 200ng/mL	200,3	217,7	208,1	202,3	201,7	215,1
Post-spiked serum sample 1ng/mL	1,1	n.a	1,0	1,3	1,0	1,2
Post-spiked MeOH sample 1ng/mL	1,0	1,2	1,1	1,0	1,0	1,1
Post-spiked serum sample 5ng/mL	5,0	5,2	5,1	5,4	5,00	5,4
Post-spiked MeOH sample 5ng/mL	4,5	5,1	4,6	5,0	5,0	4,8
Post-spiked serum sample 10ng/mL	9,9	10,3	9,8	10,0	9,0	10,3
Post-spiked MeOH sample 10ng/mL	9,8	10,1	10,0	10,0	9,7	10,1
Post-spiked serum sample 100ng/mL	100,3	102,4	100,0	101,5	100,3	103,6
Post-spiked MeOH sample 100ng/mL	100,0	101,4	101,0	101,7	100,7	102,3
Post-spiked serum sample 200ng/mL	202,5	215,6	216,7	219,1	214,6	230,2
Post-spiked MeOH sample 200ng/mL	241,4	265,2	261,6	256,8	253,9	269,4

* n.a = not found any detectable peaks for the compound.

Table 21. Recovery (%) in pre-spike samples (1-200ng/mL) compared to post-spiked serum blind samples (1-200ng/mL).

	BPS Results	BPF Results	BPE Results	BPA Results	BPAF Results	BPZ Results
Name	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**
Sample 1 ng/mL	93,9	n.a	105,4	126,1	91,9	85,2
Sample 5 ng/mL	100,5	112,4	98,4	107,1	100,5	100,7
Sample 10 ng/mL	110,8	135,6	119,3	113,7	119,3	112,6
Sample 100 ng/mL	101,8	108,5	108,4	103,1	104,1	108,5
Sample 200 ng/mL	98,9	101,0	96,0	92,3	94,0	93,4

*Accepted recovery (%) range from 70-110.

Table 22. Recovery (%) in pre- spike samples (1-200 ng/mL) compared to post-spiked methanol (MeOH) samples (1-200ng/mL).

	BPS Results	BPF Results	BPE Results	BPA Results	BPAF Results	BPZ Results
Name	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**	**Rec. (%)**
Sample 1 ng/mL	100,4	n.a	95,7	167,2	88,2	93,2
Sample 5 ng/mL	112,8	114,2	108,1	117,4	101,5	113,5
Sample 10 ng/mL	111,6	138,4	117,2	113,6	110,9	115,3
Sample 100 ng/mL	102,1	109,6	107,3	102,9	103,6	109,9
Sample 200 ng/mL	83,0	82,1	79,5	78,8	79,4	79,9

*Accepted recovery (%) range from 70-110.

Table 23. Results of the measurements for matrix blank samples with ISTD in ng/mL.

	BPS results	BPF results	BPE results	BPA results	BPAF results	BPZ results
Name	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**
Matrix blank sample 1	0,04	0,01	0,8	0,5	0,06	0,02
Matrix blank sample 2	0,04	0,02	0,8	0,4	0,07	0,09
Matrix blank sample 3	0,02	0,02	0,8	0,7	0,06	0,07
Matrix blank sample 4	0,2	0,007	0,7	1,4	0,03	0,07

Table 24. Calculate and determine LOD and LOQ by matrix blank samples with ISTD in ng/mL.

	BPS results	BPF results	BPE results	BPA results	BPAF results	BPZ results		
Name	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Start volume (mL)**	**End volume (mL)**
Matrix blank sample 1	0,01	0,003	0,3	0,2	0,02	0,01	0,5	0,2
Matrix blank sample 2	0,02	0,009	0,3	0,2	0,03	0,04	0,5	0,2
Matrix blank sample 3	0,01	0,006	0,3	0,3	0,02	0,03	0,5	0,2
Matrix blank sample 4	0,05	0,003	0,3	0,6	0,01	0,03	0,5	0,2
Average	0,02	0,005	0,3	0,3	0,02	0,02		
Standard deviation (SD)	0,02	0,003	0,02	0,2	0,007	0,01		
LOD	0,07	0,01	0,4	0,8	0,04	0,06		
LOQ	0,2	0,03	0,5	2,1	0,09	0,1		

Table 25. Results of the measurements for 18 real samples from Argentina with ISTD in ng/mL.

	BPS results	BPF results	BPE results	BPA results	BPAF results	BPZ results
Name	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**
Serum sample 1	0,2	n.a	0,7	0,1	0,1	0,07
Serum sample 2	0,1	n.a	0,5	0,8	0,1	0,08
Serum sample 3	0,1	n.a	1,0	0,4	0,2	0,1
Serum sample 4	0,07	n.a	0,9	0,2	0,1	0,02
Serum sample 5	0,1	n.a	0,4	0,2	0,09	0,2
Serum sample 6	0,1	n.a	0,8	0,7	0,1	0,2
Serum sample 7	0,1	n.a	0,7	0,5	0,1	0,7
Serum sample 8	0,1	n.a	0,6	0,7	0,06	0,09
Serum sample 9	0,2	n.a	0,9	0,5	0,07	0,2
Serum sample 10	0,7	n.a	1,1	0,8	0,1	0,1
Serum sample 11	0,5	n.a	0,9	0,2	0,1	0,1
Serum sample 12	0,5	n.a	0,7	0,4	0,09	0,1
Serum sample 13	0,5	n.a	0,7	1,2	0,1	0,04
Serum sample 14	0,5	n.a	0,8	0,4	0,1	0,2
Serum sample 15	0,6	n.a	0,8	0,5	0,1	0,05
Serum sample 16	0,5	n.a	0,8	0,2	0,1	0,07
Serum sample 17	0,5	n.a	0,7	0,7	0,09	0,1
Serum sample 18	0,5	n.a	0,8	0,7	0,07	0,04

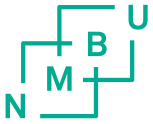
Table 26. Results of the measurements for pre-spiked serum sample 500 ng/mL (n = 6) for calculation of precision and robustness.

			BPS Results	BPF Results	BPE Results	BPA Results	BPAF Results	BPZ Results
Date	**Name**	**Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**
12.02.2024	Rep mix 1	500	471,3	489,9	491,2	480,5	536,9	494,0
13.02.2024	Rep mix 2	500	473,1	512,3	500,1	489,7	542,5	503,0
28.02.2024	Rep mix 3	500	450,8	478,0	499,9	481,1	529,9	506,3
29.02.2024	Rep mix 4	500	448,2	466,0	490,7	473,3	527,8	495,7
01.03.2024	Rep mix 5	500	455,5	466,3	496,4	473,3	533,7	517,0
13.03.2024	Rep mix 6	500	494,8	493,0	497,6	497,1	497,6	493,8

Table 27. Recovery (%) of pre-spiked serum sample (500ng/mL) compared with theoretical known concentration.

		BPS Results	BPF Results	BPE Results	BPA Results	BPAF Results	BPZ Results
Name	**Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**	**Final Conc. (ng/mL)**
Rep mix 1	500	94,3	98,0	98,2	96,1	107,4	98,8
Rep mix 2	500	94,6	102,5	100,0	97,9	108,5	100,6
Rep mix 3	500	90,2	95,6	100,0	96,2	106,0	101,3
Rep mix 4	500	89,6	93,2	98,1	94,7	105,6	99,1
Rep mix 5	500	91,1	93,3	99,3	94,7	106,7	103,4
Rep mix 6	500	99,0	98,6	99,5	99,4	99,5	98,8

* Lower limit: 40, upper limit: 110



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