Norwegian University
of Life Sciences

Master's Thesis 2020 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Verification of Nerve Agent Exposure in Human Serum

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Abstract

Nerve agents are the most toxic chemical warfare agents known. They have been used at several occasions despite that the Chemical Weapon Convention (CWC) prohibits the production, storage, and use of these chemicals. Nerve agents affect the nervous system after they are absorbed through inhalation or skin exposure. On suspicion of nerve agent exposure, it is vital to get this verified and get the right treatment fast for the exposed persons. The study that is presented in this thesis, deals with the development of a method to determine nerve agent exposure by the analysis of human blood serum. The method is based on three parts; isolation of Butyrylcholinesterase (BuChE), enzymatic digestion of the protein, and analysis of the target peptide by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Magnetic beads coated with antibodies, specific for BuChE, were used for the isolation of BuChE in serum. Enzymatic digestion was evaluated and optimized by examining the denaturation conditions, enzyme, and digestion solution. Analyses of the target peptide were performed on LC-MS with switch technique and trap-column with backflush.

Cross-linking of the antibodies showed a significant increase in the yield of BuChE from human serum. The optimized enzymatic digestion conditions were 0.2 mg/mL pepsin in 2 % formic acid. Pepsination resulted in a nonapeptide $FGAS_{198}AASAG$ with *m/z* 796.3477, which is suitable for LC-MS analysis. S_{198} is the binding site for the nerve agents, which makes it the target peptide. The LC conditions were optimized focusing on the timing in the switching technique, column dimensions and the mobile phase composition. The reduction of the inner diameter of the separation column, from 2 mm to 1 mm, resulted in a 10-fold increase in the peak intensity of the BuChE target peptide. The mobile phase delivered by the loading pump was optimized to 2.5 % formic acid and 2.0 % acetonitrile (ACN). In the mobile phase delivered by the analytical pump, 2.5 % formic acid and a gradient from 5-90 % ACN over 10 minutes was used. This resulted in the optimal peak intensity of the BuChE target peptide. The loading time was chosen to be 0.5 min, followed by backflush to the separation column, prior to switch back at 4 minutes for the exclusion of the larger peptide with a longer retention time.

The nerve agent adducts VX-BuChE and GF-BuChE were detected with excellent analytical sensitivity when the human BuChE was fully inhibited. Qualitative validation is vital to confirm if the method is fit for its purposes. The limit of detection was not estimated. Hence,

the intensity of the target nonapeptide with fully inhibition of human BuChE indicates that less than 10 % inhibition can be detected.

Sammendrag

Nervegasser er de mest toksiske av de kjemiske stridsmidlene som er kjent. De har blitt benyttet i flere anledninger til tross for at den kjemiske våpen konvensjonen (CWC) forbyr produsering, lagring og bruk av disse kjemikaliene. Nervegass reagerer raskt på nervesystemet etter å ha blitt absorbert via luftveier eller ved hudeksponering. Ved mistanke om nervegasseksponering er det viktig å få dette verifisert, og at de eksponerte personene får rask behandling. Studien som er presentert i denne oppgaven, håndterer utviklingen av en metode for bestemmelse av nervegasseksponering ved analyse av blodserum hos mennesker. Metoden tar for seg tre deler; isolering av Butyrylcholinesterase (BuChE), enzymatisk nedbrytning av proteinet og analyse på væskekromatografi-tandem massespektrometer (LC-MS/MS). Magnetiske kuler dekket med antistoff, spesifikt for BuChE, ble benyttet for isolering av BuChE i serum. Enzymatisk nedbrytning ble evaluert og optimalisert ved å undersøke betingelser for denaturering, enzym og løsning for nedbrytningen. Analyse av peptidet ble utført på LC-MS ved bruk av svitsjeteknikk og trap-kolonne med backflush.

Krysslinking av antistoffer resulterte i en signifikant økning i utbytte av BuChE i humant serum. De optimale betingelsene for enzymatisk nedbrytning var 0.2 mg/mL pepsin i 2 % maursyre. Pepsinering resulterte i nonapeptidet FGAS$_{198}$AASAG med *m/z* 796.3477, som egner seg for MS analyse. S$_{198}$ er bindingsetet for nervegass, som dermed utgjør det ønskede peptidet for analysen. LC betingelsene ble optimalisert med fokus på tiden i svitsjeteknikken, kolonnedimensjoner og mobilfasesammensetningen. Redusering i indre diameter av seperasjonskolonnen, fra 2 mm til 1 mm, resulterte i en tidobling i intensitet av det ønskede nonapeptidet fra BuChE. Mobilfasen levert av loading pumpen ble optimalisert til 2.5 % maursyre og 2.0 % acetonitril (ACN). For mobilfasen, levert av den analytiske pumpen, ble den optimalisert til 2.5 % maursyre og en gradient fra 5.0-90 % ACN over 10 minutter. Dette ga optimal intensitet av ønsket BuChE peptid. Loading tiden ble bestemt til å være på 0.5 min, etterfulgt av backflush til separasjonskolonnen, før svitsjing tilbake ved 4 min for å ekskludere de større peptidene med lang retensjonstid.

Nervegass adduktene VX-BuChE og GF-BuChE ble detektert med utmerket analytisk sensitivitet, da human BuChE var fullstendig inhibert. Kvalitativ validering er viktig for å bekrefte at metoden passer til dens hensikter og for identifisering av peptidet. Deteksjonsgrensen ble ikke estimert. Til tross for dette, indikerte intensiteten ved fullstendig inhibering av human BuChE at mindre enn 10 % inhibering kunne bli detektert.

Acknowledgement

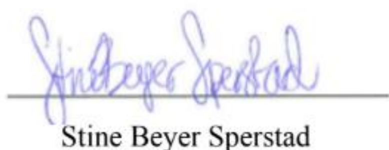
The work presented in this thesis was performed at the Norwegian Defense Research Establishment (Forsvarets forskningsinstitutt, FFI) for *Analyselaboratorier for kjemiske trusselstoffer*. This thesis represents 60 ECTS of a 120 ECTS master's degree in chemistry at Norwegian University of Life Science (NMBU) at Faculty for Chemistry, Biotechnology and Food Science (KBM).

I would like to thank Bent-Tore Røen, my main supervisor at FFI, for giving me the opportunity to write this thesis and for excellent guiding through the year. Your knowledge and work are a big inspiration to me. I would also thank the employees in the chemistry department at FFI for the helpful guidance at the workplace and always be very accommodating.

I would like to thank my supervisor at NMBU, Dag Ekeberg, for helping me during the writing process and the guiding through the COVID-19 situation.

Lastly, I would like to thank my family and friends for the support through my five years of studying. Most of all, I would like to thank my boyfriend for the encouragement and always be there for me when needed.

Ås, 01.06.2020



Stine Beyer Sperstad

Contents

Abstract I

Sammendrag III

Acknowledgement V

| List of figures .... - 1 -

| Abbreviations .... - 2 -

1 | Introduction .... - 4 -

1.1 History of nerve agents .... - 4 -

1.2 Studies for verifications of nerve agent exposure .... - 6 -

1.3 The aim of this study .... - 8 -

2 | Theory .... - 9 -

2.1 Nerve agents .... - 9 -

2.2 The toxic properties of nerve agents .... - 10 -

2.3 Biomarker .... - 11 -

2.4 Immunomagnetic separation .... - 12 -

2.5 Liquid chromatography .... - 14 -

2.5.1 Mobile phase .... - 15 -

2.5.2 The analytical column .... - 16 -

2.5.3 Column switching .... - 18 -

2.6 Mass spectrometry .... - 19 -

2.6.1 Electrospray ionization .... - 20 -

2.6.2 Quadrupole .... - 21 -

2.6.3 Time of flight .... - 22 -

2.6.4 Microchannel plate detectors .... - 24 -

3 | Experimental .... - 26 -

3.1 Materials and chemicals .... - 26 -

3.2 Preparation of solutions .... - 30 -

3.2.1. Tune mix - 30 -

3.2.2 Denaturation of BuChE pepsinated in different acids - 30 -

3.2.3 Trypsin digestion of BuChE - 31 -

3.2.4 Wash buffers and solutions for immunomagnetic separation - 32 -

3.3 Preparation of nerve agent exposed Human Butyrylcholinesterase - 33 -

3.4 Isolation of Human Butyrylcholinesterase from blood serum - 34 -

3.4.1 Cross-linking of bead-antibody complex - 34 -

3.5 Enzymatic digestion of Human Butyrylcholinesterase - 34 -

3.5.1 Denaturation of HuBuChE - 34 -

3.5.2 Enzymatic digestion - 35 -

3.5.3 Optimization of pepsin digestion in different solvents - 35 -

3.5.4 Reusing of magnetic bead - 36 -

3.6 Liquid chromatography - 36 -

3.6.1 Examination of analytical columns - 36 -

3.6.2 LC-conditions for the C4 XBridge column, for optimization of the sample preparation - 37 -

3.6.3 LC-conditions with column switching for the developed method - 38 -

3.7 Mass Spectrometer parameters - 40 -

4 | Results and Discussion - 41 -

4.1 Preliminary studies - 41 -

4.1.1 Determination of the enzyme for enzymatic digestion - 42 -

4.1.2 Examination of denaturation procedures and acid for enzymatic digestion - 43 -

4.1.3 Identification of Butyrylcholinesterase target peptide - 45 -

4.2 Isolation of Butyrylcholinesterase from human blood serum - 46 -

4.3 Enzymatic digestion of Human Butyrylcholinesterase - 49 -

4.3.1 Optimization of the concentration of FA under pepsination of HuBuChE - 49 -

4.4 Development and optimization of the LC-method - 50 -

4.4.1 Determination of analytical separation column - 50 -

4.4.2 Optimization of gradient elution - 53 -

4.4.3 Optimization of the column switching - 57 -

4.4.4 Optimization of injection volume - 60 -

4.5 Examination of VX and cyclosarin exposure - 62 -

4.6 Mass Spectrometry Parameters - 64 -

4.6.1 ESI ion source parameters - 64 -
4.6.2 TOF mass analyzer parameters - 65 -
4.6.3 Development of an MS/MS method - 66 -
4.7 Validation of an analytical method - 66 -
4.7.1 Specificity - 67 -
4.7.2 Limit of detection (LOD) - 68 -
4.7.3 Precision - 69 -
4.7.4 Stability - 69 -
4.8 To summarize; the developed method for detection of nerve agent adducts when exposure is suspected - 70 -

5 | Conclusion - 73 -
6 | Reference - 75 -

| List of figures

Figure 1.1. Schematic procedure of the method development. ....- 8 -
Figure 2.1. Structure and IUPAC names of the most common nerve agents. ....- 9 -
Figure 2.2. Immunomagnetic separation presented in four steps ....- 13 -
Figure 2.3. A schematic illustration of a liquid chromatography system................................- 15 -
Figure 2.4. Van Deemter equation presented with illustration of the terms.**Feil! Bokmerke er ikke definert.**
Figure 2.5. The principle of a 10_2 vial column switching technique....................................- 18 -
Figure 2.6. Schematic figure of a mass spectrometry system with two analyzers....................- 19 -
Figure 2.7. Principle of an electrospray ionization ion source in positive mode......................- 20 -
Figure 2.8. Principle of the quadrupole..- 22 -
Figure 2.9. Principles of a high-resolution TOF..- 23 -
Figure 2.10. Microchannel plate detector ....- 25 -
Figure 3.1. Illustration of the setup of the column switching with a 10-ports..........................- 38 -
Figure 4.1. EIC of target peptide performed on Water XBridge C4 analytical column.............- 42 -
Figure 4.2. Graphical presentation of the peak intensity of target peptide pepsinated in 0.3M HCl and 5% FA with Guanidine.HCl denaturation.. ....- 44 -
Figure 4.3. MS spectrum of target peptide from pepsinated BuChE.......................................- 46 -
Figure 4.4. EIC of the target peptide performed with cross-linked anti-BuChE. ....- 47 -
Figure 4.5. EIC of the target peptide performed with reusing of the beads-antibody complex..- 48 -
Figure 4.6. EICs of target peptide performed on C18 (2.0x100) and on C4 (2.0x100) column.- 51 -
Figure 4.7. EIC of the target peptide performed on C18 (ID 2mm) and C18 (ID 1mm). ....- 53 -
Figure 4.8. Graphical presentation of the yield of target peptide performed with different start concentrations of ACN with the time of ACN gradient.. ....- 54 -
Figure 4.9. EIC of target peptide performed with different start concentrations and gradients. - 55 -
Figure 4.10. Graphical presentation of the peak intensity of target peptide performed with 0.05%, 0.1%, and 0.2% FA delivered by the analytical pump. ....- 57 -
Figure 4.11. EIC of target peptide performed with different loading time ....- 58 -
Figure 4.12. BPC of analyses of pepsinated HuBuChE with switching to loading position........- 60 -
Figure 4.13. Graphical presentations of the peak area and peak intensity of target peptide plotted as a function of the injection volumes ....- 61 -
Figure 4.14. EIC of unexposed target peptide and 100% inhibition of VX- and GF-adducts......- 63 -

Abbreviations

AChE	Acetylcholinesterase
ACN	Acetonitrile
BPC	Base Peak Chromatogram
BuChE	Butyrylcholinesterase
CID	Collision induced dissociation
CWC	Chemical weapons convention
DC	Direct Current (Quadrupole)
DMP	Dimethyl Pimelimedate
DTT	Dithiothreitol
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
FA	Formic Acid
FFI	Norwegian Defense Research Establishment/ Forsvarets forskningsinstitutt
FWHM	The peak full width at half maximum
GA	Tabun
GB	Sarin
GC	Gas Chromatography
GD	Soman
GF	Cyclosarin
HCl	Hydrogenchloride
HPLC	High Performance Liquid chromatography
HuBuChE	Human Butyrylcholinesterase
ID	Inner diameter
IMPA	*O*-isopropyl methylphosphonic acid
IMS	Immunomagnetic separation
LOD	Limit of detection
MPA	methylphosphonic acid

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
m/z	Mass to charge ratio
OPCW	Organization for the prohibition of chemical weapons
OPNA	Organophosphorus nerve agent
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.05 % Tween
qTOF	Quadrupole-Time of Flight
RF	Radio frequency
RPLC	Reversed phase liquid chromatography
VR	Russian VX
TIC	Total ion chromatogram
TOF	Time of Flight
US	United States
VX	American VX
WW II	World War II

1 | Introduction

1.1 History of nerve agents

Chemical warfare agents are toxic chemicals with properties that cause death or harm on organisms, and are associated with military use [1]. By using munition or devices to spread the toxic chemical, they can be distributed over a larger area. The organization for the Prohibition of Chemical Weapons (OPCW) defines toxic chemicals as *"any chemical which through its chemical action on life processes can cause death, temporary incapacitation or permanent harm to humans or animals"* [2]. Nerve agents are organophosphorus compounds and are the most toxic chemical warfare agents [3]. As chemical weapons, they are classified as weapons of mass destruction, meaning that they have the potential to cause death at low doses to civilians in one attempt [4, 5].

In a study for new insecticides in 1936, the development of the first nerve agent was discovered in Germany. Chemist Gerhard Schrader found an interest in the organophosphorus compounds that led to the synthesis of 2000 compounds. Among these, the discovery of the nerve agent known as tabun (GA) took place, and a year later, the nerve agent sarin (GB) [6, 7]. Despite the production and stockpiles of these nerve agents, the Germans did not use these agents in World War II (WWII) because of the lack of protection in case they got a returning attack [6, 8]. The US and English laboratories studied organophosphorus compounds during WWII as well. Still, it was first after WWII when the projects of the Germans became known that the compounds got military significance. Further research was done in France and the Soviet Union, as well as in the US and England. The studies did not only improve the knowledge of protection against nerve agents but they developed a new class of organophosphorus nerve agents (OPNA) by the English and US studies [8]. The nerve agents were called V-agents, where the most toxic compound discovered was VX [7].

The Chemical Weapons Convention (CWC) prohibits chemical warfare from being developed, produced, stockpiled, and used [2]. The convention aimed to achieve a world free of chemical warfare. This convention was open for signatures 13th of January 1993 and entered into force 29th of April 1997. Still, there have been documented exposures of chemical warfare agents after this contract was signed. In the 1990s, the mass destruction weapons were an increased threat by terrorists [8].

In 1995 in Tokyo, the Aum Shinrikyo cult attacked the subway with sarin as an act of terrorism [9]. The cult broke plastic bags containing fluid with 30 % sarin in rush hours a Monday morning in the subways. The subway cars were full of people, where all of the passengers were headed to the center of governmental offices [10]. Six people died within 2 hours after the exposure, and six died the days after, while 5000 people got injured [9]. In the ongoing conflict in the Syrian Arab Republic, there were several attacks with nerve agents in the spring and summer in Damascus in 2013 [11]. Three hospitals in Damascus got 3600 injured with symptoms from nerve agents where 335 died [12, 13]. Several attacks have been confirmed in Syria after this. 24th and 30th of March in 2017, the OPCW confirmed the use of sarin in the conflict area of Ltmaneah as well [14, 15]. In Great Britain 2018, a "newcomer" nerve agent called novichok poisoned the double agent Sergei Skripal and his daughter Yulia when Yulia visited her father. They were poisoned through the skin after they were exposed to novichok behind the door handle to Sergei Skripal's house. They were found on a bench after eating dinner in a severe state of poisoning but survived after long treatment in the hospital [16].

These incidents show that nerve agents have been used not only in military conflicts but also in acts of terror and attacks on civilians. In these incidents, the nerve agents had different routes of exposure and absorption through the respiratory system or skin.

1.2 Studies for verifications of nerve agent exposure

In situations with the use of nerve agents, it is crucial to verify the exposure and provide the patient with the right treatment as fast as possible. The nerve agents bind and inhibit essential enzymes in the human body called cholinesterase, where acetylcholinesterase (AChE) is the most important.

Research on nerve agent exposure and their biomarkers has mostly been studied in blood and urine. The studies of nerve agent exposure over the years can be divided into three major groups of the method development: (1) measuring AChE activity, (2) measuring hydrolyzed products, and (3) measuring nerve agent adducts. The first studies to determine nerve agent exposure were performed by Ellman et al. and published in 1961 [17]. Their work is based on measuring the enzyme activity of AChE in human blood [17, 18]. The method used a colorimetric determination for measuring the enzyme activity. When nerve agent inhibits AChE, it reduces their enzymatic activity. Due to the large variety of AChE concentrations in individuals, the enzyme activity must decrease with 20 % to be certain of an exposure [9].

In the following decades, more advanced instrumentation for more sensitive detection methods were developed. Minami et al. detected in 1997 the hydrolysis products of sarin, *O*-isopropyl methyl phosphonic acid (IMPA), and ethyl methyl phosphonic acid (EMPA), from urine samples of the victims from the poisoning in Tokyo subway attack. The method was performed on gas chromatography-flame ionization detector and used trimethylsilyl derivatization [19]. A year after, Noort et al. published a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for detecting the same poisoning accident from Tokyo by analyzing the hydrolysis product of sarin, IMPA, with no derivatization required [20]. The disadvantages of determine exposure by the hydrolyzed product is that they rapidly excreted, and search for more persistent biomarker began [9].

In the last decades, studies for methods based on protein adduct as a biomarker for nerve agent exposure was developed. These methods on protein adducts of nerve agents were developed and analyzed on LC coupled with MS. Protein adducts have a longer residence time in the bloodstream than the hydrolyzed nerve agents. Two significant protein adducts for nerve agents are those occurring when the nerve agents bind to AChE and Butyrylcholinesterase (BuChE) [9]. Fidder et al. published in 2002 an LC-MS/MS method to determine nerve agent exposure by analyzing the nerve agent adducts with BuChE, after enzymatic digestion. A denaturation procedure was used, and different enzymes for digestion of BuChE were investigated. The specific masses formed for the target peptide with the different nerve agent adducts made it possible to determine an exposure [21]. Noort et al. (2006) developed a new method based on nerve agent adduct attached to BuChE, analyzed with LC-MS/MS. The method used a procainamide affinity gel for purification of BuChE. The identity of the nerve agents could be found and identified by the fragmentation of the BuChE peptide using this method [22]. In 2010, Sporty et al. developed another LC-MS/MS method for analyzing nerve agent adducts [23]. Instead of procainamide affinity gel, Sporty et al. used immunomagnetic separation to isolate BuChE from serum. In 2018, Lee published a study in which the affinity gel purification and the immunomagnetic purification of nerve agent adducts attached to BuChE were compared [24]. The immunomagnetic separation gave a 5-fold higher intensity of the nerve agent adducts than purification on procainamide affinity gel.

1.3 The aim of this study

The overall aim of this study was to develop and validate a method to determine the exposure of nerve agent adducts. The method provides sample preparation by isolation of nerve agent adducted proteins from human serum and a sensitive and specific analysis on LC-MS and LC-MS/MS. The three main steps in the development of a method for nerve agent adducts are:

- Isolation of protein from blood serum
- Enzymatic digestion method
- LC-MS/MS method for qualitative analysis of exposed human serum.

For a more detailed explanation of each step, see figure 1.1. The goal was to make this method robust and sensitive, which can be further used by FFI in the future in case of suspected nerve agent exposure.

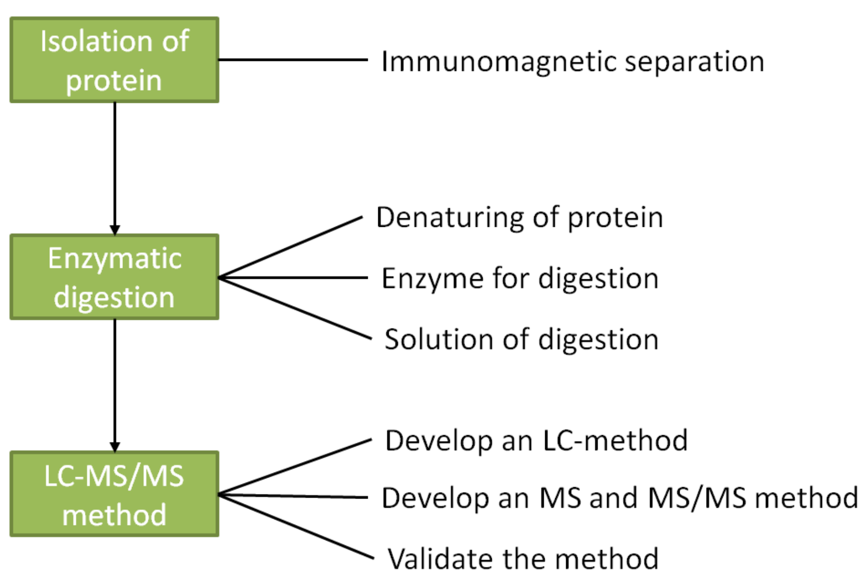


Figure 1.1. *Schematic procedure of the method development. The three main steps with sub-steps of the procedure are presented.*

2 | Theory

2.1 Nerve agents

Nerve agents are a group of organophosphorus compounds, that got their name by their toxicity on the nervous system. Nerve agents are ester derivates of phosphonic acid with a substituent group of either cyanide, fluoride, or thiolate [25]. At room temperature, the nerve agents are clear liquids [26]. These organophosphorus compounds are categorized into two subgroups; G and V agents. The most known G agents are sarin (GB), tabun (GA), soman (GD), and cyclosarin (GF). Of the V agents, the most known are VX and Russian VX (VR) [27]. The structure and IUPAC names of the most common nerve agents are presented in figure 2.1.

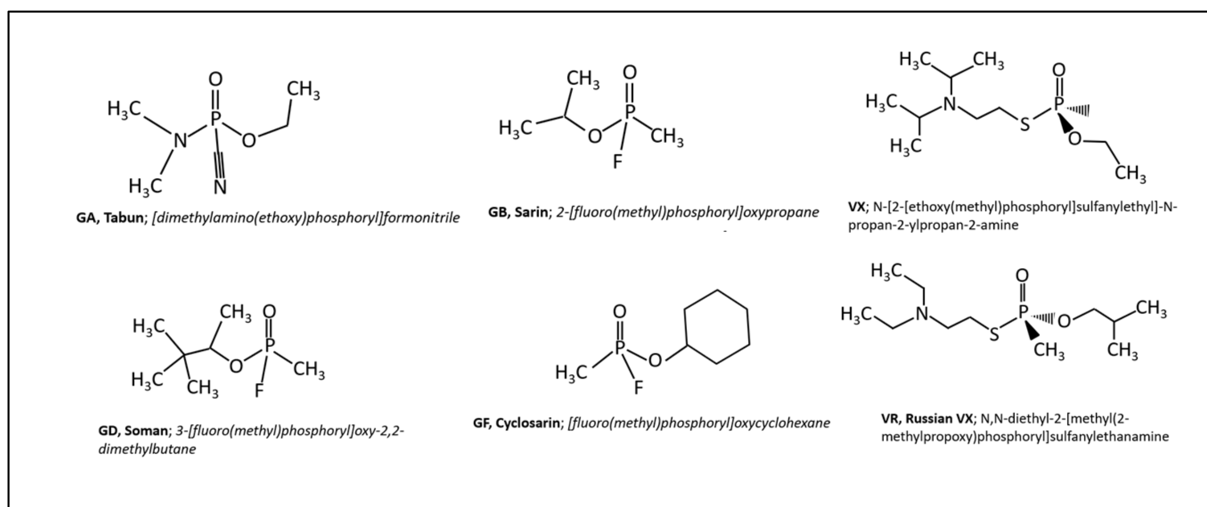


Figure 2.1. *Structure and IUPAC names of the most common nerve agents.*

All nerve agents have a nucleophilic leaving group where the bond to the phosphorous can easily be cleaved by a nucleophilic reagent [8]. Both V and G agents are comparatively stable, highly toxic, and act rapidly when they first have been absorbed. Nerve agents act through the respiratory system when absorbed in the vapor phase. In the liquid phase, they are absorbed through the skin [26]. Sarin is the most volatile nerve agent and has a volatility of 16 091 mg/m^3 at 20 ºC, and at the other end of the scale, it is VX with 10.5 mg/m^3 at 25 ºC. Sarin can be described as a volatile liquid compared to water with a volatility of 23 010 mg/m^3 at 20 ºC. Tabun, soman and cyclosarin have a volatility of 328 (at 20 ºC), 3900 (at 25 ºC) and 438 mg/m^3 (at 20 ºC), respectively [28].

Nerve agents are highly toxic in small doses for humans in liquid and gaseous phases. VX is the most toxic nerve agent and acts primarily through the skin. VR is analog to VX and is absorbed primarily through the skin, while the more volatile G agents act primarily through inhalation [8]. Sarin and soman are deadliest through inhalation, while VX is most toxic through skin [8]. The lethality dose (LD_{50}), where 50 % of the exposed individuals die of the injuries, is displayed in table 2.1.

Table 2.1. 50% lethality dose of the most known nerve agents by inhalation and skin contact. LD_{50} values are taken from Engman et al. [8].

Nerve agent	Minutes of exposure	LD_{50} by inhalation (mg min/m^3)	LD_{50} by skin contact (mg/ individual)
Sarin	2	83	1700
Soman	2	35	300
VX	2	6.4	5

2.2 The toxic properties of nerve agents

The nervous system provides communication between receptors and effectors in an organism. Electrical impulses pass through the nerve cell from the receptor to effectors like muscles and glands. Chemical messengers called neurotransmitters passes the signal from one nerve cell (pre-synaptic nerve cell) across the synapse to another nerve cell (post-synaptic membrane) [29].

Acetylcholine is a neurotransmitter in the cholinergic synapse in the peripheral and the central nervous system [30]. During the neurotransmission, acetylcholine releases from the nerve into the synapse. In the synapse, acetylcholine binds to the acetylcholine receptor on the postsynaptic membrane [4]. After transferring the signal to the nerve cell, AChE is responsible for the termination of acetylcholine. AChE inactivates acetylcholine by catalyzing its hydrolysis. Acetylcholine binds to the active site $Serine_{203}$ on AChE and is hydrolyzed to acetate and choline [4]. Choline is then taken up by the pre-synaptic membrane and is synthesized to acetylcholine in the nerve cell.

Nerve agents are substrate analogs to acetylcholine and inhibit AChE. Organophosphates phosphorylate the active site S_{203} on AChE and inactivate the enzymatic activity, leading to the termination of the hydrolyzation of acetylcholine after transferring the chemical signal to the post-synaptic membrane [30]. When the termination step of acetylcholine is blocked, the concentration of acetylcholine increases, leading to a synaptic block [4]. In a synaptic block, signals will continually be transferred to the receptor. After a few seconds, the receptors will get a steam of continually signals. The synaptic block will result in overstimulation of muscarinic and nicotinic receptors [9]. Symptoms for a nerve agent exposure are cramps, breathing difficulties, and releasing saliva and running eyes. Continual contractions will occur in the muscle cells and lead to cramps. In the respiratory musculature, cramps could cause breathing difficulties and coughing, while in the gastrointestinal musculature it will cause vomiting [8]. For the glands cell, nerve agent exposure will lead to increasing salivation by releasing large amounts of secretion. The degree of these symptoms depends on the amount of nerve agents blocking the acetylcholine's degradation [8].

2.3 Biomarker

A biomarker is a biological response, either to an exposure or an effect. Biomarkers indicate a change in the biological response that is influenced by exposure to a specific substance or a group of substances. The response could be by either a decrease or an increase in enzymatic activity or the presence of a substance in the organism. Biomarkers, as an effect, measure the adverse effect of exposure and indicate the degree of toxicity. The biological response of a biomarker is required to be reproducible and accurate for chemical exposure [31]. The range of biomarkers goes from highly specific to the non-specific [29].

Inhibition of AChE is specific for organophosphate exposure, since there are no other chemical compounds that bind irreversibly to AChE [29]. BuChE belongs to the cholinesterase family and is a known biomarker for exposure of organophosphates. As well as with AChE, organophosphate binds to BuChE. The function of BuChE is unknown, but it is structurally related to AChE [3]. The interest in measuring the exposure by isolating BuChE is related to the higher amount in plasma in contrast to AChE, and that AChE is usually attached to erythrocytes. The concentration of BuChE is approximately 80 nM in serum [32]. In contrast, the amount of AChE is ~3 nM in the erythrocytes [32]. BuChE circulates in the blood for

several days and has a half-life of 12 days. Therefore, nerve agent adducts on BuChE is detectable several weeks after exposure [9, 33].

2.4 Immunomagnetic separation

Immunomagnetic separation (IMS) is a method for isolating proteins by using superparamagnetic beads coated with antibodies. The beads are coated with protein G that conjugates to the antibodies. Antibodies are synthesized proteins that respond to the presence of a foreign substance called an antigen. Antibodies have specific and high affinity to their antigen [34]. IMS is an affinity separation technique that is selective to a protein or a group of proteins and can remove multiple matrix components [35]. This method has rapidly increased in clinical research and molecular biology [36]. Superparamagnetic beads are solid (non-porous) and coated with silane derivates covalently bonded to minimize non-specific binding. The use of antibodies as ligand makes specific binding interactions between molecules and can rapidly isolate the target molecule [37]. IMS is an effective method to isolate only the protein of interest from a biological sample that usually contains a complex mixture of proteins such as blood and urine [23].

The steps in the IMS are illustrated in figure 2.2. Biological sample containing BuChE is added to the immunomagnetic beads coated with anti-BuChE. BuChE will bind covalently to the antibodies. The magnetic properties of the beads make it easy to remove the nonbonding components by placing the tube containing the beads on a magnet. The beads will adhere to the magnet, and the supernatant can easily be removed without touching the beads towards the wall of the tube. The BuChE is attached to the beads and will only elute by an elution buffer, or they can be enzymatically digested while attached to the beads.

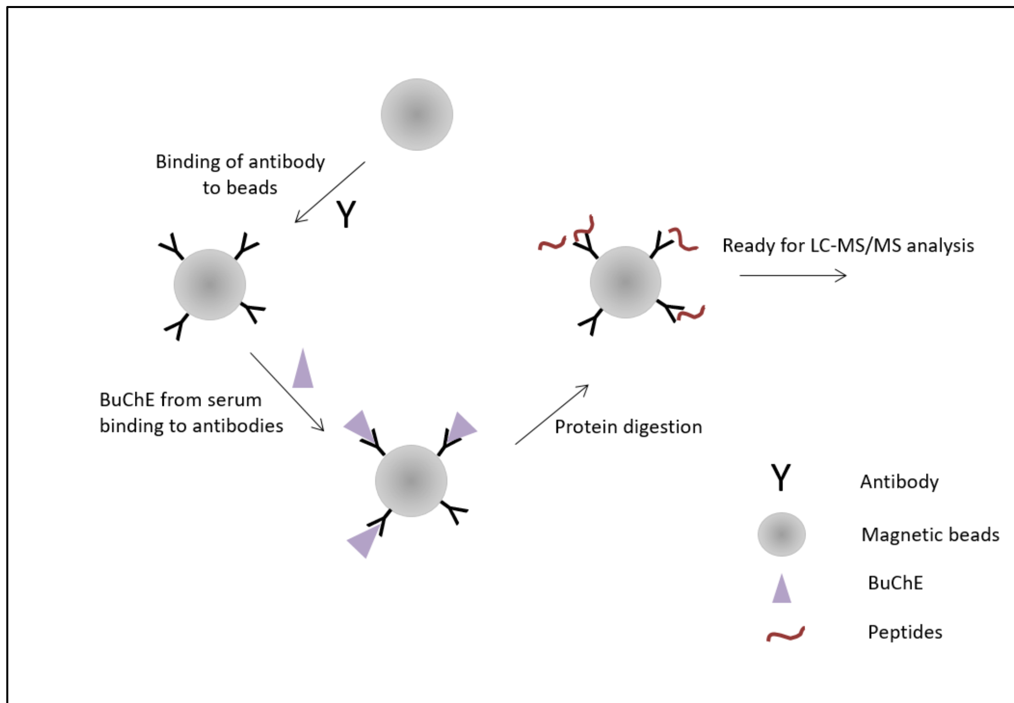


Figure 2.2. *Immunomagnetic separation presented in four steps. (1) antibody binding to bead (2) Protein bind to antibody-bead complex (3) Peptides are made from protein digestion (4) Peptides ready for LC-MS/MS analysis.*

Protein consists of carboxylic acid and amine side chains that, by different pH, can be positively or negatively charged. The large size of proteins can make detection and quantification of proteins difficult, especially on an MS. Enzymatic digestion is a technique that fractionates proteins into peptides using enzymes to break the peptide bonds. The enzyme chosen for enzymatic digestion usually has a relatively high cleavage specificity and cleaves the same bonds in the protein each time [34]. In the analysis of peptides carried out after enzymatic digestion, the peptides that consist of the same amino acid sequence usually comprise one peak in the MS due to a low distribution of charge. The mostly used detector for detection and quantification of peptides is MS/MS by providing structural and mass information [38]. In protein analysis on MS, usually coupled with LC, the ion source produces multiple-charged ions and allows large molecules to be analyzed. Due to the charge distribution within the peptide, multiple-charged ions from the same peptide obtain variations in the mass-to-charge *(m/z)* value in the mass spectrum. The distribution for the same protein over several peaks makes the quantification of the protein difficult [35]. The limitations with protein analyses with MS are the range of *m/z* in the instrument that usually is lower than the mass of the protein [39]. Therefore, enzymatic digestion is preferred for protein analysis.

2.5 Liquid chromatography

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a separation and quantification technique that enables temperature labile compounds to be detected on a mass spectrometer. LC-MS/MS has many advantages compared to the spectroscopic and electrochemical detection techniques. MS/MS has a higher selectivity and only requires that the analyte is ionizable, in contrast to UV/VIS, that requires the analyte to contain one or more chromophores [40]. The use of a chromatographic separation coupled with mass spectrometry has become more prevalent in peptide analysis. For peptides, this technique can determine the amino acid sequence and the peptide mass.

High-performance liquid chromatography (HPLC) separates analytes by their distribution between a mobile phase and a stationary phase. The stationary phase is solid, while the mobile phase is liquid and moves along the stationary phase. The HPLC usually operates in two modes; normal-phase or reversed-phase. The most used HPLC principle is reversed-phase liquid chromatography (RPLC), where the stationary phase is non-polar and has a polar mobile phase. The normal-phase technique consists of a polar stationary phase with a less polar mobile phase. The stationary phase is usually silica-based with different functional groups, depending on the analytes that are being separated and the polarity of the phase [38]. An HPLC system contains a mobile phase reservoir, a pumping system, a sample injector, an analytic column, and a detector, presented in figure 2.3. The pump system is usually a dual-headed reciprocating piston pump that delivers constant pressure on the mobile phase [38]. The sample injector is where the liquid sample is introduced to the liquid mobile phase.

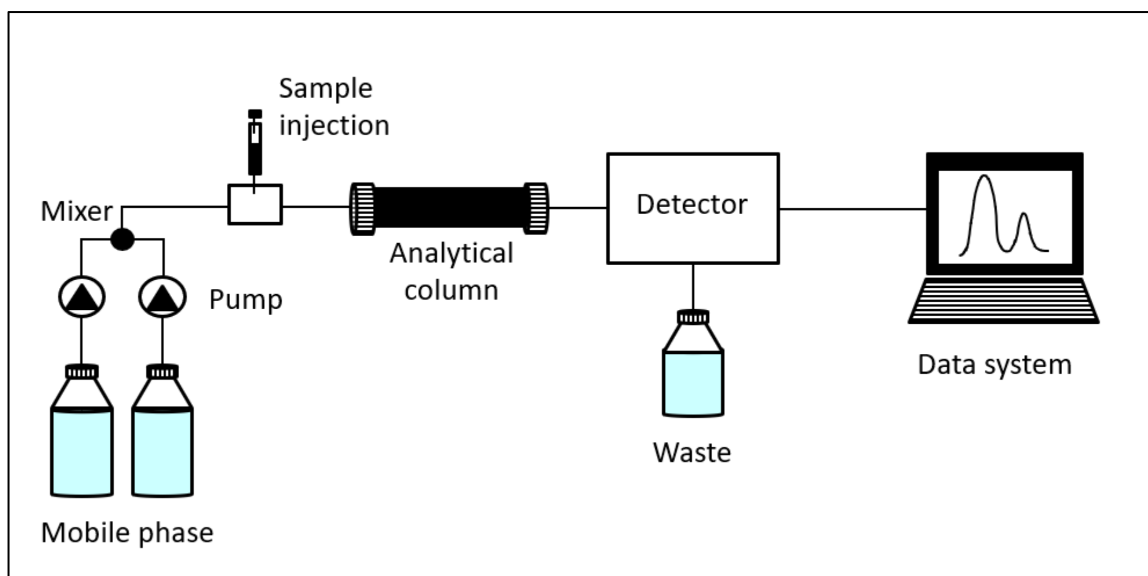


Figure 2.3. *A schematic illustration of a liquid chromatography system consisting of a mobile phase reservoir with a pump and mixing system, that will pass through the sample injector and carry the analytes to the analytical column where the separation occurs before they enter the detector.*

2.5.1 Mobile phase

In HPLC, the mobile phase is an essential factor for the separation of analytes. The mobile phase consists of a binary or ternary mixture of solvents and is based on intermolecular forces for the separation in the column. For RPLC, the mobile phase is more polar than the stationary phase and is usually a mixture of water and an organic solvent [38]. Additives like acids, bases, and buffers are also common and can set the pH and improve sensitivity and separation of the analytes. It is also essential that the mobile phase is compatible with the detector. LC, coupled with MS in RPLC, requires a volatile polar organic solvent such as methanol, acetonitrile (ACN), or tetrahydrofuran. Additives help the ionization process in the ion source in the MS with protonation or deprotonation of the analytes. Additives compatible with LC-MS analysis are ammonium hydroxide and ammonium acetate for high pH. For lower pH, formic acid (FA) is compatible and used for LC-MS analysis [41].

The mobile phase can perform with isocratic or gradient elution. For isocratic elution, the mobile phase composition is constant throughout the analysis, while the composition of the mobile phase change during the analysis for gradient elution [42]. For gradient elution in RPLC, the content of organic solvent increases over time for elution of the less polar compounds in the column [35]. By the influence of the increased organic solvent, the gradient can produce narrow peaks due to the increasing strength of the mobile phase [42].

2.5.2 The analytical column

The analytical column, or the separation column, is where the separation of the analytes takes place. The distribution of the target analyte between the mobile phase and the stationary phase determines the retention time in the column [38]. The factor that influences the separation most in HPLC is the choice of stationary phase in the column [43]. For a satisfactory separation and detection of small concentrations, choosing the right column with a proper stationary phase is essential.

The analytical columns in HPLC are usually packed columns with porous or non-porous particles. Factors that influence the separation and improve the peak shape of the analytes are particle size and column length [44]. The influence that these factors have on the separation can be expressed by the van Deemters equation, which explains the effect of column parameters and linear velocity by the plate height H that is proportional to the chromatic band. Van Deemters equation is displayed in figure 2.4, where u_x is the linear velocity, and *A*, *B,* and *C* are constants for a given column with the same length and stationary phase. The optimum linear velocity is when the lowest value for H is achieved [35].

Figure 2.4 illustrates the functions of the different constants in the equation. A is the multiple paths constant, known as eddy diffusion, and describes the different paths the analytes can obtain in the column due to the stationary phase particles. Smaller stationary phase particles give less Eddy diffusion, hence, a narrow peak in the chromatogram. B/u_x in the equation is the longitudinal diffusion [45]. When the analytes are entering the column, it occurs as a concentrated narrow band in the column. The band is broadening as the analyte is transported throughout the column and diffuses to a broader and less concentrated band as a function of

random motions of the analytes. Longitudinal diffusion is inversely proportional to the linear velocity. A higher flow gives a higher linear velocity; hence, less longitudinal diffusion will occur. Cu_x term is the resistance in mass transfer. The dispersion occurs when the analytes continuously and reversely transfer from the mobile phase to the stationary phase. The mass transfer is dependent on the linear velocities where the dispersion of the analyte increases with the linear velocity [35].

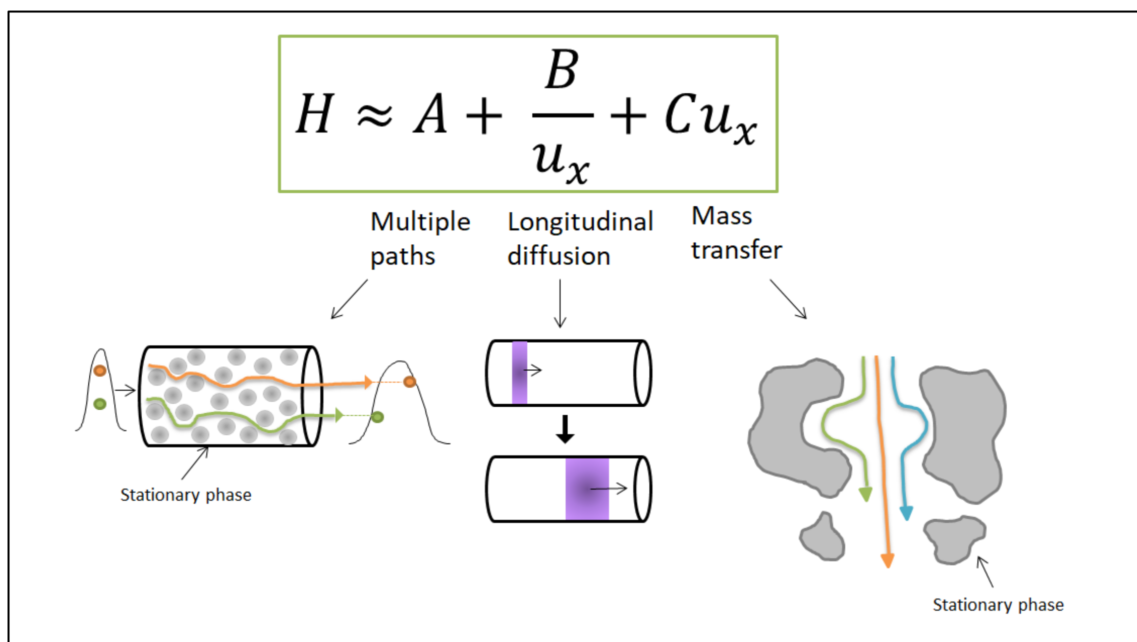


Figure 2.4. *Van Deemter equation presented, with the three terms; Multiple paths (A), Longitudinal diffusion (B), and Mass transfer (C).*

The size of the stationary phase particles in packed HPLC-columns is between 1.5 µm and 5 µm [35]. Smaller particles give a smaller diffusion path of the analytes in the column and will also reduce mass transfer, hence, the potential to reduce plate height and increase efficiency [44]. Columns with an inner diameter (ID) at 2.1 mm are becoming the most common and are compatible with MS because of its low mobile phase flow [35]. The optimal linear velocity is a compromise between what is optimal for the B term and C term. For achieving narrow chromatic band, the stationary phase, column length, particle size, and linear velocity are essentials.

2.5.3 Column switching

Trap column is used for clean-up and pre-concentration before the sample enters the analytical column and can either be on-line or off-line. The trap column is placed before the analytical column, either directly connected or connected through a switching valve. The switching valves can be used for column switching. For performing column switching, the trap-column and the analytical column can be connected to a two-position vial with at least six ports that can be used as a 6-2 switching system [46]. In this thesis, a 10-2 switching system is used and contains one loading position and one injection/analytical position, respectively, presented as position 1_2 and 10_1 in figure 2.5. Figure 2.5 carry out that only six of the ten ports are utilized.

The advantage of this technique is that salts and other molecules are introduced to the trap-column and passed to waste. By introducing the analytes to the analytical column, the trap column can be bypassed again, and a washing step can be performed with a higher flow that will exclude to entering the analytical column and the MS [47]. Column switching gives, therefore, enhancing analytical sensitivity [46]. The switching technique can provide a better sensitivity in the analysis by reducing the ID of the column, and still injecting the same volume of the sample in the trap-column, which increases the concentration of the analyte.

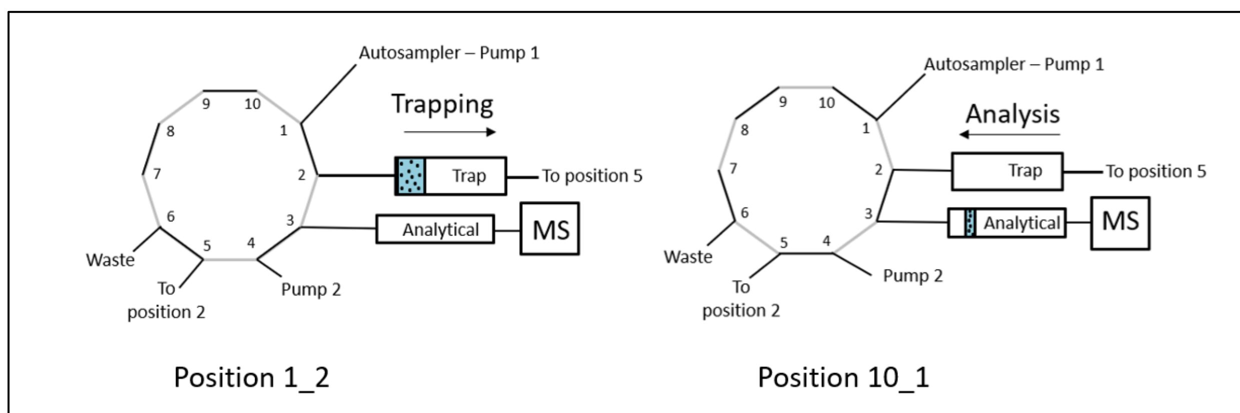


Figure 2.5. *The principle of a 10_2 vial column switching technique with a trap column with backflush. Analytes are introduced to the trap-column, and when switching from position 1_2 to 10_1, the analytes are introduced to the analytical column with backflush from the trap-column.*

2.6 Mass spectrometry

The principle of mass spectrometry (MS) is to separate ions by their mass-to-charge ratio, *m/z*. An MS operates in a high vacuum were only the ionized molecules in the gas phase will be detected. MS consists of an ion source, one or two mass analyzers, and a detector, presented in figure 2.6. In the ion source, the molecules are being ionized, before entering the mass analyzer where the ions are separated. The three most used mass analyzers are quadrupole, time of flight, and orbitrap [39]. After the separation, the ions will enter the detector that converts ions into a detectable signal that measures the abundance [39].

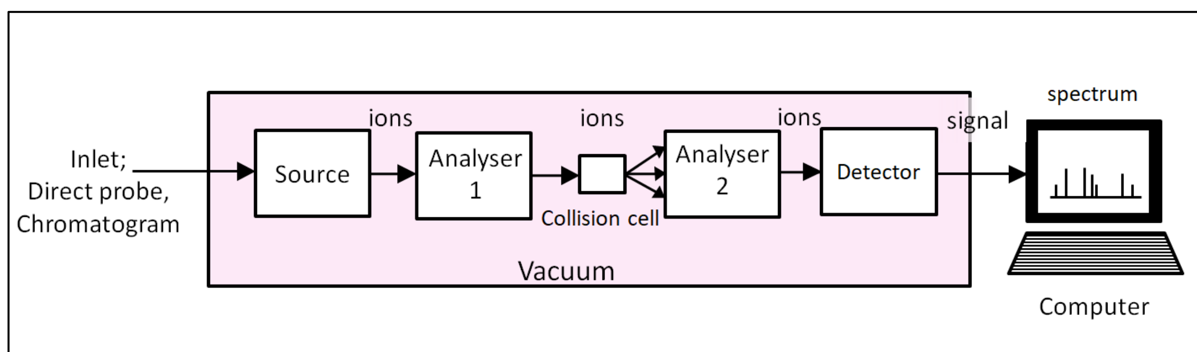


Figure 2.6. *Schematic figure of a mass spectrometry system with two analyzers. The mass spectrometer operates under a vacuum. The ionization occurs in the ion source and separated or isolated in mass analyzer 1 and 2 before the ions convert to a detectable signal given as a spectrum.*

Tandem MS or MS/MS is an indirect method to identify and obtain structural information. MS/MS contains two mass analyzers where the first mass analyzer isolates characteristic compounds, precursor ions. The precursor ions are introduced to the collision cell where a neutral collision gas fragments these ions when colliding with the gas or other ions. The collision cell fragments the ions by collision-induced dissociation (CID) and is placed between the first and the second analyzer [48]. The second mass analyzer separates the fragmented ions before they pass to the detector. The method developed in this thesis used quadrupole coupled with TOF (qTOF), and these mass analyzers are described below.

2.6.1 Electrospray ionization

Electrospray ionization (ESI) is a soft ionization technique. ESI operates in atmospheric pressure and can perform in both positive and negative modes [35]. It transfers ions or neutral compounds from the chromatography column into ions in gaseous phase [49]. ESI is an interface that makes the analytes in the LC compatible with the MS. The principle of ESI mechanism can be divided into three steps: droplet formation, desolvation, and gas-phase ion formation, as presented in figure 2.7.

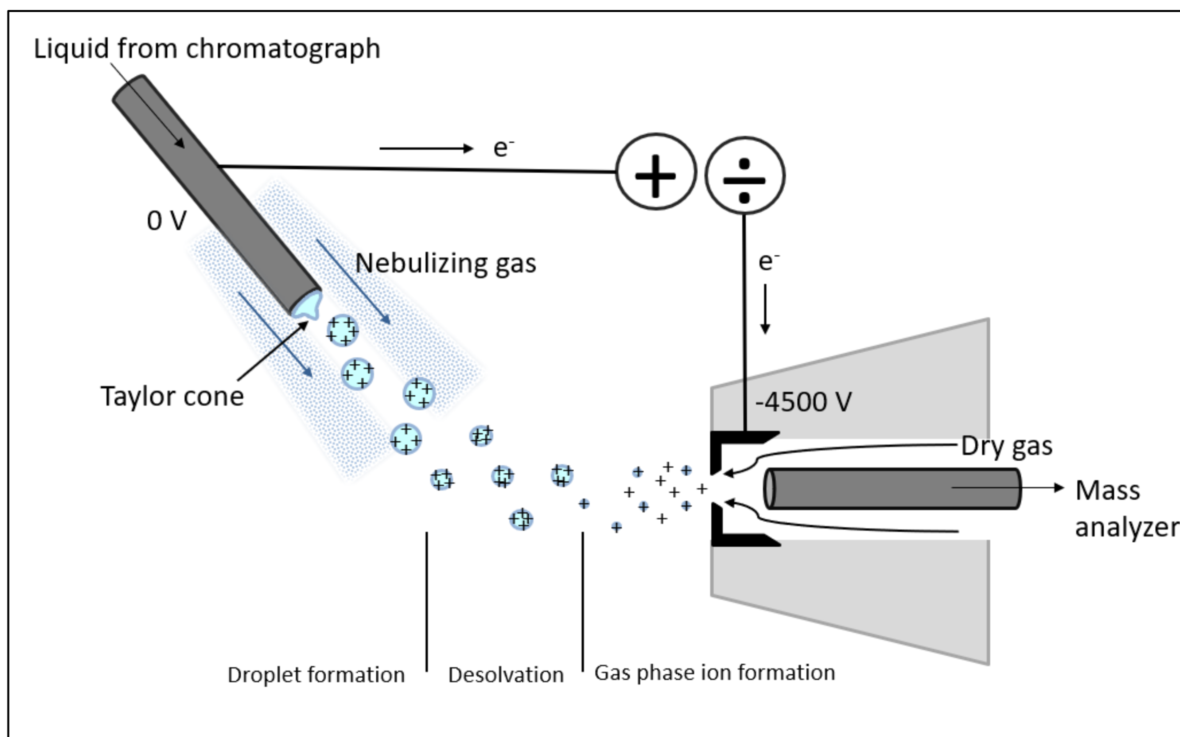


Figure 2.7. *Principle of an electrospray ionization ion source in positive mode. Liquid from the liquid chromatography is introduced to the ion source and ionized, and analytes are converted to gas-phase ions before entering the mass analyzer.*

The sample from the chromatographic column is introduced into the ion source through a nebulizer capillary held at 0 V. A counter electrode is held at -4.5 kV at the inlet to the MS. Between the nebulizer capillary and the inlet to the MS, an electric field is obtained due to the high potential differences. At the outlet of the nebulizer capillary, the liquid will produce positively charged ions that attract the negatively charged counter capillary and make a charge separation at the liquid surface. A coaxial nebulizing gas, typical nitrogen (N_2), flows among the nebulizer and acts as a shield to focus the direction of the liquid. When the pressure is

higher than the surface tension in the liquid, a Taylor cone occurs, and a spray of droplets appears [38, 50].

The droplets consist of ions and solvent, and in the electric field, the solvent evaporates. Evaporation of the solvent reduces the size of the droplets, making the charge density on the surface so large that columbic forces take place and break the droplets further. When the kinetic energy inside the droplets reaches a critical point, the droplets eject into the gaseous phase. The ions are sampled by a sampling cone and transferred to the mass analyzer. A heated drying gas flowing in the opposite direction is used to aid volatilization and ionize the analytes while the uncharged materials are carried out [50].

2.6.2 Quadrupole

A quadrupole mass analyzer separates the ions by the stability of their trajectories in an oscillating electric field. Quadrupole is made up of four metallic cylindric rods with a constant direct current (DC) potential and an alternate radio frequency (RF) [35]. The four rods are connected in pairs parallel to each other in X and Y direction, presented in figure 2.8. Two rods will be positively, and two will be negatively charged. However, with an oscillating RF voltage, the positive rod will become negative over time and then return to positive voltage [48].

Positive ions enter the quadrupole in direction Z from the ion source and will be attracted to the rods with negatively charged voltage. Since the RF voltage is periodic, the ions will be attracted to and repelled by the rods over time. The electric field makes the ions oscillate in a trajectory within the four rods in XY direction. If the ions do not collide with the rods before the RF potential changes, the ions will change direction and continue towards the other rods that now have negative voltage in an oscillating formation, presented in figure 2.8. With a particular DC and RF voltage, ions with a specific *m/z* value will reach the detector. The ions that pass between the rods (in XZ direction) or collide with one of the rods will be neutralized and will not be detected. There are no forces along the Z direction, only the flight time of the ions in between the rod that determine the time inside the quadrupole [51]. If the DC is 0 V on the quadrupole, the quadrupole will act as an ion guide that transmits all the ions from the ion source to the second mass analyzer or detector.

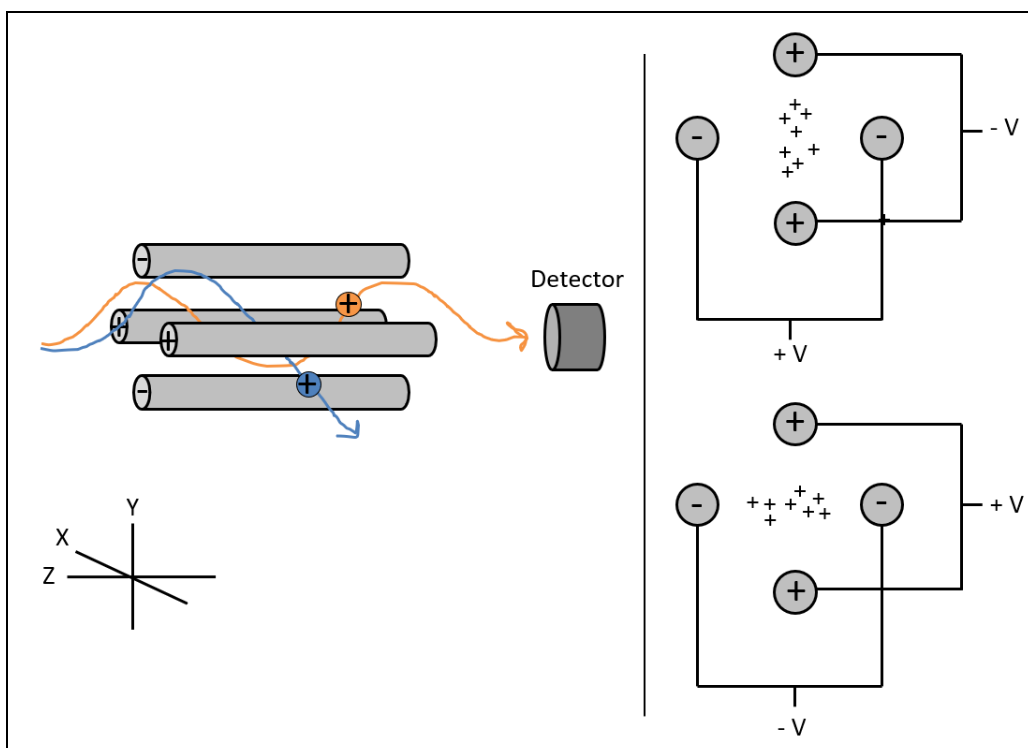


Figure 2.8. *Principle of the quadrupole with the direction X, Y, and Z (to the left) and the ions are focusing in XY direction of the two stages in the RF voltage changes (to the right).*

2.6.3 Time of flight

Time of Flight (TOF) mass analyzer separates ions by their drifting time in a field-free region with no electric or magnetic field. The separation is based on that ions with the same *m/z* have the same kinetic energy when accelerated in an electric field, and the ions with different masses enter the detector at different times. The ions are characterized by the distribution of their mass and velocities when the ions have the same kinetic energy [35, 52]. The main components in high-resolution TOF are the orthogonal accelerator stage, a reflector, and a detector, presented in figure 2.9. The high-resolution TOF has a high *m/z* range at 10,000 (compared to quadrupole with an *m/z* range at 4,000) and is compatible with detecting larger molecules such as peptides.

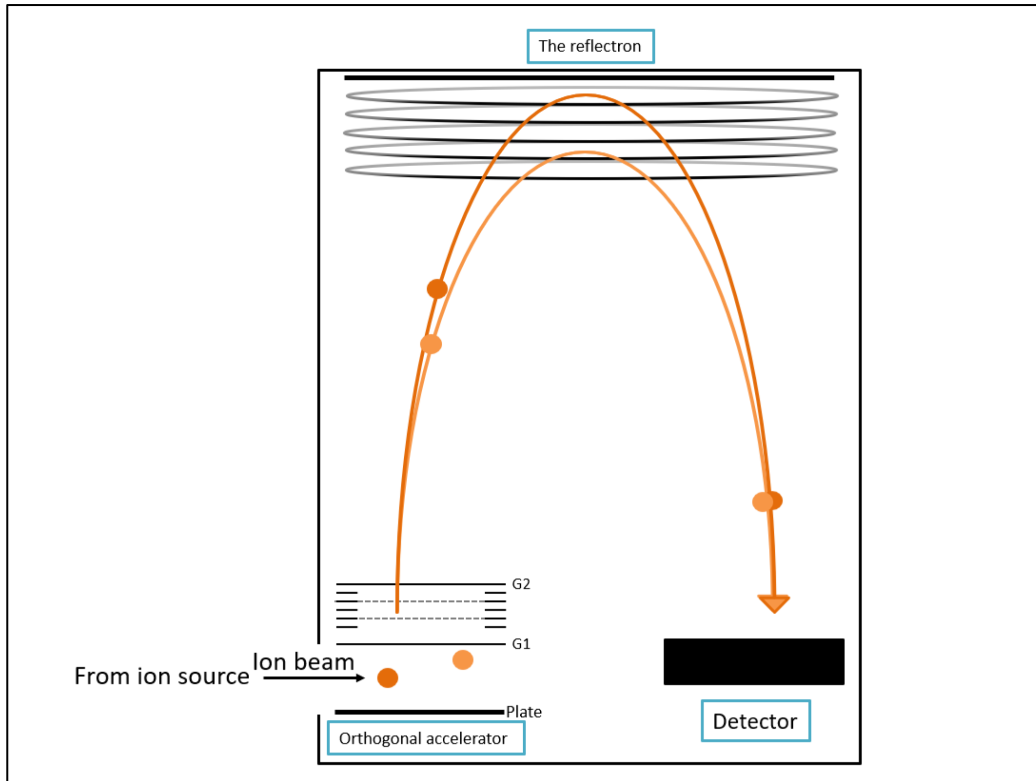


Figure 2.9. *Principles of a high-resolution TOF containing an orthogonal accelerator stage, a reflector, and a detector. The orange circles illustrate ions with the same m/z that, with different accelerations out of the orthogonal stage, will enter the detector simultaneously.*

The ions are subjected to the same acceleration energy. However, the different *m/z* values will give the ions different acceleration speed out of the accelerator. They will separate the ions by their *m/z* values in the TOF flight tube. The difference in velocities of the ions in the TOF can be presented by equation 1 [52].

$$qU = \frac{1}{2}mv^2 \qquad (1)$$

Where q is the ion charge, U is the acceleration voltage, m is the ion mass, and v is the velocity of the ion. The orthogonal accelerator consists of a package of electrodes stacked over each other with a plate at the bottom with a ground voltage at 0 V. Grid 1 (G1) is the electrode nearest the plate and obtains the same voltage as the plate. The ion beam enters the TOF between the plate and grid 1 and moves in their original direction. At one point, the plate gets an injection pulse that makes the ion travel orthogonal from their original direction [39]. When

they pass through grid 1, they accelerate further to grid 2 (G2) that is stacked above grid 1 and then enters the field-free region where the separation of ions occurs. When the ions have accelerated through grid 2, the plate voltage returns to 0 V for the next ion beam to enter the orthogonal acceleration stage. The ions in the field-free region are drifting towards the reflector. The orthogonal accelerator reduces the difference in kinetic energy from the ion source by accelerating the ions in a different direction than they initially entered the TOF. The reduction in the difference in kinetic energy leads to reducing the difference in time of ions with the same *m/z* values to reach the detector [35].

The reflector is placed behind the field-free region at the opposite side of the orthogonal accelerator. A reflector is a series of hollow rings with an electric potential. These rings have increasing positive potential that will repel the positive ions that enter the reflector. The ions with the same *m/z* value but different kinetic energy will penetrate the reflector differently. Ions with higher kinetic energy will spend more time in the reflector than those with lower kinetic energy [39]. The main task of a reflector is to minimize the difference in kinetic energy between the ions with the same *m/z* value to improve resolution. Ions with the same mass but different kinetic energy will penetrate the reflector differently and normalize the kinetic energy when they reach the detector at the same time. [50]. Thus, the ions with the same *m/z* will reach the detector simultaneously, as illustrated in figure 2.9.

2.6.4 Microchannel plate detectors

A detector converts the ion signal into an electrical signal that is proportional to their abundance. The microchannel plate is one of the most used detectors in TOF mass spectrometry. Because of the high mass range for the TOF, the detector requires large area and rapid response to achieve proper resolution and accuracy of *m/z* value with high sensitivity [52]. The microchannel plate consists of a glass plate containing parallel cylindric channels, as presented in figure 2.10. The diameter of the channels ranges from 4 to 25 µm and with a few millimeters in length. From one channel center to the next, the distance is usually between 6 to 32 µm. Inside the channels, the wall is coated with a semiconductive layer.

The ions enter the channels and will collide with the channel wall. The semiconductive layer converts the ions to secondary electrons that will collide to the wall and achieve more electrons. A difference in voltage is applied across the plate to achieve a flow in electrons. The voltage also supplies the electrons necessary to the continuous multiplication process. At the output of the plate, a metal anode collects the flow of electrons, where the output signal is proportional to the input signal [39].

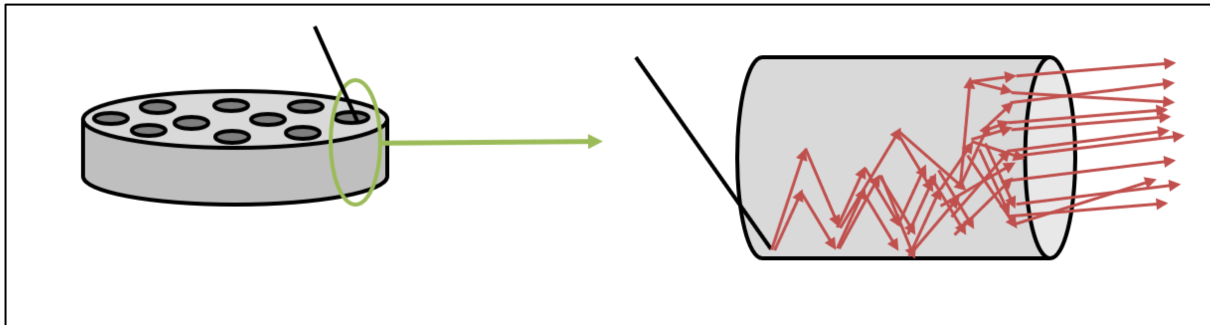


Figure 2.10. *Microchannel plate detector (to the left) showing the principle of converting an ion to an electric signal (to the right).*

3 | Experimental

3.1 Materials and chemicals

Blood serum from employees at FFI where used in this thesis and stored at -70 ºC before use. The serum is a mix from 11 persons (5 men and 6 women) in one batch aliquoted to different tubes, containing the same amount of HuBuChE.

Table 3.1 Enzymes used in this thesis with degree of purity, producer and CAS nr.

Enzymes	Degree of purity	Manufacturer	CAT nr.
Butyrylcholinesterase	≥900 units/mg protein, 10-20% protein	Merck KGaA, Darmstadt, Germany	9001-08-5
Pepsin	≥250 units/mg solid	Merck KgaA, Darmstadt, Germany	9001-75-6
Trypsin	≥10,000 BAEE units/mg protein	Merck KgaA, Darmstadt, Germany	9002-07-7

Table 3.2 Antibodies used in this thesis with the dilution degree, producer and CAT no.

Antibody	Dilution	Manufacturer	CAT nr.
HuBuChE- Monoclonal IgG1	1: 200	Invitrogen, Thermo Fisher Scientific	HAH002-01-02

Table 3.3 Nerve agents used in this thesis with dilution degree, producer and CAS no.

Nerve agent	Dilution in isopropanol	Manufacturer	CAS
Cyclosarin	3 µg/mL	Supported by FFI	329-99-7
VX	3 µg/mL	Supported by FFI	50782-69-9

Table 3.4 List of chemicals used in this thesis with degree of purity, producer and CAS no.

Chemical	Degree of purity	Manufacturer	CAS no.
Acetonitrile (ACN)	99.9 %, HPLC grade	Merck KgaA, Darmstadt, Germany	75-05-8
Ammonium bicarbonate (NH_4HCO_3,)	≥ 99.0 %	Merck KgaA, Darmstadt, Germany	1066-33-7
Citric Acid	≥99.5 %	Merck KgaA, Darmstadt, Germany	77-92-9
Dimethyl pimelimidate (DMP)	≥99.0 %	Merck KgaA, Darmstadt, Germany	58537-94-3
Dynabeads^{TM} Protein G	30 mg/mL beads in PBS	Invitrogen, Thermo Fisher Scientific, Rockford, USA	-
Formic Acid	98-100 %	Merck KgaA, Darmstadt, Germany	64-18-6
Hydrogen Chloride	30 %	Merck KgaA, Darmstadt, Germany	7647-01-0
Isopropanol	99.5 %	Merck KgaA, Darmstadt, Germany	67-63-0
Milli-Q water	HPLC grade	In house produced	-
Phosphate buffered saline (PBS)	-	VWR Chemicals International AS	-
Sodium iodoacetate	≥ 98 %	Merck KgaA, Darmstadt, Germany	305-53-3
Triethanolamine buffer solution	-	Merck KgaA, Darmstadt, Germany	-
Tween® 20	≥40% (GC)	Merck KgaA, Darmstadt, Germany	9005-64-5

Table 3.5 List of the equipment used in this thesis with manufacturer

Equipment	Manufacturer
Analysis weight: AutoChem AT200	Mettler Toledo, Ohio, USA
Autosampler vials: DWK Life Sciences MicroLiter 76 Series™ 9mm, 300 µL	Thermo Fisher Scientific, Rockford, USA
Centrifuge Tubes: Amicon Ultra – 0.5 Centrifugal filter Unit, 10 kDa Cut off	Merck Millipore, Massachusetts, USA
Eppendorf tubes: 1.5 mL, Microcentrifuge Tube, Polypropylene	Axygen Scientific Inc., California, USA
Incubator: Termarks 51L	Termaks as, Bergen, Norway
Magnetic rack: DynaMag™-2 Magnet	Thermo Fisher Scientific, Rockford, USA
Pipettes: Labsystem	BT Lab Systems, Missouri, USA
Sarstedt tubes: 15 ml, 120 x 17 mm, polypropylene	Sarstedt AG & co. KG, Nümbrecht, Germany
Sentrifuge: Kubota 1700	Kubota Corporation, Tokyo, Japan
Shaker and mixing plate: Rotamax 120	Heidolph Instrumens GmbH & CO. KG, Schwabach, Germany
Table sentrifuge: Mini Star	Avantor Inc, Radnor, USA
Water purification system: Milli-Q® Advantage A10. Pre-equipped with Q-POD. Filter: Millipak® Express 40 0.22 µm	Merck Millipore, Burlington, USA
Whirlmixer: MS 3 basic	IKA®, Staufen, Germany

Table 3.6 List of the instrumentation used in this thesis with belonging programs and specifications.

Instrument	Name	Fabricant
Liquid Chromatography		**Dionex Corporation, Idstein Germany**
LC system	Ultimate 3000 RSCL	
Auto sampler: WPS-3000 RS	Auto sampler includes: 100 µL syringe, 100 µL sample loop, 100 µL buffer loop, 2-position, 6-port injection valve suitable for pressures < 103 MPa (15000 psi).	
Flow manager: FLM 3100	Flow manager with two 10 ports micro switching valves, Split flow and column temperature.	
Pump: DGP-3600M	Dual low-pressure gradient pump. Two separate pumps are installed in one enclosure (2x3 solvents).	
Solvent racks: SRD-3600	Dual-gradient analytical pumps without degasser.	
LC software	Chromeleon 7.3	
Tandem Mass spectrometer		**Bruker Daltonics, Bremen, Germany**
MS system: MicroTOF-Q II mass spectrometer	Contains: The Apollo II Electrospray Ion Source, the quadrupole MS/MS-stage, the vertically arranged Time of Flight mass spectrometer, the vacuum system (including the rough pump).	
LCMS software: Coupling Chromatography to Mass spectrometry	Compass Hystar	
MS software	otofControl	
Data analysis program	Compass DataAnalysis 4.1	

3.2 Preparation of solutions

3.2.1. Tune mix

For tuning and calibrating the MS, a tuning mix was used. The tuning mix is a solution of different chemicals for achieving good mass accuracy. ESI-L Low concentration tuning Mix (Agilent Technologies, Santa Clara, USA) was used and added directly to the MS with a syringe pump before analyzing the samples.

3.2.2 Denaturation of BuChE pepsinated in different acids

BuChE 0.2 mg/mL stock solution

2 mg BuChE from equine serum was weighed and dissolved in 0.5 M Tris.HCl, 0.3 M HCl, or 5 % FA, depends on its use, to a total amount of 0.2 mg/mL BuChE in an Eppendorf tube in 1 mL solution. BuChE solved in 0.5 M Tris.HCl was prepared for analyses with denaturation with Guanidine.HCl by aliquot 200 µL to eight Eppendorf tubes, where four parallels were pepsinated in 0.3 M HCl and four parallels were pepsinated in 5 % FA. Four aliquots of 200 µL 0.2 mg/mL BuChE in 0.3 M HCl was prepared and added to four Eppendorf tube for pepsination in 0.3 M HCl. From the stock solution of 0.2 mg/mL BuChE in 5 % FA, six aliquots of 200 µL were transferred to each Eppendorf tubes. Two of the aliquots was performed with organic denaturation. For the two negative controls, the BuChE was excluded in one performed with denaturation and one with no denaturation.

Pepsin 2 mg/mL stock solution

Pepsin, from porcine gastric mucosa, was prepared by dissolving 2 mg pepsin in 1 mL 5 % FA or 0.3 M HCl in an Eppendorf tube approximately 30 minutes before use. The pepsin solution was whirl mixed and stored at room temperature and diluted to 0.2 mg/mL for the pepsination.

Pepsination of HuBuChE performed in different concentrations of FA, a stock solution of 2 mg/mL pepsin in 5 % FA were diluted to 0.2 mg/mL pepsin in 0.5 % and 2 % FA for pepsination. For the investigation of the influence the FA concentration had for the yield of BuChE target peptide, two parallels of BuChE pepsinated in each of the three FA concentrations were prepared.

Dithiothreitol, 1.26 mg/mL

Dithiothreitol (DTT) was prepared by dissolving 2 mg DTT in 10 M Guanidine.HCl to an Eppendorf tube. 630 µL was transferred to a new Eppendorf tube where 370 µL 10 M Guanidine.HCl was added to a total concentration of 1.26 mg/mL DTT. The solution was whirl mixed and stored at room temperature. The 2 mg/mL DTT solution was diluted to 0.63 mg/mL in 5 M Guanidine.HCl to the samples for the denaturation of BuChE.

Guanidine.HCl, 10 M buffer

Guanidine.HCl 10 M was prepared by dissolving 0.95 g Guanidine.HCl (s) in 1 mL Milli-Q water in an Eppendorf tube. Guanidine.HCl 10 M was prepared the same day or the day before use and stored at room temperature. For denaturation, 10 M Guanidine.HCl was diluted to 5 M in the BuChE samples.

Hydrogen Chloride, 3M

3 M Hydrogen chloride (HCl) was prepared from a 30 % HCl solution. For a total volume of 5 mL, 1.25 mL Milli-Q water was added to a 15 mL sarstedt tube. Added 1.585 mL HCl 30 % gently and, subsequently, added the rest of the Milli-Q water (2.165 mL) to the total volume of 5 mL.

Sodium iodoacetate, 1.42 mg/mL

1.42 mg Sodium iodoacetate was dissolved in 1 mL Milli-Q water in an Eppendorf tube. The solution was whirl mixed until sodium iodoacetate was fully dissolved and stored at room temperature.

3.2.3 Trypsin digestion of BuChE

BuChE 0.2 mg/mL stock solution, in 50 mM NH_4HCO_3

For BuChE solution containing 0.2 mg/mL, was 2 mg BuChE from equine serum dissolved in 50 mM NH_4HCO_3 for digestion with trypsin. Two aliquots of 200 µL 0.2 mg/mL BuChE in 50 mM NH_4HCO_3 were transferred to Eppendorf tubes.

Trypsin 2 mg/mL stock solution

Trypsin, from bovine pancreas, was prepared by dissolving 2 mg Trypsin in 1 mL 50 mM NH_4HCO_3 in an Eppendorf tube. The solution was whirl mixed and stored at room temperature.

NH_4HCO_3, 50mM

11.85 mg NH_4HCO_3 was dissolved in 3 mL Milli-Q water in a 15 mL sarstedt tube and whirl mixed. The solution was made the same day or the day before use and stored at room temperature.

3.2.4 Wash buffers and solutions for immunomagnetic separation

Anti-BuChE in PBST, 50 ng/mL

For 2.5 mL 50 ng/mL Anti-BuChE in PBST, 2.375 mL PBST was added to a 15 mL sarstedt tube. Subsequently, 125 µL Anti-BuChE (1 mg/mL) was added and dissolved. The solution was made fresh before use.

Wash buffers for isolation with Dynabeads

Citric Acid, 100mM

Citric Acid, 100 mM, was prepared by dissolving 96 mg Citric Acid in 5 mL Milli-Q water in a 15 mL sarstedt tube and whirl mixed. The solution was made the same day or the day before use and stored at room temperature. Two parallels were used for the investigation of reusing bead-antibody complex. Two negative controls were prepared by pepsinate the used beads without adding a new serum sample.

Phosphate-buffered saline (PBS)

One tablet of PBS was dissolved in 10 mL Milli-Q water from Millipore with a 10x PBS concentration in a 15 mL sarstedt tube. The 10x PBS solution was diluted with a factor 10 in 15 mL sarstedt tube and stored at room temperature.

Phosphate-buffered saline with 0.05% Tween 20 (PBST)

For the preparation of 7 mL PBS w/ 0.05 % tween, 700 µL 10x PBS was dissolved in 6.265 mL Milli-Q water in a 15 mL sarstedt tube. Subsequently, 35µL Tween 20^{TM} was added to the solution and whirl mixed. PBST was stored at room temperature before use.

Cross-linking reagent

Dimethyl Pimelimedate (DMP) 5.4 mg/mL

DMP 5.4 mg/mL was prepared by dissolving 8.1 mg DMP in 1.5 mL triethanolamine buffer in an Eppendorf tube and whirl mixed. The solution was made the same day it was used and stored at room temperature. Four parallels of cross-linked beads and four parallels without cross-linking were prepared for investigation of the effect of cross-linking of bead-antibody complex. Two negative controls were prepared, one without adding a serum sample and one without adding the anti-BuChE.

3.3 Preparation of nerve agent exposed Human Butyrylcholinesterase

Human Serum was incubated with the nerve agents VX and GF. The nerve agents were supported by FFI, diluted in isopropanol with a concentration of 3 µg/mL. For preparation of exposed serum, 1.2 mL Human serum was added to two Eppendorf tubes. Subsequently, 20 µL VX and 20 µL GF were added to each serum sample. The serum samples containing 50 ng/mL nerve agents was incubated for approximately 1 hour with shaking on Rotamax 120 shaking plate at 80 rpm before they were ready for analysis.

3.4 Isolation of Human Butyrylcholinesterase from blood serum

The procedure starts with preparation of the magnetic Dynabeads^{TM} Protein G conducted according to the manufacturer's protocol [53]. The amount of serum analyzed per BuChE antibodies (anti-BuChE) from clone 3E8, and the concentration of anti-BuChE relative to the beads, were given by Sporty et al. 2010 [23]. The procedure was conducted with 100 µL magnetic Dynabeads solution transferred to an Eppendorf tube. The Eppendorf tube was placed on DynaMag^{TM} magnetic rack, where the beads will adhere to the magnet, hence, separate the supernatant from the beads. The supernatant was discarded, and the beads were washed with 3x 200 µL 1x PBS. Subsequently, 400 µL 50 ng/mL anti-BuChE in PBST was added to the beads. The beads were incubated with the anti-BuChE solution overnight at room temperature with shaking on Rotamax 120 shaking plate at 80 rpm. After incubation, the supernatant was discarded, and the beads were washed with 2x 200 µL triethanolamine buffer.

3.4.1 Cross-linking of bead-antibody complex

The cross-linking procedure was conducted, according to Sporty et al. (2010) [23]. After the washing step with triethanolamine buffer, 200 µL 5.4 mg/mL DMP was added and incubated for 30 minutes at room temperature with shaking on Rotamax 120 shaking plate at 80 rpm. Subsequently, the supernatant was discarded, and the beads were washed with 3x 100 µL PBST.

3.5 Enzymatic digestion of Human Butyrylcholinesterase

3.5.1 Denaturation of HuBuChE

Denaturation of BuChE with Guanidine.HCl reagent

The denaturation with 5 M Guanidine.HCl was conducted on BuChE from equine serum in 0.5 M Tris.HCl, and the procedure was performed without the beads in 1.5 mL centrifuge tubes with 10 kDa cutoff filter. For 200 µL 0.2 mg/mL BuChE, 200 µL 10 M Guanidine.HCl was added containing 1.26 mg/mL DTT. Subsequently, the solution was incubated for 60 minutes at 50 °C, with a total volume of 400 µL of the sample. After incubation, 20 µL 1.42 mg/mL sodium iodoacetate was added to the solution and incubated for a further 60 minutes at 50 °C. The sample was centrifuged down in Kubota 1700 centrifuge for 20 minutes at 12000 rpm prior

to enzymatic digestion of BuChE. The filtrated sample was transferred to Eppendorf tubes. The concentration of DTT and Sodium iodoacetate was used according to Fidder et al. (2002) [21].

Denaturation of BuChE with organic solvent

Denaturation with organic solvent was performed on 0.2 mg/mL BuChE from equine serum solved in 5 % FA in the Eppendorf tube. For 200 µL BuChE in 5 % FA, 100 µL ACN was added to the Eppendorf tube and incubated for 15 minutes at 70 °C with the tube sealed. After 15 minutes, the solvent was vaporized to dryness with nitrogen flow at 70 °C. To the vaporized sample, 135 µL 5 % FA was added prior to enzymatic digestion.

3.5.2 Enzymatic digestion

Enzymatic digestion of BuChE was performed with either 0.2 mg/mL pepsin solved in 5 % FA or with 0.2 mg/mL Trypsin in 50 mM NH_4HCO_3. 15 µL 2 mg/mL pepsin or trypsin were added to the BuChE sample containing 135 µL 5 % FA or 50 mM NH_4HCO_3, respectively. Enzymatic digestion was performed overnight at 37 °C. After the enzymatic digestion, the supernatant was transferred directly to autosampler vials for analyzing with LC-MS.

3.5.3 Optimization of pepsin digestion in different solvents

Optimization of pepsin digestion in different acids

A stock solution of 2 mg/mL pepsin in 5 % FA and a stock solution of 2 mg/mL pepsin in 0.3 M HCl was prepared approximately 1 hour before the digestion. The digestion was performed overnight with a total volume of 150 µL.

Optimization of pepsin digestion with different concentrations of formic acid

Optimization of FA concentration for pepsin digestion was utilized by diluting the stock solution of 2 mg/mL pepsin in 5 % FA to FA concentrations 5 %, 2 %, and 0.5 %. 135 µL 5 %, 2 %, and 0.2 % FA were added to each Eppendorf tube and, subsequently, 15 µL 2 mg/mL pepsin in 5 % FA was added to each sample for a total volume of 150 µL for the pepsination of HuBuChE.

3.5.4 Reusing of magnetic bead

After the pepsination of HuBuChE and the supernatant was transferred to autosampler vials, an elution buffer, 100 mM Citric Acid, was used to investigate reusing the bead-antibody complex. The used beads were cross-linked from the previous sample preparation. The beads were washed with 3x 200 µL 100 mM Citric acid. 200 µL PBST was added to the beads and stored at 4 ºC overnight. PBST was discarded, and a 500 µL serum sample was added and conducted with no denaturation and was pepsinated in 5 % FA.

3.6 Liquid chromatography

3.6.1 Examination of analytical columns

Investigation of the analytical columns were performed on an Ultimate 3000 RSCL HPLC (Dionex Corporation, Idstein Germany). Two mobile phase reservoirs are used in this method development and consist of three channels each; HPLC-grade water (A), an organic modifier (B), and an additive (C), which are displayed in table 3.7. The mobile phase reservoir is delivered by the analytical pump and enters the analytical column and is presented as the analytical mobile phase reservoir. For the analytical columns tested, the column dimension, chemicals, and manufacturer, are displayed in table 3.8.

Table 3.7. Mobile phase channels A, B, and C used in this thesis for both mobile phase reservoirs.

Mobile phase reservoir	
A	Milli-Q water (HPLC-grade)
B	Acetonitrile
C	2 % formic acid in Milli-Q water (HPLC-grade)

Table 3.8. Analytical and trap columns investigated in this thesis with their properties and manufacturer.

Column	Description	Manufacturer
C4 XBridge	2.1 x 100 mm column Particle size 3.5µm, 300 Å	Waters. Massachusetts, USA
C18 Gravity Nucleodur	2.0 x 100 mm column Particle size 1.8µm, 110 Å	Macherey-Nagel GmbH & Co. Dueren, Germany
C18 Acclaim	1.0x 150 mm column Particle size 3 µm, 120 Å	Dionex. California, USA
Trap column C18	2.0 x 10 mm column Particle size 1.8µm, 110 Å	Macherey-Nagel GmbH & Co. Dueren, Germany

3.6.2 LC-conditions for the C4 XBridge column, for optimization of the sample preparation

For the development and optimization of the sample preparation procedure, a C4 XBridge column (2.1x 100 mm) was used for the analyses of the BuChE target peptide. The method consisted of one pump, analytical pump, and the analytical mobile phase reservoir. The total run time was set to 23.5 minutes and performed with the LC-conditions displayed in table 3.9. For the analyses, 10 µL sample was injected.

Table 3.9 LC-parameters performed on C4 XBridge analytical column.

LC method condition				
Analytical pump				
Flow rate	200 µL/min			
Time	23.5 min			
Gradient	Time	A%	B%	C%
	0.0	90	5.0	5.0
	1.0	90	5.0	5.0
	11.0	5.0	90	5.0
	16.0	5.0	90	5.0
	16.5	90	5.0	5.0
	23.5	90	5.0	5.0

3.6.3 LC-conditions with column switching for the developed method

The optimized LC parameters for verification of the target peptide from pepsinated HuBuChE are displayed in table 3.10 and performed with the C18 trap column (2.0 x10 mm) and C18 Acclaim analytical column (1.0 x150 mm). A 10 ports dual switch was used in this method with the setup illustrated in figure 3.1, where eight of the ten ports are utilized. Two mobile phase reservoirs, delivered by the loading pump and the analytical pump, were used in this method. The mobile phase reservoir delivered by the loading pump contains the same solvents displayed in table 3.7. 20 µL sample was introduced for analysis with the developed method.

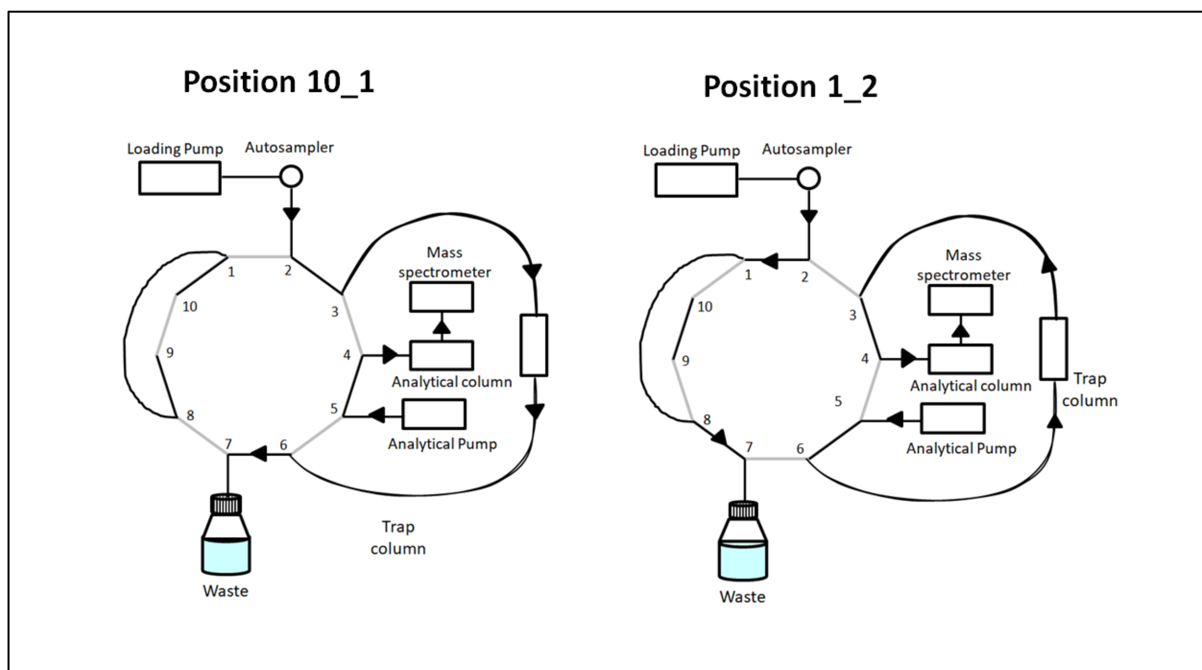


Figure 3.1. *Illustration of the setup of the column switching with a 10-ports used in this method. Loading pump is loading the analytes on the trap-column, and by switching from position 10_1 to 1_2, the analytical pump will by backflush in the trap-column introduce the analytes to the analytical column and the mass spectrometer.*

Table 3.10 Optimized LC parameters used with C18 trap column and C18 Acclaim analytical column

LC method condition				
Analytical pump				
Flow rate	50 µL/min			
Time	28.5 min			
Switch time	Time (min)	From	To	
	0.5	Loading	Analytical	
	17.0	Analytical	Loading	
Gradient	Time	A%	B%	C%
	0.0	90	5	2.5
	2.0	90	5	2.5
	12.0	5	90	2.5
	18.0	5	90	2.5
	18.5	90	5	2.5
	28.5	90	5	2.5
Loading Pump				
Flow rate	200 µL/min			
Isocratic	Time	A%	B%	C%
	0.0	95.5	2.0	2.5
	7.0	95.5	2.0	2.5
	7.5	7.5	90	2.5
	12.5	7.5	90	2.5
	13.0	95.5	2.0	2.5
	28.5	95.5	2.0	2.5

3.7 Mass Spectrometer parameters

The mass spectrometry used in this method was MicroTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany) with ESI operating in positive ion mode. The MS method performed on the analyses of BuChE target peptide are displayed in table 3.11.

Table 3.11 MS parameters used in this thesis before optimization

MS method conditions	
Ionization mode	Positive mode
Ion source ESI	
Capillary voltage	4500 V
Dry gas	6.0 L/min
Dry temperature	200 °C
Endplate offset	500 V
Nebulizer	1.5 Bar
Mass analyzer TOF	
Collision energy	10.0 eV
Collision RF	600 V
Transfer time	70 µs
Pre pulse storage time	10.0 s

4 | Results and Discussion

This chapter describes and discusses the results for the development of a method for the verification of nerve agents in human serum. The development of this method is primarily performed by the isolation of HuBuChE from serum, followed by enzymatic digestion and detection of the HuBuChE target peptide performed with LC-MS. A preliminary study on enzymatic digestion conditions and identification of target peptide was performed with BuChE from equine serum before the HuBuChE was analyzed. For the following section, preliminary studies and method development are presented and discussed.

The development of this qualitative method has almost been successfully done. However, because of instrumental problems with the MS followed by the COVID-19 international pandemic crisis, the laboratory work was ended in January 2020. Hence, the final part of the method development and method validation could not be performed. The importance of the parameters for the remaining method development and the qualitative validation is discussed later in this section.

4.1 Preliminary studies

Preliminary studies were performed with BuChE from equine serum to identify the BuChE target peptide, investigate the effect of denaturation of BuChE, and the choice of the enzyme for enzymatic digestion. The preliminary studies for this method examined whether trypsin or pepsin, and in which acid solution, gave the most optimal digestion of BuChE. Peak intensity of BuChE target peptide was also taken in consideration when deciding which enzyme to use and the effect of denaturation.

4.1.1 Determination of the enzyme for enzymatic digestion

For enzymatic digestion, trypsinization and pepsination of BuChE were tested to investigate which of them gave the most sensitive target peptide with LC-MS. Trypsinization of BuChE gives a target peptide containing 29 amino acids; SVTLFGES$_{198}$AGAASVSLHLLSPGSHSLFTR, where S$_{198}$ is the active serine site. Unmodified target peptide of BuChE digested with trypsin has a molecule weight (MW) of 2929.282. A nonapeptide, FGES$_{198}$AGAAS, is given by pepsination of BuChE. The unmodified target nonapeptide obtained from pepsination has MW of 796.3477. These target peptides are identical obtained by BuChE from equine serum and BuChE from human serum. The optimal enzyme activity for trypsin is at pH 7.8-8.7, and pepsin has an optimal enzymatic activity at pH 1.5-2.0 [54, 55]. Different solutions were necessary to maintain the enzyme activity and for optimization of the activity. For enzymatic digestion with pepsin, the solvent was 5 % FA. For trypsin, 5 % FA was switched out with 50 mM NH_4HCO_3.

Pepsination and trypsinization of 0.2 mg/mL BuChE were performed with no denaturation. The extracted ion chromatogram (EIC) of the pepsinated BuChE target peptide is presented in figure 4.1 and gave a peak with a retention time of 2.8 min. The trypsinated BuChE samples were investigated for the *m/z* 2199.17 (EIC not shown). The BuChE target peptide obtained from trypsinization was not detectable. The large BuChE target peptide may give charge distribution in the spectrum and lead to a lower intensity of the peptide due to the signal distribution. From these observations and considerations, enzymatic digestion of BuChE was performed with pepsin. The nonapeptide obtained from pepsin digestion is hereafter called the BuChE target peptide.

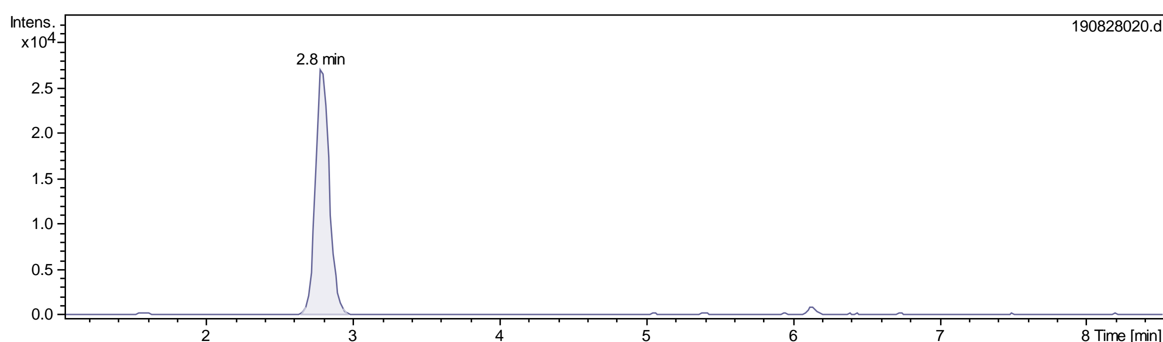


Figure 4.1. *EIC of target peptide (m/z 796.35) from pepsinated BuChE from equine serum. Experiment is performed on Water XBridge C4 analytical column.*

4.1.2 Examination of denaturation procedures and acid for enzymatic digestion

For enzymatic digestion with pepsin, pepsination in 0.5 M HCl and 5 % FA were investigated for the effect of the acids on the yield of HuBuChE target peptides. The concentration of HCl was chosen to give a pH value of around 2. Denaturation of the protein changes its three-dimensional structure. An unfolded protein is generally easier to digest. Therefore, denaturation with Guanidine.HCl was investigated and compared to the sample preparation with no denaturation procedure prior to pepsin digestion in 0.5 M HCl and 5 % FA.

Denaturation with Guanidine.HCl and pepsination in different acids were analyzed together to determine if they influenced each other to obtain the highest yield of the BuChE target peptide. A graphical presentation of the average peak intensities from the EICs of the target peptide from pepsinated BuChE with the standard deviation (SD), is presented in figure 4.2. The yield of the target peptide from pepsination of BuChE in 5 % FA, was significantly higher than the yield of the target peptide from pepsination of BuChE in 0.5 M HCl. The target peptide from pepsination in 5 % FA with denaturation had a yield six times higher than the target peptide from pepsination of BuChE in HCl with denaturation. By comparing the samples with no denaturation, the yield of target peptide from pepsination in FA was 2-fold higher than the yield of target peptide from pepsination in HCl. There were no significant differences in the yield of the BuChE target peptide between samples that were denatured and not denatured when the enzymatic digestion was performed in 5 % FA. For the method development, pepsin digestion was performed in 5 % FA.

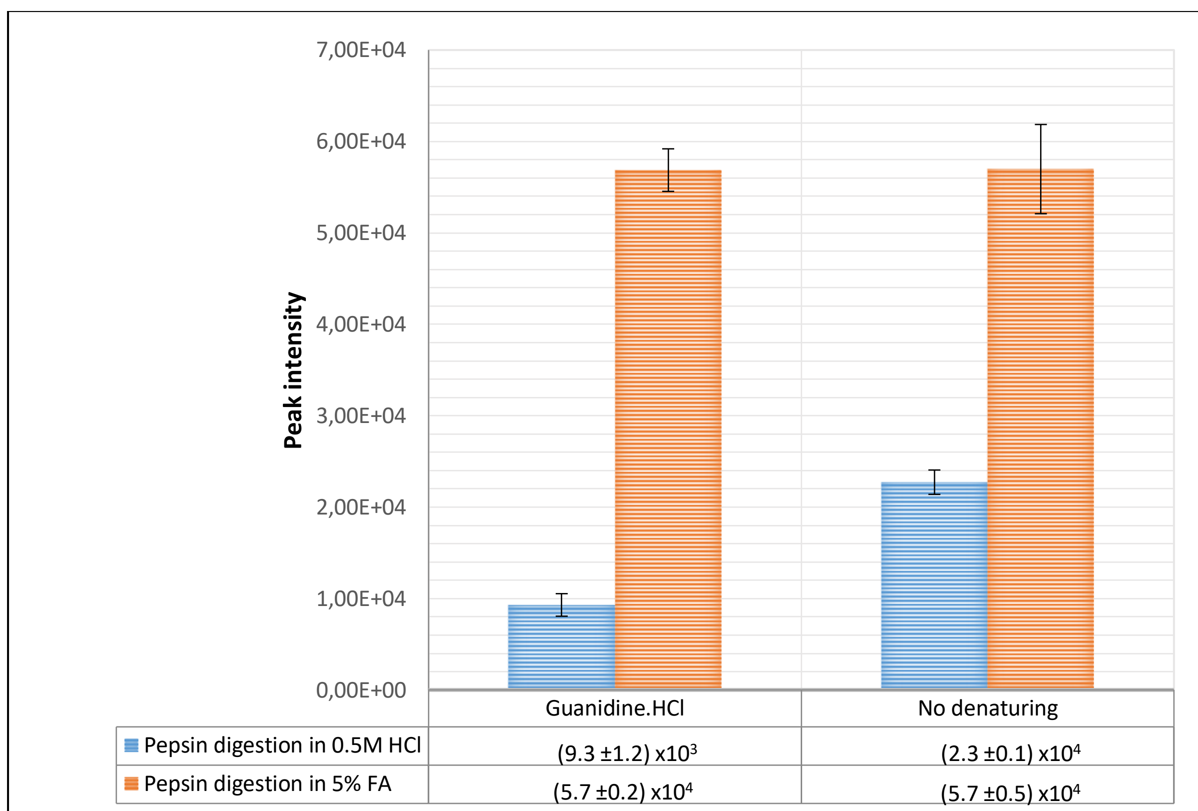


Figure 4.2. *A graphical presentation of the average peak intensity of BuChE target peptide (n=4) when pepsinated in 0.3M HCl (blue) and 5 % FA (orange) with Guanidine.HCl denaturation and no denaturation. Error bars are presented as the black line of each pole that represents the standard deviation of the parallels from each procedure.*

Denaturation with organic solvent was also examined to determine if it increased the yield of the target peptide from pepsination of BuChE in 5 % FA. The analyses of BuChE was performed with an organic solvent and with no denaturation procedure, where two parallels of each were analyzed. The analyses were investigated with 0.1 mg/mL of BuChE from equine serum. The peak intensity and peak area for the target peptide from pepsination of BuChE are displayed in table 4.1. Denaturation with organic denaturant gave no tendency in better signal for target peptide from pepsination of BuChE, than the target peptide from BuChE performed with no denaturation. The organic denaturant also gave a more substantial background noise in the chromatogram (BPC not shown). Since the organic denaturation did not affect the peak intensity of the target peptide from pepsination of BuChE, this process was unnecessary and time-consuming. For the optimized method, pepsination of BuChE in 5 % FA was used with no denaturation first.

Table 4.1 Peak intensity and area for HuBuChE target peptide with denaturing with organic solvent (n=2) and no denaturing (n=2) during the sample preparation

Nonapeptide BuChE	Intensity	Area
No denaturation	2.6×10^4	178 042
Denaturation with organic solvent	2.4×10^4	163 614

4.1.3 Identification of Butyrylcholinesterase target peptide

Identification of the BuChE target peptide was performed with LC-MS/MS to investigate the amino acid sequence in the peptide identified by the peak at 2.8 minutes for *m/z* 796.35 in the chromatogram. It was a prerequisite that the target peptide was unambiguously identified prior to sample preparation of HuBuChE from serum samples. The target peptide from pepsinated BuChE was analyzed. A simple MS/MS method was developed to only transfer the ions with 796.35 ±2 *m/z* units between 2.0 to 3.5 minutes through the quadrupole to the collision cell. The MS spectrum of the isolated target peptide from pepsination of BuChE is presented in figure 4.3 with amino acid sequence GAAS identified. Despite the poor fragmentation, the amino acid sequence G, A, A, and S are given with high mass accuracy and match the target peptide sequence. From these observations, the BuChE target peptide was identified with *m/z* 796.35 and with a retention time at 2.8 minutes carried out on the XBridge C4 column.

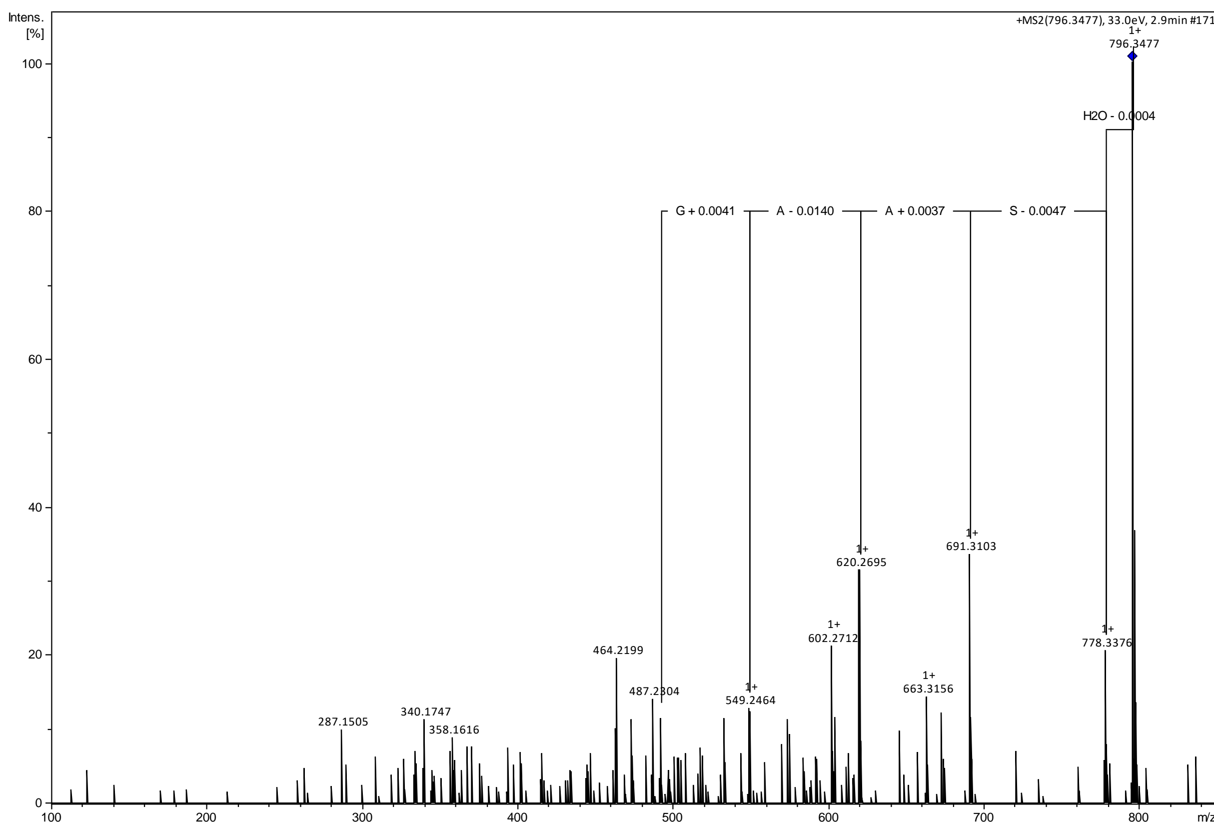


Figure 4.3. *MS spectrum of target peptide (m/z 796.35) from pepsinated BuChE from equine serum. Experiments were performed on ESI-qTOF. The amino acid sequence G, A, A, and S identify the nonapeptide FGES$_{198}$AGAAS presented with their accuracy of the mass.*

4.2 Isolation of Butyrylcholinesterase from human blood serum

Isolation of HuBuChE from blood serum was carried out by immunomagnetic beads conjugated to protein G with a high affinity for the anti-BuChE. The aim was to isolate HuBuChE from most of the other substances in the serum sample, prior to further sample preparation and LC-MS analyses. Cross-linking of bead-antibody complex was investigated to determine if it gave a higher yield of HuBuChE bound to the antibody-coated beads compared to the bead-antibody complex with no cross-linking. Cross-linking can improve the binding between the beads and the antibodies by making it more stable and last longer. Hence, the antibodies do not coelute with the target peptide or elute during the washing steps. Since the active site serine is not attached to the beads when covalently bonded, and the pepsination was conducted on the beads, the method could circumvent an elution buffer for elution of the peptides from the antibodies.

EICs of the target peptide from pepsinated HuBuChE isolated with anti-BuChE cross-linked to the beads and isolated with no cross-linking of anti-BuChE to the beads are presented in figure 4.4. Isolation of HuBuChE from serum with cross-linking gave significantly higher intensity of the target peptide obtained from pepsinated HuBuChE compared to the HuBuChE isolated without cross-linking of the antibody-beads complex. In isolation with cross-linking, the peak intensity of target peptide from pepsinated HuBuChE was $(4.86 \pm 0.02) \times 10^4$, and in isolation without cross-linking the peak intensity of target peptide from pepsinated HuBuChE was $(2.22 \pm 0.02) \times 10^4$. By cross-linking the antibody-bead complex, the peak intensity of the target peptide increased to the double. The binding of anti-BuChE to the beads was shown to be stronger when they have been cross-linked and, therefore, gave a higher yield of HuBuChE. The analysis of the negative control without antibody did not detect any target peptide from pepsinated HuBuChE, since no HuBuChE were attached to the beads without antibody. From these observations, the method with cross-linking of the antibody-bead complex was selected and performed with all the further samples analyzed.

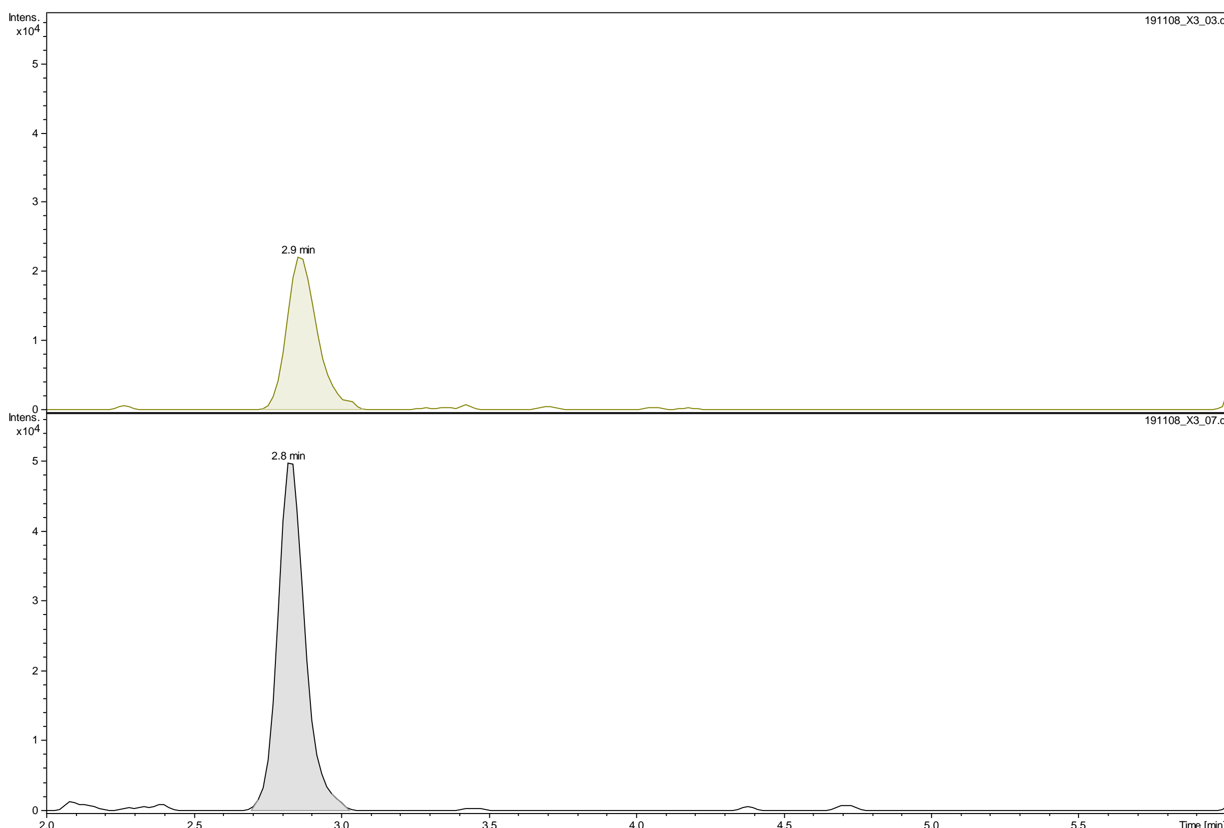


Figure 4.4. *EIC of the HuBuChE target peptide (m/z 796.34) from pepsination after immunomagnetic separation from serum. Experiments are performed with cross-linked anti-BuChE (lower chromatogram) and without cross-linking (upper chromatogram).*

The possibility to reuse the magnetic beads and the anti-HuBuChE were investigated. An elution buffer consisting of 100 mM citric acid was used for elution of the HuBuChE peptide attached to the beads. Reusing the anti-BuChE and magnetic beads would reduce the cost of the analyses. A concern was that cross-contamination or loss in the yield of HuBuChE due to reusing the antibody-bead complex could occur. The analyses of target peptide from pepsinated HuBuChE before and after reusing the beads with a fresh serum sample are presented in figure 4.5. The target peptide from pepsinated HuBuChE was not detected in the analyses after reusing the beads. Negative control samples containing the beads without a new serum sample added, but with pepsination in 5 % FA (EIC not shown), were also analyzed. The control samples did not detect any HuBuChE target peptide either. It is uncertain if the elution buffer breaks the bond in the antibody-bead complex, the antibody-HuBuChE complex, or none of them. Further analyses were not performed due to the time and the focus on the development of the method. Based on the observations, it was decided that the beads and antibody would not be reused.

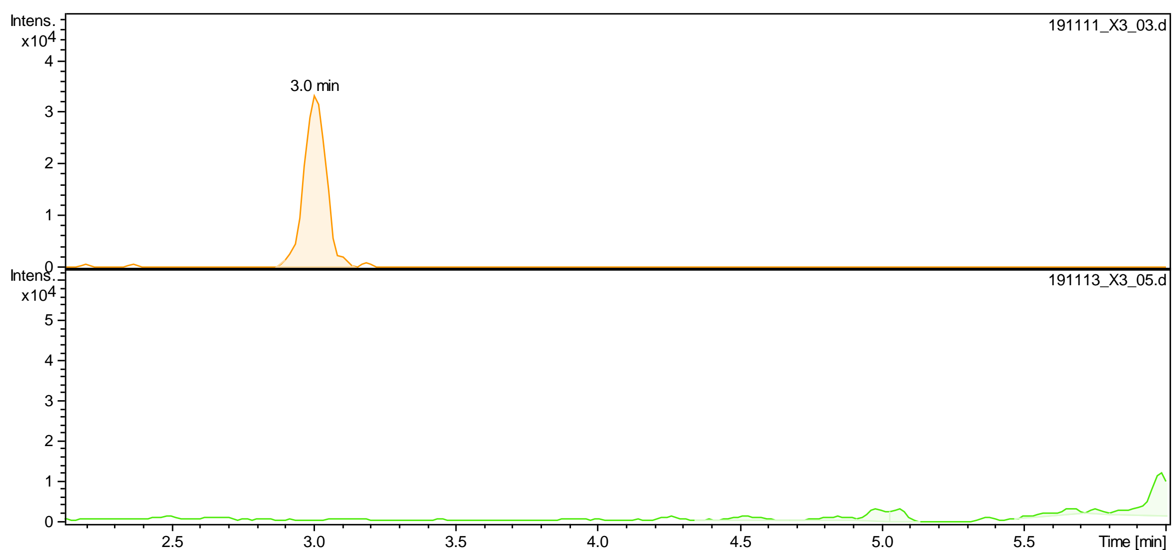


Figure 4.5. *EIC of the target peptide (m/z 796.34) from pepsinated HuBuChE after immunomagnetic separation from serum. Experiments are performed with crosslinking (upper chromatogram) and reusing of the same beads-antibody complex with new serum sample (lower chromatogram).*

4.3 Enzymatic digestion of Human Butyrylcholinesterase

Enzymatic digestion makes the proteins suitable for MS analysis by breaking the protein into analyzable peptides. Peptide analysis gives less charge distribution and will have a more efficient ionization in the ESI; hence, it gives a better signal-to-noise (S/N) of the target peptide in the chromatogram. This makes it easier to identify adduct on a peptide compared to adduct on a protein. Based on the preliminary studies, pepsination in FA was chosen for the enzymatic digestion of HuBuChE. The further optimization of the pepsination procedure was done by determining the FA concentration that gave a better yield of the target peptide of HuBuChE.

4.3.1 Optimization of the concentration of FA under pepsination of HuBuChE

From the preliminary studies, FA was preferred as the pepsination solvent for the digestion of BuChE. The digestion procedure was further optimized by investigating three different concentrations of FA for the pepsination of HuBuChE. Pepsin was solved in 5 % FA and diluted to 0.5 % and 2 % in prior for the pepsination of HuBuChE. The peak intensities and peak areas of the target peptide from pepsinated HuBuChE performed in 0.5 %, 2 %, and 5 % FA are displayed in table 4.2. There was no tendency of difference in the measured yield of target peptide from pepsinated HuBuChE in the different FA concentrations. The FA concentration will, at one point, give a decreasing effect of the yield of target peptide for the pepsinated HuBuChE. The pepsin activity is pH dependent and, therefore, with a given pH value, it would be inactive or reduced in activity that will reduce the yield of the target peptide [56]. Pepsination in 2 % FA will ensure that the concentration is not at an endpoint. Therefore, the choice of FA concentration for enzymatic digestion was 2 % FA for this method.

Table 4.2. Peak intensity and area from LC-MS determination of the HuBuChE target peptide when pepsin digested in different FA concentrations. The table shows the mean values of two parallels from each concentration.

FA concentration	Intensity	Area
5 % FA	1.9 x10^4	130 600
2 % FA	1.7 x10^4	115 454
0.5 % FA	1.8 x10^4	133 947

4.4 Development and optimization of the LC-method

A good chromatographic analysis will achieve separation of the target peptide from pepsinated HuBuChE from the unretained solvent front and the larger peptides with longer retention times. By achieving this, possible ion suppression can be avoided. The steps in developing the LC-method was first to determine the analytical separation column, prior to optimization of the mobile phase compositions and gradient elution. Subsequently, optimization of the switch time and the injection volume was investigated.

4.4.1 Determination of analytical separation column

In order to enhance the peak intensity of the target peptide from pepsinated HuBuChE, the LC method was optimized by examination of the analytical column. Smaller particles in the stationary phase and smaller ID are expected to give narrow peaks with a better separation and higher signal of the HuBuChE target peptide if the injection volume remains the same. Table 3.8 displays an overview of the columns investigated in this thesis, with column information. The C4 XBridge (2.1 x100 mm) column from Waters was used for the screening process and the development of the sample preparation. This column was selected to achieve a robust and straightforward LC-MS method for the optimization of the sample preparation.

For the introduction of the C18 trap-column to the method, the C4 XBridge column was replaced by the C18 Gravity Nucleodur. The replacement of the C4 XBridge with a C18 column generally gives a higher retention time of the target peptide. The stationary phase in the trap-column and the analytical column are generally the same, since the importance of achieving a reasonable retention in the trap-column is equally important as in the analytical column. If the retention of the target peptide is not equally good in the trap-column, it will give a more extensive peak broadening, and the loss of the target peptide can occur. The ID of the analytical column remained the same (2.0 mm). The signal intensity of the BuChE target peptide performed on the C4 XBridge and the C18 Gravity Nucleodur was investigated. The pepsination of BuChE from equine serum was used. EICs of the target peptide from pepsinated BuChE performed on the C18 Gravity Nucleodur column, and the C4 XBridge column are presented in figure 4.6. As predicted, the C18 Gravity Nucleodur gave a better yield of target peptide from pepsinated BuChE.

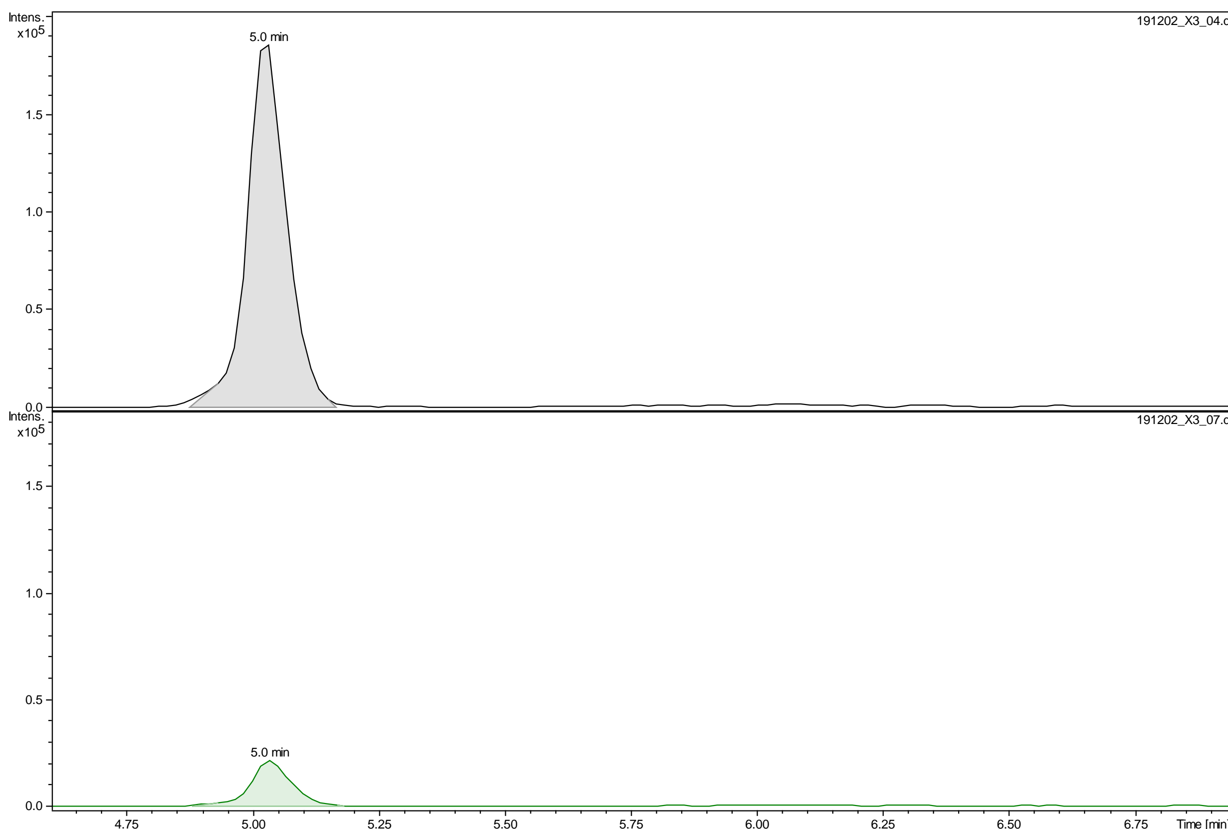


Figure 4.6. *EICs of target peptide (m/z 796.35) from pepsinated BuChE from equine serum. Experiment without switch technique performed on C18 Gravity Nucleodur 2.0x 100 mm column (upper chromatogram) and performed on C4 XBridge 2.0 x100 mm column (lower chromatogram).*

The reduction of the ID in the analytical column was investigated for the determination of the impact on the peak intensity of the target peptide from pepsinated HuBuChE. Based on theoretical values, reducing the ID will increase intensity when the same injection volume is introduced. Since MS is concentration-dependent, the maximum peak height is related to the maximum concentration in the detector. The effect of reducing the transection of the column can be illustrated by equation 2. The transection of the columns is investigated, and therefore, the length h can be excluded. If the flow volume in the columns reduces with the same factor as given in equation 2, the peak intensity of the HuBuChE target peptide in the chromatogram will theoretically be identical.

$$V = \pi r^2 h \tag{2}$$

Comparing the two columns, when the injection volume and stationary phase are equal, the peak intensity of the target peptide will increase with a factor of four. The linear flow will theoretically reduce with a factor four when the ID reduces from 2 to 1 mm. The analyses of the same samples of pepsinated HuBuChE were performed on the C18 Gravity Nucleodur (2x 100 mm) and the C18 Acclaim (1x 150 mm) column with the switching technique. The retention time of the target peptide from pepsinated HuBuChE was expected to increase by the introduction of the trap column. The flow rate was reduced from 200 µL/min to 50 µL/min when the analyses were performed on the C18 Acclaim column. EICs of the peptide analyzed on both the C18 Gravity Nucleodur and the C18 Acclaim column are presented in figure 4.7. The peak intensity of the target peptide from pepsinated HuBuChE increased by the reduction of the ID of the analytical column. The target peptide from pepsinated HuBuChE had a peak intensity of 1.2×10^4 and 1.1×10^5 for C18 Gravity Nucleodur and C18 Acclaim, respectively, when analyzing the same sample. The switching technique made it possible to inject the same volume of the sample without increasing the peak width of the target peptide from pepsinated HuBuChE.

The peak intensity of the target peptide increased not only with a 4-fold, but 10-fold. The 10-fold increase in peak intensity can be explained by a better separation of the target peptide from the other peptides in the samples which may have given less ion suppression and, therefore, better peak intensity in the chromatogram. The retention times of the HuBuChE target peptide are presented in figure 4.7 with a retention time of 5.45 and 10.42 minutes when performed on the C18 Gravity Nucleodur and the C18 Acclaim, respectively. For the retention time at 10.42 minutes, the mobile phase may have a higher amount of ACN when the target peptide elutes compared to the performance on the C18 Gravity Nucleodur. In general, a higher amount of ACN in the mobile phase will give a better ionization yield of the target peptide in the ESI that will result in higher peak intensity of the target peptide in the chromatogram or MS spectra.

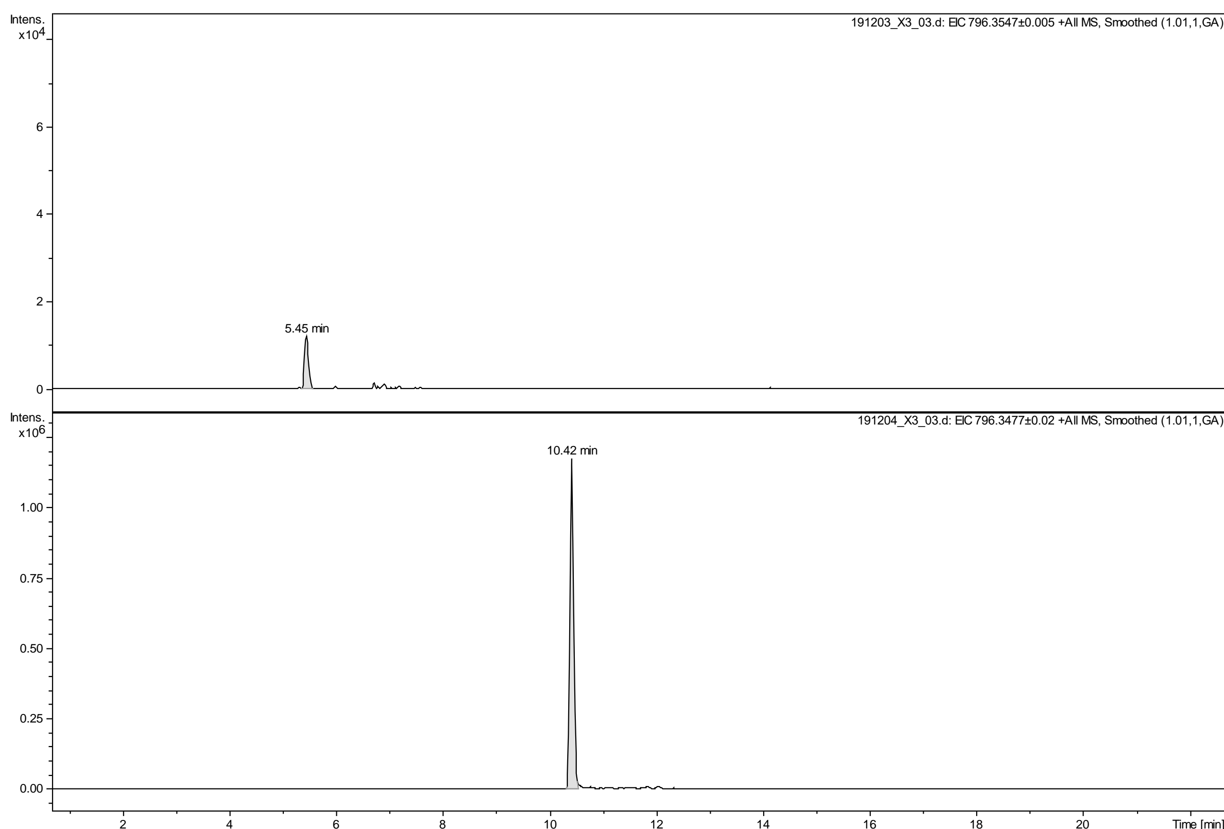


Figure 4.7. *EIC of the target peptide (m/z 796.35) from pepsinated BuChE from equine serum. Experiments of the same sample performed on C18 Gravity Nucleodur ID 2mm (upper chromatogram) and performed on C18 Acclaim ID 1mm (lower chromatogram). Both experiments were performed with the switch technique.*

4.4.2 Optimization of gradient elution

The mobile phase composition is an essential factor for separation and for achieving a high intensity of the target peptide analyzed with LC-MS. For optimization of the mobile phase, the organic solvent and additives must be compatible with MS and containing few non-volatile salts that suppress the ionization of the target peptide. ACN and FA are both suitable and compatible with ESI-MS and are used for this method. The optimal concentration of ACN and FA in the mobile phase was investigated. The optimization of the mobile phase aimed to achieve a high peak intensity of target peptide from pepsinated HuBuChE by obtaining lower background noise in the chromatogram and minimize the co-elution of substances with the target peptide. Since the sample preparation is the most time-consuming step, the time of the LC-MS method will not influence the total analysis time.

For evaluating the ACN concentration, the start concentration and the gradient time up to 90 % ACN was investigated. Analyses of target peptide from pepsinated HuBuChE were performed with start concentrations of 2.5 %, 5.0 %, and 10 % ACN with different gradients. The gradients (per min) to a final concentration of 90 % ACN are plotted against the peak intensity of the target peptide obtained from analyses with different start concentrations of ACN and are graphically presented in figure 4.8. The highest peak intensity of the target peptide from pepsinated HuBuChE was conducted with a start concentration of 5 % ACN with a gradient of 10 minutes. If a shorter gradient time were requested, the start concentration of choice would be 10 % ACN due to the highest intensity of the target peptide for the faster gradients. The peak intensity of the target peptide conducted with the start concentration of 2.5 % ACN gave a mainly lower intensity compared to the other start concentrations with the same gradient. This can be explained with a lower ACN in the ESI that will reduce the ionization of the target peptide from pepsinated HuBuChE. The optimal method was performed with 5 % ACN start concentration with a 10 minutes gradient, in which the target peptide from pepsinated HuBuChE had a peak intensity of 6.45 x10^4.

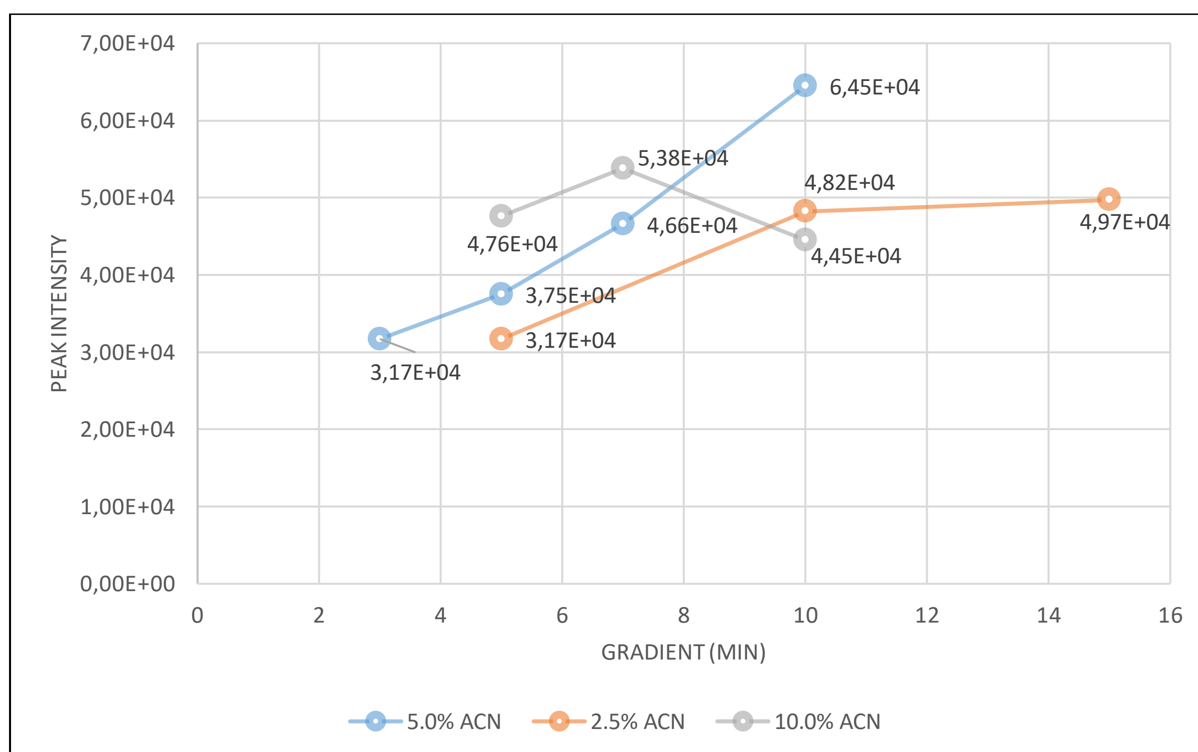


***Figure 4.8.** A graphical presentation of the yield of target peptide (m/z 796.35) from pepsination of HuBuChE after immunomagnetic separation from serum. Experiments were performed with different start concentrations of ACN with the time of gradient to 90 % ACN. ACN start concentrations 2.5 %, 5.0 %, and 10.0 % are given as orange, blue, and grey lines in the graph.*

As previously mentioned, the high amount of ACN in the ESI will improve the ionization and resulting in increasing the yield of the target peptide. This can explain the highest peak intensity of the target peptide was taken out with a mobile phase containing 5 % ACN with a gradient of 10 minutes. The retention time of the target peptide carried out by the different ACN concentration and gradients are presented in figure 4.9. The retention time variation due to the ACN concentrations and the gradient indicates the importance of examination the ACN concentration in the mobile phase. The higher amount of ACN gives a higher ionization in ESI, hence, higher peak intensity of the target peptide. The probability of higher coelution occurs due to the short retention time of the target peptide delivered by the 10 % ACN with gradient 5 and 7 minutes. This can be explained by the increased mobile phase strength. Other peptides will suppress the target peptide and decrease the peak intensity, despite the higher amount of ACN in the ESI when the target peptide is being ionized. Since the sample preparation is the time-consuming part, the ACN concentration chosen was 5 % start concentration with a gradient from 5-90 % ACN over 10 minutes.

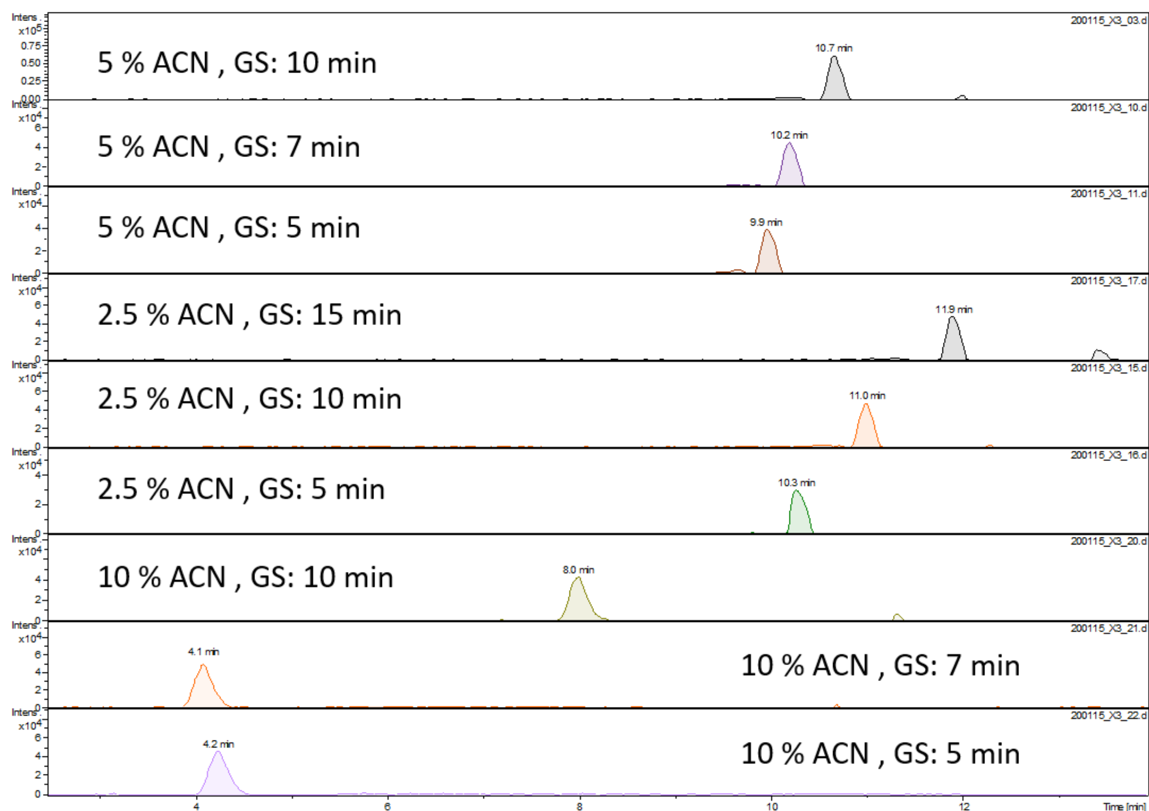


Figure 4.9. *EIC of target peptide (m/z 796.35) from pepsination of HuBuChE from immunomagnetic separation from serum. Experiments performed with different start concentrations and gradients. GS is the gradient time from start concentration to 90 %. The retention time for the different ACN concentration and gradient are presented.*

The concentration of ACN in the mobile phase delivered by the loading pump and the analytical pump may differ. However, it is important to have less or the same ACN concentration in the loading mobile phase compared to the analytical mobile phase since the ACN in the trap-column will enter the analytical column. A higher ACN concentration in the mobile phase delivered by the loading pump will give a poor focus of the analyte at the start of the gradient in the analytical column and give a broad peak. The optimization of the ACN delivered by the loading pump was investigated together with the examination of the switching time. By observing the effect of ACN on the retention time of the HuBuChE target peptide, the analyses were performed with 2.5 % ACN delivered by the loading pump before further optimization.

After optimization of the gradient, the concentration for the additive, FA, was evaluated. FA improves the chromatographic peak shape of peptides by reacting as an ion-pair reagent and provide an acidic environment in the mobile phase. In the acidic environment, the free silanol-groups in the stationary phase will protonate which would otherwise interact with the protonated amino acid- groups on the peptide and lead to peak broadening. As ion-pair reagents, FA will prevent too strong interactions between the free silanol-groups in the stationary phase and the peptide. The concentrations 0.05 %, 0.1 %, and 0.2 % of FA in the mobile phase were investigated with the optimized ACN gradient. The aim was to determine the optimal FA concentration to achieve the highest yield of HuBuChE target peptide. The peak intensities of the target peptide from pepsinated HuBuChE carried out with 0.05 %, 0.1 %, and 0.2 % FA in the mobile phase are graphically presented in figure 4.10. The analyses performed with 0.05 % FA gave the highest peak intensity of HuBuChE target peptide. There was observed a decreasing peak intensity of the target peptide by the increasing FA concentration. From these observations, 0.05 % FA in the mobile phase was chosen for the optimized method.

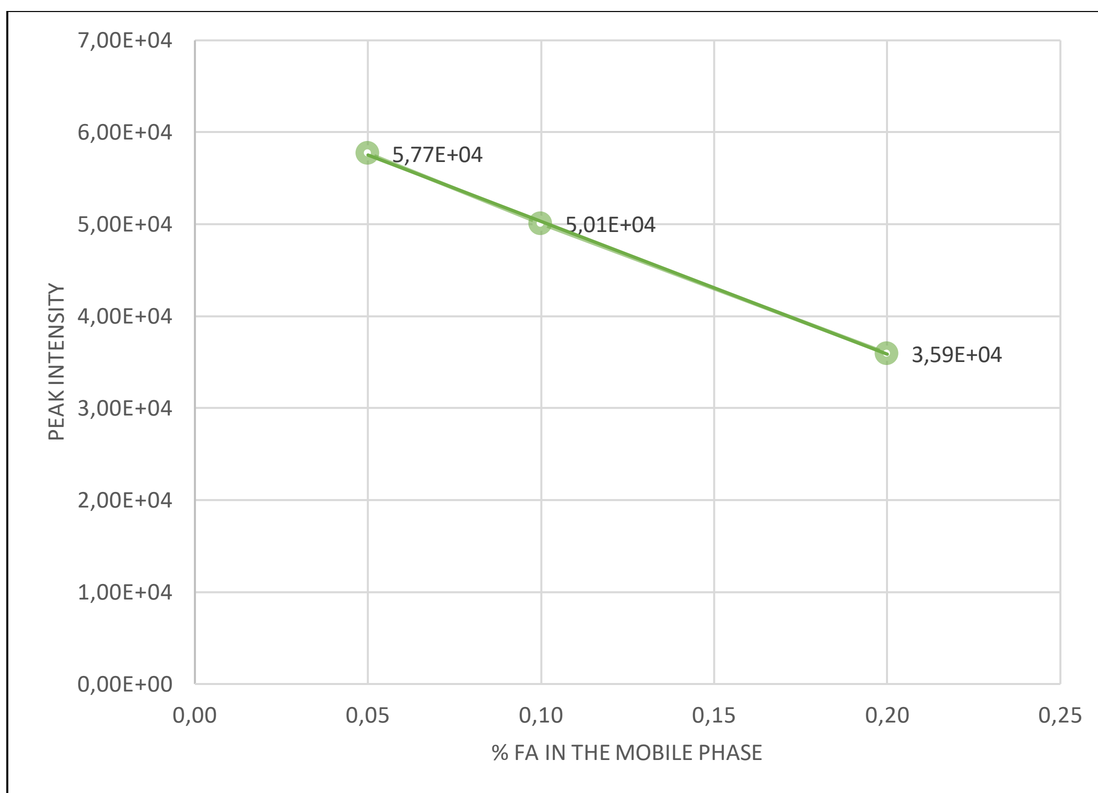


***Figure 4.10. A** graphical presentation of the average peak intensity of target peptide (m/z 796.35) from pepsinated HuBuChE after immunomagnetic separation from serum. Experiments are performed with 0.05 %, 0.1 %, and 0.2 % FA delivered by the analytical pump (n=2).*

4.4.3 Optimization of the column switching

To guard the analytical column and for loading the sample on the trap column, the time of the switch technique is essential. Since there is no MW cut-off filtration during the sample preparation, the column switching aimed to exclude the large peptides and salt to enter the analytical column. Salts are generally the first molecules that will elute from the column, and the large peptides will normally elute from the column at a longer retention time. Two switching times were determined; switching from loading to analytical position and then back to loading position again.

The aim of the switching procedure from loading to the analytical position was to concentrate the HuBuChE target peptide in the trap-column before entering the analytical column and remove salts prior to the separation. Three different switch times were tested; 0.25, 0.5, and 1.0 minute. The EICs of target peptide from pepsinated HuBuChE analyzed with the different switch times are presented in figure 4.11 (EIC (a), (b), and (c)). The peak intensity of HuBuChE target peptide was 1.7 x10^4, 6.4 x10^4, and 0 for loading time 0.25, 0.5, and 1.0 min, respectively, with a mobile phase consisting of 2.5 % ACN and 0.05 % FA. The significant highest peak intensity was the HuBuChE target peptide analyzed with a loading time of 0.5 min.

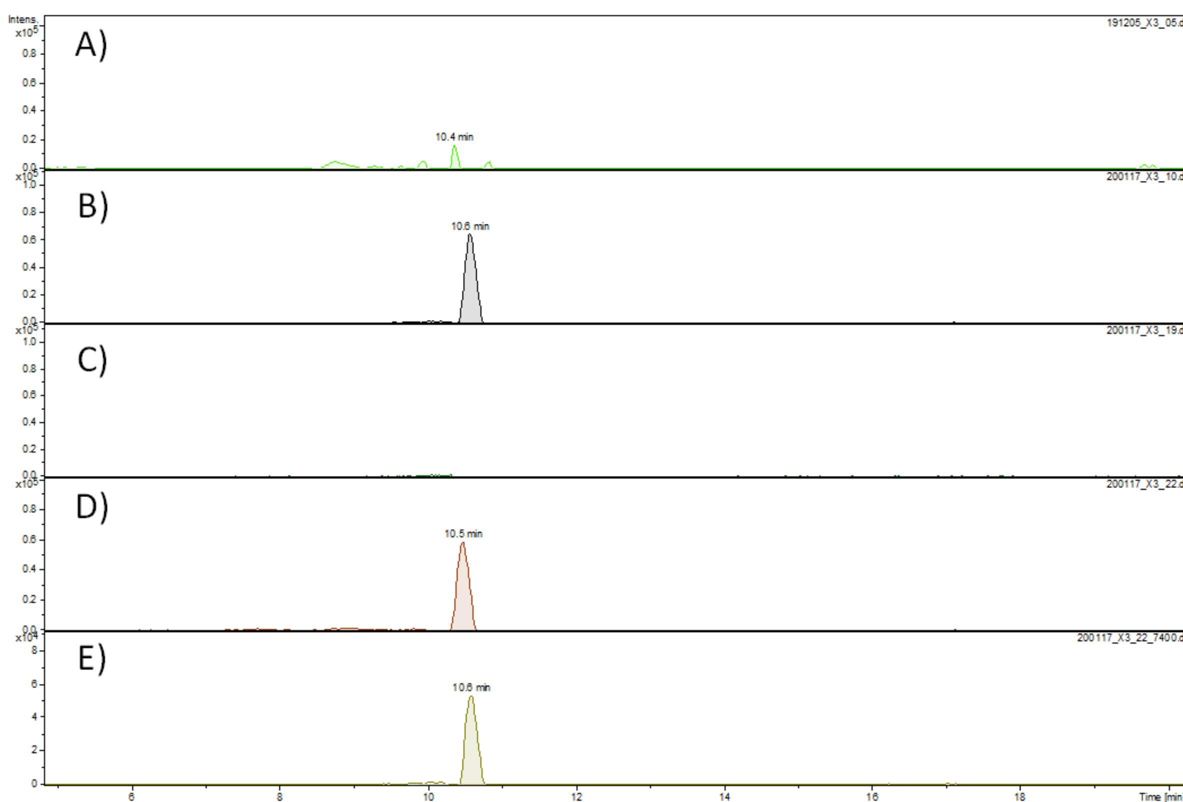


Figure 4.11. *EIC of target peptide (m/z 796.35) from pepsinated HuBuChE after immunomagnetic separation from serum. Experiments are performed with different loading time with MP 2.5% ACN and 0.05 % FA. (a) Loading time 0.25 minute (b) loading time 0.5 minute (c) loading 1.0 minute. (d) and (e) is respectively loading time 0.5 and 1.0 minute with MP 1.5% ACN and 0.05% FA.*

The concentration of ACN delivered by the loading pump, is an essential factor in ensuring that all of the amount of HuBuChE target peptide enters the analytical column. The amount of ACN in the trap column can reduce the peak broadening and change the retention time on the column. For determination of the robustness of the loading time, the loading time 0.5 and 1.0 minute with a mobile phase consisting of 1.5 % ACN, and 0.05 % FA was investigated. The EICs of these analyses are presented in figure 4.11 (EIC (d) and (e)). The peak intensities of the target peptide from pepsinated HuBuChE were 5.8 $x10^4$ and 5.3 $x10^4$ for loading time 0.5 and 1.0 minutes, respectively, and gives no tendency in the peak intensity when the ACN concentration decreases. As presented in figure 4.11, the target peptide occurs in the EIC of the sample with a loading time of 1.0 minute when ACN concentration is 1.5 % and not 2.5 %. For the switch time after 0.5 minute, the HuBuChE target peptide still occurs in the analyses. The mobile phase delivered by the loading pump was chosen to be 2 % ACN to ensure that the peptide will be detected. Therefore, switching at 0.5 minutes from the loading to the analytical position with 2 % ACN concentration in the mobile phase delivered by the loading pump was used for this method.

The second step was to determine the switching time from the analytical position to the loading position after the analyte has entered the analytical column. By determination of the optimal switching time, the method can exclude the large peptides that have a retention time after the HuBuChE target peptide from entering the analytical column. The first analyses using the switch technique was performed with switching at 17.0 minutes. Analyses of HuBuChE target peptide was performed with switch times between 17.0 and 1.0 minute. Base peak chromatogram (BPC) for switching at 17.0, 8.0, and 4.0 minutes are presented in figure 4.12. A decrease was observed in peaks with retention time longer than 15 minutes when switching time was changed from 17.0 to 8.0 minutes. A further decrease was observed between switching at 8.0 compared to 4.0 minutes. Switching back to the loading position earlier than 4.0 minutes, increased the probability of losing the target peptide. The peak intensity of the target peptide from pepsinated HuBuChE gave no tendency of difference between switching at 4.0 and 17.0 minutes. The conclusion was, therefore, to switch after 4.0 minutes from the analytical position to the loading position.

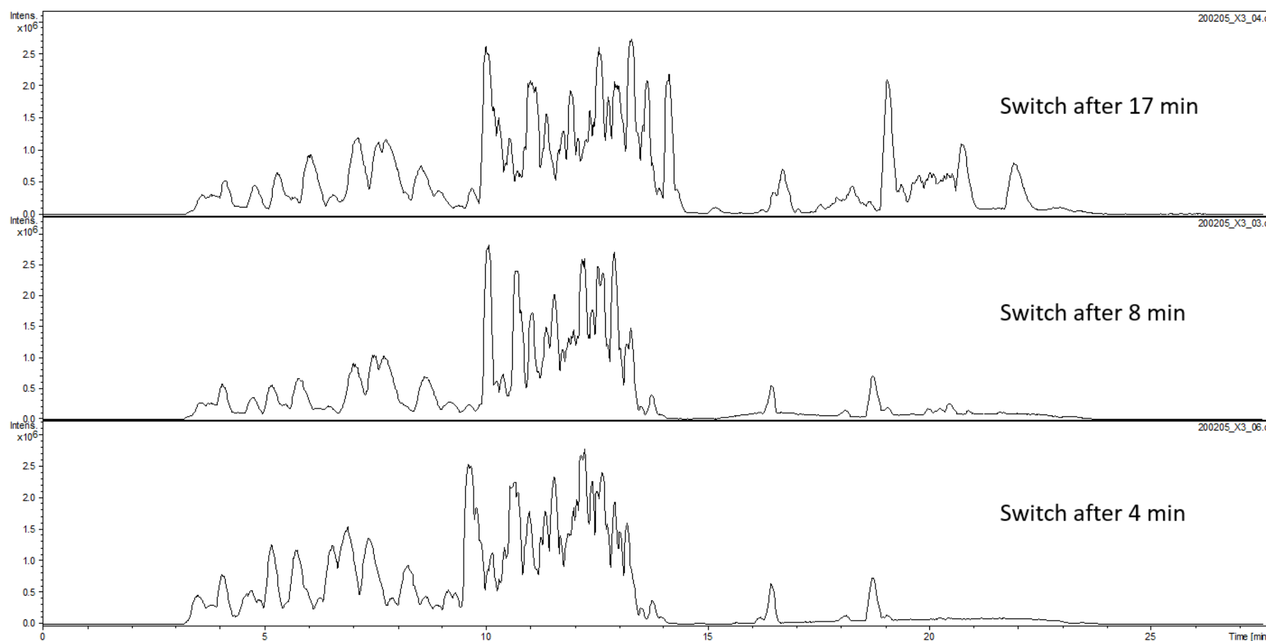


Figure 4.12. *BPC of analyses of pepsinated HuBuChE after immunomagnetic separation from serum. Experiments are performed with switching to the loading position at 17 min, 8 minutes and 4 minutes and are given in the upper, middle and lower BPC, respectively.*

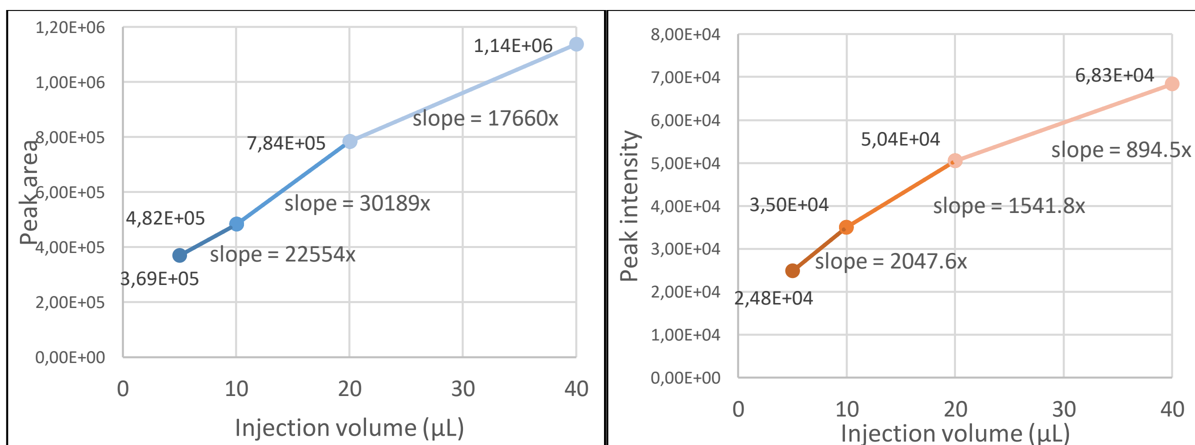
A gradient of ACN was made for the mobile phase delivered by the loading pump, due to the switching at 4 minutes from analytical to loading positions. During the analyses, the gradient delivered by the loading pump was performed to make a washing step for the trap column. This ACN gradient was performed from 2 % to 90 % in the trap column and held for 5 minutes before entering the start composition of the mobile phase. The additive concentration remained the same during the whole analysis. A higher flow could be employed by using the mobile phase delivered from the loading pump for washing, by changing from 50 µL/min to 200 µL/min, and therefore a better clean-up.

4.4.4 Optimization of injection volume

The injection volume is an important factor for the peak intensity and peak broadening of the target peptide. By double the injection volume, the peak area and peak intensity of the target peptide will ideally double as a linear function. At one point, the intensity and the area will not have a linear relationship with the volume injected. By this function, the peak of the target peptide will increase in width and not in intensity. Injection volume 5 µL, 10 µL, 20 µL, and 40 µL were investigated. The aim was to find the injection volume that gives the highest peak

intensity of the target peptide from pepsinated HuBuChE as possible and still maintain a narrow peak shape. The analyses of HuBuChE target peptide with different injection volumes were performed on the C18 Acclaim column with an optimized switching technique. The peak intensity and the area were plotted against the injection volume and presented in figure 4.13. The slope did not increase by 1 as hoped but is still useful for the determination of injection volume for both peak area and peak intensity. For the peak area and the peak intensity of the target peptide from pepsinated HuBuChE, the slope between 10 µL and 20 µL gave the highest value, and from 20 µL to 40 µL, the slope decreased with a factor 2.

Between 20 µL and 40 µL, the peak intensity and peak area of the target peptide tend to have a less increasing factor. The full peak width at half maximum (FWHM) for the target peptide, was 0.2 minute for injection volume 5 µL, 10 µL and 20 µL, but for 40 µL, the FWHM was 0.3 minute. The FWHM shows that the peak broadens by injecting 40 µL sample. The analyses of HuBuChE target peptide with an injection volume of 40 µL shows a tendency of a higher noise eluting with retention time earlier compared to the HuBuChE target peptide from the analyses with the other injection volumes. The higher noise eluting before the target peptide will reduce the S/N-ratio and reduce the HuBuChE target peptide sensitivity. Injecting less sample volume, it will strengthen the instrument. By these observations, the optimal injection volume for this method was 20 µL.



Figures 4.13 *Two graphical presentations of the peak area (blue line) and peak intensity (orange line) of target peptide (m/z 796.35) from pepsinated HuBuChE from immunomagnetic separation from serum. The peak areas and peak intensities are plotted as a function of the injection volumes 5 µL, 10 µL, 20 µL and 40 µL.*

4.5 Examination of VX and cyclosarin exposure

The importance of the HuBuChE target peptide coeluting with minimal peptides and the reduction in ion suppression for achieving a high peak intensity has been described in the previous sections. The avoidance of co-elution and ion suppression is also essential for the detection of nerve agent adducts that will occur during a nerve agent exposure. The yield of nerve agent adducts of HuBuChE target peptide and their retention time were examined. This was a preliminary test to check if the method was also suitable for the target peptide with nerve agent adducts attached and to determine its retention time range with these adducts. One serum sample was incubated with 50 ng/mL VX, and one serum sample was incubated with 50 ng/mL cyclosarin and aliquoted to two parallels each. Incubation with 50 ng/mL nerve agents will, according to literature, give a 100 % inhibition of HuBuChE [32]. The analyses of nerve agent exposed serum samples were performed on the C18 Acclaim column, and the C18 trap column before the optimization of the LC method was investigated. According to Sporty et al. [23], the nerve agent adducts on HuBuChE target peptide have a retention time between the retention time of VX- adducts and GF-adducts for the most common nerve agents. The *m/z* values for the VX- and GF- adducts, and the MW of the nerve agents are displayed in table 4.3.

Table 4.3. *m/z* values for $[M+H]^+$ of unexposed HuBuChE target peptide and NA-adducts of HuBuChE target peptide, represented together with molecular weight of the nerve agents.

Analyte	Molecule weight of nerve agent	Precursor ion for target peptide with NA-adduct and without NA-adduct $[M+H]^+$
HuBuChE	-	796.3477
VX-HuBuChE	267.4	902.3655
GF-HuBuChE	180.2	956.4052

The investigation of the nerve agent-adducts of HuBuChE target peptides is based on the values in table 4.3. The EICs of the unexposed and exposed HuBuChE target peptides with VX and GF are presented in figure 4.14. The nerve agents exposed serum sample was investigated wherever the unexposed target peptide from pepsinated HuBuChE was present, but it was not detectable. Since the unexposed HuBuChE target peptide was not detected, there was a 100 % inhibition of HuBuChE when exposed to 50 ng/mL nerve agent. The yield of VX-HuBuChE target peptide and GF-HuBuChE target peptide given in intensity of the peak were 7.69 x10^4 and 1.00 x10^5, respectively. The inhibition of HuBuChE can easily be reduced to 10 %

inhibition and still be detectable. The S/N ratios of the peaks of the target peptides of VX-HuBuChE and GF-HuBuChE were 822 and 1756, respectively. The observations of the S/N ratio for the nerve agent-adducts of the target nonapeptide indicate that 1 % inhibition might be detectable with an optimized method. This indicates that the method is sensitive to its purposes.

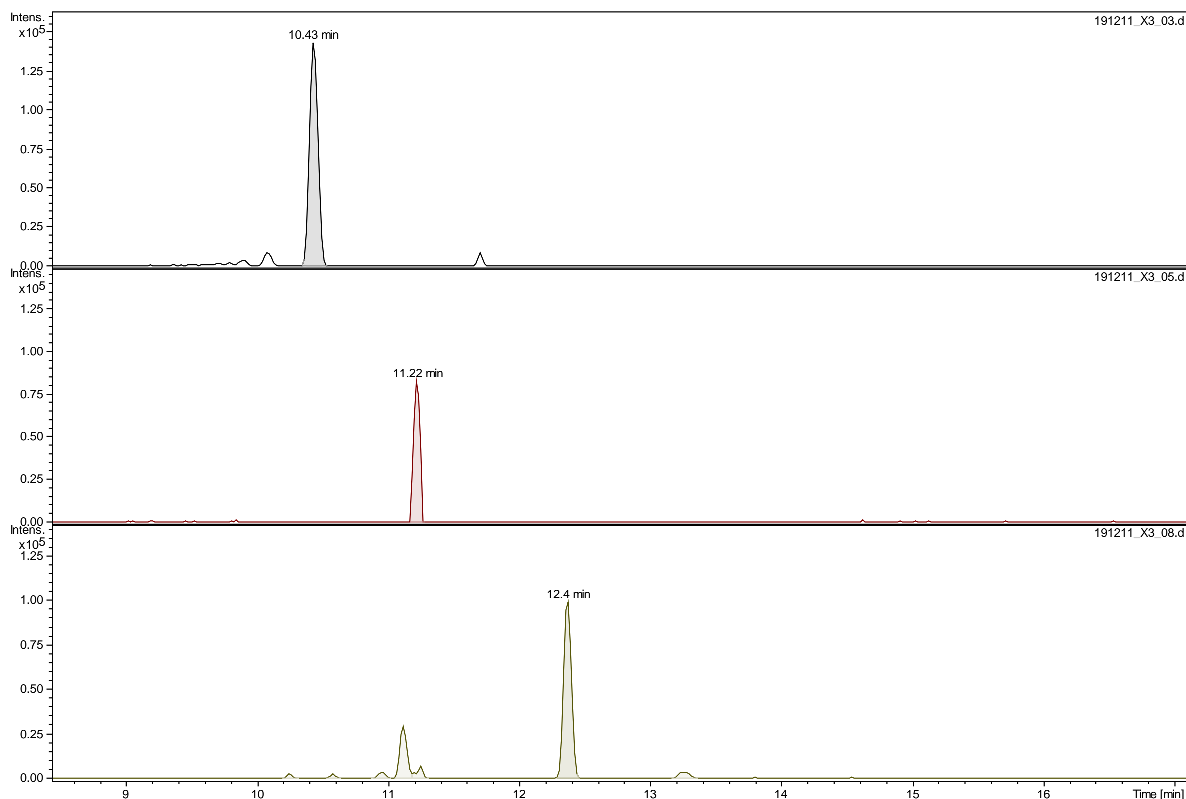


Figure 4.14. *EIC of target peptide (m/z 796.35) and NA-adducts of VX and GF attached to target peptide (m/z 902.36 and m/z 956.40) from pepsinated HuBuChE after immunomagnetic separation from serum. Experiments are performed with unexposed target peptide (upper chromatogram), and 100% inhibition of VX-adducts (middle chromatogram) and GF-adducts (lower chromatogram).*

When nerve agent adducts is bound to either AChE or BuChE, it can undergo a degradation over time called aging. Aging is a dealkylation process of the nerve agent-adduct by hydrolyzation [11]. The aged adduct has a different *m/z* value and can differ somewhat from the retention time of the nerve agent adducts of the BuChE target peptide. For analyzing serum samples from suspected exposures, these aging adducts will also be essential to determine, as they still indicate nerve agent exposure. The analyzed adducts attached to HuBuChE are still stable, since a reactivation cannot occur anymore. Reactivation is the release of adduct from the HuBuChE target peptide.

4.6 Mass Spectrometry Parameters

As a result of the COVID-19 situation and the instrumental problem before the virus outbreak, optimization of MS parameters, development of MS/MS method, and the validation were not able to be accomplished. Therefore, these sections only describe the theory and purpose with these parameters.

Optimization of the MS parameters is essential for the yield of detection of the target peptide from pepsinated HuBuChE, where the ion source and mass analyzer parameters are of interest. The parameters in the ion source determine the ionization of the HuBuChE target peptide. The ionization is equivalent to the yield of the target peptide that will reach the MS inlet and be detected. For single MS mode in the qTOF, the quadrupole operates as an ion guide without DC voltage (0V), and the separation of the ions is carried out only in the TOF for the development and optimization for the sample preparation and the LC-method. An MS/MS analysis was used in the preliminary study for the identification of the peptide. For optimization of the MS parameters, it is preferable to investigate the ESI parameters prior to the TOF parameters.

4.6.1 ESI ion source parameters

The ion source parameters determine the degree of ionization of the analytes in the liquid flow entering the MS. The optimal parameters differ from ion source to ion source due to variations in the construction of the ESI sources, such as the angle and placement of the nebulizer relative to the MS inlet. The ESI parameters are the capillary voltage, endplate offset, pressure of the nebulizing gas, the dry gas flow rate, and the dry gas temperature. The manufacturer of the MS generally has a list of default parameters dependent on the liquid flow rate entering the ESI, which can be used as a guide for optimization. These standard settings are not specified for one type of analyte, and the optimal parameters can vary from peptides from biological samples to small molecules with a low mass. Therefore, the yield of detected analytes is dependent on these parameters. For positive ionization mode, the capillary voltage is typically 4 kV larger than the end plate offset. For negative ionization, the voltage difference is reversed. These voltages decide the attraction for the ions to the MS inlet and the droplet formation of the liquid. If the surface tension is too low, it may not occur a Taylor cone that formats the liquid to droplets.

The nebulizer is a vital factor for the spray production of the liquid and a steady ion stream from the chromatographic outlet to the MS inlet. When the fine spray and the steady ion stream to the MS, reasonable sensitivity can be achieved. The dry gas flow rate and the dry gas temperature are dependent on each other and will both usually increase with increasing flow rate on the liquid entering the ESI. The dry gas aids the volatilization and carries out uncharged materials from entering the MS. When the liquid is introduced to the ESI in a higher flow rate, the need for a higher dry gas flow rate and temperature occur, so the uncharged materials do not enter the inlet, and the volatilization is complete before the entering.

For the optimization of the ESI parameters, it would be reasonable to investigate the yield of target peptide with the default parameters for the flow rate of the method used. This can be performed by increase and decrease the values for the following MS methods. The optimized method can be obtained by investigating the intensity of the target peptide for the various values. After the parameters of the ESI have been evaluated, the parameters for the TOF would be evaluated.

4.6.2 TOF mass analyzer parameters

The TOF parameters has an impact on the *m/z* values that will be introduced to the TOF. The most important parameters for the TOF, employed in this thesis, are transfer time and pre-pulse storage. Transfer time and pre-pulse storage determine the time of the ions that accelerates from the collision cell to the acceleration in orthogonal direction in the flight tube. The setting of these parameters determines the *m/z*-ratio of interest for the method. If the time is short, the small ions will accelerate to the flight tube. The ions with high *m/z* value will not reach the orthogonal accelerator stage, hence, will not accelerate to the flight tube. If the time is too long, only the ions with high *m/z* value will accelerate to the flight tube and be detected. The small ions will pass the orthogonal accelerator and not enter the flight tube.

4.6.3 Development of an MS/MS method

For identification of the nerve agent adducts of the BuChE target peptide, an MS/MS method would be developed. A simple MS/MS method was performed in the preliminary studies, but MS/MS method should be developed for the nerve agent adducts of BuChE for identification. Analyses of nerve agent adducts of the HuBuChE target peptide performed with the optimized LC-MS method will give the retention time for the different nerve agent adducts. In the MS/MS method, the quadrupole will isolate a given *m/z* value (precursor ion) that will be transferred to the collision cell. The isolated precursor ions will collide with a neutral collision gas (typically N_2). The collision will convert the translational energy in the ions to internal energy that will lead to fragmentation. The fragmentation of the ions will be given by a product-ion spectrum that can confirm the nerve agent adduct for the different NA. For the development of the MS/MS method, the collision energy is considered as an essential factor. This factor will determine the degree of fragmentation, which will either give poor or high fragmentation. I.e., for the development of MS/MS method; the VX-HuBuChE target peptide has a retention time of 11.22 minutes. The time range from 9.5 to 13.0 minutes would be performed with the quadrupole isolating the ions with *m/z* 902 ±2. Different collision voltage would be investigated. A reduced fragmentation will give a high intensity of the *m/z* value of the precursor ion. In contrast, a high fragmentation will give a low precursor ion intensity in the spectra and a higher yield of the product ions. For fragmentation of a peptide, it would be preferable that fragments identifying the amino acid sequence occurred in the spectra.

4.7 Validation of an analytical method

Validation of a method ensures the analyst that the method is reliable, and the results fit for their purposes. The validation parameters depend on the purpose of the method, i.e., whether the methods are qualitative or quantitative and which techniques and instruments are used. Qualitative chemical analyses of a method describe the presence of a substance in the sample. The quantitative chemical analysis method describes the yield of the substance present in the sample [35]. For a qualitative method, the validation usually requires determination of the specificity/selectivity, the limit of detection (LOD), precision, and stability. These terms are given by *The validation of drug methods* from the United Nations Office of Drugs and Crime (UNODC) validation guide [57]. The validation of quantitative methods includes more parameters than the above mentioned. Different analytical methods require different

parameters and have different acceptance criteria. Some terms for validation of qualitative methods are described briefly in the following sections (4.7.1-4.7.4).

The primary purpose of this method was to examine nerve agent adducts when an exposure is suspected, and if so, which nerve agent is exposed. Therefore, the focus of the validation would be to determine the qualitative parameters.

4.7.1 Specificity

Specificity is the method's ability to identify analyte(s) of interest in the matrix under the conditions for the method [57]. The method will be considered if it is suitable for the determination of the presence of the analyte(s) and can be referred to as the degree of selectivity [58]. A critical purpose of specificity is to avoid false negatives and false positives. False-positive results detect the presence of nerve agents, even though no nerve agents are present. False negative results are not detecting the nerve agents presence, despite that the nerve agents are present in the sample. False negative leads to enormous consequences, both for the exposed humans and the exposer of the nerve agent. For the exposed humans, they can get the wrong treatment and, in the worst-case scenarios, lead to death. For the exposer of the nerve agent, it is a violation of the CWC, an international convention, which is illegal. Therefore, there must be a certainty that the method does not show false results.

For the immunomagnetic separation, antibodies specific for HuBuChE were used. The use of antibodies gave a high specificity and already had a low binding capacity with other proteins and substances in the serum sample. There was investigated whether the beads could be reused. From the observations and to minimize the risk of carry-over of the HuBuChE, the beads were not reused. A blank matrix should be analyzed from different sources to determine the absence of different substances that may interfere with the same retention time, and have similar *m/z,* as unexposed HuBuChE target peptide and the nerve agent adducts of HuBuChE. Since the sample preparation is very specific, the sample will hopefully only contain BuChE and pepsin in 2 % FA. In this case, peptides from the HuBuChE and peptides from pepsin will be in the sample when analyzed. The other matrix components will be carried out during the washing steps before the pepsin digestion.

After the investigation of the retention time of the different nerve agent-adducts and serum samples from different sources, the examination of unknown serum samples containing nerve agent adducts of HuBuChE and unexposed HuBuChE could be done. The determination of false positive and false negatives would be investigated. The accuracy of the *m/z* value of the different nerve agent adducts determines whenever the method obtains a good specificity/selectivity or not. The accuracy is dependent on the mass analyzers used. The QTOF used in this thesis has a better accuracy than i.e., triple quadrupole, but is less sensitive and robust. For the identification of unknown analyte, QTOF could be preferred, but for the sensitivity, the triple quadrupole could be a better option [59].

4.7.2 Limit of detection (LOD)

Limit of detection (LOD) is the lowest concentration that can be identified with a given certainty. This parameter can easily be affected by small changes in the instrumental conditions or the method. Therefore, the LOD value must be given at any time when the instrumental conditions change [57]. If the measured analytical value is lower than LOD, the value is usually referred to as " less than the limit of detection", not "zero" [58]. For this method, the LOD should be sufficiently low due to detect low inhibition of HuBuChE. One method is to describe LOD as 3x signal-to-noise (S/N=3) or S/N= 3.3, but S/N=3 is acceptable [57].

The LOD can be expressed as the percent of inhibition of HuBuChE for this method. The detection of VX-adducts and GF-adducts gave a 100 % inhibition of HuBuChE, when the serums were incubated with 50 ng/mL VX and GF. The samples can be diluted with serum without incubation of nerve agents to investigate the lowest inhibition degree the method can detect. If a 1:100 dilution can be detected, based on LOD's criteria, the method can detect a 1 % inhibition of HuBuChE. In other words, if the S/N is ≥ 3 for 1 % of inhibition of HuBuChE, the criteria for LOD will be fulfilled. The LOD must be examined for the known nerve agent adducts. There are different methods to determine and estimate the LOD, but this method is mostly used for chromatographic and MS analyses.

4.7.3 Precision

Precision describes the closeness of agreement of a series of measurements obtained from the same sample with the same analytical conditions [60]. The precision of a method reflects the random error that can occur in the method, and is usually expressed as a standard deviation or standard uncertainty [35]. Two main terms under precision are reproducibility and repeatability. Repeatability is the precision obtained when analyzing the same sample with the same analysis conditions in a short time. Reproducibility is the precision under repeatable conditions. For qualitative analyses, the precision is investigated under repeatable and reproducible conditions [61]. The precision determines whenever the method can repeatedly analyze the same sample with the same condition in a short interval and can reproduce the same analytical information.

After finding the LOD for an exposed sample, a minimum of 10 replicate samples of the known nerve agents should have an amount between 1.25 x LOD and 2 x LOD for investigation of the precision. The acceptable precision for the lower concentrations should be 20 %. When the LOD of the inhibition of BuChE is found, the precision will be to detect the inhibition in a required number of replicates of the samples.

4.7.4 Stability

The stability parameter tells us how stable the analyte is during the whole analysis. For the determination of the stability of the analyte, one batch with identical samples are stored for different times and under different conditions. The samples are then analyzed under the same conditions. For this method, two different stability factors can be investigated; the stability of the nerve agent adduct in serum and their stability after the pepsination. The stability of the nerve agent adduct in serum would be investigated by incubating serum with nerve agents. Aliquots of the incubated serum are stored under different conditions and time before they are analyzed. Optionally, the samples are stored under different conditions and time after the sample preparation and prior to the injection on the LC-MS.

4.8 To summarize; the developed method for detection of nerve agent adducts when exposure is suspected

Detection of nerve agent exposure in serum was performed with LC-MS. From the preliminary studies, pepsination gave a higher yield of BuChE target peptide and was easier to obtain rather than from trypsinization. The pepsin digestion of HuBuChE gave the nonapeptide FGES$_{198}$AGAAS with an *m/z* value of 796.3477. For isolation of HuBuChE, magnetic Dynabeads coated with anti-HuBuChE and cross-linked with 5.4 mg/mL DMP in triethanolamine buffer was used. A 500 µL serum was added to a 100 µL bead-antibody complex. Enzymatic digestion was performed with 0.2 mg/mL pepsin in 2 % FA with no denaturation of the HuBuChE first. The analyses of the HuBuChE target peptide were performed on the C18 Acclaim analytical column after the sample was introduced on a C18 trap-column with backflush elution. The performance was carried out by a switching technique with two LC pumps and two mobile phase reservoirs. The loading pump operated with an isocratic mobile phase containing 2.0 % ACN and 0.05 % FA with a flowrate of 200 µL/min through the trap-column, and a washing step after the target peptide was introduced to the analytical column. The analytical pump delivered an ACN gradient from 5-90 % for 10 minutes and 0.05 % FA with a flowrate of 50 µL/min. The switch time was optimized to 0.5 minute of loading followed by backflush elution to the analytical column at 0.5 minutes, thereafter the trap column was switched to load position after 4 minutes. Bypassing the trap column was performed for the exclusion and washout of large peptides that have a retention time longer than 15 minutes in the column.

The investigation of different analytical columns with the different stationary phase and column dimension the C18 gravity Nucleodur (2 x100 mm) gave a better retention time than the C4 XBridge column (2 x100 mm), but C18 Acclaim (1 x150 mm) gave a better peak intensity of HuBuChE target peptide due to the reduced ID in the column. Due to the theoretical value, a reduction in ID from 2 mm to 1 mm in the analytical column with the same stationary phase, would increase the peak intensity of HuBuChE target peptide with a factor 4. Experimental, the peak intensity of HuBuChE target peptide had a 10-fold increase that can be explained by the reduction in ion suppression and a better separation for the HuBuChE target peptide from the other peptides in the sample. The yield of HuBuChE target peptide increased to the double from the determination of cross-linking to the optimized LC-method.

The detection of VX-HuBuChE and GF-HuBuChE target peptides gave 100 % inhibition by incubating serum with 50 ng/mL VX and GF. The investigation of nerve agent adducts was done before the optimization and can be expected to increase some in peak intensity with the fully optimized method. The method can detect inhibition levels lower than 10 %, but the LOD should be investigated to determine the lowest level of inhibition the method can detect with a given certainty.

The method was not fully developed due to COVID-19 and instrumental problems and the method was not validated. For the further work of this method, the MS parameters should be optimized for the ESI, TOF, and an MS/MS method would be developed for the identification of the nerve agent adducts of the most known nerve agents on BuChE. The function of the mass analyzer parameters and the procedure is described in section 4.6.1 and 4.6.2. For identification and the fragmentation pattern of the nerve agent adduct, an LC-MS/MS method can be established, as described in section 4.6.3. The fragmentation must carry out the amino acid sequence with high mass accuracy to identify the nerve agent adducts on the target peptide. Validation of the method would be based on the validation of qualitative analytical methods and, mostly, UNODC's validation guidance [57]. Section 4.7 described some of the essential validation parameters for this method's purposes.

5 | Conclusion

A method for the detection of nerve agent adducts in human serum is almost fully developed and validated. Immunomagnetic separation worked well for the isolation of HuBuChE and showed a high level of repeatability from the experiments performed during the method development. By the introduction of a switch technique, the C18 separation column ID could be reduced from 2 mm to 1 mm, giving a 10-fold increase in peak intensity of the HuBuChE target peptide.

The method is simple and not very laborious, with the exclusion of a protein denaturation step. The sample preparation is conducted with the advantage of HuBuChE being attached to the beads when the enzymatic digestion is performed, reducing the loss of HuBuChE during sample preparation.

The optimization of MS parameters and the qualitative validation of this method was not performed due to the COVID-19 outbreak and the instrumental problems that occurred before the outbreak. Despite this, the nerve agent adducts VX-HuBuChE and GF-HuBuChE were detected with excellent analytical sensitivity when the HuBuChE was fully inhibited. The LOD was not estimated, but the intensity of the target peptide at 100 % inhibition of HuBuChE indicates that less than 10 % inhibition of HuBuChE can be detectable.

6 | Reference

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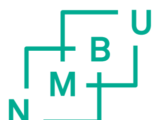

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