Liquid chromatography tandem mass spectrometry for characterization of serum albumin modifications with relevance to the human exposome

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Supervisor: **Dr. Hitesh V. Motwani, Prof. Margareta Törnqvist & Prof. Ulrika Nilsson**

Principal Investigator: **Dr. Hitesh V. Motwani**

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Abbreviations

AAs	Amino acids
AUC	Area under the concentration time curve
B(a)P	Benzo(a)pyrene
BPDE	Benzo(a)pyrene-7,8-diol-9,10-epoxide
b.w.	Body weight
CAS#	Chemical Abstracts Service number
CV	Coefficient of variation
CYP	cytochrome P450 isoenzymes
DNA	Deoxyribonucleic acid
DoE	Design of experiments
DE	Diol epoxide
EH	Epoxide hydrolase
ELISA	Enzyme-linked immunosorbent assay
ESI^+	Positive electrospray ionization
His	Histidine
HPLC	High performance liquid chromatography
hSA	human serum albumin
ICH	International conference on harmonization
Lys	Lysine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NADP	Nicotinamide adenine dinucleotide phosphate
LOD	Limit of detection
LOQ	Limit of quantification
PAH	Polycyclic aromatic hydrocarbons
PAHDE	Polycyclic aromatic hydrocarbon diol epoxide
PMF	Peptide mass fingerprinting
Pro	Proline
SA	Serum albumin
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
TIC	Total ion current

"If you can't explain it simply, you don't understand it well enough"

– Albert Einstein

Abstract

Polycyclic aromatic hydrocarbons (PAHs), originating from incomplete combustion of organic material are ubiquitous environmental pollutants. A common model compound of PAH exposure is benzo(a)pyrene (B[a]P), a genotoxic compound because of its metabolism to electrophilic metabolites known as benzo(a)pyrene-7,8-diol-9,10-epoxides (BPDEs). These highly reactive species form stable covalent bonds with serum albumin (SA), such as on His^{146} and Lys^{432}, resulting in the formation of adducts. Measurement of SA adducts can be used for biomonitoring studies of genotoxic compounds. In this project, a sensitive and selective liquid chromatography tandem mass spectrometry (LC/MS/MS) method, with a triple-quadrupole instrument, was developed for identification and quantification of digested BPDE-SA adducts, measured as (+)-anti-BPDE-His-Pro, (-)-anti-BPDE-His-Pro and (±)-anti-BPDE-Lys. Possible improvement of the enzymatic hydrolysis to release the adducts was tested by use of dithiothreitol (DTT) to reduce disulfide bridges. However, no improvement in recoveries was observed, most likely because of instability of the adducts under the experimental conditions. The LC separation was slightly modified to reduce the analysis time, i.e. to 16.5 minutes and the electrospray ionization (ESI) conditions were optimized through an experimental design strategy. An internal standard was utilized to adjust for instrumental variability and normalization of the MS response. Evaluation of the method was performed in terms of the linearity, limit of detections (LODs) and accuracy. Linearity was good, R^2>0.99, except for (+)-anti-BPDE-His-Pro, which probably due to low concentrations resulted in large variability. Coefficients of variation (CVs) were below 15 % and accuracy was 85% or higher. LODs were about one magnitude higher compared to a previously developed method by high-resolution MS (HRMS), i.e. approximately seven adducts per 10^8 molecules of SA. The observed difference in LODs were due to the different instruments used. Further, to generate reference standards of BPDE-SA, *in vitro* metabolism of B(a)P was carried out. But further investigations are required since the generated *in vitro* adducts were below or close to detection limits. To understand the formation of BPDE adducts *in vivo*, samples from B(a)P-exposed mice were analyzed, but no detectable levels were observed by the triple-quadrupole (TQ-MS) system. However, employing LC/HRMS the BPDE adducts levels in these *in vivo* samples were detected and estimated to 14amoles for (±)-anti-BPDE-His-Pro and 62amoles for (±)-anti-BPDE-Lys. The developed LC-MS/MS method is a proof-of-principle to illustrate how statistical methods can be utilized to improve the detection of target compounds in trace levels.

Popular Science Abstract

In the nature there are several environmental pollutants which may affect the human health in a negative way, for example compounds causing cancer upon long-term exposure. Polyaromatic hydrocarbons (PAHs) are typical pollutants occurring from incomplete combustion of organic material. Once these compounds enter our bodies, they may be transformed into more reactive compounds which eventually can form stable bonds to blood proteins, e.g. serum albumin (SA), or the DNA. Blood proteins can be used to monitor the exposure level of reactive compounds aiming to facilitate the detection in humans to aid in cancer risk assessment of foreign compounds. The utilization of high technology-based methods is required to measure the formation of stable protein complexes in the blood, to obtain an improved sensitivity within the measurements. Previous research in the field have gained attention due to its applicability in mice, but still needs further improvement to detect levels in the general population. In this study, a sensitive method was developed to measure the generated complex levels caused by a stable interaction between reactive compounds belonging to a foreign PAH and SA. Prior to instrumental analysis, an attempt was made to improve the efficiency of digestion by thermal degradation of the protein, but it was shown that the denaturation resulted in a reduced yield. The instrumental performance was developed by means of a systematic optimization of the response from a model compound, utilized to control variabilities between the measurements. The developed method based on variability and closeness to the true value of reference compounds were considered as acceptable. Application of the developed method was performed based on B(a)P exposed mice to facilitate the understanding about formation of modified proteins, but no detection was achieved by the present method. A highly sensitive method was on the other hand illustrating low levels corresponding to a modified protein from B(a)P exposure, and it was concluded that highly sensitive methods are crucial for detection of very low levels caused by common environmental pollutants.

Aim of this work

General aim

In this project the overall aim was to develop a sensitive and selective liquid chromatography tandem mass spectrometry (LC/MS/MS) method for simultaneous analysis of benzo(a)pyrene-7,8-diol-9,10-epoxides (BPDEs) attached to serum albumin (SA) with relevance to the human exposome. Identification and quantification of the generated adducts were performed by means of a triple quadrupole mass spectrometer (TQ-MS) in multiple-reaction monitoring (MRM) mode.

Specific aims

- Systematic optimization of the LC/MS/MS method
- Improvement of enzymatic hydrolysis by using a reducing agent (DTT) to cleave internal disulfide bridges within human SA.
- Development of an *in vitro* metabolic system to generate BPDE-SA as reference standards from metabolism of B(a)P in presence of SA.
- Application of the LC-MS/MS method for *in vivo* mice samples exposed to B(a)P and comparison with LC-HRMS measurements.

1. Introduction

Human exposure to ubiquitous environmental pollutants has been a great concern during several decades due to the incidence of modifications in either protein or DNA sequences in presence of carcinogenic compounds for instance. The probability of developing cancer might increase upon occupational exposure to foreign compounds, e.g. polycyclic aromatic hydrocarbons (PAHs). These compounds can be transformed within the liver to reactive metabolites which in presence of blood proteins may form adducts (**add**ition prod**ucts**), i.e. modified proteins. To aid in knowledge about the exposure level of these xenobiotics and the internal dose of their reactive metabolites, sensitive methods are required to measure the adduct formation *in vitro* and *in vivo*.

In the following sections, information will be provided with regards to PAHs and their transformation *in vivo*, serum albumin (SA) as a target protein to monitor modifications caused by foreign compounds as well as analytical strategies to deal with characterization and quantification of SA adducts formed during long-term exposure.

1.1 Background

Xenobiotics refer to foreign compounds for living organisms, which may bring consequences upon exposure in both animals and humans. Different classes of compounds belonging to xenobiotics are generated from either industrial emissions, or as byproducts from incomplete combustion of organic material, such as PAHs, pesticides, herbicides etc. (Ruan et al. 2006).

Adduct measurement has become a popular topic among many research groups due to its applicability for *in vivo* dose monitoring of cancer-causing agents. Stable adducts formed with SA reflect an exposure during a longer period, which is a methodological benefit since the levels of formed adducts can be extrapolated to yield information about the environmental exposure levels (Sabbioni et al. 2016).

1.2 Polycyclic aromatic hydrocarbons (PAHs)

PAHs are typical foreign compounds originating from incomplete combustion of organic material, for example vehicle exhaust, industrial emissions, char-coal grilled food, cigarette smoke, roasted and dried vegetables etc. The chemical and physical properties of PAHs are well-studied since PAHs are ubiquitous environmental pollutants found in the ambient air, water as well as in the soil. Most compounds belonging to this class are hydrophobic and non-volatile. The larger the number of aromatic rings, the less soluble in water and less volatile they are (C. Högkvist 2018).

A well-studied compound belonging to PAHs is benzo[a]pyrene, B(a)P, which through a metabolism within the liver is transformed to genotoxic metabolites known as benzo[a]pyrene-9,10-diol-11,12-epoxides (BPDEs), or more specifically (±)-anti-BPDE and (±)-syn-BPDE, or other genotoxic carcinogens (Motwani et al. 2016).

1.3 Metabolism of benzo(a)pyrene

The metabolism of B(a)P as a model compound belonging to PAHs occurs mainly inside the hepatocytes located in the liver, since it contains a wide variety of non-specific enzymes. The transformation of B(a)P is divided into phase I and phase II transformation reactions, where phase I systems generate a primary metabolite through addition of a reactive group. As the primary metabolite eventually is attached to an endogenous compound, it will form a secondary metabolite through a conjugation reaction (phase II system). This is an important step to facilitate excretion via the urine, since a secondary metabolite usually is more polar than the original structure (E. Westberg, 2015).

Three main pathways are described regarding the metabolism of PAHs, the orthoquinone-, one-electron oxidation- and the diol-epoxide pathway, where the last is commonly referred to regarding transformation of PAHs (IARC, 2010; E. Westberg, 2015).

In **Figure 1** below, B(a)P is metabolized into its corresponding metabolites in 3 steps, namely:

1. Formation of an epoxide by oxidation with cytochrome P450 (CYP1A1, CYP1A2 and CYP1B1).
2. A hydrolysis of the epoxide is performed by epoxide hydrolase (EH) into a trans-diol
3. Oxidation of a carbon-carbon double bond by means of CYPs (CYP1A1, CYP1A2 and CYP1B1) is carried out, yielding several electrophilic metabolites belonging to the class of BPDEs.

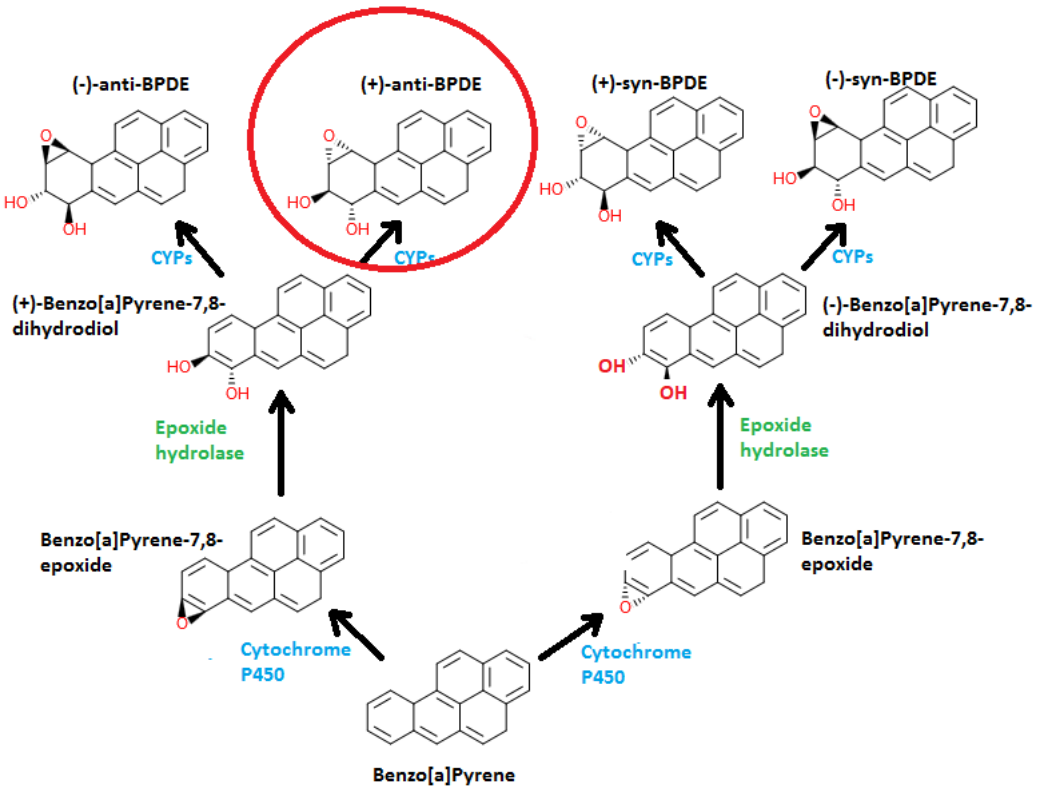


Figure 1: Metabolism of B(a)P illustrating the diol epoxide pathway, a 3-step metabolism procedure resulting in the ultimate carcinogenic compound benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE). (+)-anti-BPDE (encircled) is considered as the most potent BPDE metabolite inside a living organism.

The BPDE class is further divided into two diastereoisomers, known as anti- and syn-BPDE respectively. These are separated into enantiomers, namely (+)- and (-)-anti- as well as (+)- and (-)-syn-BPDE. Amongst these metabolites, (+)-anti-BPDE, see **Figure 1,** is considered as the most potent genotoxic metabolite associated with a high risk in developing cancer through DNA modifications *in vivo* (E. Westberg, 2015).

PAHs may contain bay- and fjord regions, where a bay region is enclosed by three aromatic rings whereas a fjord is surrounded by four rings. The organization of aromatic rings, resulting in a bay- or a fjord region, indicates the probability that a diol epoxide (DE) is prone to hydrolysis. The reactivity of PAHs containing either bay- or fjord regions depends on where a DE is formed, i.e. it becomes more resistant to hydrolysis when located close to these bay- and fjord regions compared to other parts of the molecule. A DE close to a fjord region is due steric hindrance more stable than a bay region. Previous research based on *in vivo* studies have emphasized that a DE close to a fjord remains reactive for a longer period compared to a DE protected by a bay region. (Arif et al. 1999; Stowers et al. 1984).

1.4 Serum albumin

Serum albumin (SA), a protein containing 585 amino acids with an approximate weight of 65kDa, is a blood protein with several important biological functions such as a

transport protein for essential compounds as well as maintenance of our colloidal osmotic blood pressure (Sabbioni et al. 2016).

The binding affinity to different compounds and drugs depends on different sites of SA, where for example site 1 located in a subdomain, IIA, see **Figure 2,** is attracted to large heterocyclic- and negatively charged compounds. Site 2, which is in subdomain IIIA possesses a high affinity for typically small and aromatic compounds, but also for compounds with carboxylic acid groups. (Sabbioni et al. 2016).

In **Figure 2** below, the secondary structure of SA is presented, consisting of α-helices (68%) and β-sheets (32%).

Previous research recently established that the conformation of SA based on its tertiary structure, which is maintained by specific ligands, indicates a presence of hydrophobic pockets, i.e. a probable reason why highly electrophilic metabolites are well protected from hydrolysis. One advantage by utilizing SA is the formation of stable covalent interactions, e.g. adducts, for biomonitoring purposes of genotoxic compounds *in vivo* (Sabbioni et al. 2016).

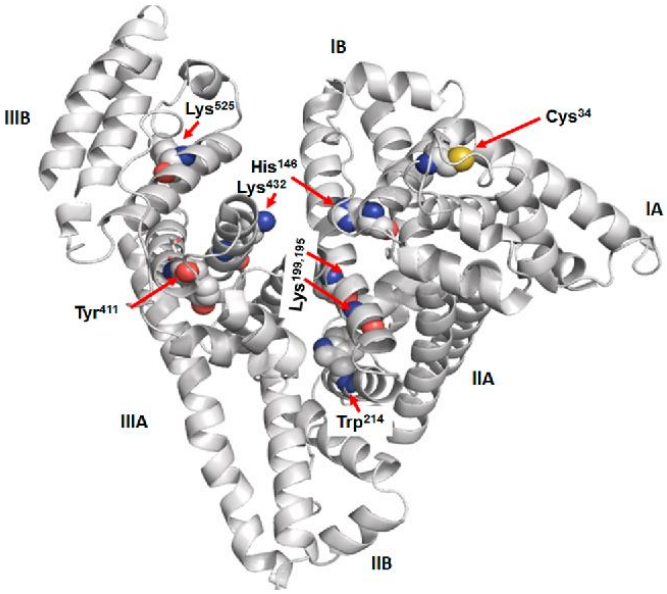


Figure 2: Illustration of the secondary structure for serum albumin maintained by a wide variety of specific ligands [Sabbioni et al, 2016]

SA is produced by the hepatocytes within the liver, approximately 200mg per kilogram of bodyweight and day, and because of that SA is considered as the main protein in blood serum. Normal human serum contains up to 60% of albumin with an average concentration of 43mg/ml. The half-life of human SA is relatively long, 19-25 days, and therefore another reason why it is suitable for biomonitoring purposes. Compared to DNA adduct formation, SA adducts are more stable for a longer period due to absence of repair systems, which makes DNA adducts less stable. (Sabbioni et al. 2016).

Since SA is produced in the hepatocytes where different xenobiotics are metabolized into reactive electrophiles, it becomes beneficial to employ SA in presence of very short-

lived electrophilic metabolites, which may react with SA instead of hemoglobin (Hb) located in the blood stream. This may facilitate measurements of such genotoxic metabolites formed in the liver to prevent an excretion as urine metabolites for instance (Sabbioni et al. 2016).

For each mole of SA, the following essential amino acids are expected:

Table 1: Relative abundance of 20 common amino acids per mole of serum albumin

Amino acid	Side chain charge (pH 7,4)	Molecular weight (Da)	# of residues per mole of SA	Relative amount (%)
Glutamate (Glu, E)	Negative	147,13	62	10,6
Alanine (Ala, A)	Neutral	89,09	62	10,6
Leucine (Leu, L)	Neutral	131,17	61	10,4
Lysine (Lys, K)	Positive	146,19	59	10,1
Valine (Val, V)	Neutral	117,15	41	7,0
Aspartate (Asp, D)	Negative	133,11	36	6,2
Cysteine (Cys, C)	Neutral	121,16	35	6,0
Phenylalanine (Phe, F)	Neutral	165,19	31	5,3
Threonine (Thr, T)	Neutral	119,12	28	4,8
Serine (Ser, S)	Neutral	105,09	24	4,1
Arginine (Arg, R)	Positve	174,2	24	4,1
Proline (Pro, P)	Neutral	115,13	24	4,1
Glutamine (Gln, Q)	Neutral	146,14	20	3,4
Tyrosine (Tyr, Y)	Neutral	181,19	18	3,1
Asparagine (Asn, N)	Neutral	132,12	17	2,9
Histidine (His, H)	Positive (10%) Neutral (90%)	155,16	16	2,7
Glycine (Gly, G)	Neutral	75,07	12	2,1
Isoleucine (Ile, I)	Neutral	131,18	8	1,4
Methonine (Met, M)	Neutral	149,21	6	1,0
Tryptophan (Trp, W)	Neutral	204,23	1	0,2

The solubility of SA is improved due to more acidic residues compared to the basic residues as shown in **Table 1,** since the overall net charge will be negative at physiological pH conditions. Due to 17 intramolecular disulfide bridges within SA, the stability properties contribute to the biological lifetime since the protein will remain intact for a longer period. Cys^{34} is the only free cysteine residue and regarded as the strongest nucleophilic site in SA. The antioxidant activity of Cys^{34} explains the probability to interact with oxidants and electrophilic metabolites, which is also a reason to the low pKa of 6.5. The relatively low pKa is associated with the ionization state of Cys^{34}, affected by the presence of 3 different residues, Tyr^{84}, His^{39} and Asp^{38} (Sabbioni et al. 2016).

1.5 Formation of adducts

Blood proteins, e.g. SA, possess the possibilities to form covalent bonds between electrophilic metabolites and nucleophilic sites within the protein. Recent studies aiming to characterize site specific positions within SA have been performed to understand the adduct formation as well as disappearance rates of electrophilic metabolites. Protein adducts are formed when electrophilic metabolites, e.g. BPDEs, are trapped by

nucleophilic sites located either on the surface or in hydrophobic pockets of SA. The interaction between the electrophilic epoxide from BPDEs and the nucleophilic site, i.e. one of the nitrogen atoms within the imidazole ring of histidine (N^{τ}) or the nitrogen located in the side chain of Lys, forms a stable covalent bond, see **Figure 3** (Day et al. 1991).

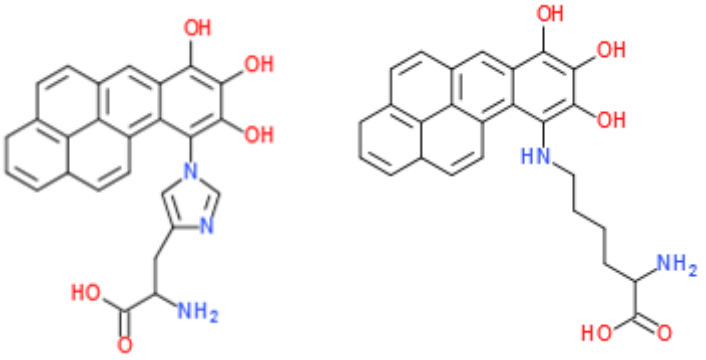


Figure 3: Illustration of the covalent bond formed between the N^{T} in the imidazole ring of histidine as well as the amine group in lysine together with an electrophilic metabolite, e.g. BPDEs. The picture is created by means of 2D sketcher by ChemDoodle web components.

Adducts to SA have been extensively studied aiming to understand the exposure level of xenobiotics. Since SA is the most abundant protein in blood serum, measurement of stable His and Lys adducts formed with SA are considered as suitable regarding biomonitoring studies of B(a)P exposure. This was illustrated in a comparison study between SA and Hb by Helleberg et al. (2000). After *in vitro* incubation of BPDE isomers in presence of SA and Hb respectively, followed by subsequent hydrazinolysis and analysis by either LC/UV, µHPLC/MS-MS or GC/MS-MS emphasized that approximately 70% of all BPDE adducts were dedicated towards the His site in SA and only 10% towards the His site in Hb (Helleberg et al. 2000).

In a study by Motwani et al. (2016), the kinetics for formation of adducts from different BPDE isomers, namely (±)-anti, (+)-anti- and (±)-syn-BPDE were investigated as well as their relative formation to the His- and Lys sites in SA. As shown in **Figure 4**, the disappearance rate of different BPDEs is illustrated which indicates different kinetics depending on the certain isomer. The rate of disappearance is related to the hydrolysis of generated metabolites as well as the reactivity towards specific nucleophilic sites in SA, e.g. His and Lys (Motwani et al. 2016).

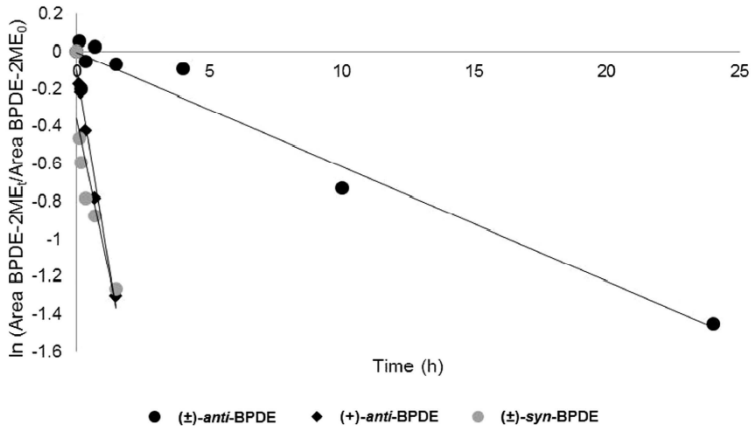


Figure 4: Illustration of the disappearance rate for different B[a]P metabolites after 24 hours incubation at 37°C, pH 7.4 (Motwani et al. 2016)

1.6 Analytical strategies to study protein modifications

To aid in our understanding of natural processes occurring within our cells as well as investigations of protein modifications with relevance to the human exposome, strategies in the field of proteomics are regarded as crucial tools for this purpose.

Different approaches regarding proteomics analysis, i.e. top-down-, middle-down- or bottom-up proteomics are essential methodologies in the field of adduct analysis. A top-down approach refers to intact proteins, i.e. a direct analysis limited to proteins <50kDa.

MS instruments are crucial for structural elucidation, preferably high-resolution MS, but both triple quadrupoles (TQ)- and quadrupole-time-of-flight (qToF) detectors are extensively used. Top-down techniques enables a limited sample preparation and a broader picture about the original structure (L. Switzar et al. 2013).

Middle-down and bottom-up strategies are commonly employed since MS analysis of peptides facilities spectra interpretation. The size of the peptides indicates whether it is middle-down, yielding peptides in the range 2-20kDa, or bottom-up proteomics, yielding peptides from 0.5-3kDa. Smaller peptides yield better MS sensitivity when using a quadrupole instrument due to higher transmission efficiency. Also, collisional induced dissociation (CID), is more efficient for smaller peptides. Database comparison of peptide sequences, e.g. with Mascot or UniProt, is also possible by liquid chromatography (LC) tandem MS analysis, resulting in an improved structure elucidation. This technique is referred to as peptide mass fingerprinting (PMF), a technique that rely on CID fragmentation, and therefore commonly applied by means of qTOF- and MALDI-TOF instruments. Identification of proteins and potential modifications with a middle-down or bottom-up approach assumes an optimal digestion prior to MS analysis (L. Switzar et al. 2013).

Development of hydrolysis methods regarding chemically induced modifications is an essential part of the analytical chain since the adduct levels becomes more reliable and reproducible. Non-enzymatic digestion, such as acidic- or alkaline hydrolysis has been utilized to obtain a complete digestion of proteins. In a previous study by H. Helleberg

et al. (2000), promising results regarding the quantification of BPDE adducts to SA were presented using hydrazine for digestion. This compound was prohibited after the 9/11 attacks due its explosive nature (Helleberg et al. 2000).

Acidic hydrolysis by means of hydrochloric acid (HCl) at high temperatures (110°C) have been utilized as an alternative to enzymatic hydrolysis, but with a drawback where the adducts may undergo further hydrolysis to form tetrols, see **section 1.7.3 MS/MS analysis** (E. Westberg 2015).

Digestion of proteins is usually performed by enzymes, e.g. trypsin, commonly employed due to its resistance to undergo autolysis. The cleavage specificity of trypsin is located to Arg and Lys residues, except from when followed by Pro as a neighboring amino acid, probably because of steric hindrance. CID fragmentation is facilitated due to the length of tryptic peptides, i.e. around 14 amino acids which includes two positively charged residues. PMF will therefore ensure a reliable identity of the protein. Despite the advantages of trypsin, there are other proteases available, for example pronase from *Streptomyces griseus,* considered as a non-specific protease containing chymotrypsin, trypsin, carboxypeptidase and aminopeptidase together with neutral and alkaline phosphatases (L. Switzar et al. 2013).

Pronase is considered a universal enzyme, since it theoretically may digest proteins into separate amino acids (Roche Diagnostics GmBH, Mannheim Germany).

1.7 Analysis of adducts from B(a)P

1.7.1 Digestion of BPDE-SA

In a study by E. Westberg et al. (2014), an analytical method was developed to obtain a sensitive method for polycyclic aromatic hydrocarbons diol epoxides (PAHDE) as adducts to SA aiming to assess the *in vivo* dosimetry of genotoxic metabolites. Investigation of enzymatic hydrolysis by means of pronase from *Streptomyceus griseus* versus alkaline hydrolysis by NaOH was performed, and subsequent analysis by LC/MS/MS. The results emphasized that alkaline hydrolysis of BPDE alkylated SA resulted in enough hydrolysis, giving the modified residues BPDE-His and BPDE-Lys, while digestion by pronase resulted in a dipeptide (BPDE-His-Pro) and even a tripeptide (BPDE-His-Pro-Tyr), probably because of steric hindrance caused by large bulky epoxides attached to nucleophilic amino acids. During the project, it was evident that the efficiency of digestion did not improve by means of RapiGest and Papain included in the mixture (E. Westberg et al. 2014).

A complete hydrolysis of modified proteins would theoretically result in lower LODs as well as an improved repeatability giving a single modified amino acid rather than a complex mixture of modified peptides to be measured. Even though alkaline hydrolysis by NaOH was shown to be complete, it was not considered as a suitable choice because a degradation of the formed adduct was observed. On the other hand, digestion by pronase demonstrated lower detection limits and was therefore regarded as the

method of choice. The enzymatic hydrolysis was also found reproducible, i.e. giving the same peptide fragments regardless of experimental day or laboratory personnel. Besides, previous methods utilizing cleavage by trypsin would be insufficient regarding the measurement of *in vivo* adduct levels. In the earlier work, it was evident that utilization of a surrogate internal standard (DBPDE-Lys) resulted in more reproducible levels (E. Westberg et al. 2014).

1.7.2 Possible improvements by using dithiothreitol and iodoacetamide

A reliable quantification of BPDE modifications in SA assumes a high recovery of digested modified peptides. Protein hydrolysis of BPDE alkylated SA with pronase has been shown to yield relatively low adduct level. A question might be whether this is due to the presence of hydrophobic pockets within SA, which might protect covalently bound adducts from hydrolysis and result in a poor efficiency regarding the overall hydrolysis. A common solution to facilitate the proteolytic digestion is accomplished by pretreatment, for example by use of dithiothreitol (DTT), which reduces disulfide bridges present in the protein. After reduction, alkylation, e.g. with iodoacetamide (IAA), of the cysteine residues is crucial to prevent regeneration of disulfides. Such a pretreatment is expected to unfold the protein so that a more complete hydrolysis is achieved (Sabbioni et al. 2016).

1.7.3 MS/MS analysis

According to Kafferlein et al. (2010), there is a need to develop sensitive and selective methods for the measurements of protein adducts from B(a)P exposure. For epidemiological studies it has been established that blood protein adducts are advantageous for biomonitoring studies with regards to the internal dose of BPDEs.

Several analytical methods, e.g. GC/MS/MS, LC/MS/MS and enzyme-linked immunoassay (ELISA) methods, have been used for the identification and quantification of protein adducts. Some of these methodologies have been designed for tetrols formed after hydrolysis of carboxylic ester adducts, i.e. covalently bound BPDEs to acidic amino acids, such as glutamic acid or aspartic acid. It has been shown that carboxylic ester adducts are prone to undergo mild acidic hydrolysis *in vivo*, which is considered as inappropriate regarding *in vivo* dosimetry. Thus, the hydrolysis removes any information regarding the identity of the adduct, which results in an uncertainty about its origin, i.e. no tag from the protein is left. To achieve an accurate and reliable quantification of adducts in the form of tetrols, it is important to first remove any free or non-covalently bound tetrols originating from electrophilic metabolites, otherwise the generated adduct levels will be overestimated (Westberg et al, 2014).

A previous study by E. Westberg et al. (2014) concluded that methodologies based on sensitive LC/MS/MS detection are required to provide structure elucidation with regards to the generated adducts. To strengthen the quantification of BPDE adducts, it is preferable to measure adducts containing a “tag”, indicating the origin from a blood protein.

1.8 Measurement of amino acids

The determination of amino acids, AAs, for example from serum or as free AAs, released after proteolytic digestion, presumes accurate and reproducible analytical methodologies, where either electrophoresis or chromatography is utilized in combination with MS, spectrophotometry or fluorescence spectroscopy. To achieve an improved sensitivity, associated with lower detection limits, derivatization is a commonly applied technique for GC separations, combined with flame ionization (FID) or electron-capture detection (ECD). In addition, GC/MS methods have been developed for derivatized AAs, providing fast separations of less than 10 min with enough reproducibility. Unfortunately, the derivatization process may result in losses and contamination of the samples (M. Calderon-Santiago et al. 2012).

Similarly, several LC/MS/MS methods for AAs have been developed either with- or without a prior derivatization. For underivatized AAs on reversed phase LC platforms, ion pairing agents, e.g. trifluoroacetic acid (TFA) or tributylamine (TBA), are required to achieve an acceptable separation. Importantly, the ion paring agent should have enough volatility not to deteriorate the MS sensitivity, but unfortunately ion suppression is a major problem associated to such agents (Krumpochova et al. 2015).

The development of analytical techniques to accomplish a direct measurement of underivatized AAs has been of a major interest since it preserves the original structure of the analyte without the drawbacks mentioned above. Hydrophilic interaction liquid chromatography (HILIC), enables a beneficial separation of underivatized AAs without the use of ion pairing agents, typically when log P < 0, since a higher log P value indicates more hydrophobic compounds. In comparison to conventional LC, i.e. both normal- and reversed phase systems, the use of HILIC platforms allows several different classes of polar compounds to be measured, such as AAs, peptides and carbohydrates as in clinical analyses of metabolic mixtures or other biological systems, such as from the soil (Guo et al. 2013). HILIC utilizes interaction between target analytes and a water layer adsorbed on polar functional groups, e.g. in aminopropyl, ethylene bridged hybrid (BEH), silica, diol and zwitterionic phases. The mobile phase composition usually starts with a higher percentage of organic phase followed by stepwise elution of an aqueous buffer solution. Due to the application for separation of polar compounds, the mechanism of the separation in HILIC is strongly influenced by the ionic strength and the pH of the eluents, indicating that method development is crucial for optimal analysis (F. Gritti et al. 2009).

The popularity of HILIC separations have increased during the last decade because of a reliable MS connection, but also due to the enhanced separation efficiency of polar compounds compared to conventional RP-LC methods (P. Jandera. 2011).

In **Appendix 13: Literature review regarding AAs analysis,** a literature review of different HILIC methods regarding analysis of AAs is presented, providing essential information about the performance related to each method.

1.9 Project plan

The main scientific aim with this diploma work (60 ECTS) was to develop a sensitive and selective LC/MS/MS method for simultaneous characterization and quantification of adducts to SA by using a TQ-MS instrument. As seen in **Figure 5**, the project is divided into two important parts of the analytical chain, i.e. optimization of the instrumental method and improvement of digestion concerning sample treatment, respectively. The main milestones are listed below:

1. A method based on HILIC chromatography and detection by a TQ-MS in positive ionization mode is to be investigated for measurement of seven underivatized amino acids from human SA. A partial evaluation in terms of linearity, precision and accuracy is to be performed.
2. The digestion yield of SA with pronase from *Streptomyces griseus* will be investigated with and without DTT and IAA treatment. Solid-phase extraction of the digested protein will be performed prior to LC/MS/MS analysis to guarantee efficient clean-up of investigated amino acids. An internal standard will be employed to correct for losses and for improved repeatability.

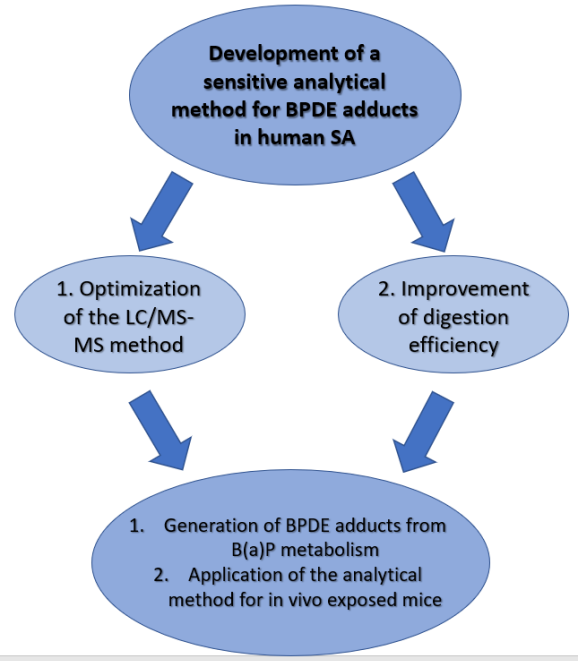


Figure 5: Overview of project including the specific aims to provide a better illustration for this work.

3. Improvements of the sensitivity and LODs of triple quadrupole MS detection in positive mode will be made based on a design of experiment approach (DoE). Chromatographic conditions will be optimized using a pentaflurophenyl (F5) column in accordance with previous research by Zurita et al. 2018.
4. BPDE adducts will be generated as reference standards through an *in vitro* metabolism of B(a)P in presence of human SA.
5. Analysis of *in vivo* precipitated SA from mice exposed to B(a)P, after 3- and 14 days of administration, respectively, will be performed using the optimized methodology.

2 Experimental

2.1 Material

2.1.1 Chemicals

Lyophilized human serum albumin (96-99%), DL-dithiothreitol (DTT), iodoacetamide (IAA), benzo[a]pyrene (B(a)P) and formic acid (FA, >95%) were purchased from Sigma Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4) and ammonium acetate (NH_4Ac) were from Merck (Darmstadt, Germany). Pronase from *Streptomyces griseus* was purchased from Roche Chemicals (Mannheim, Germany). Ammonium bicarbonate (AMBIC, 99%) was purchased from Fluka analytical. All solvents for HPLC and sample preparation were of analytical grade, namely methanol (MeOH >99%), acetonitrile (ACN, 99%) and water from Honeywell (Seelze, Germany). Dimethylsulfoxid (DMSO, 99%), hydrochloric acid (HCl, 37%), sodium acetate (NaAc), ethyl acetate and n-pentane were purchased from VWR (France). Rat liver S9 fraction (35.9mg/ml), Regensys A (sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate, potassium chloride and magnesium chloride hexahydrate) and Regensys B (nicotinamide adenine dinucleotide phosphate) were purchased from Moltox (Boone, NC in USA). All experiments were performed in Protein Lo-Bind tubes.

Table 2: A mixture of Regensys B and Regensys A was prepared as described by the supplier. The concentrations for each component are based on a final volume of 45ml that was used for the *in vitro* experiments, i.e. each component is a cofactor needed for the rat liver S9 enzymes involved in the metabolism of B(a)P.

Compounds	Amount (g)	Conc. (mg/ml)	Supplier	Cat. No	Lot No
$NaH_2PO_4 * H_2O$	2.7	58.9	Amresco	0823	1287C241
Na_2HPO_4	11.5	254.7	Chem Impex	00835	002251-01114
KCl	2.5	54.7	Amresco	0395	0625C284
$MgCl_2 * 6H_2O$	1.6	36.2	Amresco	0288	0046C146
G6P	1.5	33.8	Roche	10153079	14171728
NADP	0.2	3.4	Roche	10004669	16102524

2.1.2 Standards

Table 3: Amino acid standards obtained from Sigma Aldrich (Steinheim, Germany) were utilized for development of the HILIC/MS-MS method. An isotopically labeled internal standard was used, namely DL-His-d_3, obtained from Cambridge Isotope laboratories (Andover, MA, USA). Cone voltages and collisional energies (CE) for every compound were obtained after univariate optimization of the sensitivity.

Amino acid	CAS No.	Mw (Da)	Molecular formula	Cone (V)	Quantifier (CE, eV)	Qualifier (CE, eV)
L-Trp	73-22-3	204.23	$C_{11}H_{12}N_2O_2$	50	188 (10)	146 (15)
L-Met	63-68-3	149.21	$C_5H_{11}NO_2S$	45	133 (10)	61 (25)
L-Arg	74-79-3	174.2	$C_6H_{14}N_4O_2$	55	70 (20)	116 (15)
L-His	71-00-1	155.16	$C_6H_9N_3O_2$	50	110 (15)	83 (20)
L-Lys	56-87-1	146.19	$C_6H_{14}N_2O_2$	30	84 (15)	130 (10)
L-Thr	72-19-5	119.12	$C_4H_9NO_3$	50	74 (12)	102 (10)
L-Val	72-18-4	117.15	$C_5H_{11}NO_2$	50	72 (15)	56 (20)
His-d3	344299-50-9	158.17	$C_6H_6D_3N_3O_2$	50	113 (15)	86 (15)

2.1.3 Materials

For solid phase extraction, Sep-Pak Plus C_{18} cartridges (360mg) were purchased from Waters (Millford, MA, USA). Protein LoBind tubes (Eppendorf, Hamburg, Germany) were used for incubation of serum albumin samples. Consumables such as Pasteur pipettes, tips, test tubes, syringes etc. were all from VWR chemicals Ltd.

2.1.3 Instrumentation

An HPLC (LC-20 Prominence system, Shimadzu, Kyoto, Japan) coupled to a triple quadrupole instrument (Xevo TQ-S Micro from Waters corporation, Millford, MA, USA) was used for detection of all target compounds.

Recovery experiments of human SA were performed by measuring the absorbance at 280nm with a Hitachi U-3000 spectrophotometer (Tokyo, Japan).

2.2 Methods

Caution

For the *in vitro* metabolism in this project, B(a)P was utilized, a compound characterized as carcinogenic and mutagenic to humans. Further hazardous compounds such as dithiothreitol and iodoacetamide were employed during the sample preparation. N.b! Iodoacetamide is an alkylating agent, which may increase cancer risk upon exposure.

2.2.1 Chromatography and MS set-up for BPDE adducts

Chromatography

An LC/MS/MS method was optimized for identification and quantification of BPDE adducts to human SA after enzymatic digestion, and the BPDE adducts were successfully

identified according to their unique fragmentation pattern, see **Figure 22** (BPDE-His-Pro) and **Figure 23** (BPDE-Lys). The purified fractions from SPE were analyzed by means of HPLC coupled to a triple quadrupole MS detector. Chromatographic separation was achieved by means of a Kinetex F5 column (pentaflurophenyl, 100x2,1 mm, 2,6 µm dp, Phenomenex, Torrance, CA, USA). For the mobile phase, a binary system was utilized, namely solvent A consisting of H_2O/MeOH/HCOOH (95:5:0.1, v/v) and solvent B consisting of H_2O/MeOH/HCOOH (5:95:0.1, v/v). Gradient elution was optimized according to the following profile, (90:10) A-B during the initial 48 seconds when the flow was diverted to waste, followed by a linear gradient to 55.8% of solvent B in 10 minutes. The composition was then changed to 100% of solvent B for 3 minutes followed by re-equilibration during 3.5 minutes with the initial conditions (90:10) A-B. Mobile phase flow rate was set to 200µl/min. The total analysis time after optimization was 16.5 minutes.

MS set-up

The mass spectrometer was operated in positive electrospray ionization mode, where the conditions in the ion source were optimized according to **section 2.2.3 Optimization of MS/MS conditions for BPDE adducts**. Precursor ions were the protonated molecules, i.e. $[M+H]^+$, see **Table 4.** Quantifier and qualifier ions were chosen from a daughter ion spectrum for each BPDE adduct, where the highest peak intensity was selected as quantifier ion and the second highest as qualifier ion. The analysis was performed in MRM with dwell times optimized according to **section 2.2.3 Optimization of MS/MS conditions for BPDE adducts**. All measurements were collected in centroid mode and unit mass resolution was applied for both quadrupoles. Collisional gases utilized for fragmentation were nitrogen (N_2) and argon (Ar_2). Data analysis and acquisition of all experiments were performed with Masslynx software version 3.1 (Waters Cooperation, Milford, MA, USA).

Table 4: Target compounds analyzed in digested BPDE-alkylated human SA, with MRM transitions and collisional energies (CE) based on a previous study by J. Zurita et al. 2018. Benzyl-L-His was used as internal standard during the measurements.

Compounds	CAS No.	Mw (Da)	Molecular formula	Quantifier (CE, eV)	Qualifier (CE, eV)
Benzyl-L-His	16832-24-9	245	$C_{13}H_{15}N_3O_2$	246→91 (15)	246→200 (15)
BPDE-His-Pro	-	554	$C_{31}H_{30}N_4O_6$	555→257 (45)	555→253 (40)
BPDE-Lys	-	448	$C_{26}H_{28}N_2O_5$	449→257 (30)	449→285 (30)

2.2.3 Optimization of MS/MS conditions for BPDE adducts

The experimental design was created and evaluated by use of Modde™ (Umetrics, Umeå, Sweden) in combination with Office Excel (Microsoft Cooperation, 2010).

Regarding the method optimization related to MS detection, multiple linear regressions (MLR) were estimated to evaluate the MS responses. For an acceptable MS^2 spectra, the lower limit of the response signal from benzyl-L-His (IS), with a relative base peak intensity higher than 1.0E4 was selected. To class a target compound as identified, the variation in retention time had to be less than ±10% and m/z within ±0.5Da from theoretical m/z. Positive identification also required a match in MS^2 spectrum, i.e. intensity ratios between qualifier and quantifier ions.

To achieve a predictive DoE approach by means of a Plackett-Burman (P.B) design, eight quantitative factors were selected because of their potentially large impact on the ionization efficiency, see **Table 5**. These influential factors were simultaneously varied between their minimum (-) and maximum levels (+) throughout a maximum of 15 experiments including three center points, see experimental plan in **Appendix 5: Experimental design,** where all tested values are illustrated. To identify significant factors, a comparison was made towards so-called "dummy factors", see

Table 7, to structurally sort out such insignificant factors resulting in lower noise for the predicted model, and to identify important factors resulting in higher sensitivity.

To further optimize important parameters identified through a Placket-Burman approach, a face centered design and a full factorial design were evaluated to find a reliable model. The face centered design included a maximum of 17 experiments with three center points, while the full factorial design included a total of 12 experiments. The experimental central composite factorial (CCF) model was created by means of MLR based on experimental versus predicted MS response, and evaluated in terms of cross-validated predictability (Q^2) as well as the regression coefficient (R^2).

Table 5: Influential quantitative variables utilized for the design of experiments (DoE) approach regarding optimization of the ionization efficiency measured by LC/ESI-MS.

Quantitative variables	unit	Low value (-1)	Center value (0)	High value (1)
Dwell time	s	0,1	0,55	1
Source temp.	°C	120	135	150
Cone Voltage	V	10	30	50
Capillary voltage	kV	2	3,5	5
Desolvation temp.	°C	300	450	600
Cone gas flow	L/h	10	55	100
Desolvation gas flow	L/h	300	550	800
LC flow	µl/min	100	150	200

During the systematic optimization of the ionization efficiency, a previously developed chromatographic method by Zurita et al. (2018) was utilized with the following conditions, a Kinetex UPLC F5 column (100x2.1mm, 2.6µm dp) resulting in a

backpressure of 245 bar as well as a column temperature of 23°C, with an injection volume of 10µl.

All things considered, the optimal ESI parameters suggested by Modde software were the following, a source temperature of 150°C, a capillary temperature of 2kV, a cone voltage of 50V, a desolvation gas temperature of 500°C, a cone flow of 10L/h, a desolvation gas flow of 300L/h, a collisional energy of 15eV and a final LC flow rate of 200µl/min.

Furthermore, additional studies with regards to the dwell time were carried out by studying how the MS response varied for the utilized internal standard. The use of scan descriptors was investigated by creating certain scan windows based on where the target analytes eluted from the LC column. A dwell time of 0.5s was found to be suitable for both the internal standard as well as the target compounds, BPDE-His-Pro and BPDE-Lys.

2.2.4 Method evaluation by using reference BPDE adducts

To confirm whether the developed method is reliable and accurate there are common procedures utilized to access the validity of the method. Thus, by considering guidelines provided by the International Conference on Harmonization (ICH), the developed method was partially validated by investigating linearity, detection limits, precision and accuracy.

A reference stock solution was prepared by digesting 1mg of BPDE alkylated human SA (prepared by Motwani et al. 2016) by enzymatic hydrolysis as described in **section 2.2.6** Enzymatic hydrolysis of a reference BPDE-alkylated SA. Purification of the enzymatic digest was performed in accordance with **section 2.2.7 Enrichment of BPDE alkylated human SA** followed by evaporation under N_2 until 90µl. The solution was then reconstituted with H_2O/MeOH (90:10) in a total volume of 150µl. Following reconstitution, a working solution was prepared by dilution, 10x, which was utilized for further evaluation of linearity, precision and accuracy. The obtained adduct levels from 1mg of digested BPDE-SA was based on a previous study by Motwani et al. 2016, see **Table 6**. These levels were used for further method evaluation as well as for validation purposes.

Table 6: Generated adduct levels at His and Lys sites per 1mg of digested BPDE alkylated SA (Motwani et al. 2016)

BPDE isomer	**$A_{His\text{-}Pro}$ (pmol/mg SA)**	**A_{Lys} (pmol/mg SA)**
(±)-anti-	1.4	1.4
(+)-anti-	0.3	2.2
(-)-anti-	2.4	0.6
(±)-syn-	1.3	1.9

2.2.4.1 Evaluation of linearity for BPDE-adducts

To study the linearity and detection limits, calibration curves were prepared for (+)-anti-BPDE-His-Pro (0.5 to 1.7fmol, n=7x3), (-)-anti-BPDE-His-Pro (4.8 to 14.4fmol, n=7x3) and (±)-anti-BPDE-Lys (2.8 to 8.4fmol, n=7x3), where the amount in fmol was injected on column. A volumetric internal standard (IS, benzyl-L-His) with a concentration of 50ng/ml was spiked prior to analysis to adjust for instrumental variability during the measurements. The final concentration of IS was 5ng/ml in each calibration sample. The linearity was evaluated in terms of regression coefficients (R^2) for each isomer.

2.2.4.2 Precision and accuracy for measurements of BPDE-adducts

Precision and accuracy of the developed method was examined by evaluation of intraday variation, based on the analysis of quality control (QC) samples at 3 different concentrations (low, medium and high) within the linear range. Evaluation of intraday variability was performed by duplicate analysis of each QC level within 1 day (n=2).

Intraday precision was investigated by studying the standard deviation of 2-3 replicate injections, followed by evaluation of the accuracy through a comparison of the back calculated areas using the calibration curves and the nominal concentration of the QC samples.

To assess the instrumental capabilities in terms of sensitivity, additional investigations regarding the detection- and quantification limits (LOD & LOQ) were performed by preparation of the lowest concentration expected to detect by the instrument. Following triplicate analysis, the detection limits were calculated according to:

$$LOD = \frac{5 * STDEV}{slope\ of\ calibration}, LOQ = \frac{10 * STDEV}{slope\ of\ calibration}$$

To investigate the presence of carryover effects, a method blank consisting of H_2O/MeOH (90:10; v/v) was injected between the samples as well as the QC samples.

2.2.5 Quantification using an internal standard

To correct for instrumental variability during HPLC/MS/MS analysis an internal standard (IS) was tested for its applicability regarding adduct measurements from PAHDE. Benzyl-L-Histidine was utilized as a volumetric IS throughout the analytical measurements, even though the structure is different compared to the target compounds, BPDE-His-Pro and BPDE-Lys respectively. The choice of IS was also based on availability from a commercial point of view, i.e. since the investigated target compounds does not exist commercially and it is not a simple task to synthesize these.

2.2.6 Enzymatic hydrolysis of a reference BPDE-alkylated SA

Standard procedure (without DTT)

Digestion of 1mg BPDE alkylated SA was performed with pronase (0.9mg/mL) from *Streptomyces griseus*, prepared by x10 dilution from a stock solution (9mg/ml). This stock was prepared by weighing 1.35mg of pronase in 150µl of ammonium bicarbonate (AMBIC) solution. To yield a 1:11 ratio of enzyme-to-SA, the enzymatic digestion was

initiated by adding 100µl of pronase solution (0.9mg/ml) into a 400µl mixture containing 1mg of human SA, yielding a final volume of 500µl. The concentration of pronase was 0.18mg/mL for 1mg of SA. Incubation for 20 h at 37°C and 800rpm with interval mixing was carried out overnight.

Procedure using DTT

50mM of AMBIC solution was prepared by dissolving 39.5mg in 10ml of deionized H_2O, followed by adjusting the pH to 7.4 by acetic acid (30%). 1mg of BPDE alkylated human SA was dissolved in 185µl AMBIC and homogenized by gentle vortex. Reduction of disulfide bridges was carried out by adding 15µl of DTT (324mM in AMBIC), followed by incubation at 56°C for 60 min. After DTT treatment, 30µl of IAA solution (500mM in AMBIC) plus 70µl AMBIC was added to alkylate the cysteine residues for 45 min at room temperature covered by aluminum foil. The reaction was then quenched by adding 15µl of DTT (324mM in AMBIC) plus 85µl AMBIC solution followed by 15-min reaction in the dark. The final volume was 400µl, after which enzymatic hydrolysis was carried out by adding 100µl of pronase (0.9mg/ml in AMBIC). Incubation was carried out for 20 h at 37°C and 800rpm with interval mixing.

2.2.7 Enrichment of BPDE alkylated human SA

Following enzymatic hydrolysis of BPDE alkylated human SA, the enzymatic digest was subjected to SPE purification on a Sep-Pak Plus C18 column (360mg) from Waters (Milford, MA, USA). Conditioning of the cartridge was performed with 5ml of methanol and water respectively, followed by loading the sample obtained from enzymatic hydrolysis, see **section 2.2.6 Enzymatic hydrolysis of a reference BPDE-alkylated SA;** digest of 1mg reference BPDE-SA, dissolved in 500µl of AMBIC solution. Subsequently, the SPE sorbent was washed with 5mL of 0.5M NaAc (pH 5.0), 5ml of H_2O and 5ml of MeOH/H2O (30:70, v/v). The digested sample was then eluted with MeOH/H_2O (95:5, v/v) and evaporated to 0.09ml by a gentle stream of nitrogen. The remaining solution was completed to a final volume of 150µl with H_2O/MeOH (90:10, v/v), and thereby ready for analysis by LC/MS/MS.

2.2.8 Methods concerning analysis of amino acids (unmodified SA)

A HILIC/MS/MS system was developed for seven different underivatized AAs from digested human SA based on various research studies presented in **Appendix 13: Literature review regarding AAs analysis; Table 28**. See **section 2.2.9 Chromatographic conditions** below for further details.

A working solution was prepared by addition of 400µl of each amino acid (n=7) plus 400µl of internal standard (His-d_3) based on an original concentration of 100µg/ml of each compound, yielding a final concentration of 10µg/ml. This standard mixture was then utilized throughout the method development to find the optimal chromatographic conditions.

For the univariate approach regarding improvement of ESI parameters and collisional energies of the AAs, a stock solution of 500µg/ml was prepared and utilized for direct infusion at a flow rate of 20µl/min.

2.2.9 Chromatographic conditions for AAs

In **Appendix 3: Additional chromatograms**; **Figure 18** is illustrating an LC/MS/MS chromatogram from the method described below. Preparation of the NH_4Ac buffer solution was prepared by weighing 0.578g in 200ml deionized H_2O. The salt was dissolved, and the pH was adjusted to 5.75 by acetic acid (50%). Approximately 50ml of deionized H_2O was then added until the calibration mark (250 ml) was reached. Final concentration of the buffer was 30mM.

Chromatographic separation of underivatized AAs was carried out on an iHILICFusion column (150 x 2,1mm, 3µm dp, Hilicon AB, Umeå, Sweden). The mobile phase was a binary solvent system, with solvent A consisting of ACN/H_2O/NH_4Ac (98:1:1, v: v) and solvent B of H_2O/ACN/NH_4Ac (98:1:1, v: v). Isocratic elution was utilized with 90% of solvent B for 25 min. The flow rate was 300µl/min, column temperature was 35°C and the injection volume was set to 5µl. Evaluation of chromatographic conditions was performed as shown in **Appendix 10: Chromatographic conditions tested for separation of AAs; Table 25**.

The mass spectrometric analysis was performed with the same triple-quadrupole instrument (Xevo TQ-S Micro from Waters Corporation, Milford, MA, USA) as for the BPDE adducts, in positive ESI mode with acquisition in MRM mode. In **section 2.1.2 Standards; Table 3,** MRM parameters for the studied AAs are summarized.

2.2.10 Univariate optimization of MS/MS conditions

Variables affecting the ESI sensitivity were optimized by a univariate design by means of direct infusion in positive ionization mode at a concentration of 500µg/ml for each AA. Positive ionization mode was selected because of a higher response for the target compounds compared to negative ionization mode. Precursor ions were selected as the protonated molecules, i.e. $[M+H]^+$, followed by investigation of respective daughter ion spectra for each compound. For each spectrum, a quantifier- and a qualifier ion associated with the highest intensity were selected for further quantitative analysis in MRM mode. The collisional energies presented in **section 2.1.2 Standards; Table 3,** were selected so that 10% of the precursor ion intensity was remaining after fragmentation. Optimization of the cone voltage presented in **Table 3**, was performed for each AA by evaluation of different voltages.

The remaining MS parameters influencing the ionization efficiency were investigated by studying the signal intensity corresponding to the selected internal standard (His-d_3) through an investigation of certain ranges, namely capillary voltage: 2-5kV, desolvation temperature: 300°C-600°C, source temperature: 100°C-150°C, cone gas flow: 10-40L/h and desolvation gas flow: 300-800L/h. Unit mass resolution was used in both

quadrupoles and the best MS conditions were as follows, capillary voltage 3kV, source temperature 150°C, desolvation temperature 400°C, cone voltage 50V, cone gas flow 15L/h and desolvation gas flow 800L/h. Argon was used as collision gas in the second quadrupole and nitrogen was used as desolvation gas in the ESI source.

All measurements were collected in continuum mode and the data acquisition of all experiments were performed with Masslynx software version 3.1 (Waters Cooperation, Milford, MA, USA).

2.2.11 Method evaluation of AAs

Evaluation of the developed HILIC/ESI-MS/MS method was performed by preparation of a working solution containing the target compounds and an internal standard with a concentration of 5µg/ml. The instrumental performance was examined by employing optimal chromatographic conditions, described in **section 2.2.9 Chromatographic conditions** and the developed MS/MS method including appropriate parameters for the ESI sensitivity, see **section 2.2.10 Univariate optimization of MS/MS conditions.** The separation efficiency of the studied AAs see **section 3.3.2.1 Separation efficiency of AAs,** was evaluated by consideration of important chromatographic parameters, i.e. retention factor (k), selectivity (α), number of theoretical plates (N) and the resolution (R_s).

$$k = \frac{t_R - t_0}{t_0}$$

Equation 1: Capacity factor (k) indicating the retention of a compound on a chromatographic column, where t_R is the retention time and t_0 corresponds to where the void volume elutes.

$$\alpha = \frac{k_2}{k_1} = \frac{t_2 - t_0}{t_1 - t_0}$$

Equation 2: Selectivity factor (α) which indicates the ability to distinguish between neighboring compounds

$$N = 5.54 * \left(\frac{t_R}{w_{0,5}}\right)^2$$

Equation 3: The plate number (N) relates to the efficiency of the chromatographic column, which illustrates the performance of separation since more theoretical plates enables more equilibrations, where t_R indicates the retention time and $w_{0,5}$ the width of a peak at half height.

$$R_s = {}^1\!/_4 * \sqrt{N} * \frac{\alpha - 1}{\alpha} * \frac{k}{1 + k}$$

Equation 4: Resolution equation illustrating the influence of chromatographic parameters, namely the selectivity (α), efficiency (N) and the capacity factor (k).

The linearity was investigated by preparing a reference stock solution of seven AAs (20µg/ml) together with an internal standard (10µg/ml); see **Table 3**. Calibration standards were prepared by series dilution ranging from 2.5 to 12.5µg/ml, and 100µl of IS was spiked prior to injection, yielding a concentration of 5µg/ml. Further method validation was carried out by preparing a QC sample at a concentration of 10µg/ml (n=3),

followed by SPE enrichment prior to LC/MS/MS analysis. Interpretation of the precision was performed by interpretation of the relative standard deviation (RSD) for three measured QC samples according to:

$$RSD\ (\%) = \frac{STDEV}{Average} * 100$$

The accuracy of the developed method was calculated according to:

$$Accuracy(\%) = (1 - (\frac{Amount_{nom.} - Amount_{calc.}}{Amount_{nom.}})) * 100$$

2.2.12 Pretreatment of unmodified SA with DTT & IAA

Ammonium bicarbonate (AMBIC, 50mM, pH 7.4) solution was prepared by weighing 39.5mg in 10ml of deionized H_2O, and the pH was adjusted to 7.4 by acetic acid (98%). A stock standard solution of lyophilized human serum albumin (100mg/mL) was prepared in AMBIC (pH 7.4).

Stock solutions of DTT (324mM, 50mg/ml), and IAA (500mM, 92.5mg/ml) in AMBIC (50mM in 10mL, pH 7.4) were prepared in separate Eppendorf test tubes. Note, IAA was covered with aluminum foil since it is light sensitive. The incubation experiments were initiated by spiking 100µl of human SA solution into 6 different Eppendorf test tubes, i.e. 3 tubes dedicated for DTT treatment prior to enzymatic hydrolysis, and the remaining tubes without treatment by DTT. Test tubes without DTT and IAA were completed to 900µl with AMBIC solution. In the remaining tubes, 145µl of AMBIC solution (50mM, pH 7.4) was added simultaneously. A procedural blank was prepared by transferring 245µl AMBIC solution to a fourth Eppendorf test tube. A volume of 155µl of DTT solution was added to each tube followed by incubation at 56°C for 60 min. Then, 300µl of IAA solution was directly spiked to each tube, yielding a total volume of 700µl. The tubes were vortexed and incubated for 45 min at room temperature. IAA is light-sensitive and must be covered with aluminum foil. A volume of 45µl of AMBIC was then added to all Eppendorf tubes, followed by quenching the remaining IAA solution with 155µl of DTT solution (324mM) and vortex properly. The tubes were then left for 15 min in the dark at room temperature. The final volume of each sample was 900µl, and enzymatic hydrolysis was then carried out according to **section 2.2.13 Enzymatic hydrolysis of unmodified SA** below.

2.2.13 Enzymatic hydrolysis of unmodified SA

For digestion of human SA, pronase from *Streptomyces griseus* (9mg/mL) was prepared by weighing 6.3mg of enzyme dissolved in 700µl of AMBIC solution.

To yield a 1:11 ratio of enzyme-to-SA, the enzymatic digestion was initiated by adding 100µl of pronase solution (9mg/mL) into a 900µl mixture containing 10mg of human SA, yielding a final volume of 1000µl. Incubation was performed at 800rpm for 20 h (37°C) with an interval mixing, i.e. a 30-s pause with 5-min intervals.

2.2.14 Enrichment of amino acids by solid phase extraction

For solid phase extraction, 0.5M of sodium acetate in deionized water was dissolved in 80mL of deionized H_2O and the pH was adjusted to 5.0 by using 50% acetic acid. Approximately 20ml of deionized H_2O was added to the calibration mark (100mL). Sample digests were stored on ice prior to enrichment of digested SA, which was performed by means of Sep-Pak Plus C_{18} cartridge (360mg), without vacuum filtration. The cartridges were conditioned by 5ml of MeOH and H_2O respectively and then loaded with the sample digest (1ml). Washing was carried out by adding 5ml of 0.5M NaAc (pH 5.0) followed by 5ml of H_2O. The amino acids were then eluted from the SPE cartridge by adding 5ml of methanol/H_2O (30:70, v/v), which was collected in separate fractions and evaporated to dryness by a gentle stream of nitrogen. The samples were then reconstituted in ACN/H_2O (10:90, v/v) to 1000 µL. Samples without DTT treatment were diluted x10 and DTT treated samples were diluted x20 prior to LC/MS/MS analysis as described in **section 2.2.9 Chromatographic conditions for AAs** and **2.2.10 Univariate optimization of MS/MS conditions** above.

2.2.15 Significance test of DTT treatment

The HILIC/MS/MS method was applied to compare a control sample (10mg SA) and a DTT-treated sample (10mg SA). The samples were treated as described in **section 2.2.12 Pretreatment of unmodified SA with** and **2.2.13 Enzymatic hydrolysis**. Significance testing was then carried out to study the average amount of released AAs from human SA, which were measured by LC/MS/MS. An F-test was carried out to investigate whether the variances were similar or not, followed by a t-test (either assuming unequal- or equal variances) to study whether the average amounts of amino acids were similar or not.

2.2.16 Isolation of human SA

Isolation of human SA was evaluated through precipitation experiments by means of MeOH, acetone and saturated ammonium sulphate respectively. Briefly, after incubation of human SA dissolved in 400µl of PBS buffer (pH 7.4) with 100µl of rat liver S9 fraction for 60 min, either MeOH or acetone was added in a ratio of 1:4 (0.5ml SA:2ml solvent). Precipitation was carried out on ice for 20 min followed by subsequent centrifugation at 4500g, 4°C for 10 min.

A third precipitation method based on a previous study by E. Westberg et al. (2014) was tested by means of saturated ammonium sulphate precipitation. The procedure following incubation of human SA dissolved in 650µl of PBS buffer (pH 7.4) with 100µl of rat liver S9 fraction for 60 min, started with an addition of 140µl 1M HCl to reduce the pH to 4. Precipitation was performed by slow addition of 1ml saturated ammonium sulphate during stirring, followed by precipitation for 20 min. Centrifugation at 4500g, 4°C for 10 minutes was performed followed by purification by 1.5ml MeOH, 1.5ml EtOAc and 1.5ml pentane. Centrifugation was performed at 1600g, 4°C for 10 min between

each purification step. The precipitate was left to dry overnight and reconstituted in AMBIC (50mM, pH 7.4) the following day.

2.2.17 Evaluation of human SA precipitation

To investigate the precipitation efficiency of human SA, the absorbance was measured at 280nm with a Hitachi U-3000 spectrophotometer (Tokyo, Japan); see **Figure 12**, to determine the recovery of added SA, namely 1mg/ml and 10mg/ml respectively. The obtained concentration was calculated through Lambert-Beers law and compared to the nominal values. The extinction coefficient (Ɛ) according to Uniprot database was approximately 34445$M^{-1}cm^{-1}$.

2.2.18 I*n-vitro* alkylation of SA

2.2.18.1 S9 fraction preparation

A microsomal S9 fraction from Moltox (Boone, NC, USA) was used for the metabolic *in vitro* studies of B(a)P. The procedure includes homogenization by dissolving 1g of liver tissue in 3mL isotonic potassium chloride (0.15M KCl), followed by centrifugation at 9000g for 20 min, 4°C. Notably, when ordering the S9 mixture commercially, it is important that the mixture have been stored below -70°C. The metabolic enzymes are instable and lose its activity fast at normal temperatures (M. Registre et al. 2016).

2.2.18.2 Metabolism of B(a)P in presence of SA

A stock solution of B(a)P (37.5mM) was prepared by dissolving 4.4mg in 465µl dimethyl sulfoxide (DMSO). NADPH buffer solution was prepared by dissolving 153mg of Regensys B in 45ml of Regensys A as described by the supplier, see **Table 2**. Phosphate buffer saline (PBS) was prepared by dissolving 0.4g pf KH_2PO_4 and 1.9g of Na_2HPO_4 in 100mL volumetric flask with deionized H_2O. The pH was adjusted to 7.4 by acetic acid (50%). Human SA was prepared by dissolving 100mg in 1ml pf PBS buffer (pH 7.4).

Day 1 -

The experiment was initiated by mixing 50µl of S9 microsomal fraction (homogenized rat liver), 462µl of the NADPH solution, 128µl of PBS buffer (pH 7.4) and 100µl of human SA in 6 Eppendorf test tubes i.e. 3 tubes dedicated for B(a)P and 3 without B(a)P (controls). Pre-incubation at 37°C and 800rpm was carried out for 3 min. To yield a final concentration of 0.5mM B(a)P, 10µl of 37.5mM B(a)P was added to 3 Eppendorf test tubes, yielding a final volume of 750µl. To the control samples, 10µl of pure DMSO was added. The mixture was then incubated at 37°C for 60 min. Immediately after incubation, 130µl of 1M HCl was added to reduce the pH aiming to quench the incubation. Protein precipitation by slow addition of 1ml ammonium sulphate (saturated solution in deionized H_2O, 5x200µl), followed by precipitation on ice for 20 min. The test tubes were then centrifuged at 4500g for 10 min (4°C) and the precipitate was washed with 1.5ml each of methanol, ethyl acetate and pentane, followed by subsequent centrifugation at 1600g for 10 min (4°C) after each wash. The precipitate

was then left to dry overnight covered with a filter tissue to evaporate any remaining pentane.

Day 2 –

Without DTT treatment: The precipitate from day 1 was reconstituted in 400µl AMBIC solution (50mM, pH 7.4), followed by gentle vortex to homogenize the isolated protein. Enzymatic hydrolysis was carried out according to **section 2.2.6 Enzymatic hydrolysis of a reference BPDE-alkylated SA.**

With DTT treatment: The precipitate was reconstituted in 245µl AMBIC solution (50mM, pH 7.4) and vortexed properly to homogenize the protein. Reduction of disulfide bridges was carried out by spiking 155µl DTT (324mM in AMBIC), followed by incubation at 56°C for 60 min. After DTT treatment, 300µl of IAA solution (500mM in AMBIC) was added to alkylate the cysteine residues for 45 min at room temperature covered by aluminum foil. The reaction was then quenched by spiking with 155µl of DTT (324mM in AMBIC) plus 45µl of AMBIC solution, followed by 15 min of reaction in the dark. The final volume was 900 µl, and enzymatic hydrolysis was then carried out according to **section 2.2.6 Enzymatic hydrolysis of a reference BPDE-alkylated SA**.

2.2.19 *In vivo* measurements of B(a)P exposed mice

As a part of a previous study by J. Zurita et al. (2018), SA samples from exposed mice treated by B(a)P from the national Hellenic Research Foundation in Athens, were analyzed for adduct levels. Briefly, male mice had been treated by an injection of B(a)P dissolved in tricaprylin (100mg/kg of b.w.), and then killed by asphyxiation after 1, 3, 7, 14 and 28 days of exposure. The blood was collected followed by centrifugation in heparinized tubes for plasma isolation. The samples were then properly stored at -20°C during transportation to Sweden, where the blood plasma was isolated by addition of saturated ammonium sulphate (J. Zurita et al. 2018).

In vivo precipitated mice SA, obtained after 3- and 14 days of B(a)P exposure, respectively, was measured by the developed LC/MS/MS method as described in **section 2.2.1 Chromatography and MS set-up for BPDE** and **2.2.3 Optimization of MS/MS conditions for BPDE adducts.** Briefly, extracts from 1mg of precipitated mice SA was dissolved in 400µl of AMBIC (50mM, pH 7.4). A volume of 100µl of pronase solution (0.9mg/ml) was then added to yield a final volume of 500µl. The mixture was then incubated for 20 h at 37°C with interval mixing. Enrichment of the samples was performed as described in **section 2.2.7 Enrichment of BPDE alkylated human SA,** followed by LC/MS/MS analysis.

2.2.20 Statistical analysis and data evaluation

All collected data was evaluated and treated by means of Microsoft Excel, version 2010, except those data obtained during the experimental design, as described in **section 2.2.3 Optimization of MS/MS conditions for BPDE adducts** above.

2.2.21 Calculations and raw data

All calculations performed in this thesis and the corresponding raw data are summarized in **Appendix 11: Excel calculations and raw data**. The raw data are also located on the Masslynx workstation (WS) located in the control room within the MS laboratory at Stockholm University. The files are stored at the following path; C:/Masslynx/Tim.pro/Data/.

3. Results and discussion

3.1 LC/MS/MS of BPDE adducts

3.1.1 Chromatography and MRM selection for BPDE adducts

Chromatography

Chromatographic separation was obtained on an F5 column in accordance with a previously developed method by J. Zurita et al. (2018). Chromatographic conditions were further optimized in terms of flow rate and gradient conditions, aiming to improve the sensitivity while enabling a shorter analysis time. A flow rate of 200µl/min combined with gradient elution resulted in a total analysis of 16.5 min, as described in **section 2.2.1 Chromatography and MS set-up for BPDE**. All target compounds were separated within a 9.5 min run, but to rinse the chromatographic column extra time was necessary. In **Figure 6,** are shown the LC/MS/MS chromatograms in MRM mode regarding the studied BPDE adducts and the utilized IS (Benzyl-L-His).

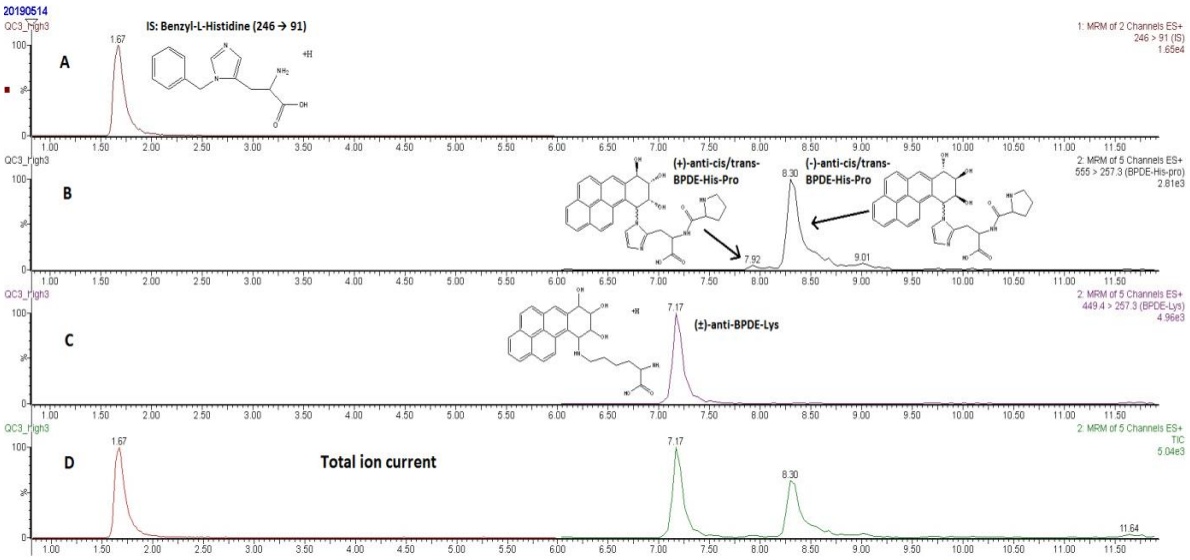


Figure 6: LC/ESI-MRM chromatograms illustrating the separation efficiency of studied target compounds from the standard mixture of BPDE alkylated human SA. (A): Benzyl-L-His (0.05µg/ml), (B): (+)-anti-cis/trans-BPDE-His-Pro (1.7fmol) and (-)-anti-cis/trans-BPDE-His-Pro (14.4fmol), (C): (±)-anti-BPDE-Lys and (D): Total ion current (TIC).

MRM selection of studied BPDE adducts

In **Table 4**, the utilized MRM transitions are presented after evaluation of their respective product ion spectrum, presented in **Appendix 4: Mass spectrum; Figure 22** and **Figure 23** respectively. Identification of the target compounds was based on their retention times and the intensity ratio of quantifier and qualifier ions.

3.1.2 Optimization of ESI parameters

Benzyl-L-His was used as a model compound to improve the sensitivity during ionization of target compounds. This was because BPDE alkylated SA as reference standards are not commercially available. Benzyl-L-His was aimed for a design of experiment approach to identify and optimize crucial ESI parameters influencing the sensitivity. The optimized MS method was applied to measure BPDE-His-Pro and BPDE-Lys, even though the structure is different compared to Benzyl-L-His, see **Appendix 8: Target compounds Figure 32.** The Plackett-Burman design enabled a systematic identification of important parameters by calculation of fitting coefficients (B_1→ B_8) presented in **Table 7**. Note, 3 extra coefficients (b_9, b_{10} and b_{11}) were estimated due to matrix requirements, usually referred to as *"dummy factors" (DF)*. A comparison between the highest *DF*, b_{11} in this case, with each fitting coefficient was performed to assess the most influential instrumental parameters, as seen in **Table 7**, where b_3, b_5 and b_8 were selected. These terms correspond to the cone voltage, desolvation temperature and the chromatographic flow rate, indicating a large impact on the ionization efficiency.

Table 7: Fitting coefficients (B_1→ B_8) estimated through the Plackett-Burman design. DF indicates a "dummy factor", which are additional factors required to make a square matrix. B_{11} is the highest coefficient among all three DFs, and it is used to identify important parameters, B_1→ B_8 influencing the ionization efficiency.

ESI parameter	B-coefficient	Value	Significant?
-	b0	1.08E+07	YES
Dwell time	b1	1.46E+06	YES
Source temp.	b2	1.19E+06	YES
Cone voltage	**b3**	**2.06E+06**	**YES**
Cap. Voltage	b4	8.88E+04	NO
Desolv. Temp.	**b5**	**2.60E+06**	**YES**
Cone gas flow	b6	1.42E+06	YES
Desolv. Gas flow	b7	7.90E+05	NO
LC-flow	**b8**	**3.87E+06**	**YES**
-	b9	6.93E+05	*DF*
-	b10	7.96E+04	*DF*
-	**b11**	**1.11E+06**	***DF***

Coefficients b_3, b_5 and b_8 were selected for a second experimental design aiming to optimize the MS response by a systematic change of instrumental settings. Two

mathematical models were evaluated, i.e. a full factorial- and a face centered design. The following two models were used to predict a new set of data including five experiments, see

Table 8Error! Reference source not found.**.**

Model A – 2^3 full factorial design:

$$y = \beta_3 x_3 + \beta_5 x_5 + \beta_8 x_8 + \beta_{35} x_3 x_5 + \beta_{38} x_3 x_8 + \beta_{58} x_5 x_8 + \beta_{358} x_3 x_5 x_8$$

Model B – Face centered composite design:

$$y = \beta_3 x_3 + \beta_5 x_5 + \beta_8 x_8 + \beta_{33} (x_3)^2 + \beta_{55} (x_5)^2 + \beta_{88} (x_8)^2 + \beta_{35} x_3 x_5 + \beta_{38} x_3 x_8 + \beta_{58} x_5 x_8$$

Considering the sum of residuals, calculated as the absolute difference between predicted- and experimental MS response, it was possible to conclude which of the selected models that corresponds to the most suitable prediction with regards to a new set of data. The sum of residuals for model A was 1.41E7, while for model B approximately 7.88E5. The overall sum of residuals for model B are smaller than the sum of residuals for model A, which indicates that model B provides a better estimation for a new set of experiments.

Table 8: Comparison between 2^3 full factorial design and a face centered design in terms of residuals related to each estimated model. The experimental plan corresponds a new set of experiments.

Exp No.	x3 Cone	x5 Desolv. temp	x8 Flow	Residual 1 (Model A)	Residual 2 (Model B)
1	10	200	200	1.09E+06	5.09E+04
2	50	200	200	7.61E+05	5.13E+03
3	10	600	200	4.47E+06	1.65E+05
4	50	600	200	4.47E+06	3.81E+05
5	30	400	200	3.30E+06	1.86E+05

The normal probability plot in **Appendix 5: Experimental design**; **Figure 24**, shows whether the calculated residuals, i.e. the deviation between predicted- and experimental response follows a normal distribution or not. Since the plot of all residuals remains on a straight line between -4 to 4 studentized standard deviations, it was possible to conclude that the calculated residuals came from a normally distributed set of data.

In **Figure 7** below, the experimental MS response (y-axis) have been plotted against the predicted MS response (x-axis) to graphically illustrate the predictive power of the quadratic model. A good predictive model will result in data points remaining on a straight line with a regression coefficient close to one. It seems reasonable to say that the estimated quadratic model can be used as a predictive tool to investigate the response of new experimental data.

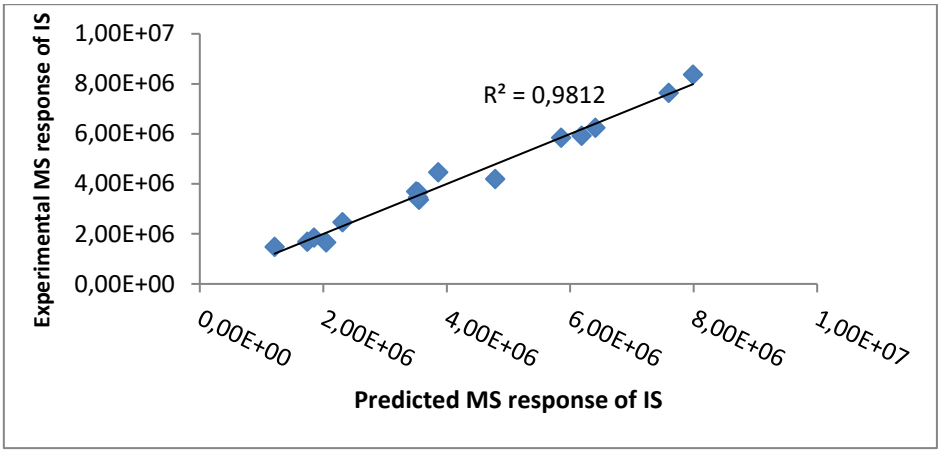


Figure 7: Predicted vs experimental response for the CCF design based on the three most influential factors (x_3, x_5 and x_8) selected from the Plackett-Burman design.

To investigate whether a model is predictive and reliable for a new set of data, there are a few statistical options to consider during the evaluation, namely the regression coefficient (R^2), the predictive cross validation (Q^2), model validity and the reproducibility, see **Appendix 5: Experimental design**; **Figure 25**. A good model will involve higher bar graphs, preferably as close to 1 as possible, with exception to the reproducibility which may need further investigation in a case when the value is almost 1. The estimated quadratic model in this experimental approach resulted in a large reproducibility, which indicates that the response for the replicates are identical, i.e. with a pure error approximately 0 under the same experimental conditions. Considering the value of R^2, a value of 0.98 was obtained, which relates to the percentage of variation according to the response, i.e. a higher R^2 explains a better model. The predictive cross validation (Q^2) equals 0.82, also indicates a reasonable model estimation, i.e. the ability to predict new experimental data. Regarding the model validity, a value larger than 0.25 means that there is no lack of fit in the estimated model, i.e. the error relating to the model is in the same range as the pure error.

The coefficient plot presented in **Figure 8** indicates the influence of each factor relating to scaled and centered variables (-1, 0 and 1), which facilitates a comparison between the factors. By considering the size of each factor it is possible to estimate how much the response will change by changing a specific parameter, for example factor x_5 which corresponds to the desolvation temperature. This factor brings a large impact on the experimental response as it changes between the minimum and maximum values. To determine whether a certain parameter is significant or not, the confidence interval must be investigated, i.e. it should not cross 0 to be significant. In **Figure 8** below, the following factors, namely x_3 (β_3), x_5 (β_5) and x_{33} (β_{33}) are significant variables with regards to their confidence limits. Factor x_8 (β_8; LC flow rate) indicates an insignificant influence regarding the response, and therefore it was decided to keep it constant for the further optimization, i.e. at 200µl/min.

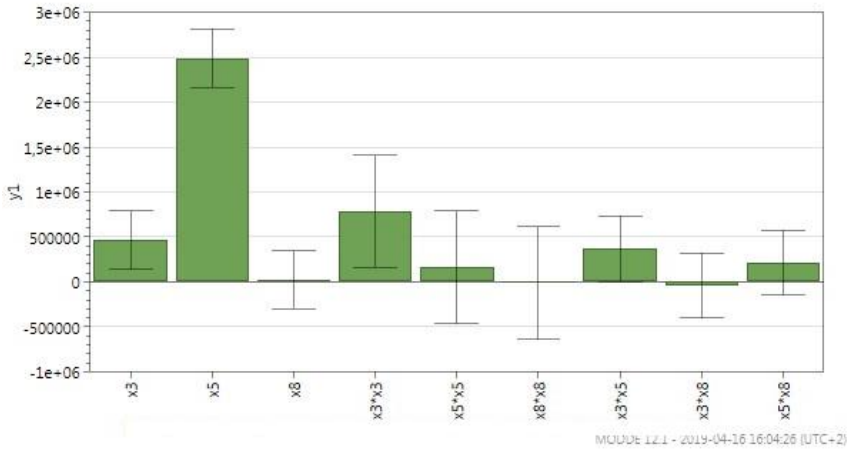


Figure 8: Regression coefficients estimated by multiple linear regressions (MLR) by means of a face centered design, which are related to scaled and centered variables.

In the response contour plot, presented in **Figure 9**, several regions illustrating different experimental responses are presented depending on how the value of the instrumental parameters x_3 (cone voltage) and x_5 (desolvation temp.) varies between their minimum and maximum values (-1 to 1). By considering the contour plot, the maximum experimental response is obtained when x_3 and x_5 are operated at their maximum levels, i.e. at a cone voltage of 50V and a desolvation temperature of 600°C. Thus, the same result was as well achieved by running the optimizer add-on in MODDE software, see **Appendix 6: Optimization of MS sensitivity; Figure 26.**

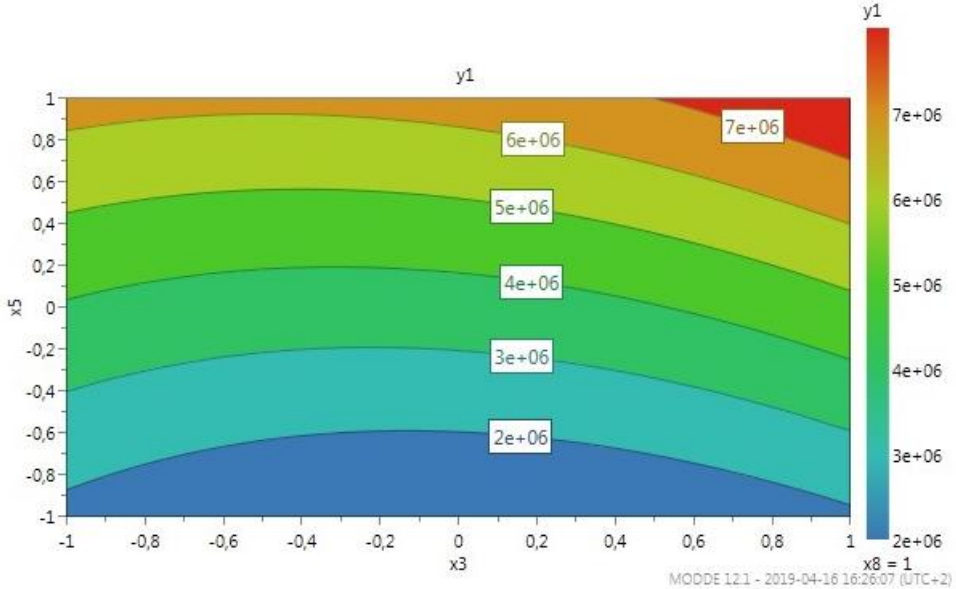


Figure 9: Response contour plot illustrating the maximum response obtained within the selected ranges for the 3 most influential parameters (x_3, x_5 and x_8). Note, the third factor x_8 (LC flow rate) is kept constant at 200µl/min.

3.1.3 Comparison to previously developed methods

The developed MS method based on the response for benzyl-L-His (IS) was applied to a sample of 1mg digested BPDE-SA to investigate possible improvements in comparison

to a previously developed MS method by J. Zurita et al. (2018), see **Table 9.** The difference was only slightly improved by a factor of two, which again is related to the difference in structure regarding benzyl-L-His and the target compounds, BPDE-His-Pro and BPDE-Lys. Anyway, the main scientific aims were to state the advantages by experimental design using benzyl-L-His as a model compound, which must serve as a foundation for future optimization of the target analytes. Additional illustrations are given in

Appendix 6: Optimization of MS sensitivity**; Figure 27** and **Figure 28,** regarding the differences in MS response.

Table 9: Measured MS responses obtained by the optimized MS method and a previously developed method by J. Zurita et al. (2018). Response A corresponds to the optimized method described above, and response B is obtained by using parameters described in the previously described method.

BPDE isomer	Ret. Time (min)	Response A	Response B	Ratio
BPDE-His-Pro	8.3	9.84E+05	4.06E+05	2.4
BPDE-Lys	7.2	3.72E+05	2.09E+05	1.8

3.2 Method evaluation for BPDE adducts

3.2.1 Calibration and detection limits

To evaluate the linearity and detection limits of BPDE-SA adducts, calibration samples were prepared for every BPDE isomer presented in **Appendix 1: Calibration data Figure 17,** summarized in **Table 10** below**.** The numbers of calibration points were seven, and the estimated linear ranges remained within an interval from 0.6-14.4 fmol injected on column, depending on the isomer. All calibration standards were normalized to benzyl-L-His, and the regression coefficients varied from 0.98 to 0.99, except from (+)-anti-BPDE-His-Pro which had a value of 0.92, probably due to the low adduct levels measured from this enantiomer. To reduce potential problems related to carry-over effects, the calibration standards were injected from the lowest concentration to the maximum concentration. A method blank following the highest concentration did not indicate any carry-over effects. The repeatability of the linear curves was overall acceptable, except from the lowest concentration points, which indicated a higher variability. This is probably due to the relatively small amounts measured for each BPDE isomer.

By series dilution of a working solution, prepared from 1mg BPDE-SA alkylated *in vitro*, it was possible to estimate the detection limits for each BPDE isomer presented in **Table 10.** Compared to a previously developed HPLC/MS/MS method using a reversed phase (C_{18}) column by E. Westberg et al. (2014), where the LOD was approximately 1fmol/mg SA, measured as the total adduct formation of (±)-anti-BPDE-His-Pro, the present analytical method provided lower detection limits. Thus, by the optimal LC/MS/MS conditions developed during this project as well as utilization of an IS, an LOD around 80 amoles/mg SA was obtained for (+)-anti-BPDE-His-Pro and 35 amoles/mg SA for (-)-anti-BPDE-His-Pro. The corresponding LOD for (±)-anti-BPDE-Lys was approximately 0.6fmol and 0.65fmol for (±)-anti-BPDE-His-Pro, injected on column.

Table 10: Method evaluation regarding analysis of digested BPDE-SA by means of LC/MS/MS (Xevo). The areas are normalized to the utilized IS spiked prior to injection at a concentration of 0.05µg/ml. All calibration samples were analyzed in triplicates (n=3).

BPDE isomer	Calibration curve	R2	Linear range (fmol)	RSD (%)	LOD (fmol/mg SA)	LOQ (fmol/mg SA)
(±)-anti-BPDE-His-Pro	y=0.041x + 0.0232	0.9964	2.8-8.4	5.2 ± 2.4	0.65	1.29
(±)-anti-BPDE-Lys	y=0,043x + 0.0034	0.9919	2.8-8.4	7.8 ± 7.1	0.60	1.21
(+)-anti-BPDE-His-Pro	y=0.013x + 0.0033	0.9207	0.6-1.7	4.9 ± 1.9	0.08	0.16
(-)-anti-BPDE-His-Pro	y=0.0259x - 0.0131	0.9896	4.8-14.4	5.7 ± 2.1	0.04	0.07

***Values for LOD and LOQ are presented as an average from 3 replicate injections, in fmol/mg SA.**

In a previous study by Zurita et al. (2018), LC/HRMS was employed for quantification of digested BPDE-SA using the same chromatographic column as in this project. The obtained calibration as well as the estimated LODs in that work are summarized in **Table 11.** In comparison to the present study, the obtained LODs were approximately one order of magnitude lower, i.e. 4 and 8 amoles per mg of SA for (+)-anti- and (-)-anti-BPDE-His-Pro respectively. In the previous study, it was also possible to identify (±)-syn-BPDE-His-Pro and (±)-syn-BPDE-Lys, as well as resolving (+)-anti-BPDE-Lys and (-)-anti-BPDE-Lys from each other.

Table 11: Method evaluation in a previous study by J. Zurita et al. (2018), calculated from 1mg digested BPDE-SA alkylated with human SA. The samples were analyzed by HRMS (Orbitrap).

BPDE isomer	Calibration curve	R2	Linear range (fmol)	LOD* (fmol)
(+)-anti-BPDE-His-Pro	y=134732x-842.3	0.9571	0.1-0.7	0.004
(-)-anti-BPDE-His-Pro	y=581806x + 72919	0.9927	1.3-5.7	0.008
(+)-anti-BPDE-Lys	y=4468.9x - 428.4	0.993	1.1-5.0	0.2
(-)-anti-BPDE-Lys	y=88328x + 5235.7	0.9765	0.3-1.5	0.01

****Values for LOD and LOQ are presented as an average from 3 replicate injections, in fmol/mg SA.**

3.2.2 Intraday precision and accuracy

The developed LC/MS/MS method was evaluated by preparation of QC samples at 3 different concentrations (low, medium and high) remaining within the linear range for each BPDE isomer, see **Table 12.** Each control sample was injected in triplicates (n=3) to estimate the precision (RSD) in the analysis. BPDE isomers corrected by means of IS demonstrated an RSD of 4.9 ± 1.9 regarding (+)-anti-BPDE-His-Pro, while the values for (±)-anti-BPDE-His-Pro and (±)-anti-BPDE-Lys as single peaks indicated an RSD of 5.2 ± 2.4 and 7.8 ± 7.1, slightly higher compared to (+)-anti-BPDE-His-Pro. In terms of analytical

precision this gives an indication that the repeatability was acceptable since all RSD values remained within a range from 0-15%. Considering the values obtained for accuracy, **see Table 12,** ranging from 85.4 to 108%, it is reasonable to say that the tested QC samples remained within an acceptable range (85-115%). This provides an indication that the developed method is accurate, i.e. without significant deviations from the nominal concentration.

Table 12: Intraday precision (RSD) and accuracy (%) based on QC samples at 3 different levels (n=3). The amount refers to how much that was injected on column (fmol).

BPDE isomer	QC level	Amount injected (fmol)	RSD (%)	Accuracy (%)
(+)-anti-BPDE-His-Pro	*LOW*	0.6	3.7	109
	MED	0.9	8.5	99.4
	HIGH	1.3	2.3	89.2
(-)-anti-BPDE-His-Pro	*LOW*	4.8	2.2	93.5
	MED	8.0	3.3	96.2
	HIGH	11	6.1	85.4
(±)-anti-BPDE-Lys	*LOW*	2.8	5.4	108
	MED	4.7	9.5	102
	HIGH	6.5	2.5	101
(±)-anti-BPDE-His-Pro	*LOW*	2.8	6.5	86.9
	MED	4.7	0.9	89.7
	HIGH	6.5	5.3	87.3

3.3 Digestion efficiency

3.3.1 BPDE alkylated human SA

One of the main scientific objectives in this project was to establish whether pretreatment with DTT improves the enzymatic digestion of BPDE-alkylated human SA, see protocol in **Appendix 2: Experimental protocols.** In **Figure 10,** the main target compounds, **BPDE-His-Pro** and **BPDE-Lys** treated with and without DTT are presented. It was observed that there was no significant improvement achieved by using DTT prior to enzymatic hydrolysis. By comparison of the obtained MS responses it was evident that the adduct levels to the His site were almost 250 times lower in samples treated with DTT, and approximately 800 times lower at the Lys site. Thus, DTT treatment reduced the yield of the studied adducts.

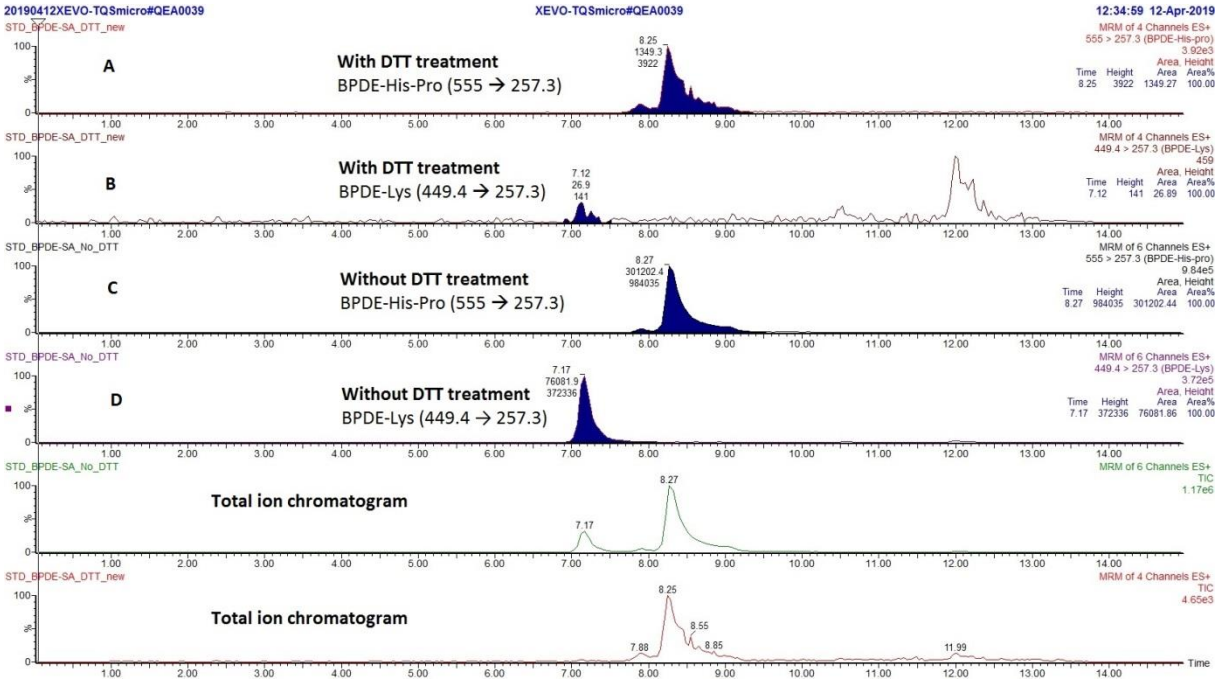


Figure 10: Comparison of BPDE alkylated human SA with and without pretreatment by means of DTT prior to enzymatic hydrolysis. Trace A and B illustrates the influence of DTT treatment on (A) BPDE-His-Pro and (B) BPDE-Lys, while trace C and D illustrates the adducts generated without the presence of DTT.

3.3.2 Unmodified human SA

3.3.2.1 Separation efficiency of AAs

Separation of underivatized AAs was achieved by means of a HILIC/ESI-MS/MS method aimed to measure the obtained amount after enzymatic hydrolysis of unmodified SA. The main purpose was to investigate potential differences related to digestion yield of AAs with and without pretreatment by DTT.

To assess the separation efficiency in terms of resolution (R_s) and selectivity (α) between the studied AAs, various chromatographic conditions were evaluated; see **Appendix 10: Chromatographic conditions tested for separation of AAs**. All studied chromatographic conditions included water and acetonitrile in the mobile phase with ammonium acetate buffer as an additive. Both gradient and isocratic elution were tested, but the isocratic elution was found to be preferable to keep repeatable elution conditions during the analysis.

Considering the tested chromatographic conditions (data not shown) and the optimized chromatogram presented in **Appendix 3: Additional chromatograms Figure 18,** it was evident that the retention of the target compounds depended on pH of the buffer as well as a suitable buffer concentration. Ammonium acetate buffer with a pH of 5.75 was chosen since this value is in the middle of the two major pKa values for AAs (M.C Santiago et al. 2012). Such conditions improve the chromatographic interaction between the stationary phase and the target compounds. In the present study there was

no investigation performed related to matrix effects caused by the NH_4Ac buffer for the studied AAs. Further details about the chromatographic efficiency are described in **Appendix 3: Additional chromatograms Table 22,** where different parameters regarding the chromatographic separation are presented. Even though it is recommended to initiate with a low concentration of H_2O for HILIC separations (M.C Santiago et al. 2012), the chromatographic separation was optimized based on a higher percentage of H_2O throughout the isocratic elution, i.e. a probable explanation why the separation was poor for many AAs, see **Appendix 10:** Chromatographic conditions tested for separation of AAs **Table 22.**

3.3.2.2 Method evaluation for AAs

Evaluation of the developed HILIC/MS/MS method was performed by preparation of calibration samples to investigate the regression coefficients for each AA. Based on the calibration curves presented in **Appendix 1: Calibration data Figure 16,** acceptable linearities were obtained for all AAs, since the regression coefficients were improved by means of His-d_3 as internal standard (R^2>0.99).

The developed method for AAs was only partially evaluated through the linearity of 6-7 calibration points (n=2), and the detection limits were not investigated for the studied AAs since the sensitivity was not of concern for this work.

3.3.2.3 SPE purification of AAs

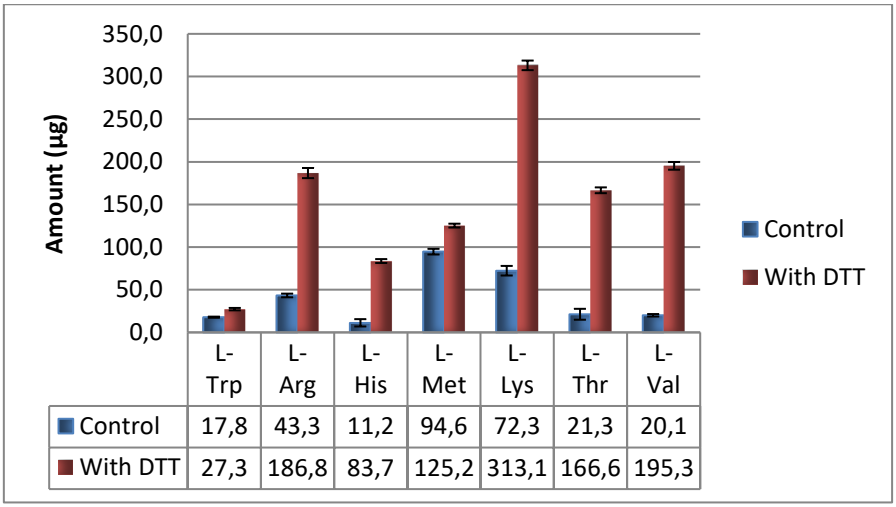
Purification of AAs by means of SPE was performed, as described in **section 2.2.14 Enrichment of amino acids by solid phase extraction** Thus, three separately prepared QC samples (10µg/ml) were subjected to SPE enrichment and the obtained elution phase (MeOH/H_2O, 30:70; v/v) was analyzed by HILIC/ESI-MS/MS. The variability between measured replicates was evaluated by the precision expressed as RSD, which remained within a range of 0-15% for all measured AAs; see **Table 13**. The accuracy on the other hand did not remain within the acceptable range (85-115%) for His, Lys, Thr and Val, but regarding the purpose of this study it was considered as enough. It was clear that our SPE purification system did not recover a suitable amount regarding the nominal values of AAs, at least by considering the poor accuracy presented in **Table 13.** Because of this, additional investigations are required to improve the clean-up of AAs.

Table 13: HILIC/ESI-MS/MS analysis of free AAs subjected to SPE enrichment as QC samples (10µg/ml). Precision interpreted as relative standard deviation (RSD) and accuracy in percentage (%).

Compound	Conc. (µg/mL)	RSD (%)	Accuracy (%)
Trp	10	0.2	98.5
Arg	10	2.3	80.8
His	10	3.3	47.2
Met	10	0.8	82.8
Lys	10	2.9	46.0
Thr	10	5.5	61.5
Val	10	3.4	32.9

3.3.2.4 Improvement of digestion

Digestion of pure SA was performed in triplicates (n=3), and the presented result is the amount (µg) of different AAs obtained after enzymatic hydrolysis, see **Figure 11**. A detailed protocol based on a previous study by C.R. Weisbrod et al. (2012) can be found in **Appendix 2: Experimental protocols; Table 17.**



	L-Trp	L-Arg	L-His	L-Met	L-Lys	L-Thr	L-Val
Control	17,8	43,3	11,2	94,6	72,3	21,3	20,1
With DTT	27,3	186,8	83,7	125,2	313,1	166,6	195,3

Figure 11: Proteolytic digestion of 10mg human SA with and without pre-treatment by DTT and IAA. The digestion was prepared in triplicates (n=3), followed by LC/MS/MS analysis. The error bars indicate the variability within a 95% confidence interval.

Hypothesis testing was carried out to investigate if there was a significant difference between control- and DTT treated samples, followed by a significance test. A t-test was used to compare the average values of both datasets based on the variances which were evaluated by conducting an F-test, according to:

H_0: No significant difference in the variances

H_1: There is a significant difference regarding the variances

According to **Table 26** in **Appendix 12 Significance testing of DTT treatment,** the calculated F value is greater than the critical F value. Thus, the null hypothesis (H_0) is therefore rejected at 95% confidence interval (α=0.05), i.e. the variances of both

treatment groups are unequal. Considering this information, a t-test was applied assuming unequal variances, namely:

H_0: No difference between the mean values of measured AAs

H_1: A significant difference of measured AAs between the groups

Persuasive, according to **Table 27** in **Appendix 12 Significance testing of DTT treatment,** the calculated t value is greater than the critical t value, so the null hypothesis is therefore rejected at 95% confidence interval, indicating that there is a significant difference related to the average amount of measured AAs. This proves our hypothesis that treatment by means of DTT improves the enzymatic digestion of unmodified SA. By considering the average amount of released AAs, **Table 27** in **Appendix 12 Significance testing of DTT treatment**, approximately 4x higher amounts were obtained by utilization of DTT reagent prior to enzymatic hydrolysis.

3.4 *In vitro* studies

3.4.1 Isolation of human SA

Isolation of human SA was investigated with different precipitation agents, namely MeOH, acetone and saturated ammonium sulphate, protocols summarized in **Appendix 2: Experimental protocols; Table 18** to **Table 21**.

Precipitation by use of MeOH and acetone, respectively, was found to be poor by visual comparison with samples precipitated by ammonium sulphate. The recoveries were not evaluated for MeOH and acetone. The SA precipitate by ammonium sulphate was evaluated for yield and purity by means of UV-Vis spectrophotometry at 280nm, see **Figure 12**. Description of the method can be found in **section 2.2.17 Evaluation of human SA precipitation.**

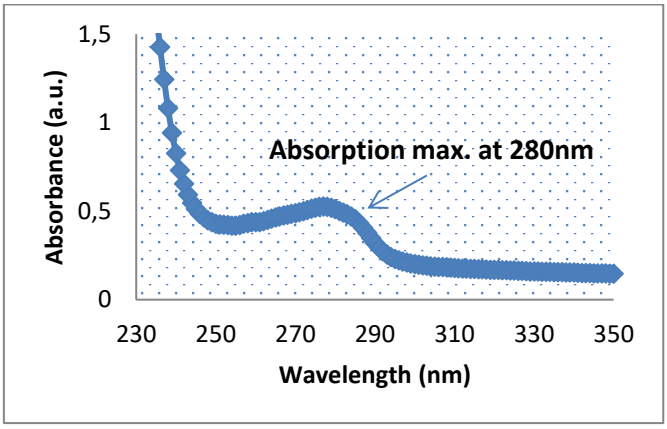


Figure 12: Spectrophotometric analysis between 230 and 350nm illustrating the absorption maximum (280nm) of 1mg human SA

The results in **Figure 13** (1mg human SA) and **Figure 14** (10mg human SA), suggest that saturated ammonium sulphate was suitable for precipitation, even though a matrix interference caused by the rat liver S9 fraction was observed during the spectrophotometric measurements. Thus, this might result in an overestimation of the SA content. To prevent the formation of salt precipitation, the precipitating step

following addition of ammonium sulphate was carried out for a maximum of 20 min (E. Westberg et al. 2014).

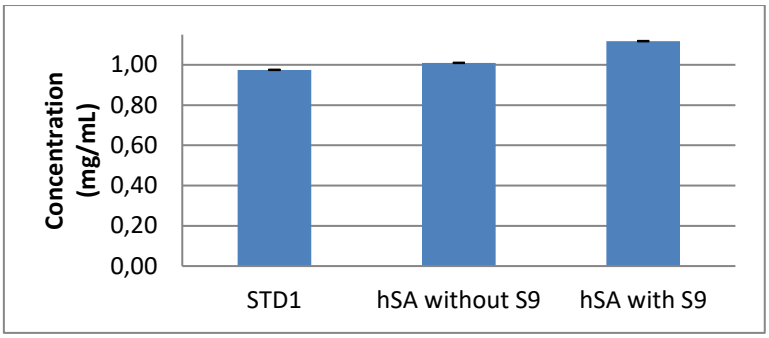


Figure 13: Protein precipitation of 1mg human SA by means of NH_4SO_4 (n=3). The concentration was measured using absorbance at 280nm as described under methods.

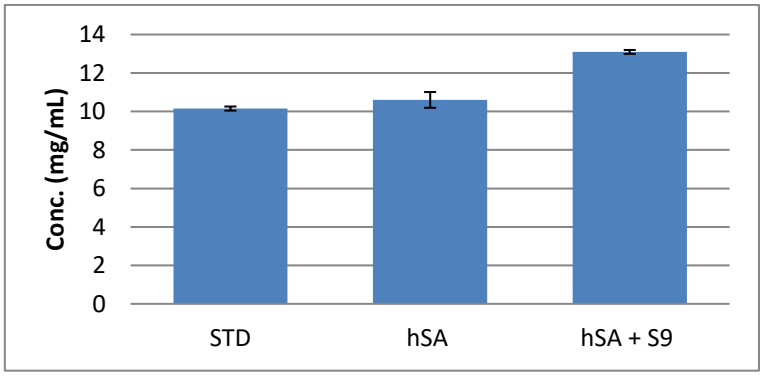


Figure 14: Protein precipitation of 10mg human SA by means of NH_4SO_4 (n=3)

3.4.2 Metabolism of B[a]P

Generation of BPDE adducts *in vitro* as reference standards was performed through metabolism of B(a)P in a liver S9 system, see **section 1.3 Metabolism of benzo(a)pyrene.** These were analyzed as modified peptides (adducts), i.e. BPDE-His-Pro and BPDE-Lys, after isolation and enzymatic hydrolysis, as described in **section 2.2.18 In-vitro alkylation of SA**. Following enzymatic hydrolysis, **section 2.2.6 Enzymatic hydrolysis of a reference BPDE-alkylated SA,** and enrichment by SPE described in **section 2.2.7 Enrichment of BPDE alkylated human SA,** subsequent analysis of the target compounds were performed by the optimized LC/MS/MS method, described in **section 2.2.1 Chromatography and MS set-up for BPDE** and **2.2.3 Optimization of MS/MS conditions for BPDE adducts.**

Unfortunately, it was not possible to detect any BPDE adducts from *in vitro* metabolized B(a)P by means of triple quadrupole mass spectrometry (TQ-MS). To investigate whether the problem was generated due to resolution issues, a high-resolution mass spectrometry (HRMS, Orbitrap) instrument was later employed for these samples. A previously digested standard of BPDE-SA (1mg) was measured by LC/HRMS; see **Appendix 7: LC-HRMS PRM chromatograms; Figure 29**. Notably, this is the same sample

previously analyzed by a low-resolution mass spectrometer (Xevo), see **Appendix 3: Additional chromatograms; Figure 19**.

A comparison of the MS responses obtained by TQ-MS (Xevo) and HRMS (Orbitrap), see **Table 14,** clearly demonstrates the advantages by using HRMS for measurements of low BPDE adduct levels generated through a metabolism of B(a)P. The MS response was approximately 20 times higher for BPDE-His-Pro, and almost 9 times higher for BPDE-Lys when using MS Orbitrap as detection instrument.

Table 14: Comparison between MS responses for 1mg digested BPDE-SA measured as either BPDE-His-Pro or BPDE- Lys using triple quadrupole- (Xevo) or high-resolution MS (Orbitrap).

BPDE isomer	Xevo	Orbitrap	Ratio
BPDE -His-Pro	9.84E+05	1.97E+07	20.0
BPDE-Lys	3.72E+05	3.30E+06	8.9

In **Appendix 7: LC-HRMS PRM chromatograms; Figure 30,** an LC/HRMS PRM chromatogram is presented, which might indicate that possible BPDE adducts were generated during *in vitro* metabolism of B(a)P. The adduct levels are still very low and it was not possible to accomplish a reliable identification of the target compounds since there was also a slight shift in retention time compared to the reference standard (BPDE-SA), and no investigation was made whether this shift was caused by matrix interferences or not.

3.5 Analysis of SA from *in vivo* exposed mice

Analysis of 1mg SA from mice exposed to B(a)P at 100mg/kg of b.w, sampled after 3 and 14 days of administration after exposure, were analyzed by the optimized HPLC/MS/MS method in MRM mode. No BPDE adducts were detected by means of the utilized LC/triple-quadrupole MS system, therefore LC/HRMS was employed instead. Both BPDE-His-Pro and BPDE-Lys were detected at approximately similar retention times compared to the reference standard, see **Figure 31**, but the absolute peak intensities were generally low, about two orders of magnitude. The presence of parent ions from BPDE-His-Pro and BPDE-Lys were observed from measurements of *in vivo* samples, see **Appendix 7: LC-HRMS PRM chromatograms**; **Figure 31** (spectra A.1 and B.1). Together with the high mass accuracy utilized by HRMS (<3.0ppm), the presence of a parent ion within a full scan MS spectrum facilitated compound identification. In **Table** 15**,** an estimation of the injected amount for BPDE-His-Pro and BPDE-Lys is given based on a single point calibration of the reference standard, **see Appendix 7: LC-HRMS PRM chromatograms; Figure 29.** No carry-over effects were observed between the injections, which was verified by injection of a method blank.

Table 15: Estimation of adduct levels from *in vivo* exposed mice 3 days after exposure. The estimation is a rough estimation based on a single point calibration by a reference standard of BPDE-SA (1mg). The STD concentration in pmol/mg SA refers to a previous article by Motwani et al. (2016). The estimated adduct levels relate to the single point calibration of B(a)P exposed mice, sampled 3 days after exposure.

BPDE isomer	STD conc. (pmol/mg SA)	Area (STD)	Area (sample)	Estimated adduct level (mol)	Estimated adduct level (fmol/mg SA)
(*±)-anti-His-Pro*	1.4	391000000	22604	1.35E-17	0.084
(*±)-anti-Lys*	1.4	39631500	10467	6.16E-17	0.372

4. Discussion

4.1 LC/MS/MS analysis of BPDE adducts

An LC/MS/MS method for quantification of BPDE-alkylated SA was improved regarding both chromatography and MS sensitivity to decrease the detection limits.

4.1.1 Triple-quadrupole mass spectrometry

Enzymatic hydrolysis of BPDE-SA adducts resulted in two major target compounds, BPDE-His-Pro and BPDE-Lys, monitored by LC/ESI-triple-quadrupole MS (TQ-MS). In the previous study by J. Zurita et al. (2018), LC/HRMS was employed for characterization purposes and quantification of BPDE adducts from B(a)P exposed mice- and human samples. As presented in **Appendix 3: Additional chromatograms; Figure 19,** the generated adduct levels from digested SA are given in pmol per mg of SA. Considering the lower adduct levels measured with LC/HRMS by J. Zurita et al. (2018), it might be a bit confusing why a low-resolution MS instrument was used in the present study, but the advantages using TQ-MS instruments are well-known, namely for highly reproducible quantification of trace levels. The profits of a higher selectivity in MRM mode usually makes TQ-MS detectors favorable compared to HRMS instruments, which is explained by the duty cycle, i.e. a fraction of time when each ion being scanned in the quadrupole, which is approximately 100% for TQ-MS instruments. For the BPDE adducts in this study, however, the high mass accuracy provided by the Orbitrap instrumentation was found to be more important for the selectivity and the signal-to-noise (S/N) than the duty cycle of TQ-MS in MRM mode.

4.1.2 Optimization of MS sensitivity

The sensitivity during ESI-MS analysis is crucial for qualitative- and quantitative measurements of target compounds. Identification of important factors and optimization is usually carried out in the beginning of a new research project. The DoE approach in this study was proven applicable since it resulted in an improved linearity of the LC/MS/MS method. The optimization of MS sensitivity was based on the response of benzyl-L-His (IS) because standard BPDE alkylated SA requires a comprehensive synthetic effort, and such synthetic standards are not commercially available. An

exception would be if BPDE adducts to SA are generated through an *in vitro* metabolism of B(a)P as reference standards.

Since ESI-MS analysis is very prone to ion suppression, there is a major risk to underestimate BPDE adduct levels during quantification. These matrix effects can be compensated for by use of isotopically labeled IS, however expensive and/or hard to obtain. With these appropriate IS, it would be possible to estimate matrix effects and further optimize ESI parameters, as well as to account for enzymatic variability during digestion.

4.1.3 Chromatographic separation of adducts

A pentaflurophenyl (F5) column was used in accordance with a previous study by J. Zurita et al. (2018), since previous research clearly established improved separation efficiency for digested BPDE alkylated SA compared to conventional RP-LC with C_{18} as a stationary phase, which was utilized in a study by E. Westberg et al. (2014). An improved chromatographic separation will aid in compound characterization and identification as well as a possibility to reduce matrix interferences in the ion source. A specific feature that characterizes F5 columns is the ability to separate both non-polar and polar compounds (mixed mode) from the same sample matrix. Using the F5 column for separation of BPDE adducts resulted in Gaussian shaped peaks for Benzyl-L-His (IS), (+)-anti-BPDE-His-Pro and (±)-anti-BPDE-Lys, see **Appendix 3: Additional chromatograms Figure 19.** A little bit worse peak shape with tailing was observed for (-)-anti-BPDE-His-Pro. More specifically, the overall separation efficiency of adducts is related to a mix between van der Waals and π-π interactions between the stationary phase, see **Figure 15**, and the aromatic BPDE structure (J. Zurita et al. 2018).

In the present study, detection of neither (±)-syn-BPDE-His-Pro or (±)-syn-BPDE-Lys was accomplished, probably because of low resolution in the TQ-MS system. Diastereoisomers of (±)-anti-BPDE-Lys were not possible to distinguish with the developed method, which most likely was related to sensitivity issues. Considering these results, the developed chromatographic method was still acceptable since enough separation was achieved between (+)-anti- and (-)-anti-BPDE-His-Pro. Measurements of (+)-anti-BPDE-His-Pro are important since the precursor (+)-anti-BPDE is regarded as the most potent metabolite generated upon exposure.

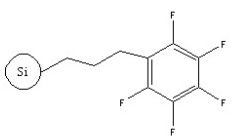


Figure 15: Illustration of stationary phase for the F5 column utilized for separation of BPDE adducts

4.1.4 DTT treatment of human SA

According to an article by Sabbioni et al. (2016), treatment by DTT possesses both advantages and disadvantages considering a complete hydrolysis of proteins. In this

work, a clear disadvantage was found related to quantification of bulky adducts within the protein, since the levels of BPDE adducts were lower after DTT treatment. One reason to this could be related to sensitivity issues at high temperatures, since BPDE adducts are known as thermolabile compounds (Sabbioni et al. 2016). The DTT treatment includes a denaturation of the protein at 56°C for 60 min which may destroy the BPDE adduct. Another possibility could be the DTT molecule which possess a strong reducing capacity, and therefore a risk to reduce bulky adducts attached to SA.

4.2 Unmodified human SA

4.2.1 Development of HILIC separation

In general, a HILIC separation usually enables reliable analysis of underivatized polar compounds compared to conventional RP-LC techniques. HILIC chromatography is also very difficult to run in a reproducible way with gradient elution, wherefore it is recommended to perform the analysis in isocratic mode to ensure stable conditions. The separation efficiency of studied AAs was found to be inappropriate considering important chromatographic parameters, presented in **Appendix 3: Additional chromatograms; Table 22.** Additional investigations with regards to the chromatographic separation of underivatized AAs are required to obtain a better resolution and selectivity. A lower content of H_2O would be worthwhile to test, since the present study utilized a very high percentage of H_2O during isocratic elution. Also, the pH of utilized buffer solution should be further investigated since the pH strongly influences the selectivity. In a previous study by J. Gao et al. (2016), a system based on HILIC chromatography was developed using 8mM NH_4HCO_3 with 0.12% FA, with optimal peak shapes at a pH of 3 and a relatively short analysis time of 11 min.

4.2.2 Digestion efficiency using DTT

Improvement of the enzymatic digestion was achieved after DTT treatment aiming to reduce disulfide bridges within the structure of human SA (unmodified), followed by IAA alkylation of sulfhydryl groups to prevent regeneration of disulfide bridges. These results were in accordance with a previous study by C.R Weisbrod et al. (2012), where internal disulfide bridges of bovine SA were reduced prior to enzymatic cleavage by trypsin. According to Sabbioni et al. (2016), pretreatment by means of DTT and IAA are commonly employed to facilitate the enzymatic hydrolysis for either trypsin or other common proteases.

Missed cleavages are a common issue dealing with enzymatic hydrolysis due to an incomplete treatment of the protein, or steric hindrance within the structure. A non-specific enzyme, e.g. pronase, is expected to digest a protein into single AAs, but due to the tertiary structure of SA, a varying degree of hydrophobic pockets may protect certain AAs or attached moieties, assuming the pretreatment is inefficient (H. Motwani et al. 2016; G. Sabbioni et al. 2016).

4.3 *In vitro* metabolism of B(a)P

An *in vitro* metabolic system for B(a)P was developed, see **section 2.2.18 In-vitro alkylation of SA**, aiming to generate BPDE adducts to SA as reference standards. This experiment was a pilot study since there are to our knowledge no previous similar research studies published to yield BPDE alkylated SA with B[a]P as the precursor. No BPDE-SA adducts were detected by the optimized LC/MS/MS method due to sensitivity issues. A systematic optimization would possibly solve the problem, for example by conducting an experimental design to optimize the ratios of B(a)P in relation to rat liver S9 and human SA. The number of generated adducts within 10mg of human SA were probably not enough to be detected by LC/MS/MS in MRM mode, but an indication was observed using LC/HRMS in PRM mode, i.e. an observation which needs further investigation. A developed approach to detect BPDE adducts to SA from *in vitro* metabolism of B(a)P would facilitate a comparison between species of metabolic rates related to B(a)P, as well as the transformation to electrophilic metabolites.

4.4 Analysis of BPDE-SA from B(a)P exposed mice

In the LC/HRMS PRM chromatograms from B(a)P-exposed mice (after 3 days of administration following exposure), see **Appendix 7: LC-HRMS PRM chromatograms**; **Figure 31,** there were two main peaks with a relatively low MS response, corresponding to extracts of 1mg SA from mice samples. According to a previous study by E. Westberg et al. (2014), it was recommended to prepare 10mg of BPDE alkylated SA for the LC/MS/MS analysis, since very low adduct levels are expected from *in vivo* samples. In addition, J. Zurita et al. (2018) prepared extracts of 10mg mice SA which were subjected to enzymatic hydrolysis followed by SPE enrichment and then subsequent analysis by LC/HRMS. Even though the detection limits of (+)-anti-BPDE-His-Pro and (-)-anti-BPDE-His-Pro were improved in comparison to the study by E. Westberg et al. (2014), it is reasonable to conclude that extracts of 1mg mice SA are insufficient to measure by LC/MS/MS with TQ-MS in MRM mode. Based on the analysis performed by means of LC/HRMS, illustrated in **Appendix 7: LC-HRMS PRM chromatograms; Figure 31,** it seems reasonable to say that a high mass accuracy is crucial for detection of BPDE adducts in trace levels.

5. Conclusions

An analytical method including sample preparation and LC/MS/MS detection was developed and evaluated for detection of BPDE adduct levels in SA and partially validated in agreement with the ICH guidelines. The present project should be considered as a proof-of-principle illustrating the power of experimental design to improve the ionization efficiency of a model compound, i.e. benzyl-L-His (IS), and eventually also BPDE-His-Pro and BPDE-Lys by use of a TQ-MS instrument. The obtained LODs in this project was improved in comparison to a previously developed HPLC/MS/MS method employing low-resolution MS, indicating that the present

methodology would be applicable for measurements of *in vivo* exposed mice, and eventually also humans by using LC/HRMS.

The following main milestones were achieved during the development of the LC/MS/MS method, namely:

- Improvement of ESI sensitivity by conducting a DoE approach through a multivariate evaluation of important parameters influencing the ESI conditions
- A suitable chromatographic separation of (+)-anti-BPDE-His-Pro, (-)-anti-BPDE-His-Pro and (±)-anti-BPDE-Lys by employing the F5 column
- Optimization of the LC conditions to reduce the analysis time
- DTT treatment of BPDE alkylated SA was found to be unsuitable
- Pretreatment with DTT of unmodified SA facilitated the enzymatic digestion
- Development of an accurate LC/MS/MS method by considering precision and accuracy of the studied isomers of BPDE-SA adducts
- Improved linearity of (-)-anti-BPDE-His-Pro and (±)-anti-BPDE-Lys by utilization of a volumetric IS to correct for instrumental variability
- Generation of BPDE adducts to SA from an *in vitro* metabolic system of B(a)P was not successful, most likely due to non-optimal ratios between B(a)P, S9 and human SA.
- BPDE adducts levels in SA from *in vivo* exposed mice, sampled after 3 days of administration after exposure, were below LOD in the TQ-MS system, but were detectable by LC/HRMS

6. Perspectives

6.1 Research perspectives

Previous studies in the field of adductomics, e.g. Westberg et al. (2014), focused on the development of experimental conditions to improve the detection limits. This study was partly a continuation based on a previous work by Zurita et al. (2018), where a selective LC/HRMS methodology was developed for characterization of SA modifications and quantification of *in vivo* exposed mice and humans. LODs at the attomole level were achieved for the investigated adducts, but the quantification of *in vivo* samples was not as expected, most likely since no IS was used during the analysis to adjust for either enzymatic- or instrumental variabilities. There is a huge demand among researchers regarding the supply of suitable analytical instrumentation aiming to identify and quantify adduct formation from *in vivo* exposed-mice or humans since these results can be used to assess the exposure levels. In the present study, the main scientific aim was to develop a methodology based on detection by means of a TQ-MS instrument applicable for measurements of *in vivo* samples. Improvements by using an IS was accomplished to adjust for instrumental variability in the response, resulting in a reliable quantification of BPDE adduct levels. Considering some implications for future work, there are still crucial things to investigate in the field, namely:

- To conduct a systematic optimization of the LC/MS/MS analysis for the target compounds, i.e. BPDE-His-Pro and BPDE-Lys, i.e. a DoE approach.
- Further evaluation of an appropriate IS to spike before enzymatic hydrolysis would be a possible improvement to adjust for variability during digestion. A suitable IS to employ would be a different PAHDE alkylated to SA compared to the one under study, as described by E. Westberg et al. (2014).
- Utilization of a column-switch set-up which can facilitate injection of larger sample amounts should be considered.
- Evaluation of the enzymatic efficiency, for example by means of a Lineweaver-Burk plot to understand whether the adduct levels may be underestimated or not
- Reduce the number of interferences by improving SA isolation through using trichloroacetic acid (TCA)/acetone during precipitation agent as described by Chen et al. (2005).
- Evaluation of SPE enrichment by choosing a more selective stationary phase, e.g. a weak-cation exchanger (WCX) to obtain lower LODs.
- Further development of the *in vitro* metabolic system by performing an experimental design based on the major parameters, i.e. the concentration of B(a)P and rat liver S9, as well as the amount of SA.

6.2 Ethical perspectives

The present work was performed based on the 3R-principles, i.e. replacement, reduction and refinement, which are considered as a foundation when dealing with animal experiments. A metabolic *in vitro* system to follow the transformation of B(a)P in presence of commercial human SA was evaluated in this work. Analysis of precipitated SA from B(a)P treated mice (3- and 14 days after exposure), obtained from the National Hellenic Research Foundation in Athens, was possible to perform due to a previous approval from the ethical committee at this institute (Ref: Prof. M. Törnqvist, ACES department, Stockholm University). Considering the 3R-principles related to future work, further optimization of the B(a)P, S9 and SA concentrations would facilitate the reduction of experimental animals. Replacement of *in vivo* samples can be achieved through a combination of *in vitro* studies and *in silico* simulations.

6.3 Societal perspectives

From a societal point of view, the development of highly sensitive and selective MS methods for quantification of stable adducts to SA caused by ubiquitous environmental pollutants is considered as a crucial part in toxicological research. If the SA adduct levels from reactive electrophiles could be properly quantified, the levels can be used to extrapolate the *in vivo* dose related to the environmental exposure. This will aid in further risk assessment of cancer-causing agents, which eventually could be a part of future guidelines and decisions how the general population can minimize exposure to

such compounds. An appropriate decision regarding a potential restriction, from either an authority or a political division, must always be preceded by scientific empirical data which clearly states the consequences related to a long-term exposure, e.g. from environmental pollutants.

7. Acknowledgements

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8. Appendix

Appendix 1: Calibration data

Evaluation of the LC/MS-MS method is described in **section 2.2.11** Method evaluation of AAs, and the results are summarized in **section 3.3.2.2** Method evaluation for AAs. Histidine-d_3 was utilized as internal standard, with MRM transition 159 → 113. Every calibration point is a mean of relative peak area from 2 injections.

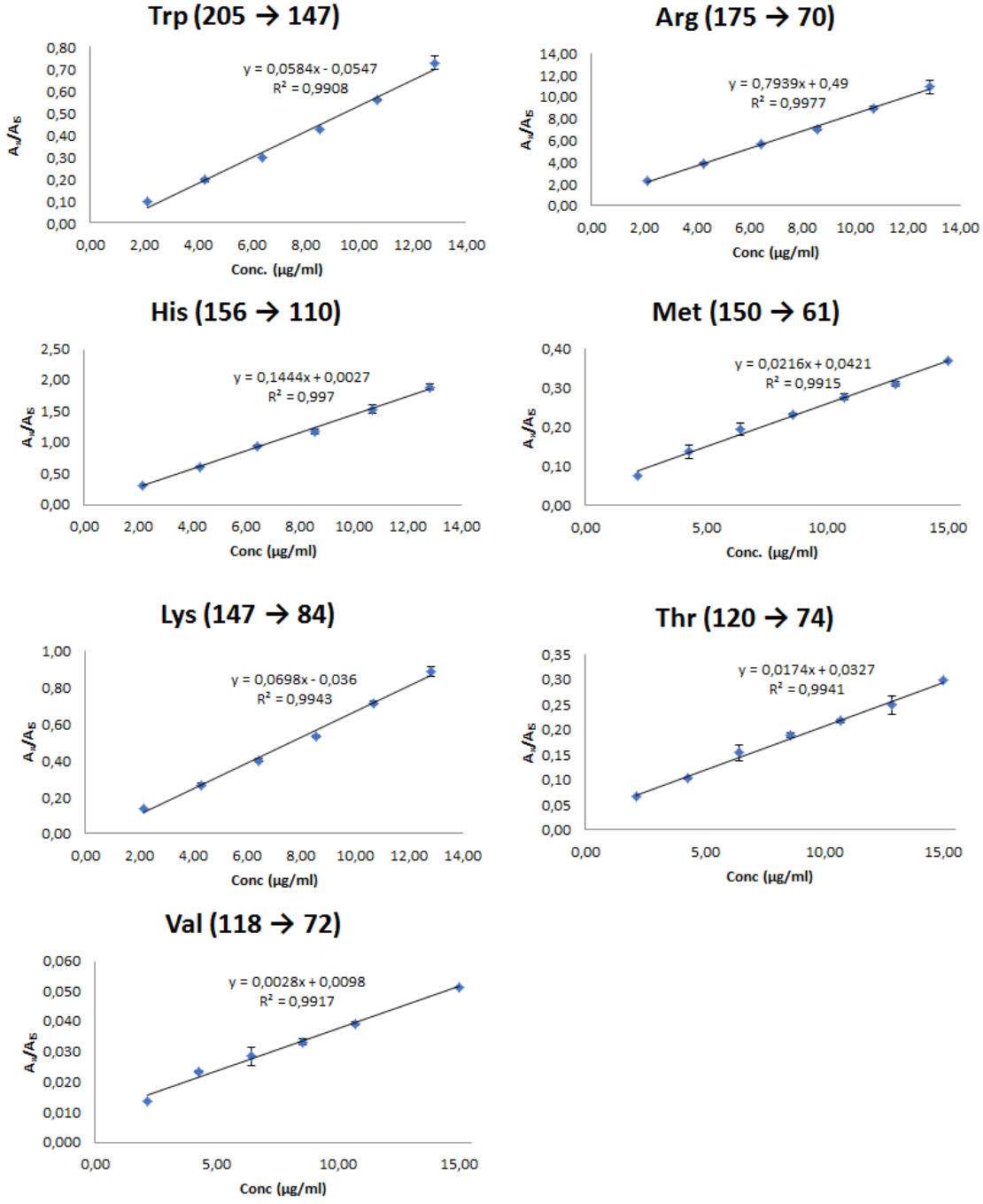

Figure 16: Calibration curves of investigated amino acids ranging between 2.5 to 15µg/ml. The response of each amino acid has been normalized towards Histidine-d_3 (IS). Measurements of 6-7 calibration samples in duplicate analysis (n=2).

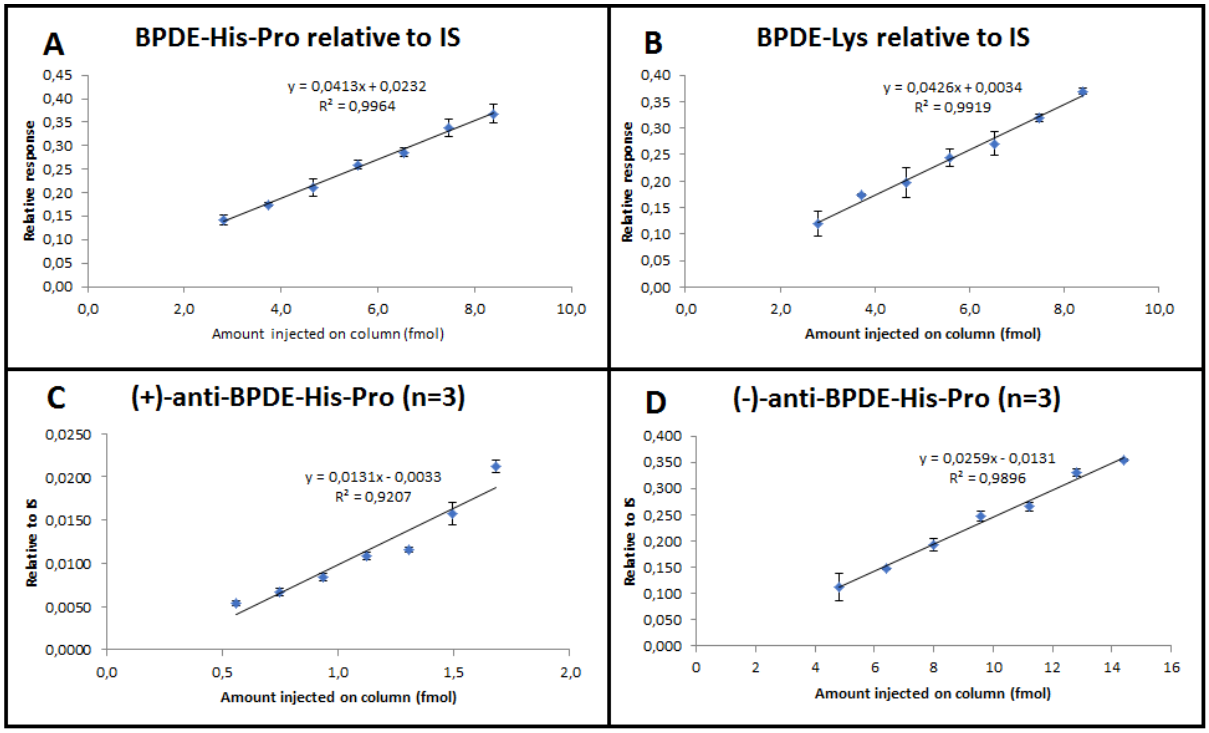

Figure 17: Regression curves for (A): (±)-anti-BPDE-His-Pro, (B): (±)-anti-BPDE-Lys, (C): (+)-anti-cis/trans-BPDE-His-Pro and (D): (-)-anti-cis/trans-BPDE-His-Pro. All target compounds have been normalized to the utilized IS, spiked prior to injection at a concentration of 0.05µg/ml. The number of replicates were 3 for all calibration standards.

Appendix 2: Experimental protocols

Table 16: *In vitro* metabolism of B(a)P followed by subsequent alkylation to human SA. The protocol was developed based on a previous study using calf-thymus DNA for trapping of reactive metabolites by C. Högkvist (2018).

In vitro metabolism of B(a)P + S9 + human SA		
Compound	**50µl S9 fraction**	**Control (without B(a)P)**
S9-fraction from rat (µl)	50	50
NADPH (µl)	462 (400+62)	462 (400+62)
100mg/ml hSA (µl)	100	100
PBS (µl	128	128
Tot. Vol (µl)	740	740
Pre-incubation for 3 minutes at 37°C		
B[a]P, 37,5mM	10	(10µl DMSO)
Conc. B[a]P (mM)	0,5	-
Tot. Vol (µl)	750	750
Incubation for 1 hour at 37°C (pH 7,5)		
Cold HCl (µl)	140	140
Tot. Vol (µl)	890	890
Protein precipitation for 20 minutes		
NH_4SO_4 (µl)	1000 (200x5)	1000 (200x5)
Tot. Vol (µl)	1890	1880
Wash the precipitate		
MeOH	1500	1500
EtAC	1500	1500
Pentane	1500	1500
Enzymatic hydrolysis of 10mg BPDE-SA for 20h		

Table 17: DTT pretreatment of 1mg BPDE-SA followed by IAA alkylation of free cysteine residues. The experimental protocol is based on a previous study by C. R. Weisbrod et al. 2012. The protocol used for 10mg human SA is simply obtained by multiplication of 10 for each reagent.

Steps	Compound	µl of hSA	Reagent (µl)	µl of AMBIC	TOT. Vol (µl)
1	DTT (324mM)	100	15.4	84.6	200
Degradation of disulfide bridges in hSA at 56°C for 60 minutes					
2	IAA (500mM)	-	30.0	70.0	300
Alkylation of free cysteine residues for 45 minutes (20°C)					
3	DTT (324mM)	-	15.4	84.6	400
Quench the remaining IAA reagent during 15 minutes under light					

Precipitation experiments of human SA by ammonium sulphate (NH_4SO_4)

- Prepare a saturated solution of ammonium sulphate in a 50ml Falcon tube
- In 3 different Eppendorf test tubes (2mL), add 100µl of 100mg/mL hSA solution respectively.

Table 18: Protein precipitation of human SA by means of NH_4SO_4, without rat liver S9 fraction

Sample	hSA (µl)	PBS (µl)	HCl (µl)	NH4SO4 (µl)	TOT. Vol (ml)
Procedural blank	-	750	140	1000	1890
1 (1mg hSA)	10	740	140	1000	1890
2 (10mg hSA)	100	650	140	1000	1890

Table 19: Protein precipitation of human SA by means of NH_4SO_4, including rat liver S9 fraction

Sample	hSA (µl)	S9 (µl)	PBS (µl)	HCl (µl)	NH4SO4 (µl)	TOT. Vol (ml)
Procedural blank	-	50	700	140	1000	1890
1 (1mg hSA) + S9	10	50	790	140	1000	1890
2 (10mg hSA) + S9	100	50	600	140	1000	1890

1. *Incubate the mixtures for 75 minutes at 37°C.*
2. *Add 140µl of 1M HCl (50µl at a time) until pH 3*
3. *Finally, add 1mL of saturated ammonium sulphate (ICECOLD) solution to precipitate the protein for 20 minutes. N.B! Important to add the precipitating agent slowly, or stepwise around 200µl at a time.*
4. *Centrifuge at 4500g for 10 minutes at 4°C.*
5. *The precipitate is washed according to the following scheme:*
6. *N.b! Remember to centrifuge the samples after addition of each solvent. This is performed at 1600g and 4°C for 10 minutes.*
    a. *1.5mL of MeOH*
    b. *1.5mL of EtAC*
    c. *1.5mL of pentane*
7. *Let the precipitate dry overnight (NOT WITH N_2)*
8. *Measure the weight of your test tubes and subtract the first weight of the Eppendorf.*
9. *Reconstitute in 1mL of AMBIC solution.*
10. *Detect the serum albumin by spectrophotometry at 280nm*

Precipitation experiments of human SA by MeOH or Acetone

(N.b! The procedure described for MeOH is equivalent for acetone)

- Prepare human SA solution by weighing 100mg SA in 1mL of PO_4 buffer
- Prepare MeOH solution in a 50ml Falcon tube
- Measure the weight of your Eppendorf test tubes
- In 3 different Eppendorf test tubes (2mL), add 10, 50 and 100µl of 100mg/mL hSA solution respectively.

Table 20: Protein precipitation of human SA by means of MeOH, without S9 fraction

Sample	hSA (µl)	PBS (µl)	MeOH (µl)	TOT. Vol (ml)
Procedural blank	-	1135	500	1.635
1 (1mg hSA)	10	1125	500	1.635
2 (10mg hSA)	100	1035	500	1.635

Table 21: Protein precipitation of human SA by means of MeOH, including rat liver S9 fraction

Sample	hSA (µl)	S9 (µl)	PBS (µl)	MeOH (µl)	TOT. Vol (ml)
Procedural blank	-	100	1035	500	1.635
1 (1mg hSA)	10	100	1025	500	1.635
2 (10mg hSA)	100	100	935	500	1.635

- *Incubate the mixtures for 75 minutes at 37°C.*
- *Add 500µl of icecold MeOH (50µl at a time)*
- *Keep the mixtures on ICE!!!!!*
- *The precipitate is the washed according to the following scheme:*
- *N.b! Remember to centrifuge the samples after addition of each solvent. This is performed at 4540g for 10 minutes.*
    1. *1.5mL of MeOH*
    2. *1.5mL of EtAC*
    3. *1.5mL of pentane*
- *Evaporation of pentane until dryness overnight (NOT WITH N_2)*
- *Reconstitute in 1mL of AMBIC solution (50mM)*
- *Detect the serum albumin by spectrophotometry at 280nm*

Appendix 3: Additional chromatograms

(A) LC-MRM chromatograms of standard amino acid mixture in deionized water prepared as described in section **2.2.8** Methods concerning analysis of amino acids (unmodified SA) . The results on separation efficiency are summarized in section **3.3.2.1** Separation efficiency of AAs**,** which was achieved after testing various chromatographic conditions (data not shown).

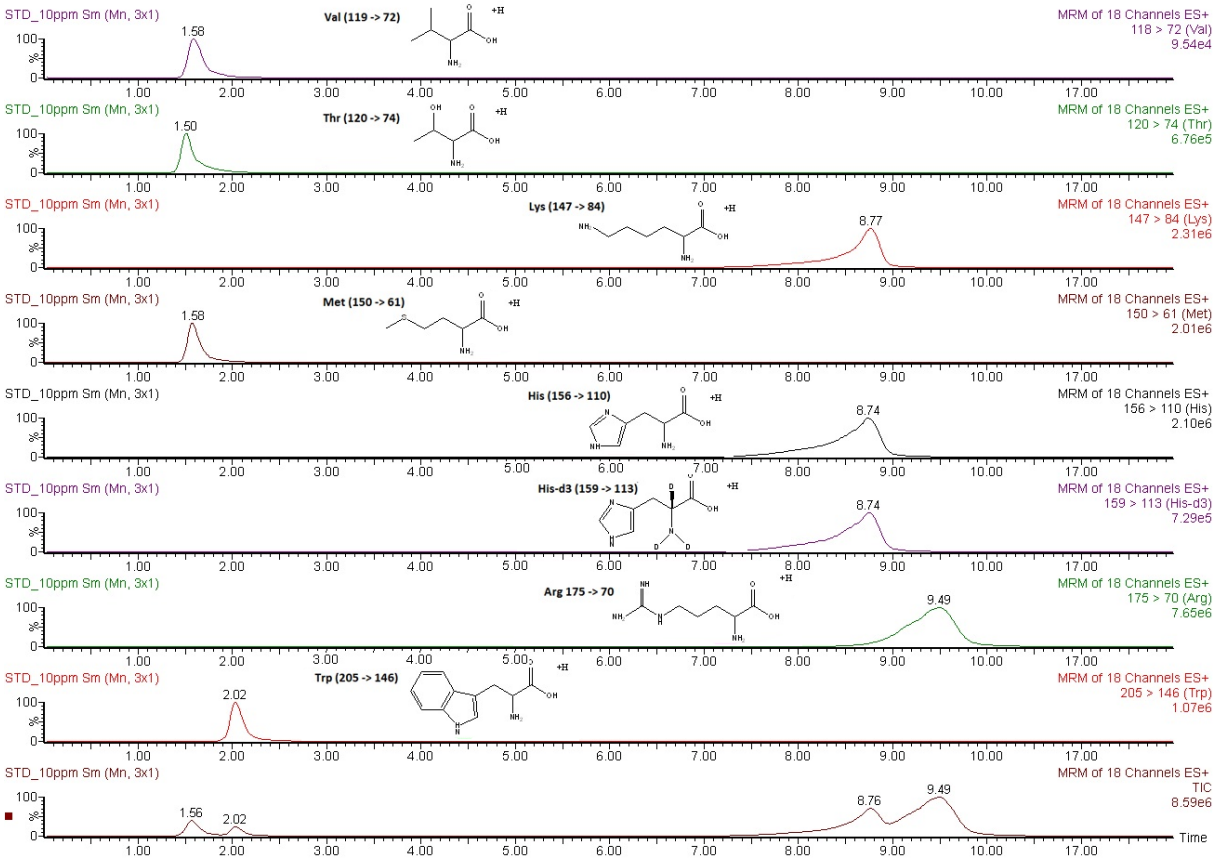

Figure 18: MRM chromatogram illustrating the separation efficiency for 7 amino acids in a standard mixture at a concentration of 10ppm of each amino acid.

Table 22: Evaluation of the separation efficiency regarding the developed HILIC-ESI-MS/MS method performed in isocratic mode at a pH of 5.75. The dead time, t0 is 1.21 minutes.

	Ret. Time			Peak width			
Amino acid	**(min)**	**k**	**α**	**(min)**	**$W_{1/2}$**	**# of plates (N)**	**Res. (R_s)**
L-Thr	1.53	0.26	1.31	0.35	0.138	681.0	0.3
L-Met	1.63	0.35	1.17	0.4	0.122	988.9	0.3
L-Val	1.7	0.40	2.00	0.4	0.168	567.3	0.9
L-Trp	2.19	0.81	3.60	0.5	0.138	1395.2	3.0
L-His	4.74	2.92	1.01	1.1	0.506	486.1	0.1
His-d3	4.79	2.96	1.01	1.1	0.522	466.5	0.0
L-Lys	4.83	2.99	1.11	0.9	0.368	954.4	0.6
L-Arg	5.23	3.32	-	1.05	0.644	365.4	-

(B) HPLC/MS-MS chromatograms illustrating 1mg digested BPDE-SA after enrichment by SPE, as described in **section 2.2.6** Enzymatic hydrolysis of a reference BPDE-alkylated SA and **2.2.7** Enrichment of BPDE alkylated human SA The injection corresponds to 1mg SA containing (A) 0.28pmol/mg SA of (+)-anti-BPDE-His-Pro, 2.4pmol/mg SA of (-)-anti-BPDE-His-Pro and (B) 1.4pmol/mg SA of (±)-anti-BPDE-Lys (Motwani et al. 2016).

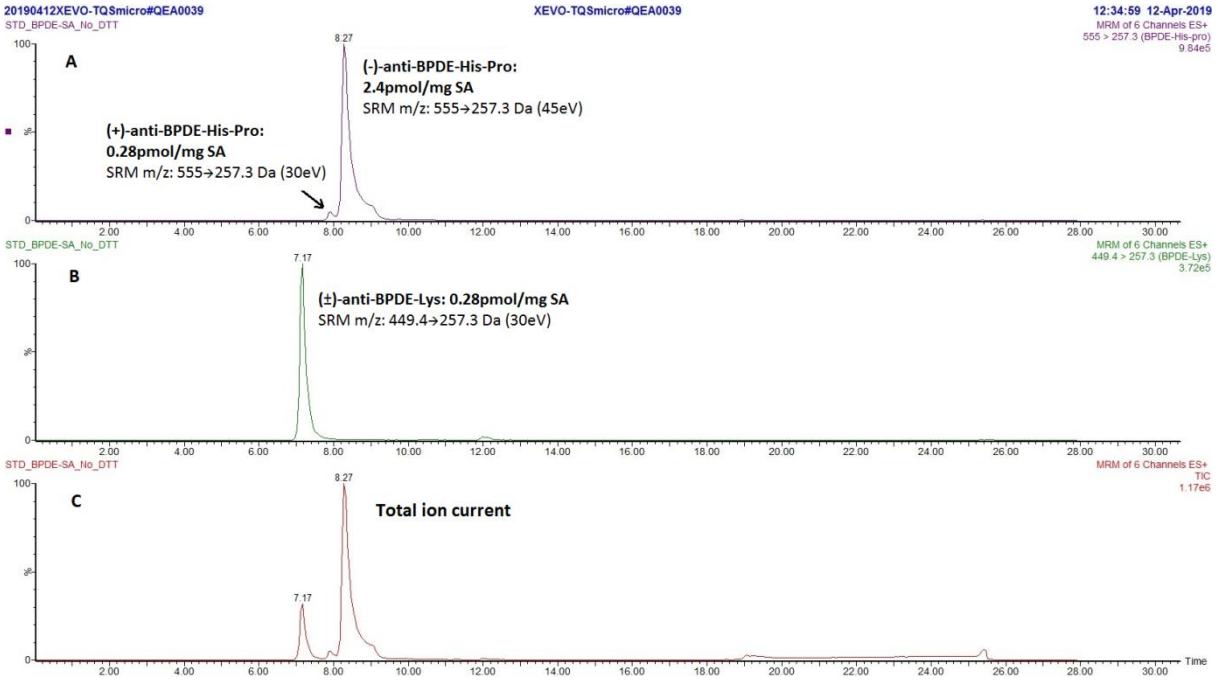

Figure 19: Analysis of 1mg digested BPDE-SA by LC/MS-MS. The standards were previously alkylated *in vitro* to generate BPDE-SA as reference standards (Motwani et al. 2016). Trace A illustrates a separation between isomeric compounds, (+)-anti- and (-)-anti-BPDE-His-Pro, and trace B illustrates (±)-anti-BPDE-Lys as a single peak.

Appendix 4: Mass spectrum

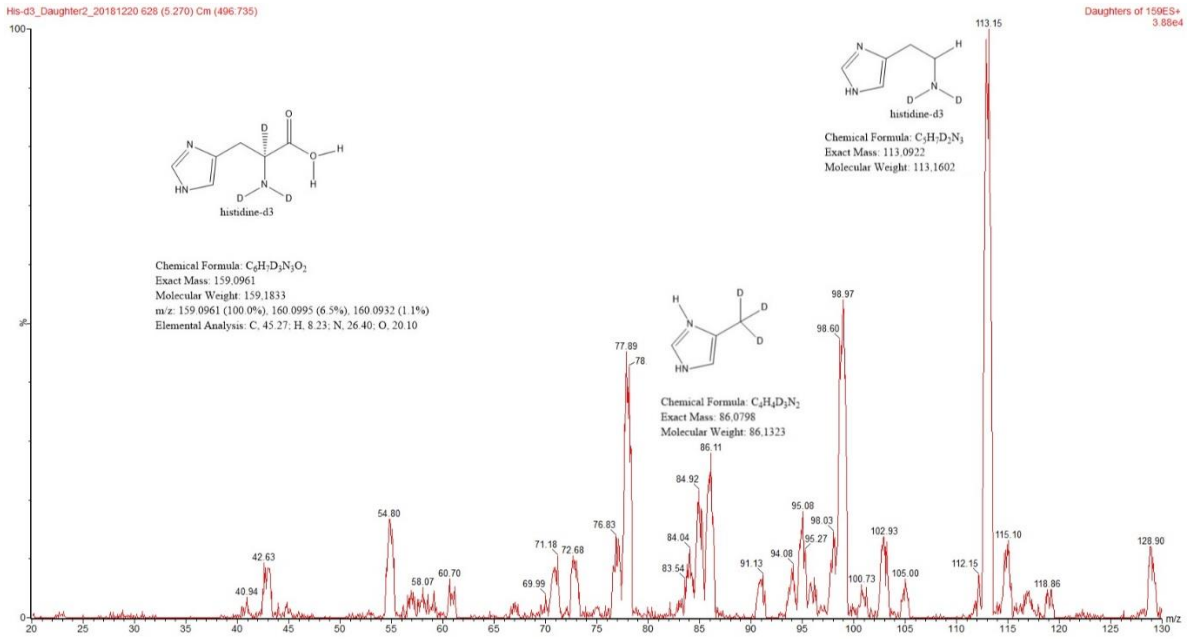

Figure 20: Representative mass spectrum of Histidine-d3 and its fragmentation pattern acquired in continuum mode. The spectrum is intended to illustrate the fragmentation pattern of amino acids, i.e. a neutral loss of H_2O followed by a loss of CO.

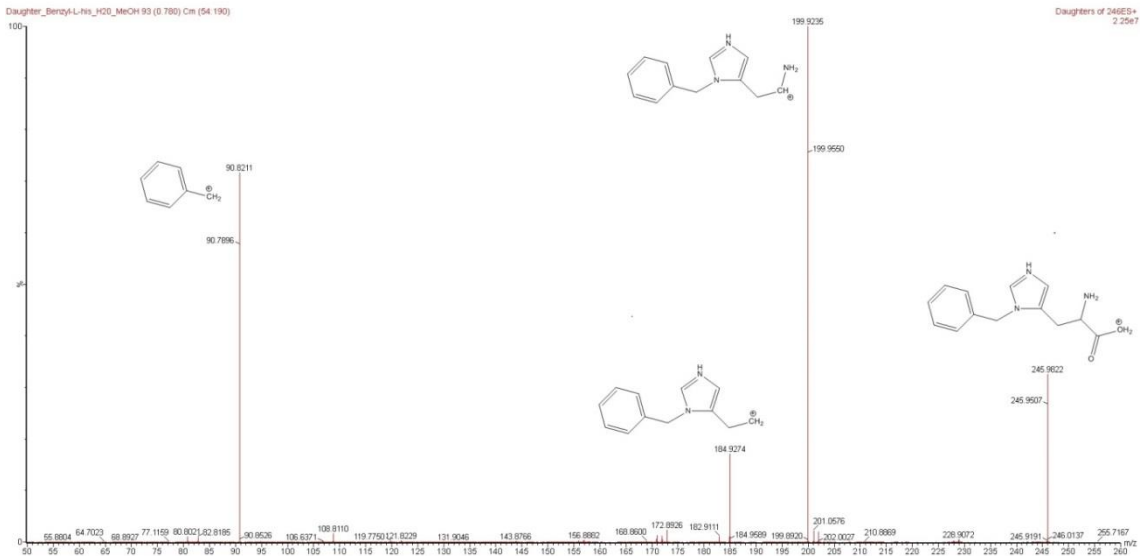

Figure 21: Fragmentation pattern for Benzyl-L-Histidine utilized as an internal standard. A daughter ion scan of m/z 246Da with a collisional energy of 15eV was performed to generate the spectrum.

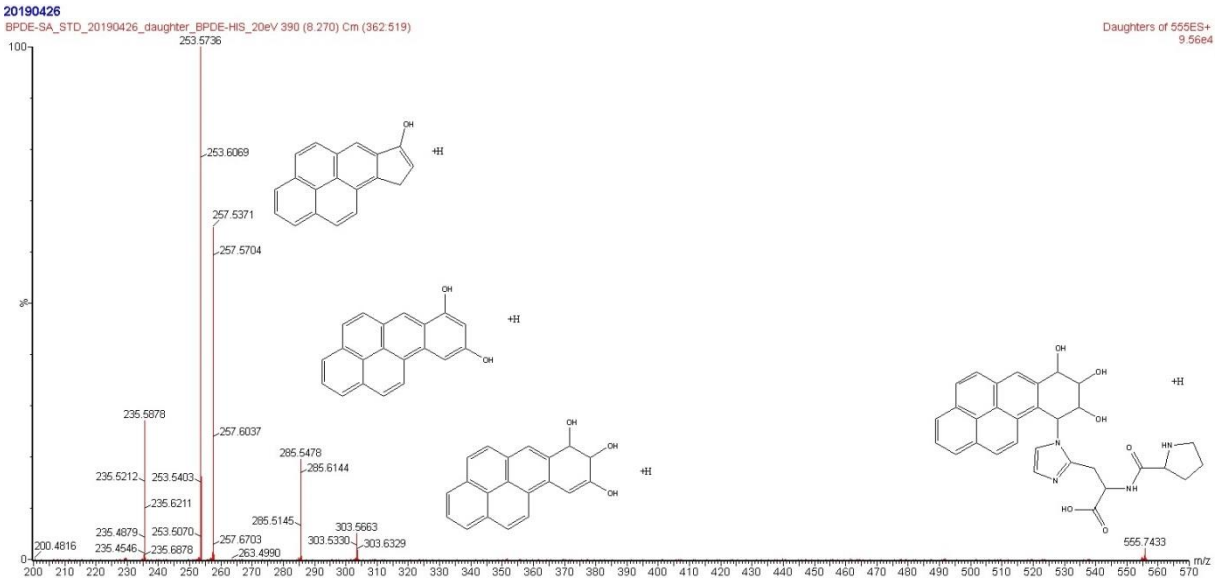

Figure 22: Fragmentation pattern of BPDE-His-Pro obtained through a daughter ion scan of m/z 555 with a collisional energy of 20eV.

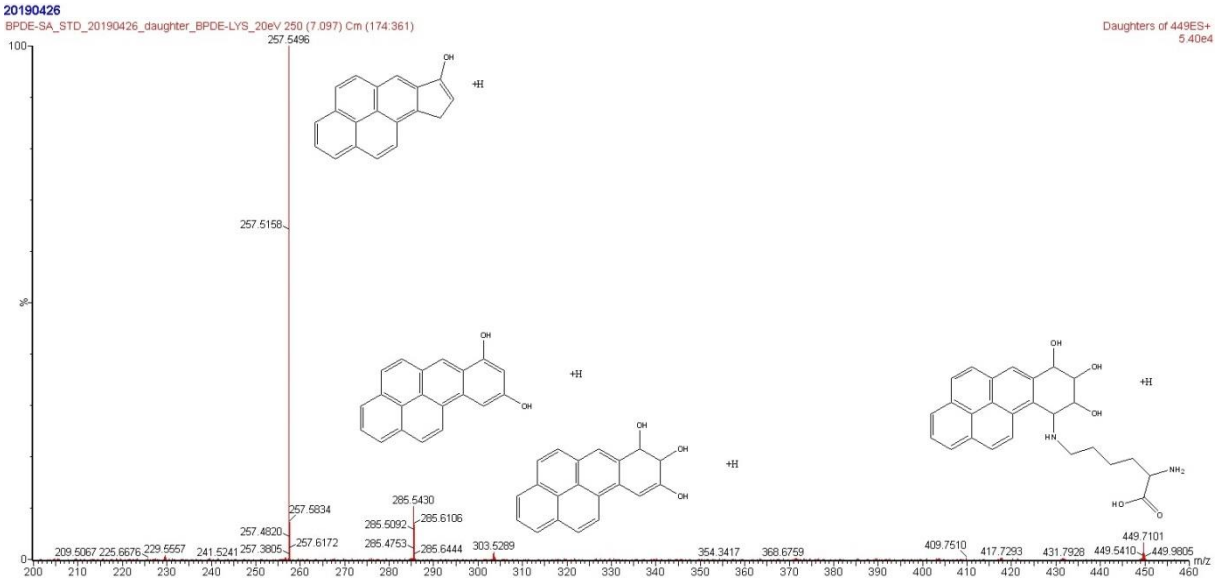

Figure 23: Fragmentation pattern of BPDE-Lys obtained through a daughter ion scan of m/z 449 with a collisional energy of 20eV.

Appendix 5: Experimental design

Table 23: Experimental matrix illustrating a Placket-Burman design including 3 center point values, i.e -1, 0 and +1. Quantitative variables from x_1 to x_8, where x_1: dwell time in seconds, x_2: source temperature, x_3: cone voltage, x_4: capillary voltage, x_5: desolvation temperature, x_6: cone gas flow, x_7: desolvation gas flow and x_8: LC flow rate.

	Quantitative factors							
	x1	x2	x3	x4	x5	x6	x7	x8
1	2	120	50	2	200	10	800	200
2	2	150	10	5	200	10	300	200
3	0.1	150	50	2	600	10	300	100
4	2	120	50	5	200	100	300	100
5	2	150	10	5	600	10	800	100
6	2	150	50	2	600	100	300	200
7	0.1	150	50	5	200	100	800	100
8	0.1	120	50	5	600	10	800	200
9	0.1	120	10	5	600	100	300	200
10	2	120	10	2	600	100	800	100
11	0.1	150	10	2	200	100	800	200
12	0.1	120	10	2	200	10	300	100
13	1.05	135	30	3,5	400	55	550	150
14	1.05	135	30	3,5	400	55	550	150
15	1.05	135	30	3,5	400	55	550	150

Table 24: Experimental plan illustrating a central composite factorial design (CCF) based on the 3 most influential factors (x_3, x_5 and x_8) selected from the Plackett-Burman design. The quantitative variables marked in bold are varied between their minimum, center- and maximum values through 17 experiments including 3 center points.

Quantitative factors								
Exp.	x1	x2	**x3**	x4	**x5**	x6	x7	**x8**
1	0.1	150	**10**	2	**200**	10	300	**100**
2	0.1	150	**50**	2	**200**	10	300	**100**
3	0.1	150	**10**	2	**600**	10	300	**100**
4	0.1	150	**50**	2	**600**	10	300	**100**
5	0.1	150	**10**	2	**200**	10	300	**200**
6	0.1	150	**50**	2	**200**	10	300	**200**
7	0.1	150	**10**	2	**600**	10	300	**200**
8	0.1	150	**50**	2	**600**	10	300	**200**
9	0.1	150	**10**	2	**400**	10	300	**150**
10	0.1	150	**50**	2	**400**	10	300	**150**
11	0.1	150	**30**	2	**200**	10	300	**150**
12	0.1	150	**30**	2	**600**	10	300	**150**
13	0.1	150	**30**	2	**400**	10	300	**100**
14	0.1	150	**30**	2	**400**	10	300	**200**
15	0.1	150	**30**	2	**400**	10	300	**150**
16	0.1	150	**30**	2	**400**	10	300	**150**
17	0.1	150	**30**	2	**400**	10	300	**150**

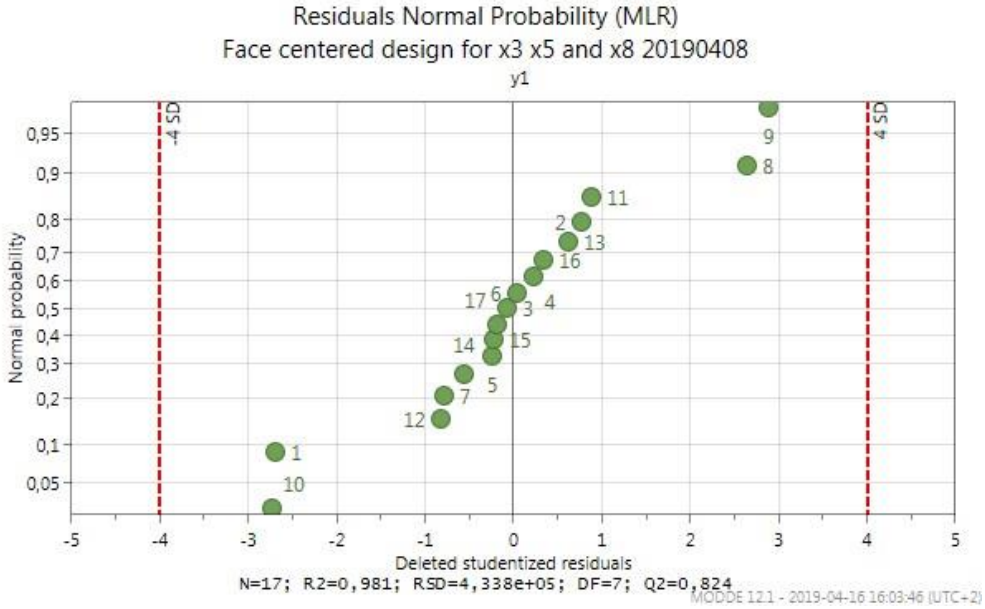

Figure 24: Assessment of normality based on the residuals for the estimated CCF model. Both the horizontal and vertical axis are transformed into logarithmic values to obtain a normally distributed set of data

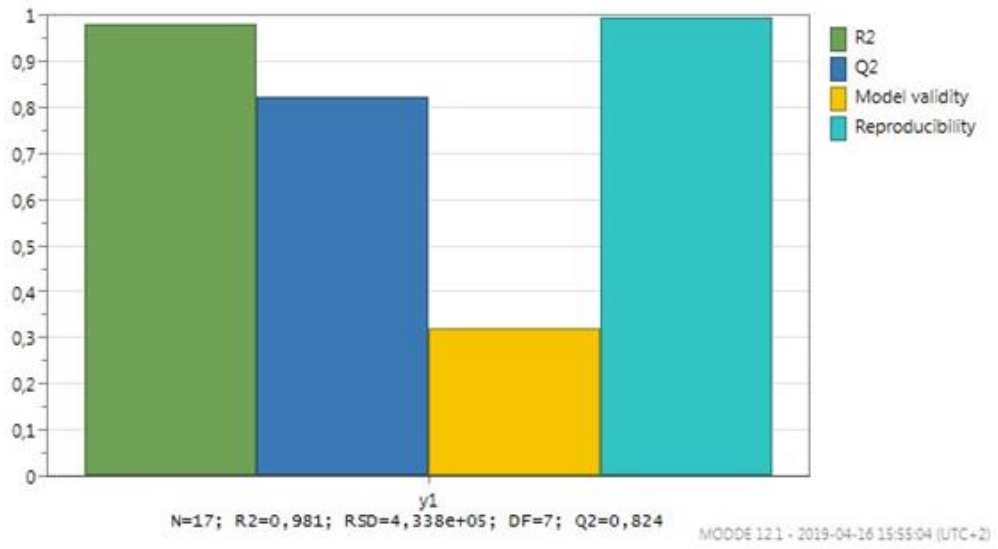

Figure 25: Evaluation of the estimated model by means of MLR in terms of R2, Q2, model validity and reproducibility.

Appendix 6: Optimization of MS sensitivity

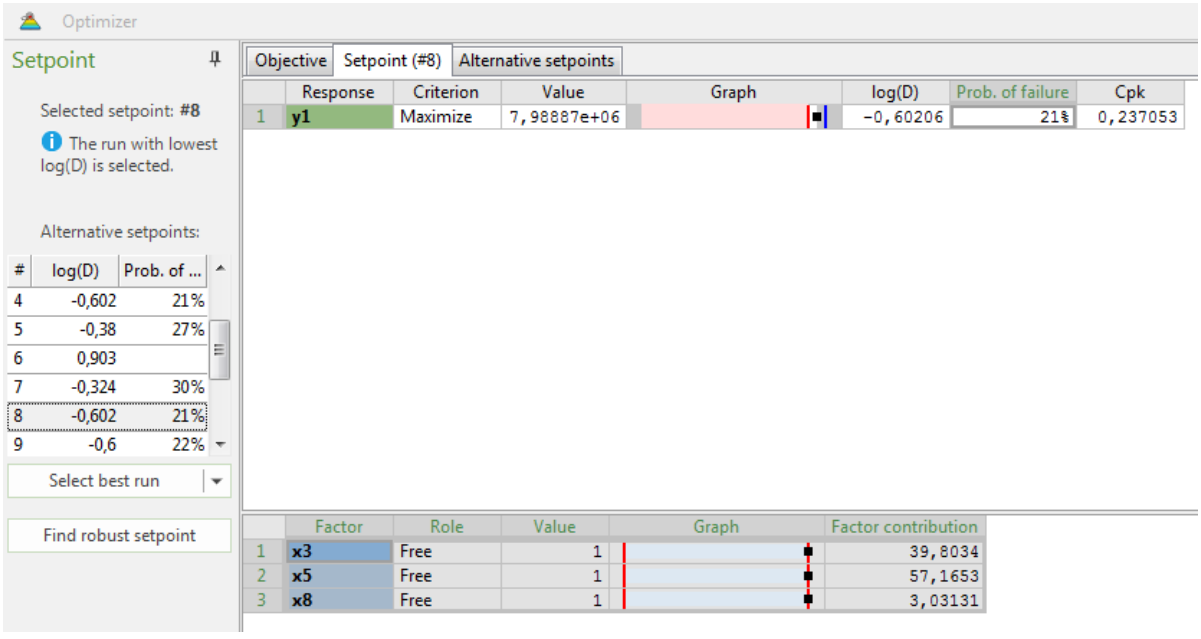

Figure 26: Optimization of MS ionization efficiency through a systematic optimization of the instrumental parameters (x_3, x_5 and x_8) by using an optimizer add-on in MODDE software by Umetrics in Sweden.

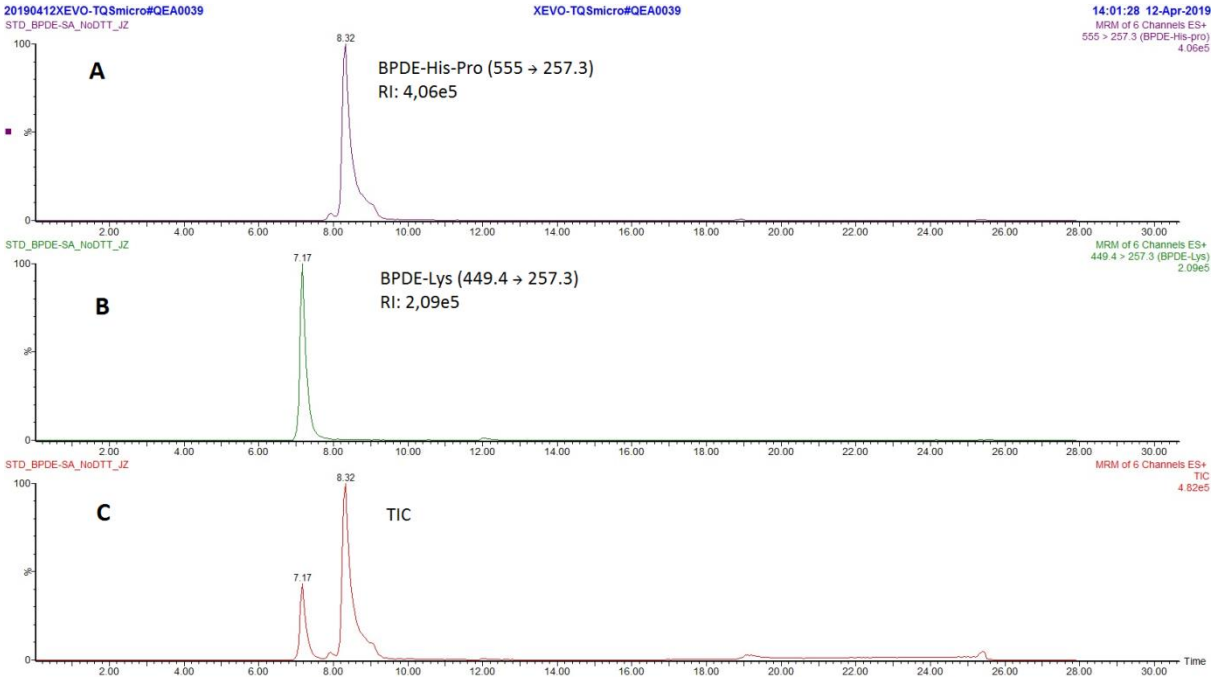

Figure 27: Analysis of digested BPDE-SA by means of a previously developed tuning method. (A): BPDE-His-Pro with a relative intensity (RI) of 4,06e5 and (B): BPDE-Lys with an intensity of 2,09e5.

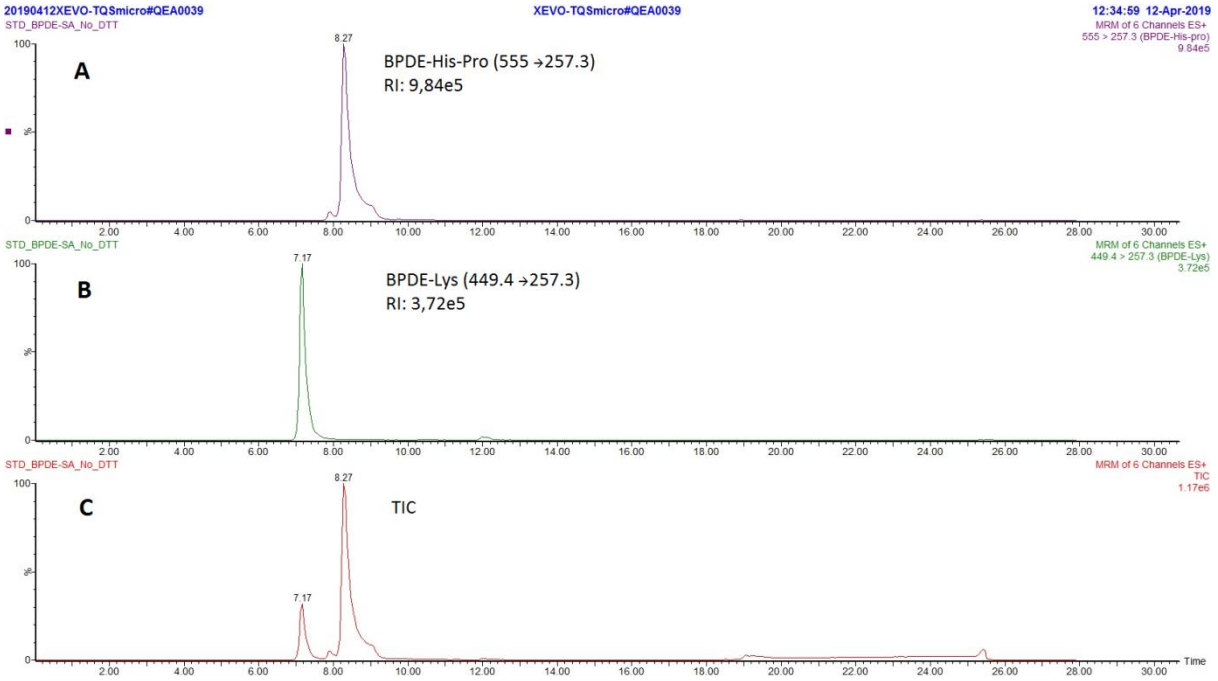

Figure 28: Analysis of digested BPDE-SA by means of the optimized MS method. (A): BPDE-His-Pro with a relative intensity (RI) of 9,54e5 and (B): BPDE-Lys with an intensity of 3,72e5.

Appendix 7: LC-HRMS PRM chromatograms

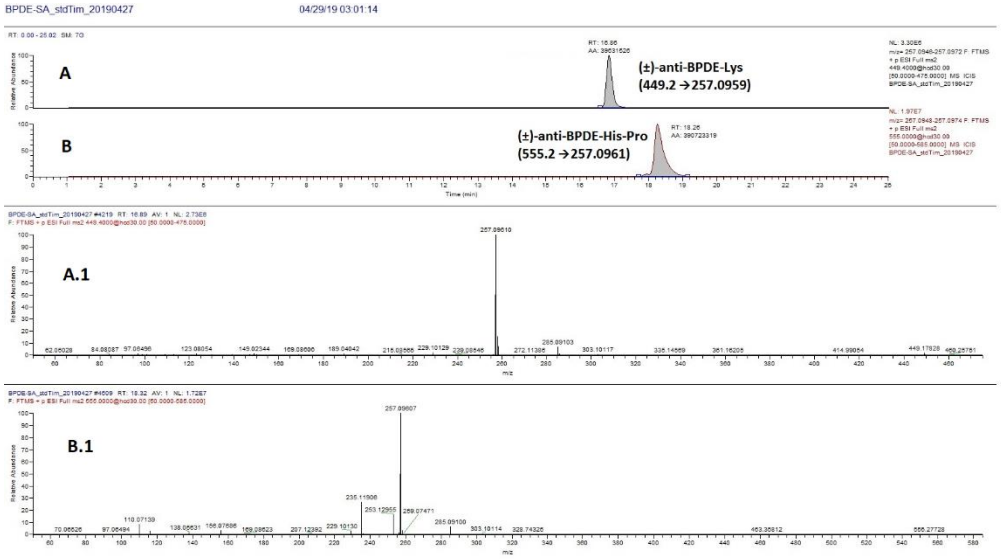

Figure 29: LC-HRMS chromatograms of 1mg digested BPDE-SA from *in vitro* alkylated human SA. (A): (±)-anti-BPDE-Lys (1.4pmol/mg SA) and (B): (±)-anti-BPDE-His-Pro (1.4pmol/mg SA). Spectrum A.1 belongs to A and spectrum B.1 belongs to B.

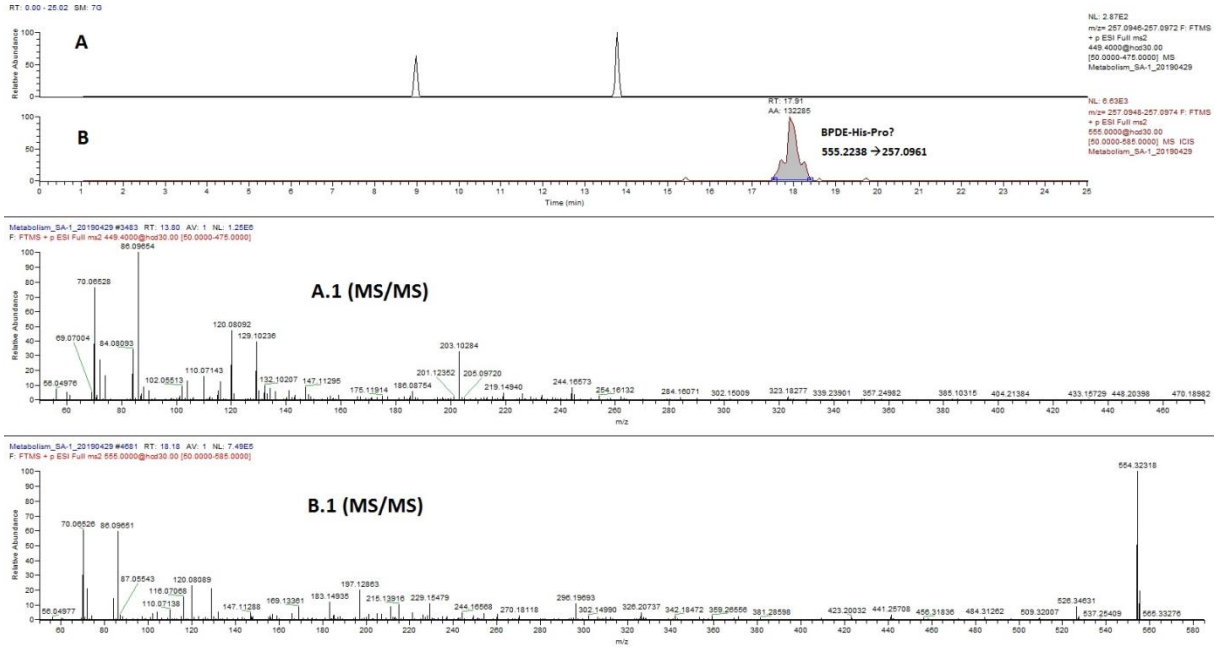

Figure 30: LC-HRMS PRM chromatograms illustrating *in vitro* metabolized B(a)P with 50µl rat liver S9 in presence of human SA. (A): PRM transition for (±)-anti-BPDE-Lys (449.2→257.0959) and (B): PRM transition for (±)-anti-BPDE-His-Pro (555.2→257.0961). Spectrum A.1 belongs to A and spectrum B.1 belongs to B.

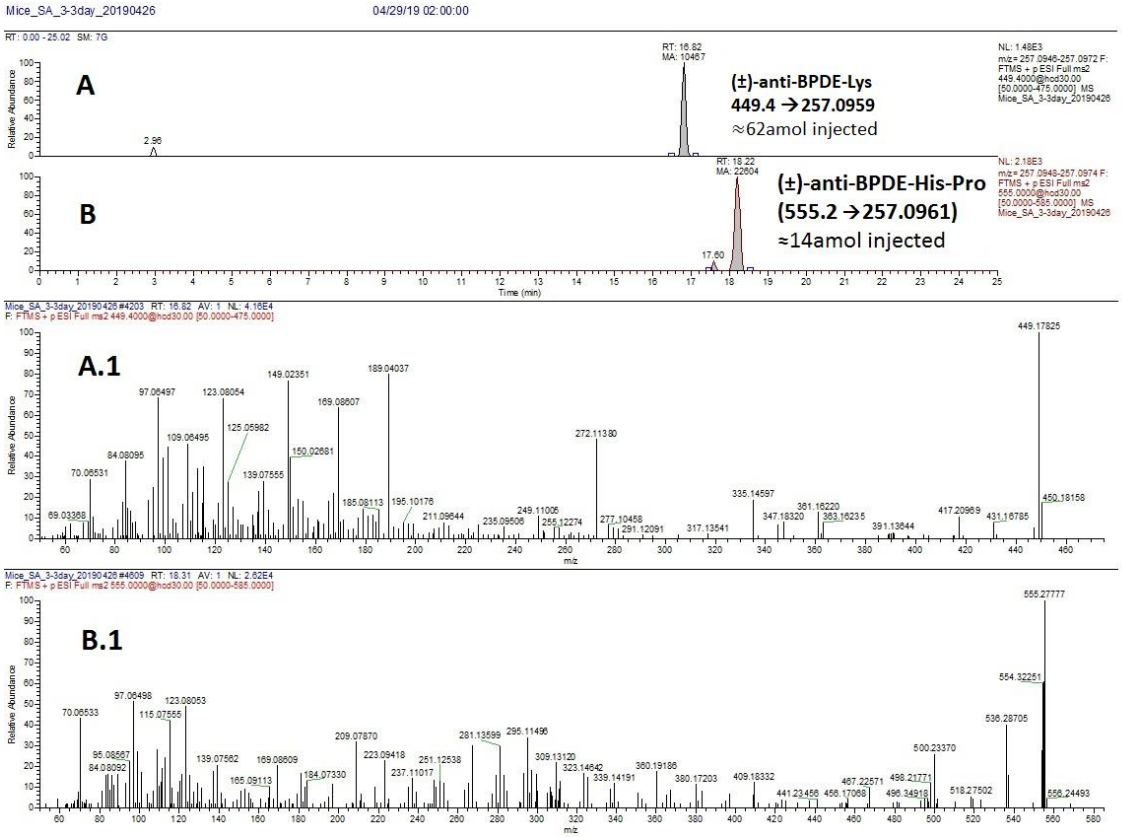

Figure 31: LC-HRMS PRM chromatograms illustrating (A): (±)-anti-BPDE-Lys (62amol injected) and (B): (±)-anti-BPDE-His-Pro (14amol injected) from 1mg BPDE-SA treated mice (100mg/kg of b.w), administered 3 days of exposure.

Appendix 8: Target compounds

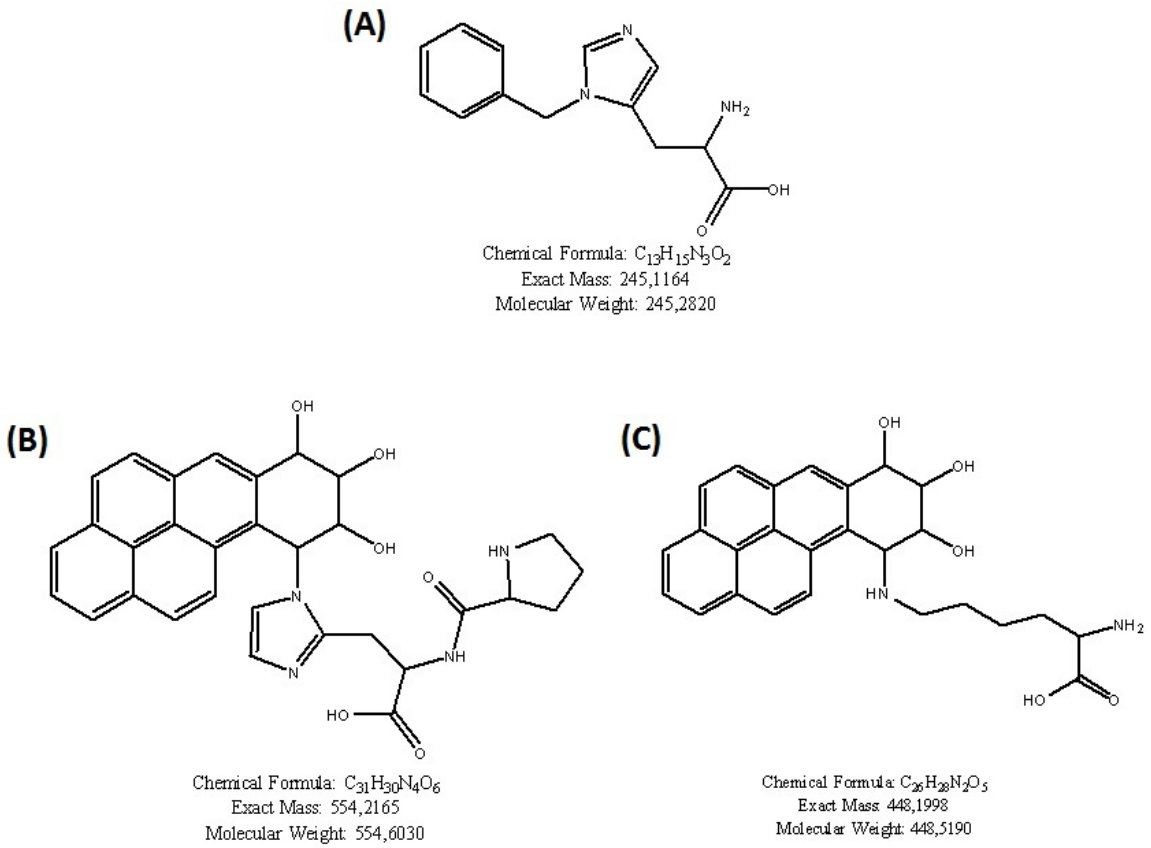

Figure 32: Illustration of target compounds measured by LC/MS/MS. (A): Benzyl-L-Histidine utilized as IS and adducts formed after enzymatic hydrolysis, namely (B): BPDE-His-Pro and (C): BPDE-Lys respectively.

Appendix 10: Chromatographic conditions tested for separation of AAs

Table 25: Various chromatographic conditions tested regarding the separation of AAs by means of iHILIC-fusion column (data not shown).

Condition No.	Solvent A (%)	Solvent B (%)	pH
1	5	95	5.75
2	10	90	5.75
3	25	75	5.75
4	50	50	5.75
5	80	20	5.75
6	90	10	5.75
7	95	5	5.75

Appendix 11: Excel calculations and raw data

The excel files and corresponding raw data, see below, are located on the following computer: **ACESC0009.ad.itm.su.se** (Tims office)

1. **Experimental design (Plackett-Burman, Face center- and full factorial design)**
   C:\Users\TimAstrom\Desktop\Master project Tim\Excel calculations + Raw Data\Experimental design

2. **Calibration, QC and LOD for BPDEs**
   C:\Users\TimAstrom\Desktop\Master project Tim\Excel calculations + Raw Data\Calibration + QC + LOD for BPDEs

3. **Calibration and Recovery of Amino Acids**
   C:\Users\TimAstrom\Desktop\Master project Tim\Excel calculations + Raw Data\Calibration and Recovery of AAs

4. **Digestion of unmodified human SA**
   C:\Users\TimAstrom\Desktop\Master project Tim\Excel calculations + Raw Data\Digested hSA without DTT 20190311_FINAL

5. **Isolation of human SA**
   C:\Users\TimAstrom\Desktop\Master project Tim\Excel calculations + Raw Data\Precipitation experiments 1 and 10mg 20181123 BLACK FRIDAY updated 20190123

Appendix 12 Significance testing of DTT treatment

Table 26: F-test for checking the variances of two groups (control and DTT treatment)

	DTT (µg)	*Control (µg)*
Mean	156.8	40.1
Variance	8339.0	1018.1
Observations	7.0	7.0
df	6.0	6.0
F	8.2	
P(F<=f) one-tail	0.0	
F Critical one-tail	4.3	

Table 27: T-test for comparison of the average values of released amino acids from (1) DTT treated sample and (2) Control samples without DTT treatment.

	DTT (µg)	*Control (µg)*
Mean	156.84	40.09
Variance	8339.01	1018.07
Observations	7.00	7.00
Hypothesized Mean Difference	0.00	
df	7.00	
t Stat	3.19	
P(T<=t) one-tail	0.01	
t Critical one-tail	1.89	
P(T<=t) two-tail	0.02	
t Critical two-tail	2.36	

Appendix 13: Literature review regarding AAs analysis

Table 28: Literature review of recent publications regarding amino acid analysis by HILIC chromatography

Article No.	Stationary phase	Eluent A	Eluent B	AT**** (min)	Detection	Amino acids	Linear range	LOD	R2	Reference	Year of publication
1	Luna-NH2 (250x2.0mm, 5µm)	NH4Ac	ACN	17	TQ-MS (ESI+)	18	0,03-100µM	1-100nM	0.973-0.999	P. Krumpochova et al.	2015
2	Luna-NH2 (250x2.0mm, 5µm)	NH_4Ac/NH_4OH/ACN	ACN	40	TQ-MS (ESI+)	20	LOD - 1.5µg/ml	1-50ng/ml	0.957-0.999	S. U. Bajad et al.	2006
3	Acquity BEH amide (150x2.1mm, 1.7µm)	ACN/NH4HCO2	ACN/NH4HCO2	40	TQ-MS (ESI+)	18	N/A	N/A	N/A	C. Virgiliuo et al. **	2018
4	Acquity BEH amide (100x2.1mm, 1.7µm)	H_2O/NH4HCO2/NH4Ac/FA	ACN/NH4HCO2/NH4Ac:FA	12	TQ-MS (ESI+)	20	0.011-68µg/ml	0.25-78.6µg/ml	>0.997	G. Zhou et al.	2013
5	ZIC-HILIC (150x4.6mm, 5µm)	200mM FA/H_2O	200mM FA/ACN	39	TQ-MS (ESI+)	20	0.005-150µg/ml	0.005-0.5µg/ml	0.9808-0.9986	S. Schiesel et al.	2010
6	Luna (100x4.6, 5µm)	H_2O/NH4HCO2	ACN/NH4HCO2	27	TQ-MS (ESI+)	8	1.9-760ng	0.6-28ng	0.969-0.996	M.C Santiago et al.	2012
7	Aquity BEH amide (100x2.1mm, 1.7µm	H_2O/ACN/FA/8mM NH4HCO3	ACN/H2O/FA/8mM NH4HCO2	12	TQ-MS (ESI+)	20	0.005-2µg/ml	1.5-30.3ng/ml	0.9922-0.9998	Gao et al.	2016
8	Aquity BEH amide (100x2.1mm, 1.7µm	H_2O/FA/10mM NH4HCO2	ACN/FA/2mM NH4HCO2	12	TQ-MS (ESI+)	20	0.01-63.4µg/ml	0.31-79.3ng/ml	0.9910-0.9999	Guo et al.	2013
9	iHILIC-Fusion (100x2.1mm, 1.8µm)	ACN/MeOH	10mM NH4HCO2/FA	15	qTOF MS	6	N/A	N/A	N/A	M. Cuykx et al.	2016
10	Atlantis HILIC silica (150x2.1, 3µm)	ACN	H2O/FA	6	Orbitrap MS (ESI+)	18	0.25-10µg/ml	N/A***	0.9977-0.9999	V. Gökmen et al.	2012
11	ZIC-HILIC (150x2.1mm, 3.5µm)	H2O/ACN/FA	ACN/H2O/FA	19	TQ-MS (ESI+)	14	0.05-10µM	0.1-12fmol	0.9454-0.9993	M. Dell'mour et al.	2010
12	iHILIC-Fusion(+) (150x2.1mm, 3.5µm)	ACN/H2O/NH4Ac	H2O/ACN/NH4Ac	20	Orbitrap MS (ESI+)	13	N/A	N/A	N/A	A. Schriewer et al.	2017