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Analysis of Constitutional Isomeric Phenethylamines and Synthetic Cathinones by Supercritical Fluid Chromatography and Tandem Mass Spectrometry

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Abstract

An ultra high performance supercritical fluid chromatography-tandem mass spectrometry (UHPSFC-MS/MS) method was developed and validated for the determination of a group of basic drugs of abuse in human whole blood. The following compounds were evaluated for the applicability for this technique: 2-, 3-, and 4-fluoroamphetamine, 2-, 3-, and 4-fluoromethamphetamine, 2-, 3-, and 4-methylmethcathinone, 2-, 3- and 4-methylamphetamine, amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine. For the validation of the method the following compounds were included: 2-, 3-, and 4-fluoroamphetamine, 2-, 3-, and 4-fluoromethamphetamine, amphetamine, methamphetamine and MDMA. The sample preparation consisted of liquid-liquid extraction using ethyl acetate : heptane (80:20, v/v). The samples were reconstituted in isopropanol before injection. Four $^{13}C_6$-labelled analogs were used as internal standards. The compounds were separated using an ethylene-bridged hybrid column (3 mm x 100 mm, 1.7 µm) by gradient elution with 40 mM ammonia in methanol and supercritical carbon dioxide.. Quantification was performed by tandem MS using multiple reaction monitoring in positive mode, applying two transitions for the compounds and the internal standards. The run time for the method was 4 min. The calibration curves had r^2 above 0.99 for all the compounds.The interday precision was below 15 % for all the 2-, 3-, 4-phenethylamine analytes for the levels above lowest limit of quantification (LOQ), while the classical phenethylamines displayed a precision below 10 % for the levels above LOQ. The intermediate accuracy was below 20 % for all the 2-, 3-, 4-phenethylamine analytes for the levels above LOQ, while the classical phenethylamines demonstrated accuracy below 10 % at all levels. However, the accuracy diverged less than 3 % for most of these levels. LOD varied from 0.007 to 0.02 µM, while LOQ ranged from 0.02 to 0.06 µM for all analytes. Matrix effects were between 67 and 81 % for all the 2-, 3-, 4-isomers, while the classical phenethylamines experienced between 88 and 93 % matrix effect. Extraction recovery was above 80 % for the 2-, 3-, 4-isomers, though the classical phenethylamines had a minimum of 50 % extraction recovery. Carry-over was measured to range between 0.34 and 0.88 % for all analytes. Intraday precision was below 15 % for all analytes, while the intraday accuracy was +/- 20 % for all analytes above LOQ. It was noted that for the conditions of this method chromatographic separation was decreased with repeated injections, proposedly caused by a silyl ether formation or alcohol adsorption on the stationary phase. Additionally, a spray pulsing effect was observed in the UHPSFC-MS interface corresponding to high back pressures.

Preface

This thesis was performed at the Norwegian Institute of Public Health (NIPH), Department of Drug Abuse Research and Method Development in collaboration with the Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science. The work was conducted from December 2014 until March 2016 as a part of the Master degree program in Chemistry at NMBU, under the supervision of scientist Dr. Thomas Berg (NIPH), department director Dr. Åse Marit Leere Øiestad (NIPH, Department of Drug Analysis) and Professor Dag Ekeberg (NMBU, Institute of Chemistry, Biotechnology and Food Science).

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Throughout my time spent at NIPH I have, occasionally, clumsily struggled with my work, but I have always been provided with plentiful of academic, technical and professional help from my coworkers. Thank you, you are all wonderful!

To my family, for making this possible. Especially my beloved brother Gunnar, I could not have completed this without you. Thank you, Kine Thorvaldsen, for all moral support. I am forever grateful to you.

Oslo, March, 2016

Marthe Lid

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Abbreviations

µ	Greek letter mu, abbreviation for micro (10^{-6})
µL	microliter
µM	micromolar
mg	milligram
mM	millimolar
σ	Greek letter sigma, symbol for standard deviation
atm	Atmospheric
ADHD	Attention deficit hyperactivity disorder
BPR	Back pressure regulation
CO_2	Carbon dioxide
CSP	Chiral stationary phase
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	Electrospray ionization
ESI+	Electrospray ionization, positive ion scanning
GC	Gas chromatography
KRIPOS	National Criminal Intelligence Service
LC	Liquid chromatography
MDMA	3,4-methylenedioxymethamphetamine
MMC	Methylmethcathinone
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NH_3	Ammonia

NIPH	Norwegian Institute of Public Health
NMBU	Norwegian University of Life Sciences
NPS	New psychoactive substances
ODS	Octadecylsilane
P	Pressure
P_c	Critical pressure
PMA	Parametoxyamphetamine
PMK	Piperonyl methyl ketone
PMMA	Parametoxymethamphetamine (1-(4-methoxyphenyl)-2-methylaminopropane)
RSD	Relative standard deviation
SD	Standard Deviation
SIRUS	Norwegian Institute for Alcohol and Drug Research
SFC	Supercritical fluid chromatography
SPE	Solid phase extraction
SSB	Statistics Norway
T	Temperature
T_c	Critical temperature
TAAR1	Trace amine-associated receptor 1
UHPLC	Ultra high performance liquid chromatography
UHPSFC	Ultra high performance supercritical fluid chromatography
UN	United Nations
UNODC	United Nations Office on Drugs and Crime
UPC^2	Ultra Performance Convergence Chromatography
WHO	World Health Organization

1. Introduction

1.1 Background

1.1.1 New psychoactive substances

New psychoactive substances (NPS) are a range of chemical compounds produced as substitutes for classical drugs of abuse. They are often referred to as "legal highs", referring to their initial lack of legislation. Although referred to as new psychoactive substances, many of these substances have been developed as medicinal drugs, but put aside due to harmful side-effects or lack of desired pharmacological effect. Despite this, compounds are being revived by clandestine drug producers lacking the concern of prior data suggesting high toxicity. There are several subclasses of NPS, of which phenethylamines and synthetic cathinones are among the largest.

Traditionally, the most common phenethylamines; amphetamine, methamphetamine and MDMA, have been consumed as recreational drugs by young consumers or by outlaw motor cycle gangs (Sanders, 2005; Armstong, 2007). However, the profile of the "typical" consumers seems to have evolved in recent years, as phenethylamines are found to act as a performance-enhancer among athletes, laborers in hard and tedious work, students, party-goers and in older polydrug abusers (WHO). A recent Norwegian study distinguished between three groups of consumers; the party-goers, the hard-workers and the people suffering from ADHD, depression or fatigue who self-diagnose and self-medicate (Pedersen *et al*., 2015). This is consistent with the findings of another recent study, where the substance was revealed as the most prevalent among incarcerated users. Two underlying causes were highlighted in the study; psychological trauma and ADHD (Shammas *et al*., 2014). Many of the inmates in the study of Pedersen *et al*. claimed to suffer from ADHD (2015) and a recent study suggested that more than half the prison population in several European and North-American countries might be suffering from this disorder (Young *et al*., 2011).

The market for narcotics appears to have evolved towards being more changeable and dynamic than in previous years. The market is in lesser extent concentrated around using plant-based drugs like cocaine and heroin, but rather on using synthetic substances, which makes it possible to produce the illicit drugs closer to the consumers (EMCDDA 2010-2015; Folkehelserapporten 2014; KRIPOS 2015; SIRUS 2010-2015; UNODC, 2014). Instead of smuggling the illegal drugs across distances and borders, drug precursors can legally be transported across borders. Precursors of many drugs are legal as they are used for several

purposes, as for the precursors of amphetamine and MDMA which can also be used in the synthesis of perfume, pesticides, insecticide, medicine and other chemical products (Reade, 2010). This eliminates the risk of hazardous long distance transport, smuggling across borders and harsh criminal penalties. Additionally, the internet facilitates rapid distribution between clandestine drug producers and consumers. The internet thus functions as a global market place, where drugs can be bought anonymously and sent in discrete, unmarked packages by mail (EMCDDA, 2015).

There is thus an ongoing cat-and-mouse game between legislators and drug suppliers. Drug suppliers react rapidly to legal measures like prohibition, resulting in substance replacement, producing a new modified version of the classical ones. This rapid evolvement of drugs on the market is challenging for forensic toxicologists, demanding relentless evolvement of new analytical methods to unveil new substances of abuse.

1.1.2 Forensic toxicology at the Norwegian Institute of Public Health

This thesis work was performed at NIPH, Domain for Forensic Sciences at the Department of Drug Abuse Research. The Department is divided into two subdivisions; Drug Abuse Research and Method Development. The group of method development conducts analytical toxicological research, focusing on development and validation of new methods for drug analysis in blood, urine, saliva, hair and dialysate.

The purpose of the domain of Forensic Science is to provide science-based, biomedical knowledge on an international level for use by the judiciary and service to the community. The Department of Drug Abuse Research and Method Development collaborates with the Department of Drug Analysis and the Department of Toxicological Analysis, which annually receives on average more than 30 000 forensic samples from the police, the judiciary, the Norwegian Correctional Services, the Norwegian Labor and Welfare Service, social and child welfare services, private companies and the health service. Data of samples received at NIPH in the period of 2010-2014 is shown in Appendix IV (Table IV a).

1.2 Aims of thesis

The aim of this thesis work was to develop and validate a method which could separate and distinguish a group of new synthetic amphetamines and their constitutional isomers by a UHPSFC-MS/MS method. The following substances where included: 2-, 3-, 4-fluoroamphetamine, 2-, 3-, 4-fluoromethamphetamine, 2-, 3-, 4-methylmethcathinone, 2-, 3-, 4-methylamphetamine, amphetamine, methamphetamine and MDMA.

The road to a finished developed method utilizing UHPSFC-MS/MS for analysis of the constitutional isomers of the analytes of interest entailed a series of partial goals. Foremost a method capable of separating the analytes was needed and a subsequent optimization. The extraction method would need to be satisfactory for extraction of the analytes from whole blood. For the method to be used in the routine laboratory of the NIPH, a method validation was required. The method's applicability for quantitative application required evaluation as well.

2. Theory

2.1 New psychoactive substances – Phenethylamines and synthetic cathinones

2.1.1 Culture of phenylethylamine and cathinone abuse

Phenethylamines are a group of substances characterized by its resemblance to the natural monoamine alkaloid phenethylamine (Figure 1). Phenethylamines are classified as stimulants, entactogens[1] or psychoactive hallucinogens by their pharmacological properties, and includes substances like amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA). They are extensively abused for their effects on the central nervous system.

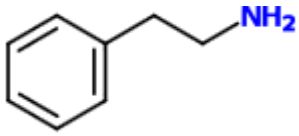

Figure 1. Base structure of phenethylamine (made at emolcules.com).

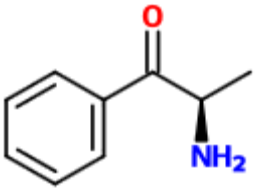

Figure 2. Base structure of cathinone (made at emolecules.com).

Cathinones are also a group of monoamine alkaloids, but differs from the phenethylamines in having a ketone functional group in its common structure (Figure 2). These substances share both structural and pharmacological similarities with the phenylethylamines, and include both natural and synthetic compounds like cathinone from khat, ephedrine and 4-methylmethcathinone, better known as mephedrone.

Psychoactive hallucinogens are known to induce psychosis, but unlike cathinones, they are thought to produce less dependence (Nichols, 2004). Phenylethylamines that have direct and indirect serotonergic agonist properties are associated with more entactogenic effects than cathinones (Tyrkkö *et al*., 2015).

Figure 3. Advertising campaign promoting the use of amphetamine as an antidepressant and diet pill (theawl.com).

[1] Entactogen is a term used to describe drugs that produce experiences of empathy and induce feelings associated thereof; emotional communion, oneness, relatedness, emotional openness.

Amphetamine was first synthesized in China in 1887, while methamphetamine was first synthesized in Japan in 1914. Production of amphetamine as a synthetic drug was first performed in 1887, but did not gain much interest until its stimulatory effects were discovered in the beginning of the 20th century by biochemist Gordon Alles (Cody, 2005). The stimulatory effects of amphetamines were extensively exploited during World War II. Allied forces were supplied amphetamine pills known under its brand name Benzedrine, while Axis and Japanese forces provided their soldiers with Pervitin, pills made from the slightly stronger substance methamphetamine. However, amphetamines were also strongly marketed for the general population. Marketing campaigns from the pharmaceutical industry promoted amphetamines as antidepressant and diet pills (Figure 3 and 4). Although uncertain efficacy and safety of amphetamines, therapeutic and recreational use increased immensely until the 1960s and was prevalent in everyday life in many countries. However, as the addictive and harmful effects became elucidated, stricter regulations were implemented and amphetamines became prescription drugs (Rasmussen, 2008).

Figure 4. Advertisement for amphetamine pills in the US, known under its market name Benzedrine (Rasmussen, 2008; California Western Medicine, April 1945)

What is bought on the street is most often made in clandestine laboratories. The synthesis results in a product with impurities like reactants, unwanted by-products and stimulant by-products, like amphetamine residues in a methamphetamine synthesis, and always consists of a mixture of drug and various other compounds. The doses are additionally "diluted" with caffeine, sugar, salts etc. to enable more profit per dose for the distributors. Besides, the manufacture results in a racemic mixture, where one of the enantiomers of amphetamine, dextroamphetamine, is more potent as a stimulant euphoriant, as it is a more potent agonist of the trace amine-associated receptor 1 (TAAR1) than levoamphetamine (Lewin *et al*., 2011). Consequently, dextroamphetamine produces greater central nervous system stimulation than levoamphetamine, roughly three to four times more, but levoamphetamine has slightly stronger cardiovascular and peripheral effects (Vaughan and Foster, 2013). Thus, both the composition and chirality profile indicates the link between the starting materials and the illicit drug synthesized by the clandestine chemist.

Another common phenethylamine is MDMA, which is often considered the drug of choice within the rave culture and is also used at clubs, festivals and house parties (Carvalho *et al*., 2012). The prevalence of MDMA experienced a peak in the 1990s and increased until year 2000, after which it stabilized (EMCDDA, 2003). In 2008-2009 a sharp decrease in the occurrence of MDMA on European markets was likely caused by successful law enforcement actions and an international cooperation between Europe and Asia by targeting the main precursor of MDMA, piperonyl methyl ketone (PMK). The market was partially reimbursed with MDMA in 2010, indicating that clandestine manufacturers found alternative precursor chemicals. Several production facilities revealed in the Netherlands and Belgium in 2013 and 2014 seems to confirm this (EMCDDA, 2015ii). Nevertheless, the temporary MDMA shortage led suppliers to adapt by selling other synthetic substances as MDMA (Glennon, 2014). This resulted in several fatal intoxications across Europe, where the victims supposedly had taken MDMA or similar substances (Dybdal-Hargreaves *et al*., 2013). Several seizures and forensic autopsies revealed the occurrence of several novel NPS in recent years. One of the more prominent was parametoxymethamphetamine, in which resulted in a total of 27 deaths in Norway in the period of 2010-2012 (Vevelstad *et al*., 2012; Al-Samarraie *et al*., 2015).

NPS are referred to as legal highs, research chemicals, plant food, bath salts etc. and they are often marked "not for human consumption" in order for the suppliers to avoid regulatory control and customs. The increasing selection and abuse of NPS has experienced a rapid growth, which could only have been possible through the internet and various other virtual social networks. A selection of the available NPS's are shown in Figure 5. There are still vast majorities in making new chemical compounds with slight modifications and each year there is a selection of novel substances appearing on the market (KRIPOS, 2014).

Figure 5. A variety of NPS seized by KRIPOS (KRIPOS, 2015).

2.1.2 Prevalence of the new psychoactive substances in Norway

The first fatal NPS incidence was reported in Norway in 2003; intake of pills thought to be ecstasy, was found to be a combination of paramethoxyamphetamine and paramethoxymethamphetamine (Refstad, 2003). Since then, there has been an increase in the Norwegian market from a limited selection of alternatives to the classical drugs to a wide selection of 101 different substances in 2014 (Figure 6). The increasing number of seizures of NPS in Norway is consistent with the overall European findings by the EMCDDA and UN World Drug Report 2015, as UNODC reported an increase in the NPS selection, peaking at a total of 450 different NPS on the European market in 2014 (UNODC, 2015).In Norway, a total of 620 seizures of NPS were made in 2014 by KRIPOS, shown in Figure 6, and 20 100 people were charged for a total of 40 300 drug offenses. Data from the Norwegian customs laboratory also show that the variety of NPS confiscated have been ever increasing the last few years and less than 10 % of the NPS packages confiscated contained of NPSs listed in the Norwegian drug list. This suggests that consumers know how to circumvent current legislation, while the market is changing in response to new regulations (Skjørsæter, personal correspondence). Men are overrepresented in the statistics of abuse and possession of narcotics, accounting for 85 % of the charges in 2014 (SSB, 2015).

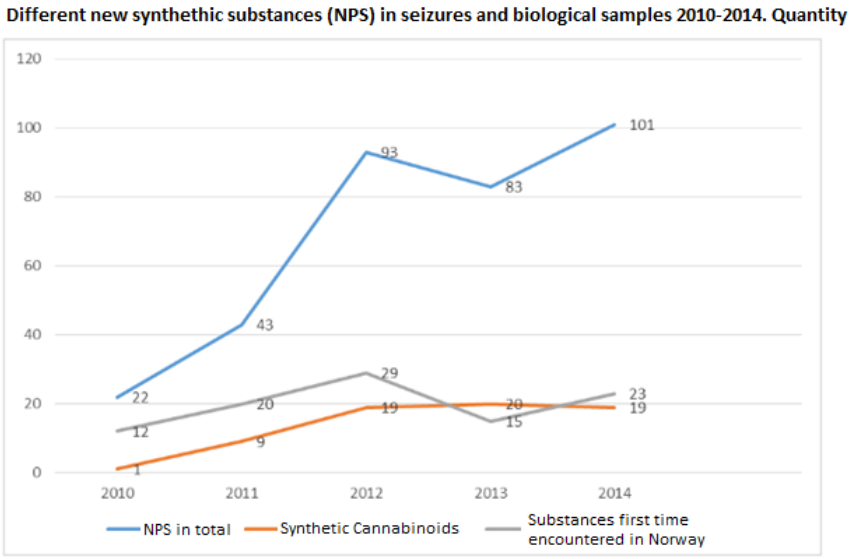

Figure 6. Graph showing the increase in NPS in Norway. Adapted from the webpage of SIRUS.

Trade and consumption of NPS is challenging to discover as the internet facilitates anonymous global trading of drugs, functioning as a cryptomarket often using cryptocurrencies like bitcoins (EMCDDA, 2015iii). In order to elucidate the extent of NPS abuse in Norway, a nightlife study was conducted in collaboration between Statistics Norway (SSB) and NIPH in 2014. The survey aimed to uncover the prevalence of drug abuse in Oslo's nightlife among 1 100 young people, aged 18-35. The study was conducted by voluntary and anonymous participation of nightlife patrons and sample collection was performed by an oral fluid sampling device. The results showed 1.4 % tested positive for an NPS during sample collection and that 7 % of the responders replied to have ever tried an NPS (Gjerde *et al*., 2016). In 2013, a school survey was conducted in the district of Ullern in Oslo, where 425 children in the age span of 14 to 16 years of age participated. 3.1 % replied to have tried an NPS at some point, while 6.3 % had been offered an NPS (SIRUS webpage, 2013). These Norwegian findings are consistent with the findings by the 2014 Flash Eurobarometer, a survey of 13 000 young adults aged 15-24 in the EU member states. 8 % of the respondents had used an NPS at least once, and 3 % within the last year (EMCDDA, 2015i).

2.1.3 Legislation concerning new psychoactive substances in Norway

The Norwegian legislation on drugs is covered by the narcotics legislation and the medicinal drugs act. In February 2013, a set of new drug regulations were adopted in Norway in an attempt to mitigate the increase of NPSs. These substances were produced as an alternative to classical drugs of abuse in an attempt to avoid current legislation. Their structure and physiological function resemble their illegal cousins, but by modifying one or a few atoms of the original substance, clandestine chemists were able to produce drugs which at first were not enforced by any law. This drug regulation defines drugs more precisely than previous and includes a list of illicit drugs, currently including ten groups of substances, phenethylamines and cathinones being two of them.

For a substance to be classified as a drug of abuse several criteria must be obeyed. The compound must have a psychoactive effect, be harmful and addictive. It must additionally cause tolerance and abstinence, and have a scattering potential (Narcotics legislation; Skretting, 1999). The Norwegian Medicines Agency (*Norwegian*: legemiddelverket) decides what substances are defined as narcotics. The NPS of this thesis became illegal in Norway in 2013. However, the legislation of 2013 included a range of new substances and their salts, stereoisomers, esters and ethers of the groups listed are also included, but still left a need for new legislation each time a new, slightly modified substance appears on the market (Narcotics legislation, Medicinal drugs act).

According to the Norwegian Medicines Agency, 10 of the 23 new substances identified in 2014, were included in the new drug legislation from 2013 by belonging to a group of similar substances, by so-called generic listing. In order for the remainder of these compounds to be scheduled, a listings proposal must be submitted for evaluation. In the meantime, they are covered by the medicinal drugs act, and consequently must obey its regulations on import, sales and marketing (SIRUS webpage, 2015).

2.2 Sample matrices

In contemporary forensic toxicological analysis, there is a range of possible sample matrices. Drug analysis of blood, urine, hair, and oral fluid has been successfully implemented with liquid chromatography (LC), in which all are represented in the analysis repertoire at NIPH. Whole blood and urine are the most common biological fluids used for drug confirmation and quantification (Chèze *et al*., 2007; Marin *et al*., 2008; Øiestad *et al*., 2007; Gergov *et al*., 2009; Øiestad *et al*., 2011; Montesano *et al*., 2014).

Urine contains both the drugs taken and its metabolites, but does not indicate current impairment, as drug concentration in urine does not immediately increase after intake. Therefore drug testing is most commonly used for determination of abuse and not of impairment, e.g. in traffic incidents. Drug detection in urine has the advantage of being relative non-invasive compared to blood withdrawal, but requires that the specimen collection must be done under surveillance of authorized personnel. Urine has a longer detection window compared to blood, up to several days after intake, and is mainly applied for workplace drug testing, drug testing of prisoners, drug abuse rehabilitation programs or clinical drug screenings (Gjerde *et al*., 2011).

For most drug analytes, blood concentration decreases quite rapidly after intake, and blood sample collection is invasive and requires medical personnel. Nevertheless, blood is most often the specimen of choice when measuring, quantifying and interpreting concentration of drugs and correlating metabolites, as it shows the best correlation between drug concentration and pharmacologic effects. This can be attributed to blood being the only biological specimen, except for cerebrospinal fluid, that reflects drug concentration in the brain (Gjerde *et al*., 2011). Blood can produce an immediate approximation of the level of drug thereby facilitating the use of cut-off values for later confirmatory analysis, i.e. avoiding too many false positives (Skopp, 2004; Birkler *et al*., 2012; Langel *et al*., 2014).

Analysis of blood samples requires extensive knowledge about the composition, properties and functions of the blood. Whole blood is a complex mixture of solubilized proteins, dissolved fats, solids and suspended cells (Chargaff and West, 1946; Wolf, 1967; Skopp, 2004; Manzone *et al*., 2007). The main cell types of blood are red blood cells (erythrocytes), white blood cells (leukocytes) and blood platelets (thrombocytes). Red blood cells constitute more than 90 % of the total cell number and are the main cause of the relatively high viscosity

of the blood. The blood cells are normally distributed in the extracellular fluid plasma, due to the continuous motion of the blood. However, the blood cells immediately sink in stagnant plasma, due to greater mass density. Furthermore, blood produces three layers when centrifuged for the same reasons as shown in Figure 7.

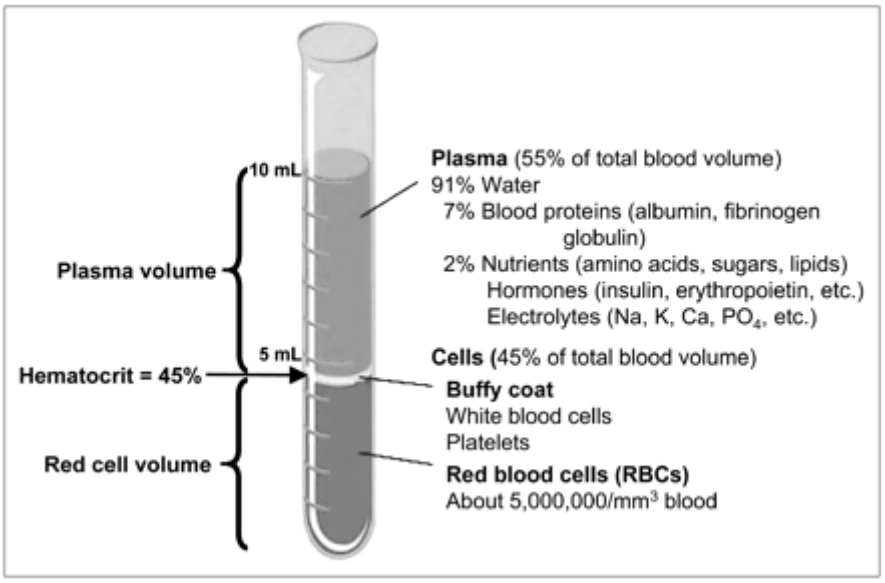

Figure 7. The components and relative proportions of blood (Manzone *et al.*, 2007)

Many matrix components are thus present in blood to cause interference with the response in MS. Serum proteins can reduce sensitivity by binding to analytes, endogenous phospholipids can cause significant matrix effects (MEs) and anticoagulants added to blood to prevent coagulation can affect extraction and analysis (Judis, 1980; Wu *et al.*, 2008).

2.3 Sample preparation of forensic samples

The choice of sample preparation technique is critical for the analysis, as it is error-prone and inherently the most labor-intensive part of the analytical process. Ideally, the process should be simple, rapid, remove possible interfering compounds, give high recovery, and concentrate anayte(s) of interest.

In order to perform a robust and efficient chromatographic and mass spectrometric analysis of biological samples it is important to get rid of possible contaminants and also to retain as high a percentage of analyte as possible. All biological samples contain a complex mixture of substances. For the method in question blood was chosen as sample agent. Among the most prominent contaminants in blood is phospholipids, which is the main component of cell membranes and are ubiquitous in blood samples. Phospholipid removal was considered to be an important evaluation point when considering different extraction methods, as phospholipids are considered to be one of the most troublesome components of bioanalytical samples when performing chromatography coupled to tandem mass spectrometry to avoid smudging and contaminating the MS (Wu *et al*., 2008). A possible co-elution could possibly result in ion-suppression or enhancement effects of the MS signal that would cause variability and disrupt the accuracy of the methods result.

2.3.1 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is an abstraction of an analyte from one liquid phase to another liquid phase (a two-phase distribution of a solute), usually from an aqueous solution to an organic solvent. The mechanism underlying LLE is based on transferring the analyte from matrix compounds to an appropriate liquid phase based on their relative solubilities in two different immiscible liquids.

2.3.2 Solid phase extraction

Solid phase extraction (SPE) is a mechanism based on the analyte binding with great affinity to a sorbent solid phase. The ability of the analyte to bind to the solid phase makes it quite easy to extract it from the sample matrix. The method procedure requires several steps;

conditioning, loading of sample, washing and elution of analytes. When the analyte binds with higher affinity to the sorbent then the matrix components, it is possible to wash the sorbent to exclude unwanted compounds which have less affinity for the sorbent. The analyte can be eluted from the sorbent by using an elution solvent and collected for analysis.

2.3.3 Protein precipitation

A simple sample preparation approach for biological fluid is protein removal by protein precipitation. Protein denaturation can be performed using acids, heat, ultrafiltration membranes, or by using organic solvents. Protein precipitation partly removes phospholipids present in the plasma and serum of blood, depending on the organic solvent used. Studies have shown that methanol extracts contain 40 % more phospholipids compared to acetonitrile (Bylda *et al*., 2014; Ferreiro-Vera *et al*., 2012).

2.4 Chromatography – From chromatographic ABC to SFC

2.4.1 Chromatographic ABC

Chromatography is a collective term used to describe a physical method applied for analyte separation in mixtures where the components to be separated are distributed between two phases; the stationary and mobile phase. A successful chromatographic separation occurs as a result of repeated sorption and desorption acting during the movement of the sample components along the stationary bed, and the separation is due to differences in the distribution constants of the individual sample components.

The Russian botanist Mikhail Tswett is generally credited for the first development of chromatography around year 1900. Using a column of calcium carbonate, he successfully separated green leaf pigments like chlorophyll, carotenes, and xanthophylls. The name of the technique given by Tswett literally means "color writing", referring to the initial components analyzed, which due to their different colors created colorful bands (Tswett, 1906).

For LC and SFC, the stationary phase used is generally a porous, granular powder in the form of a dense homogenous bed packed into a tube, referred to as a column, able to withstand the operating pressures normally employed. The sorbents are usually porous solids of high surface area, a similar solid modified by bonding a ligand to its surface or used as a support for a thin film of liquid, or an inert sorbent on a controlled pore size (packed column). Alternatively, the stationary phase can be distributed as a thin film or layer on the wall of an open tube of capillary dimensions leaving an open passageway through the center of the column. In order to elute the substances of interest a solvent is added to the mobile phase referred to act as a displacer. The displacer must have a higher affinity for the stationary phase than the sample. The displacer drives the adsorbed components progressively along the column, each component displacing the one in front, until they are eluted in the same order in which they adsorbed on the column; the least strongly retained being eluted first.

The chromatographic process provides information summarized in a chromatogram, a record of the concentration or mass profile of the sample components as a function of the movement of the mobile phase. Information can readily be extracted from the chromatogram, which includes an indication of sample complexity based on the number of observed peaks,

qualitative identification of sample components based on the accurate determination of peak position, quantitative assessment of the relative concentration or amount of each peak, and an indication of column performance.

In chromatography, the term theoretical plate is commonly used as a measure of efficiency in a separation process. The term describes a hypothetical stage in which two phases establishes an equilibrium with each other, also referred to as an ideal stage, theoretical stage or equilibrium stage. Separating substances in a chromatographic process thus depends on series of equilibrium stages, where an increased number of theoretical plates mean increased efficiency. In the same way theoretical plates arises from the concept of equilibrium stages, as does plate height. Plate height is numerically equal to the absorption bed length divided by the number of theoretical plates in the absorption bed and is inversely proportional to plate number (Poole and Poole, 1991).

As an extension of these terms, the van Deemter equation (eq. 1) describes the plot of a theoretical plate as a function of mobile phase velocity (van Deemter *et al*. 1956) for packed columns. The equation describes three factors of band broadening; eddy diffusion, longitudinal diffusion and resistance to mass transfer. The equation is a hyperbolic function that predicts the optimum velocity (Figure 8).

$$H = 2\lambda * d_p + \frac{2\gamma * Dm}{u} + \frac{f(k) * dp^2 * u}{D_m} \qquad \text{eq.1}^2$$

[2] H = Plate height, λ = packing factor, d_p = particle diameter, γ =obstruction factor, u =mobile phase linear velocity, f(k) = function of the retention factor, D_m = diffusion coefficient of the solute in the mobile phase.

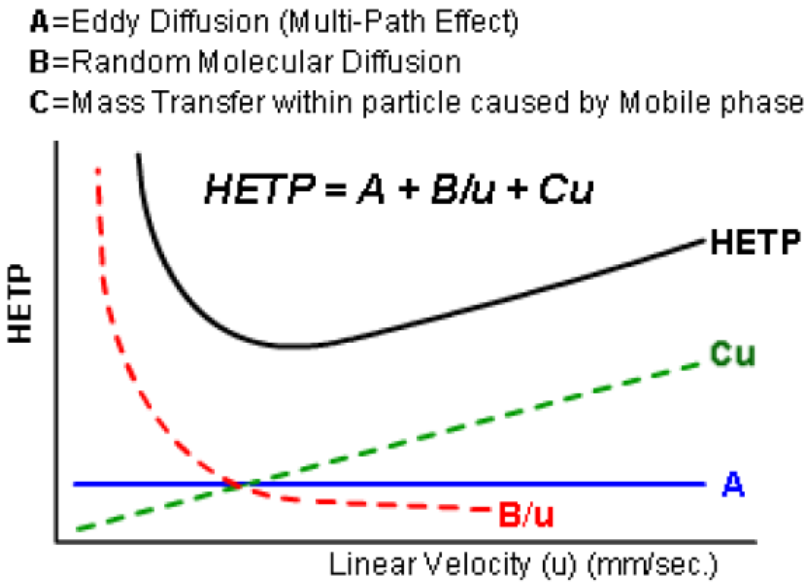

Figure 8. Van Deemter plot showing the contributions from the different terms of the van Deemter equation (Chromacademy).

The Basics of Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) is a chromatographic technique using supercritical or subcritical fluid as a mobile phase, referring to the conditions of the fluid being above or just below the critical point, respectively. The technique is used for the analysis and purification of most low to moderate size molecules, and even thermally labile molecules. One of the most successful applications of supercritical fluid chromatography has been the separation of enantiomers on chiral stationary phases (CSP) (Khater *et al*., 2013a; Lee *et al*., 2014). Properties making SFC favourable for chiral separations include lower operating temperatures compared to GC, higher diffusivity of the solutes and reduced viscosity of the eluent compared to traditional LC. Lower operating temperatures reduces the probability of racemization of either the analyte or the CSP, eliminating the need for a derivatization agent during sample preparation and often resulting in improved enantioselectivity (Petersson *et al*., 1994). Higher diffusivity of the solutes and reduced viscosity of the eluent offers higher efficiency and shorter analysis time compared to LC.

The principles of SFC are similar to those of LC, differing in the use of carbon dioxide (CO_2) as the mobile phase. For proper solvation of the analytes in the mobile phase, an organic solvent is used as a solvent and a displacer. In SFC, the solvent is referred to as a modifier, a term which is specific to SFC. SFC holds several theoretical advantages, such as low

viscosity, high density and elevated analyte diffusion coefficient. These properties enables fast analysis at high linear velocity and higher chromatographic resolution can easily be achieved in SFC with long columns compared to LC, whilst keeping the column-pressure drop at a reasonable level (Kaczmarski *et al*., 2012; Grand-Guillaume Perrenoud *et al*., 2014; Poe *et al*., 2014; Lesellier et al., 2014b). In recent years instrumentation with sub-2-µm particles have been attainable for SFC applications as well, often referred to as UHPSFC, which further enhances the potential kinetic performance of SFC as shown in Figure 9 (Lesellier *et al*., 2011; Khater *et al*., 2013b; Biba *et al*., 2014; Grand-Guillaume Perrenoud *et al*., 2014).

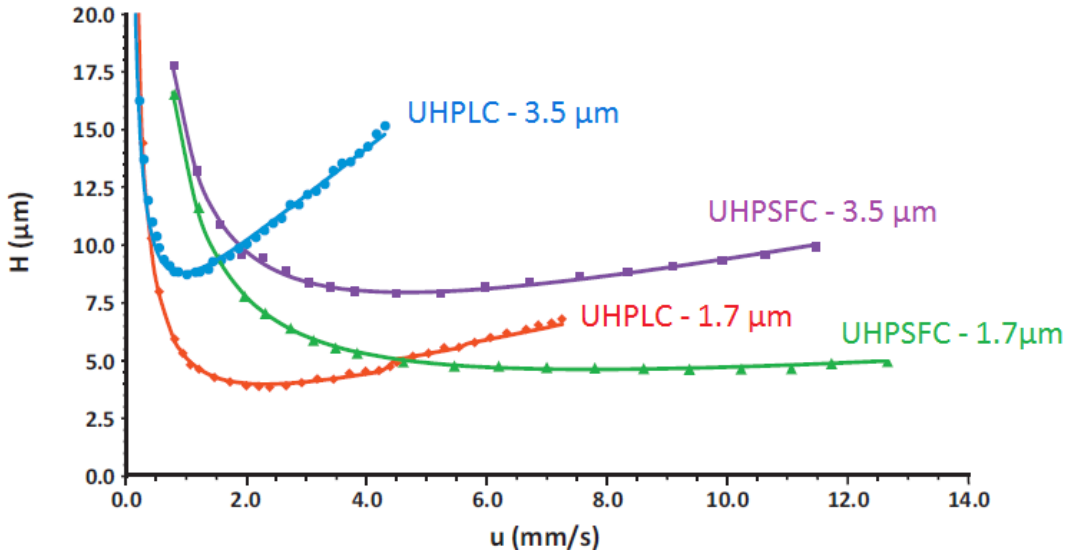

Figure 9. Kinetic performance expressed in van Deemter curves for butylparaben for UHPLC and UHPSFC systems equipped with 1.7 and 3.5 µm particles (adapted from Grand-Guillaume Perrenoud *et al*., 2012b).

2.4.2 History of supercritical fluid chromatography

A supercritical fluid is an element or a compound above its critical point, the critical point being defined by its critical temperature and pressure (Figure 10), where there is no change in state as pressure is increased or heat is added (Klesper, 1978; Fountain *et al*., 2014). It is a misleading name, in that it implies enhanced properties.

The critical point was identified in 1822 by Baron Charles Cagniard de la Tour, while conducting experiments involving the discontinuities of the sound of a flint ball in a sealed cannon barrel filled with various fluids at various temperatures. De la Tour was able to observe a reduction to vapor in a space from two to four times the original volume of the

sample (de la Tour, 1822 and 1823). Nevertheless, the nature of the transition was not understood until 1869 when Andrews performed quantitative measurements on CO_2. The term "critical point" was first applied by Andrews to describe the phenomenon associated with the liquid-vapor transition (Andrews, 1869).

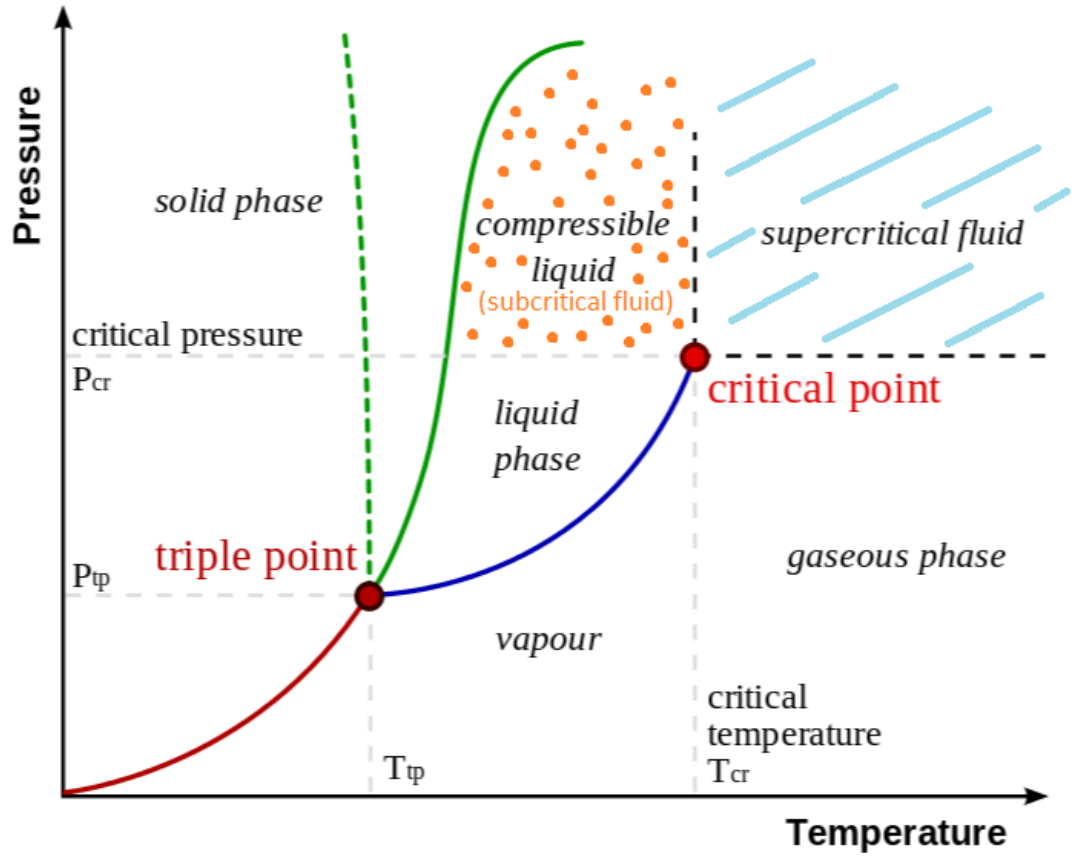

Figure 10. Phase diagram showing the critical point and progression towards subcritical and supercritical properties (adapted from Wikipedia and modified in Paint).

The properties of elements or compounds beyond the critical point were however not considered for many years. Supercritical fluids remained a curiosity and were not considered to have an analytical potential until Jim Lovelock proposed a chromatography method, at a GC conference in 1957, utilizing supercritical fluid as a mobile phase. He suggested the name "critical state chromatography". SFC was first performed by Klesper *et al.* in 1962 (Klesper *et al.*, 1962; Guiochon and Tarafder, 2011), and subsequently they reported a new SFC system equipped with a mechanical back pressure regulator that could control the pressure independent of the flow rate (Saito, 2013). Karayannis and Corwin showed in 1968 how to independently control the column back pressure and the flow rate and described a UV detector with a cell operating under high pressure (Karayannis *et al.*, 1968). During the same time period Sie and colleagues published a series of articles on what they referred to as high-

pressure gas chromatography (HPGC) with supercritical CO_2 as the mobile phase. The articles discussed fluid-solid and fluid-fluid separation modes (Sie and Rijnders, 1967). In 1969, Giddings and colleagues emphasized the importance of carrying out gas chromatographic separation under extremely high pressures, up to 2000 atm (which they called dense gas chromatography), and implied a possible convergence with classical LC, many years before the title unified chromatography was identified and reported by Ishii *et al*. (1988) and more recently by Chester and Pinkston (1998). Gouw and Jenthoft (1970) developed a pressure-programmed SFC, applied to separate a wide molecular weight range samples of polynuclear aromatic hydrocarbons and of styrene oligomers. The instruments for SFC were not as sophisticated and advanced as those for HPLC. Yet, the latter took over in the late 1970s while the former remained a niche mode of chromatography until today/to this day. Klesper and Hartmann developed preparative SFC and used it to purify oligomers of styrene, which they also analyzed by mass spectrometry (Klesper and Hartmann, 1978). However, SFC was not recognized as a useful method until the 1990s due to the concurrent arrival of HPLC, which at the time produced a more versatile and robust analysis (Saito, 2013).

Currently, CO_2 is the most widespread supercritical fluid used in SFC. It displays the advantages over other fluids, as shown in Table 1, of having smooth critical conditions of temperature and pressure (31°C and 1073 psi) fully compatible with chromatographic instrumentation. It is also relatively safe (non-toxic, non-flammable), inert and quite cheap.

Table 1. Beneficial properties of CO_2 as supercritical fluid mobile phase in SFC

Advantages of using CO_2 in SFC:
Easily achievable critical conditions; 31°C and 1073 psi (Grand-Guillaume Perrenoud *et al*., 2014)
Fully compatible with current chromatographic instrumentation
Non-toxic
Non-flammable
Inert
Cost efficient
Easily attainable
Highly compressible
Good compatibility with MS
Low viscosity, thus making it easy to achieve high efficiency

The very high density of the carrier gas at these pressures allows intermolecular forces to become very high, permitting the extraction of macromolecules into the gas phase. Giddings *et al*. (1969) used helium, nitrogen, CO_2, and NH_3 as carrier gases and separated nucleosides,

nucleotides, and purines, proteins, peptides and amino acids, sugars, terpenes, and steroids. However this work was unfortunately not pursued by the authors. Later research related to SFC has been almost exclusively dedicated to CO_2 as mobile phase (Guiochon and Tarafder, 2011). Although other compounds also possess the ability to be in a supercritical state, few can compete with CO_2 in its range of beneficial properties (Berger, 1997). Additionally, most studies have focused solely on pressure and mobile phase density, rather than different possible organic modifiers and additives (Blackwell *et al*., 1999; Brunelli *et al*., 2008; Fogwill and Thurbide, 2008; Kato *et al*., 2009; Hamman *et al*., 2013; Lee *et al*., 2014). For now there is still limited knowledge concerning the molecular interactions between solutes, mobile phases and stationary phases, but hopefully the burst of interest in this field will lead to new insights in near future.

SFC recent rise in popularity occurred in 2008 after a long period of being a niche method, due to an acetonitrile shortage, which forced researchers and engineers to develop alternative strategies. SFC was considered a valuable option, because its mobile phase requirements are drastically different from those of conventional chromatographic techniques (Nováková *et al*., 2014).

The name SFC is thus often inadequate as the conditions often are below critical value for the mobile phase fluid. The term subcritical fluid chromatography is alternatively used, but is equally inadequate as a subcritical fluid (with $P<P_c$ and $T<T_c$) is actually a liquid (Figure 10). The controversy surrounding the name of SFC is described in Appendix III. The re-emergence of SFC the last few years has been possible due to the arrival of equipment that is significantly improved. SFC instrumentation slowly evolving over a number of decades in combination with the many improvements in LC equipment this century has resulted in well-designed, highly efficient, highly robust and user-friendly SFC-instrumentation.

2.4.3 Applications of supercritical fluid chromatography

Klesper and colleagues attempted to separate porphyrin derivatives that could not be analyzed by GC because they begin decomposing at temperatures where their vapor pressure is too low to allow for elution (Klesper *et al*., 1962). Today SFC is a more universally applied method and it is often orthogonal to the more commonly used UHPLC in pharmaceutical analysis (de la Puente *et al*., 2011; West and Lesellier, 2008a and b; Lemasson *et al*., 2015b). This is crucial in method development as verification of the method should also be done in an

instrument with a different technique than the instrument used in the analytic method to verify analytical results.

SFC is considered to be superior for chiral separations (Stringham and Blackwell, 1997; Phinney, 2000; Stringham, 2005; Plotka *et al*., 2014; Lesellier and West, 2015). The potential for chiral separations lies in that it is possible to adjust several parameters simultaneously; temperature, back pressure, modifier type, gradient, additive type and additive concentration. It should be noted that both temperature and back pressure afflict the density of the mobile phase fluid. Fine tuning an SFC method gives more possibilities to adjust the separation, producing separation for many analytes which cannot be analyzed with regular LC or GC methods. SFC being water-free or nearly water free is an advantage for separating analytes that are hygroscopic or which degrade in the presence of water.

SFC is thus interesting for its analytical applications, but also due to its green profile. Compared to other solvents utilized in analytical chemistry, it produces far less adverse effects to human health, safety, biodegradability and the environment (Plotka *et al*., 2013).

2.4.4 Conditions of supercritical fluid chromatography

Few analytes are soluble in the apolar supercritical CO_2 alone, so different organic solvents are often included. The organic solvent is often referred to as a modifier, when added to the SFC mobile phase. Addition of modifier increases the solvating power of the mobile phase for better solubility of polar analytes, by increasing the polarity of the mobile phase in SFC (Bartmann and Schneider, 1973; Strubinger *et al*., 1991b; Tarafder, 2016). Polar modifiers used in combination with polar stationary phases can produce good resolution and symmetrical peaks. The modifier can also affect the retention, by changing the density of the mobile phase, but probably has limited effect. Using an alcohol modifier in combination with a silica-based column can lead to blockage of active sites on the stationary phase or alcohol adsorption onto the surface of the stationary phase over time, leading to alteration of the chemical environment the analytes are subject to under chromatographic elution. Adsorbed modifier molecules can also lead to a modification in the net volume of the stationary phase, which alters the phase ratio of which the analytes are subject to under the chromatographic separation (Janssen *et al*., 1989; Strubinger *et al*., 1991a and b; Heaton *et al*., 1994; Gurdale *et al*., 2000; Lesellier *et al*., 2002; de la Puente *et al*., 2011; Desfontaine *et al*., 2015; Fairchild *et*

al., 2015; Lesellier and West, 2015; Tarafder, 2016). When sufficient modifier is added, the elution strength increases, resulting in elution of the analyte when the level of the interaction between the analyte and the mobile phase is equal to that of the analyte and the stationary phase. Different modifiers are compatible with SFC conditions and provide a range of possibilities in optimizing the separation of analytes by differing the polarity of the mobile phase (Figure 11).

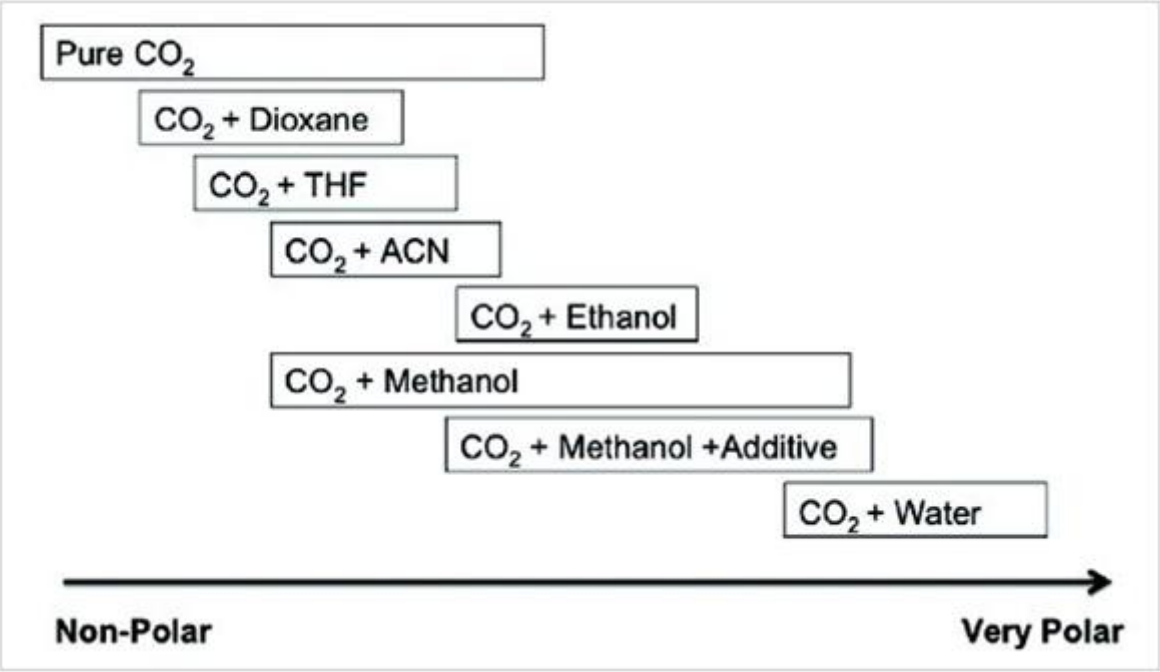

Figure 11. Schematic overview of the polarity ranges of the mobile phase combination in SFC-MS (Kott, 2013).

Although many possible solvents can be used as modifier in SFC, the most commonly applied solvent has so far been methanol. The combination of supercritical CO_2 and methanol yields a mobile phase with a very nonlinear solvent strength. The empirical P' scale, originally intended for normal phase HPLC, indicates that the addition of only 2 % methanol produces a fluid with a polarity expected from 10 % methanol. The phenomenon of enhanced solvent strength in SFC is caused by clustering of the polar modifier molecules. This creates pockets with a polarity higher than what might be expected. Polar solutes tend to be solvated within such clusters (Berger, 2007).

The conditions are probably subcritical when a sufficient amount of modifier is added, producing two distinct phases in the system. This is the result of a much higher critical point of the modifier. The total critical point of the binary mixture can be simplistically estimated as the arithmetic mean of the critical temperatures and pressures of the two components, as shown in equation 2 and 3:

$T_c\,mix = x_1T_{c,1} + x_2T_{c,2}$ (eq. 2)

$P_c\,mix = x_1Pc_{,1} + x_2P_{c,2}$ (eq. 3)

X is the mole fraction of the respective component, whereas T_c and P_c refers to the critical temperature and pressure of the indicated component, respectively. Calculating the arithmetic mean only gives approximate results, as it does not take into account the fact that the solvents may experience interactions with each other, varying from different types of modifiers. Estimating critical point for the binary mixture can be done more accurately by using equations of state, e.g. the Lee-Kesler or the Peng-Robinson equations, or by using group contribution methods (Poole and Poole, 1991). Saito (2013) estimated the critical temperature and pressure of CO_2-methanol mixtures, where 5 % methanol in CO_2 was predicted to be at 51 °C and 105 bar (1523 psi) (Figure 12). In short, when the pressure and temperature is below the critical values of the binary mixture, the mobile phase is actually a simple mixture of liquefied CO_2 gas and organic solvent. This mobile phase nevertheless has an advantage over ordinary liquid mobile phases in having lower viscosity and easy recovery of the sample solute by decompression (Saito, 2013; Poe *et al*., 2014).

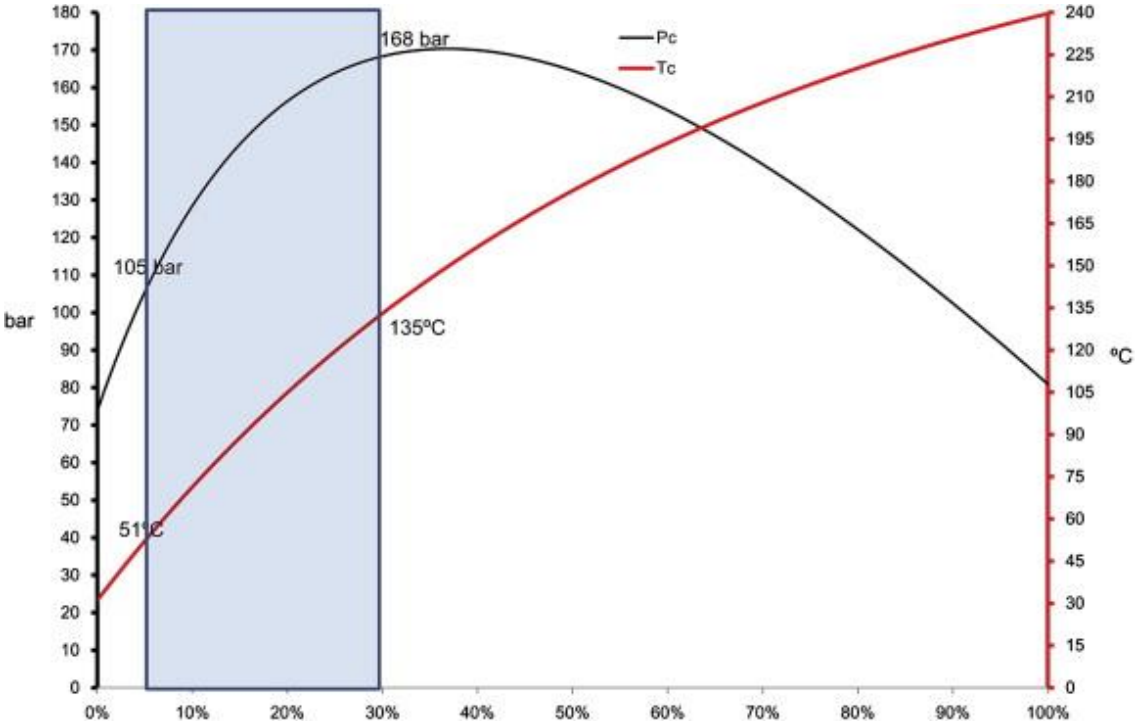

Figure 12. Phase diagram indicating the relationship between CO_2 and methanol in SFC by calculating the critical temperature, pressure and mass % of a CO_2-methanol mixture (Saito, 2013).

Most of the active pharmaceutical ingredients abused today are basic, as for the analytes of interest in this thesis work. When analyzing these basic entities, an additive is often required

added to the modifier, as the basic sites on the molecules not only become ionized, but also interact with the silanol groups of the silica support (Neue *et al*., 2004; Hamman *et al*., 2011). The result can be broad peaks that provide very little, if any, resolution. Since the CO_2 – methanol mixture is acidic, with a pH that might be nearly 4-5, basic compounds will predominately be in their cationic, protonated form (Wen and Olesik, 2000; West, 2013; Lemasson, *et al*., 2015a). Acidic compounds on the other hand, will mainly be in their anionic form, depending on their pK_a values. Basic additives with a pH above the pK_a of the analyte will restore the neutral state of basic species and deprotonate acidic species. Addition of an additive can also affect the retention by creating interactions with the analytes and thus increase the analytes' affinity for the mobile phase. The additive can also compete with the analytes for the hydrogen bonding donor and acceptor sites of the stationary phase (Ren-Qi *et al*., 2012; Desfontaine *et al*., 2015). Under the acidic conditions created by the CO_2 – alcohol mixture, any ionisable group on the stationary phase could be charged. The silanol groups could become partly anionic, while amino or pyridine bonded ligands could be partly cationic, resulting in a probable poor robustness (West, 2013).

The use of packed column SFC for basic analytes favors the use of NH_3 additives, for the promotion of solubility and for the compatibility to MS detection (West, 2013). The effect of an organic modifier alone is usually insufficient to overcome the chromatographically deleterious effects of residual silanol groups and to enable the elution of very polar or basic compounds. The role of an additive includes providing coverage of the active sites and changing the stationary phase and mobile phase polarity.

Water is an alternative additive, either as the only additive or as a second additive in the mobile phase mixed with another additive. Water has very low solubility in supercritical CO_2 (~0.1 %, w/w), due to its high dipolar nature in contrast with the apolar CO_2 (Tassaing *et al*., 2004; Oparin *et al*., 2005; Nováková *et al*., 2014). Nevertheless, a small amount of water is miscible with supercritical CO_2 when combined with an organic modifier. Water has twice the hydrogen bonding ability of methanol and becomes acidic in contact with CO_2 as shown in Figure 13.

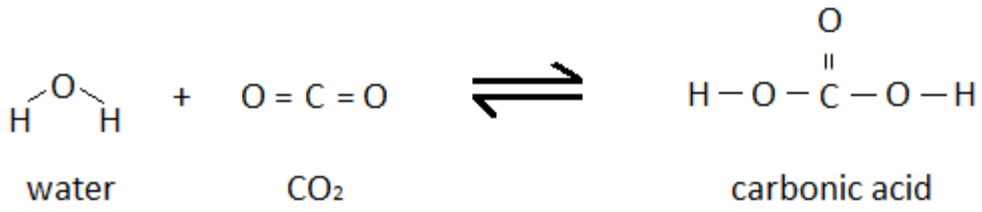

Figure 13. Equilibrium of water and CO_2, creating carbonic acid (adapted from Nováková *et al*., 2014).

The addition of water could improve the elution of polar analytes and is increasingly used as an alternative additive in SFC (Taylor, 2012; Lemasson *et al*., 2015a; Nováková *et al*., 2015; Pauk *et al*., 2015).

SFC is mostly operated in normal-phase mode, which functions by using polar-polar interactions between analytes and the stationary phase. The stationary phases most commonly used in SFC were initially developed for HPLC use, thus based on silica with different bonding chemistries with a wide number of functional groups (alkyl, phenyl, cyano, amino, propane-diol etc.) or coated with polymers (West and Lesellier, 2006a,b,c; West and Lesellier, 2008b; West *et al*., 2012a; da Silva *et al*., 2013). The ethylene bridged hybrid (BEH) stationary phase was originally developed for HPLC and UHPLC, and is now marketed as suitable for SFC analysis due to its durable nature. The stationary phase is based on BEH particles, which has no ligand bonded to the surface (Figure 14). Without ligands, the analytes are provided access only to silanols and the BEH particle has been proven to exhibit high chemical and physical stability. An unbonded particle simplifies the interaction between analytes and provides a single source of retention on the stationary phase. The particles are made of a hybrid of silica and polymers, creating a durable material which is known to withstand high pH and the high pressures of UHPLC as well.

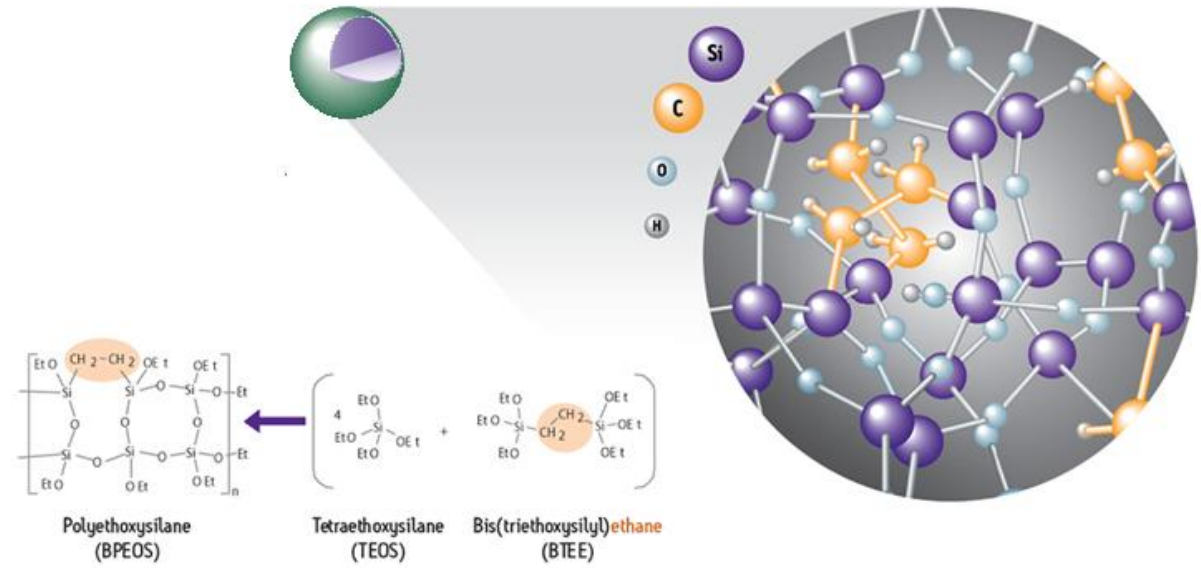

Figure 14. The stationary phase chemistry of BEH (waters.com).

The TORUS columns of Waters corp. are modified BEH particles. The silanol seats are ligated with 2-picolylamine (2-PIC, Figure 15), diethylamine (DEA, Figure 16), or 1-aminoanthracene (1-AA, Figure 17). The compounds are meant to shield the silanol seats from the direct or indirect bonding of modifier or additive, which over time reduces the plate number of the column.

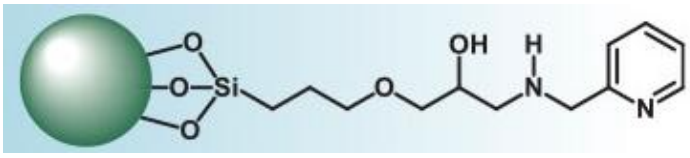

Figure 15. The stationary phase chemistry of 2-PIC (waters.com).

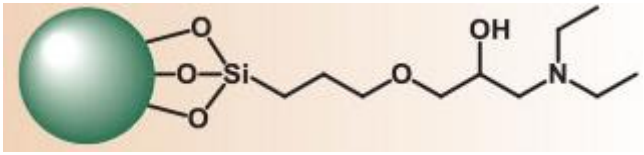

Figure 16. The stationary phase chemistry of DEA (waters.com).

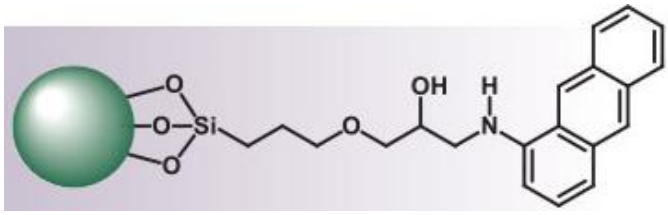

Figure 17. The stationary phase chemistry of 1-AA (waters.com).

The chiral column from Waters corp. with a amylose tris(3,5-dimethylphenyl)carbamate (AMY1, Figure 18) stationary phase is made to distinguish between enantiomers, stereoisomers, metabolites and other compounds with high resemblance (Figure 17). The stationary phase consists of repeated amylose tris(3,5-dimethylphenyl)carbamate units, which consists of a single enantiomer of the saccharide, creating a single-enantiomer stationary phase of which the analytes will have different affinity towards (Li et a., 2010). Normally, such CSPs are applied for the distinction of enantiomers, but a recent study has illustrated the benefits of SFC using CSPs in method development for separating multicomponent mixtures of closely-related achiral analytes as well (Regalado and Welch, 2015).

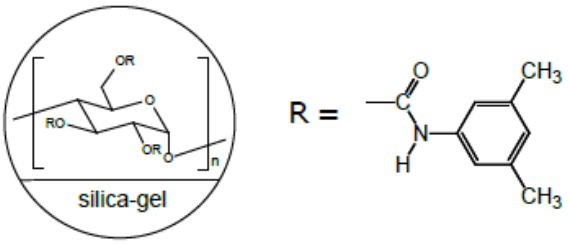

Figure 18. The stationary phase chemistry of amylose tris(3,5-dimethylphenyl)carbamate (adapted from Chiraltech).

It is advantageous that there is such a large variety of stationary phases which can be used in SFC without much precaution. However, the greatest difference between LC and SFC is in the nature mobile phase being with and without water, respectively. Thus, the knowledge acquired about these stationary phases in LC is generally of little guidance when working with supercritical fluids (West and Lesellier, 2008b).

2.4.5 Challenges of supercritical fluid chromatography

There have been a few technical issues that have limited adoption of SFC technology, first of which is the high pressure operating conditions. High-pressure vessels are expensive and bulky, and special materials are often needed to avoid dissolving gaskets and O-rings in the supercritical fluid. A second drawback is difficulty in maintaining pressure (back pressure regulation). Whereas liquids are nearly incompressible, so their densities are constant regardless of pressure, supercritical fluids are highly compressible and their physical properties change with pressure - such as the pressure drop across a packed-bed column. Currently, automated back pressure regulators can maintain a constant pressure in the column even if flow rate varies, mitigating this problem. A third drawback is difficulty in gas/liquid separation during collection of product. Upon depressurization, the CO_2 rapidly turns into gas

and aerosolizes any dissolved analyte in the process (Smith, 1999; Hicks *et al*., 2016; Fountain *et al*., 2014; Lesellier and West, 2015).

The back pressure regulation (BPR) has been one of the biggest pit falls of traditional SFC. As back pressure greatly affects the mobile phase density and consequently the analyte solvation and retention as well, its control has been key to improve the instrumentation. The challenges have been caused by multiple factors; poor pressure monitoring at the BPR, slow-to-respond feedback loops, low-resolution stepper motors, poor control of pressure and flow at the pump, and degradation of BPR components over time (Fountain *et al*., 2014).

2.5 Mass spectrometry

MS is a technique that identifies molecules based on their mass-to-charge-ratio (*m/z*) of their molecule fragments. MS is one of the most common techniques used in analytical chemistry to detect analytes. The spectra obtained by MS can be used to quantitatively or qualitatively determine analyte existence in an unknown sample, find the elemental or isotopic signature of an analyte, the masses of molecules, and to elucidate the chemical structures of molecules.

MS works by separating the ions in a sample based on the fact that they will behave differently depending on their mass-to-charge ratio. There is a variety in the different physical principles upon which MS can be based on. In general all are divided into three steps: A sample introduction system where the analytes are ionized, a mass analyzer that separates ions based on their m/z ratio, and a detector that collects the ions.

Ionization, separation and detection can be performed in several ways. In this study ionization was performed by ESI+, separation was performed by a triple quadrupole mass analyzer, and detection was performed by a photomultiplicator.

2.5.1 Electrospray ionization

Electrospray ionization (ESI) is performed with a high electrical field. The analyte is introduced into the MS in a volatile mobile phase, in this case CO_2 and methanol. The flow passes through a narrow capillary and when exiting the capillary the flow is exposed to an electrical potential between the capillary tube and a counter electrode. The potential required depends on the surface tension of the solvent. Opposing forces between like charges make the

droplet expand until it ruptures, producing an aerosol made up from many small droplets with electrical charge. The aerosol passes an inert drying gas (typically N_2), typically at 500-650 °C, which evaporates and eliminates the solvent. With sufficiently high surface tension, ions from the droplets are released and are carried into the MS. Figure 19 shows the schematic overview of an ESI process. The ESI is considered a relatively soft ionization technique, denoting that mainly unfragmented molecular ions are produced. Both positive and negative ions can be produced by ESI via the electrochemical oxidation and reduction. The reactions depend on concentration of analyte, and the amount of ions detected per time unit is limited by the reactions.

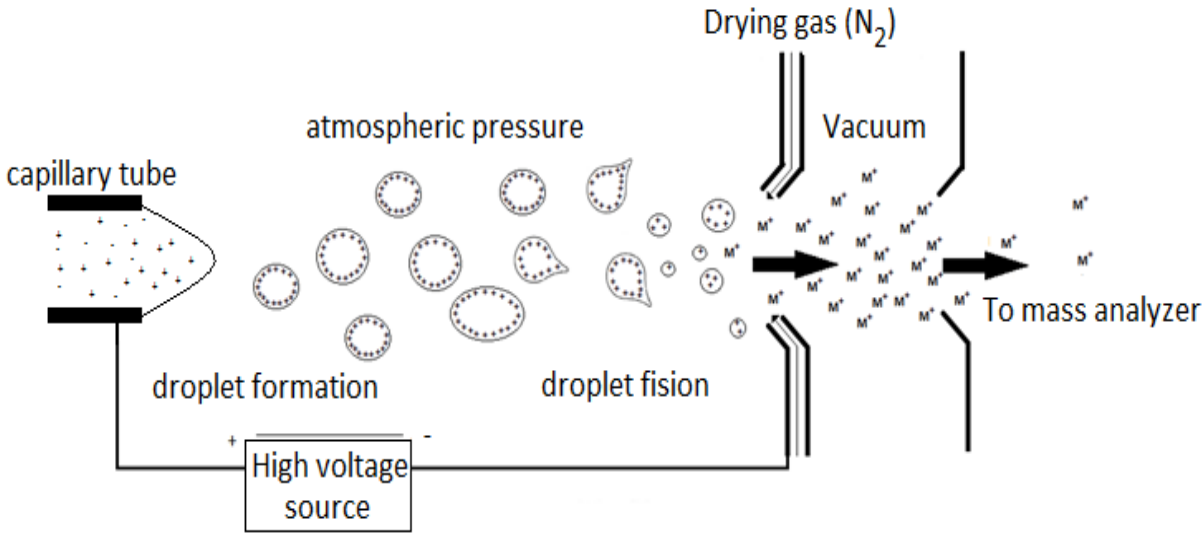

Figure 19. Schematic drawing of an ESI (adapted from Ho *et al.*, 2003)

2.5.2 Quadrupole mass analyzer

Work performed in the late 1800s in the search of a model to describe the fundamental matter was undertaken by chemists and physicists such as Dempster, Aston and so on. Independently, they made advances in the field of physics, and more specifically concerning the behavior of small particles like atoms, molecules and electrons when exposed to ionization and electrical or magnetic fields, which lead to the first model of a mass spectrograph. Their fundamental work lead to combination of insights which provided a foundation for the creation of the important technique of mass spectrometry. The modern deflection-type instruments and techniques all bear some resembles to the original models of Dempster, Wein, Thomson and Aston (Dempster, 1918; Aston, 1927; Münzenberg, 2013; Sharma, 2013). The resemblance is in the deflection of charged particles by a combination of electrical and magnetic fields, but the apparatuses first developed by Aston and Dempster bear an even stronger resemblance to the modern-day instruments.

In practice it is difficult to achieve stable and spatially uniform magnetic fields, in particular with permanent magnets due to fluctuations in thermal and magnetic energy, even in a thermally stable environment. Many factors affect magnet stability include time, temperature, reluctance changes, adverse fields, radiation, shock, stress, and vibration. These variations lead to mass resolution degradation and calibration drifts. In the early 1950s Paul accomplished using alternating quadrupolar electric fields; hence the instrument was named Quadrupole Mass Spectrometer (Paul and Steinwedel, 1953). This clever design eliminated the problems associated with permanent magnets, was highly stable and provided excellent mass resolution. The Quadrupole Mass Spectrometer operates by applying the elegant mathematic equation of Mathieu (1868), which describes the passage of ions through the alternating quadrupolar electric field, as shown in Figure 20.

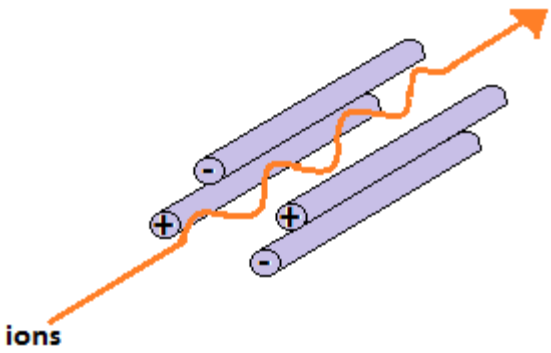

Figure 20. Passage of ions through a quadrupole.

The instrument consists of four cylindrical rods with a specific ratio of rod diameter-to-spacing, which make it easier to attain adequate hyperbolic shaping of the rods.

2.5.3 Tandem mass spectrometry

By MS/MS it is possible to increase sensitivity compared to using MS only. The technique uses two consecutive mass analyzers, which opts for selective examination specific ion fragmentations in mixtures of ions.

Three quadrupoles connected linearly is known as a triple quadrupole tandem mass spectrometer (Figure 21). The first (Q_1) and third (Q_3) quadrupoles functiones as mass filters, while the second (Q_2) is employed as collision cell. The first quadrupole is used to select a precursor ion. In the collision cell, the analytes are subject to radio-frequency potential only. By using an inert gass, such as argon or nitrogen, the analytes can collide and dissociate to form distinctive daughter ions. The daughter ions produced in the collision cell can be filtered in the third quadrupole (Hoffmann and Stroobant, 2007).

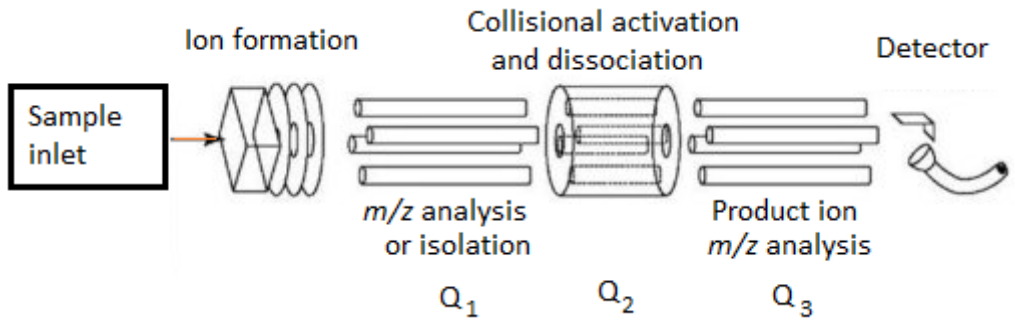

Figure 21. Schematic drawing of a triple quadrupole instrument (adapted from wageningenur.nl).

3 Experimental

3.1. Ultra high performance supercritical fluid chromatography

The SFC instrument (Figure 22 and 23) applied in this thesis work was an Acquity Ultra Performance Convergence Chromatography (UPC^2), which was introduced by Waters (Milford, MA, USA) in 2012. The UPC^2 instrument is a modified Acquity UHPLC system, equipped with an efficient, integrated device to compress and chill the CO_2 before entering the system. Integrating this device is preferable compared to a separated unit, as the path between the chilling unit and the pump can result in density variations (Fountain *et al*., 2014). Good control of both pressure and temperature is achieved by cooling of the pump heads with a Peltier module, which results in less density variations and thus improved repeatability and sensitivity. The instrument is also equipped with a dual stage back pressure regulator (BPR), enabling better pressure control within the system. Dual-stage refers to the design of the BPR performing a combination of an active and a static pressure control. The static BPR ensures that the pressure is kept above a minimum, while the active BPR enables greater control to fine-tune the back pressure. To improve robustness, the static component of the BPR is heated to mitigate problems of ice formation as CO_2 expands adiabatically. The maximum pressure and flow of the instrument are 6000 psi and 4 mL/min, respectively (Fountain *et al*., 2014; Desfontaine *et al*., 2015).

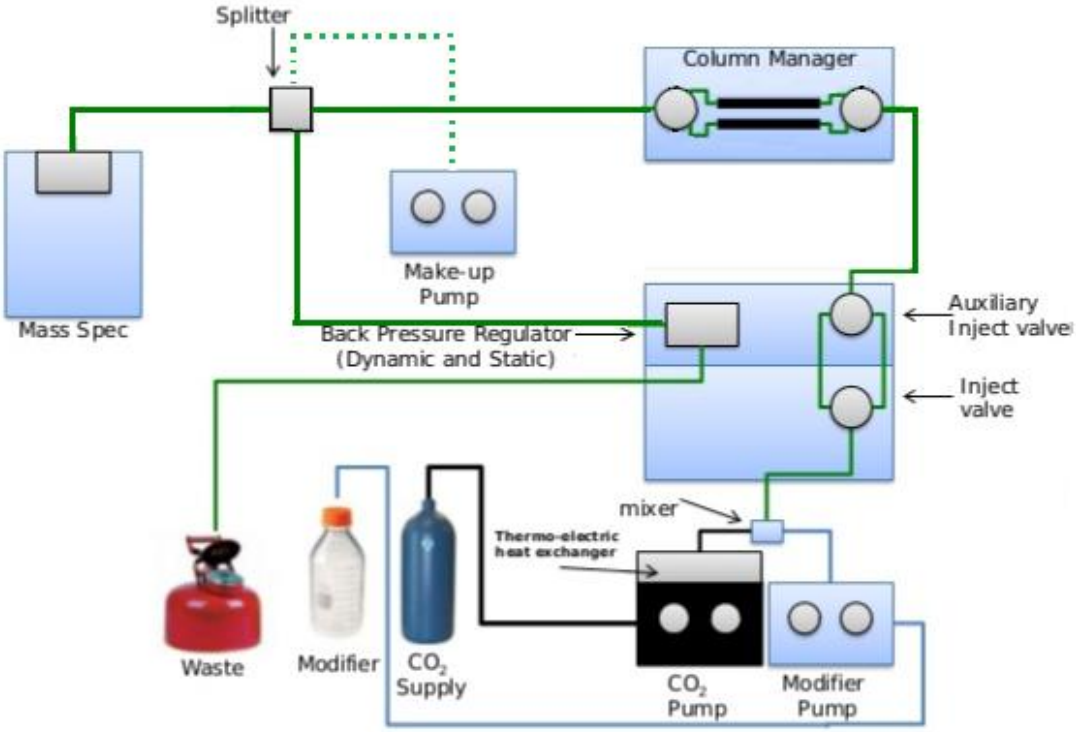

Figure 22. Schematic overview of the UPC^2-instrument, modified version of the one from the Waters to more accurately render the instrument used (modified from the schematic overview from the webpage of the Waters corp.)

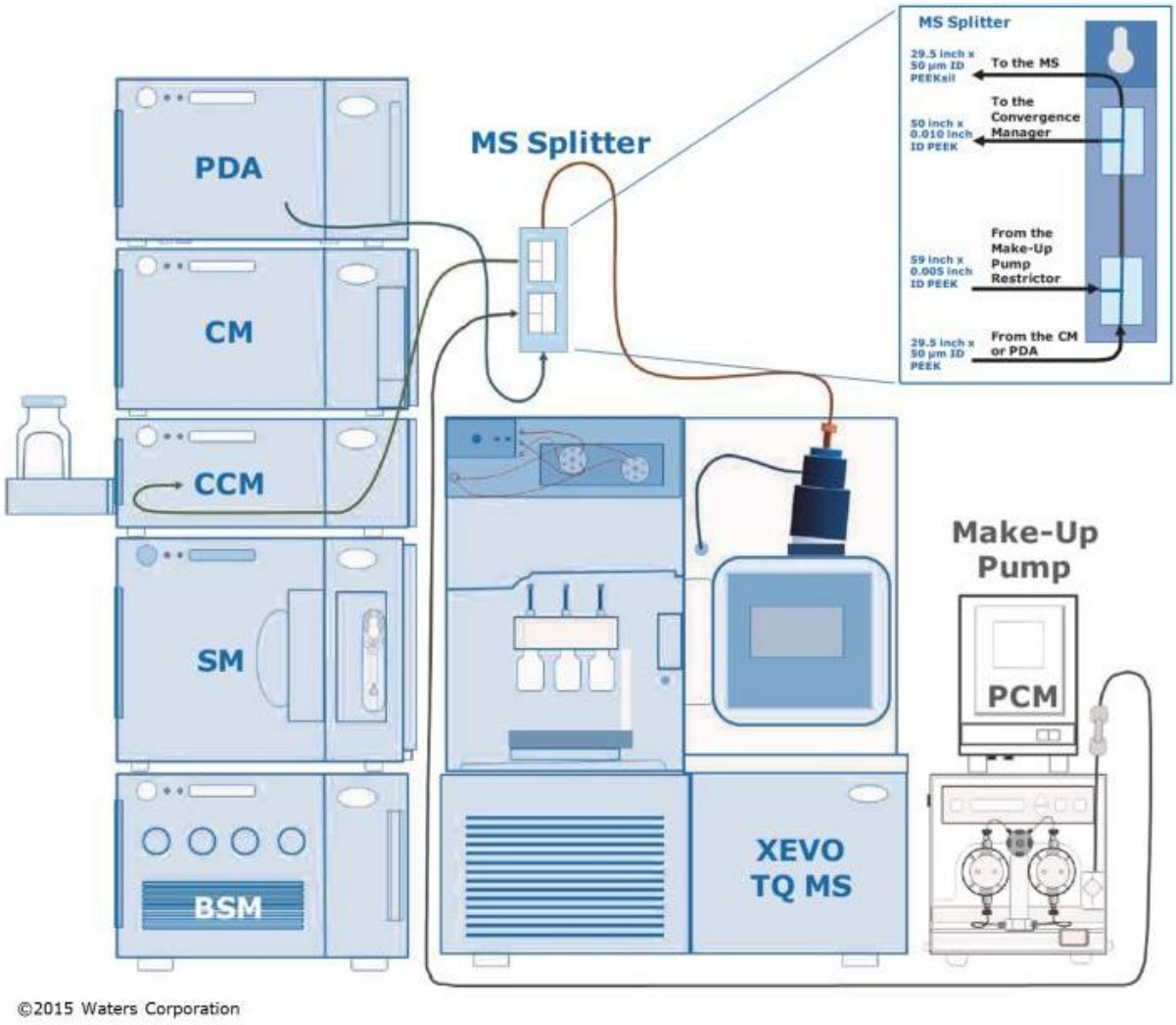

Figure 23. The SFC instrumentation coupled to a MS from Waters corp., including a binary solvent manager (BSM), Sample Manager (SM), Convergence Chromatography Manager (CCM), Column Manager (CM), Photodiode Array (PDA) Detector (not including in the instrument used in this thesis work), Make-Up Flow Pump with additional Pump Control Module (PCM).The MS splitter is used to efficiently merge the SFC components with optimum MS detection. Insert: overview of the MS splitter (Fogwill, 2015).

3.1.1 Chromatographic screening conditions

In order to investigate the selectivity, different stationary phase chemistries and mobile phases were tested. The chromatographic screening conditions used for UHPSFC are listed in Table 2, while Table 3 shows the UHPLC conditions used for the analysis of phospholipids and 2-, 3-, 4-methylamphetamine.

Table 2. UHPSFC instrument parameters.

Injector	Mode	Partial loop with needle overfill
	Injected volume	0.5 µL
Solutions	Weak wash	Isopropanol and heptane (90:10, v/v)
	Strong wash	Methanol
	Seal wash	Methanol
	Washing solvent	Methanol
Sample	Solvent	Isopropanol
	Volume	60 µL
Mobile phase	Supercritical fluid	CO_2
	Modifier	Methanol
	Additive	40 mM NH_3 (dissolved in methanol)
	Gradient components	A: CO_2 B: Methanol with 40 mM NH_3
	Gradient program	Time (min) Component A/B Initial 98 % A : 2 % B 0.2 98 % A : 2 % B 0.3 90 % A : 10 % B 2.0 76.5 % A : 23.5 % B 2.5 60 % A : 40 % B 3.5 60 % A : 40 % B 3.6 98 % A : 2 % B
	Flow	2 mL/min
	Back pressure	1800 psi
	Makeup flow	0.3 mL/min
Column	Stationary phase	BEH (Waters corp.)
	Dimensions	3.0 mm x 100 mm, 1.7 µm particle diameter
	Temperature	60 °C
	Extra column volume	59 µL (Grand-Guillaume Perrenoud *et al*., 2013)
	Dwell volume	40 µL (Grand-Guillaume Perrenoud *et al*., 2013)

Table 3. UHPLC instrument parameters

Injector	Mode	Partial loop with needle overfill
	Injected volume	1.0 µL
Solutions	Weak wash	Water and methanol (95:5)
	Strong wash	Water and methanol (10:90)
	Washing solvent	Methanol
Sample	Solvent	Isopropanol
	Volume	60 µL
Mobile phase	Buffer	Ammonium formate (5mM) dissolved in distilled water
	Modifier	Methanol
	Gradient components	A: 5mM ammonium formate B: Methanol
	Gradient program	Time (min) Component A/B
	Used for methylamphetamine analysis	Initial 95 % A : 5 % B 0.15 95 % A : 5 % B 0.30 90 % A : 10 % B 16.00 60 % A : 40 % B 16.50 2 % A : 98 % B 17.00 2 % A : 98 % B 17.50 95 % A : 5 % B 18.00 95 % A : 5 % B
	Used for phospholipid analysis	Initial 98 % A : 2 % B 5.00 60 % A : 40 % B 10.00 60 % A : 40 % B
	Flow	0.5 mL/min
Column	Stationary phase	BEH (Waters corp.)
	Dimensions	3.0 mm x 100 mm 1.7 µm particle diameter
	Temperature	60 °C

3.1.2 Investigated UHPSFC columns

To examine the selectivity of attainable columns, five different stationary phase chemistries were tested; Ethylene bridged hybrid (BEH), 2-picolylamine (2-PIC), diethylamine (DEA), 1-aminoanthracene (1-AA) and amylose tris(3,5-dimethylphenyl)carbamate (AMY1) from Waters corp. All the columns had dimensions of 3 mm x 100 mm, with 1.7 µm diameter of the particles, expect for the AMY1 column which had dimension of 4.6 mm x 100 mm and 2.5 µm diameter particles. All stationary chemistries of the tested SFC columns are described in chapter 2.4.4.

3.2. Tandem mass spectrometry

For the tandem MS detection a Xevo TQ-S MS from Waters corp. (Milford, MA, USA) was used. Detection was performed with positive electrospray ionization (ESI^+) in MRM mode. Table 4 shows the different MS specification used in this thesis work. Table 5-7 shows the different analyte and internal standard (IS)-transition ions, with associated mass spectrometric parameters.

Table 4. MS parameters for all experiments.

Mass analyzer			
	LM1 resolution	2.7	
	HM1 resolution	14.7	
	Ion energy 1	0.5	V
	Collision energy	2.0	(MS mode collision energy)
	Collision gas flow	0.2	
	Ion energy 2	1.0	V
	LM2 resolution	2.8	
	HM2 resolution	14.8	
MS-source			Annotation
	Capillary voltage	1.0	kV
	Desolvation gas temperature	600	°C
	Desolvation gas flow	900	L/Hr
	Cone voltage	30	V
	Cone gas flow	300	mL/min
	Source temperature	150	V

Table 5. MRM transitions and compound specific MS settings of the 2-, 3-, 4-isomers.

Compound	Precursor ion (*m/z*)		Product ion (*m/z*)	Cone voltage (V)	Collision energy (V)	Dwell time (s)
2-, 3-, 4-methylmethcathinone						
Quantifier	178.10	>	119.00	44.0	20.0	0.008
Qualifier	178.10	>	145.10	44.0	18.0	0.008
2-, 3-, 4-fluoroamphetamine						
Quantifier	154.10	>	109.00	20.0	15.0	0.008
Qualifier	154.10	>	137.10	20.0	8.0	0.008
2-, 3-, 4-fluoromethamphetamine						
Quantifier	168.10	>	109.00	20.0	18.0	0.008
Qualifier	168.10	>	137.10	20.0	10.0	0.008
$^{13}C_6$-PMMA						
Quantifier	186.16	>	127.09	20.0	14.0	0.008
Qualifier	186.16	>	155.12	20.0	14.0	0.008

Table 6. MRM transitions and compound specific MS settings of the classical phenethylamines.

Compound	Precursor ion (*m/z*)		Product ion (*m/z*)	Cone voltage (V)	Collision energy (V)	Dwell time (s)
Amphetamine						
Quantifier	136.11	>	91.05	15.0	12.0	0.008
Qualifier	136.11	>	119.09	15.0	12.0	0.008
Methamphetamine						
Quantifier	150.13	>	91.05	15.0	14.0	0.008
Qualifier	150.13	>	119.09	15.0	14.0	0.008
MDMA						
Quantifier	194.12	>	133.07	20.0	14.0	0.008
Qualifier	194.12	>	163.08	20.0	14.0	0.008
$^{13}C_6$-Amphetamine						
Quantifier	142.13	>	97.07	15.0	12.0	0.008
Qualifier	142.13	>	125.11	15.0	12.0	0.008
$^{13}C_6$-Methamphetamine						
Quantifier	156.15	>	97.07	15.0	14.0	0.008
Qualifier	156.15	>	125.11	15.0	14.0	0.008
$^{13}C_6$-MDMA						
Quantifier	200.14	>	139.09	20.0	14.0	0.008
Qualifier	200.14	>	169.10	20.0	14.0	0.008

Table 7 displays the MS settings used to scan for the parent ion of phospholipid, when evaluating different extraction techniques with respect to their ability to remove phospholipids.

Table 7. Settings for the MS analysis of phospholipids used for the evaluation of extraction agents.

Compound	Precursor ion (*m/z*)		Product ion (*m/z*)
Phospholipids			
Parent ion	184.40	>	184.40

3.3 Other equipment

The whirl mixer used was supplied by Heidolph (Schwabach, Germany). For centrifugation a Thermo Fisher Scientific Multifuge X3R was applied from VWR (Oslo, Norway). The N_2-evaporator RapidVap Vertex Evaporator was provided by Labconco (Kansas city, Missouri, USA). For the tilting of samples a tilting machine built by local technician at NIPH was applied. Nitrogen used for MS desolvation was produced by a nitrogen generator (Oxymat, AGA Norway, 0.5 % O_2).

3.4 Chemicals

The CO_2 gas was purchased from Yara Praxair (Oslo, Norway) as food grade (99.8 %), while the methanol used was LC-MS Chromasolv purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol was absolute and purchased from Merck Millipore (Billerica, MA, USA). Isopropanol, heptane, ethyl acetate, HNO_3 (65 %, dissolved in methanol), HPLC-grade methyl tert-butyl ether (MTBE) and 25 % (v/v) aqueous NH_3 for analysis was purchased from Merck as well. The NH_3 dissolved in methanol (2M) was purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile was HPLC-grade from J. T. Baker (Center Valley, PA., USA). Formic acid (98 %) was supplied by VWR (Oslo, Norway). Cyclohexane was supplied by Rathburn (Walkerburn, UK). Water used for analysis was purified by filtering deionized water on a Milli-Q filtration system (Millipore, Bedford, MA, USA). Argon gas (99.999 %) was supplied by AGA (Oslo, Norway).

2- and 3-methylmethcathione were supplied by Cayman Chemical Company (Ann Arbor, Michigan, USA), while 4-methylmethcathione and amphetamine was supplied by Sigma Aldrich (St. Louis, MO, USA). 2-fluoroamphetamine was purchased from Alfa Aesar (Heysham, Lancashire, UK). 3-, 4-fluoroamphetamine, 2-, 3- and 4-fluoromethamphetamine and the 2-, 3- and 4-methylmethamphetamine were purchased from Chiron AS (Trondheim, Norway). Methamphetamine and MDMA were purchased from Cerilliant Corporation (Round Rock, Texas, USA). The structures of the analytes of interest are shown in Figure 24. The pK_a and log P for the analytes of interest are listed in Table 8.

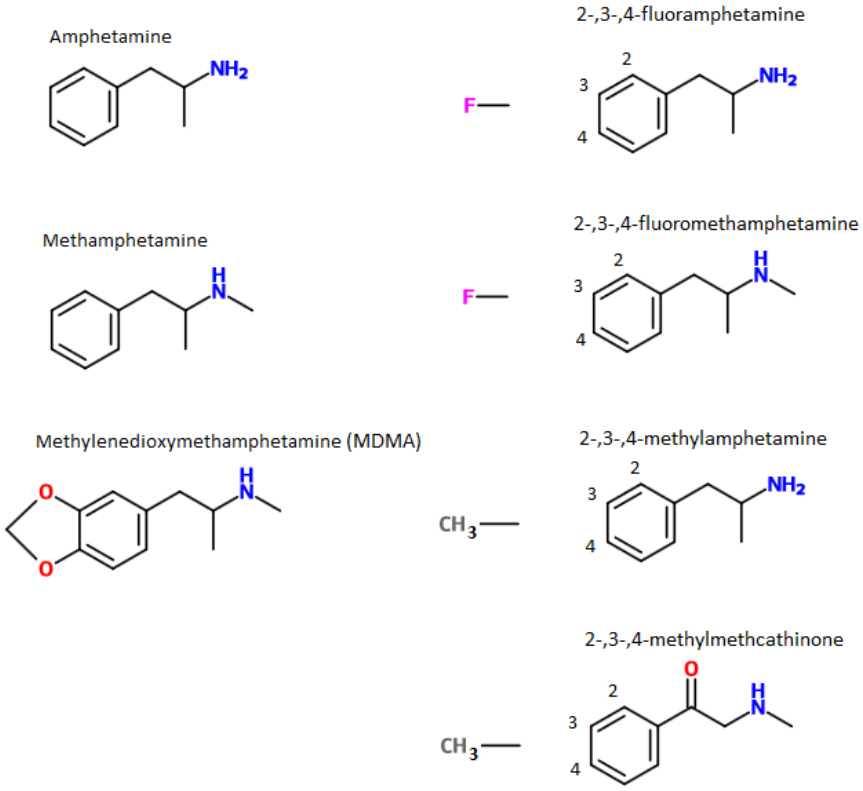

Figure 24. Structures of the analytes of interest (made at emolecules.com/ and modified in Paint with numbers)

Table 8. Table of pK_a and log P for all analytes.

Compound(s)	pK_a	Log P
Amphetamine	10.13, at 20 °C (Perrin, 1965)	1.76 (Hansch *et al*., 1995)
Methamphetamine	9.87 at 25 °C (Perrin, 1965)	2.07 (Hansch *et al*., 1995)
MDMA	10.14 (drugbank.ca)	1.07/1.27 (drugbank.ca)
2-, 3-, 4-Fluoroamphetamine	≈ 10	≈ 2
2-, 3-, 4-Fluoromethamphetamine	≈ 10	≈ 2
2-, 3-, 4-Methylamphetamine	≈ 10	≈ 2
2-, 3-, 4-Methylmethcathinone	≈ 10	≈ 2

The values of the constitutional isomers of the new designer drug versions of amphetamines are estimated to be somewhat the same as for amphetamine and methamphetamine. The small differences in structures should not affect their values, especially since the functional group of the molecule is not changed and the alteration is on the opposite side of the phenyl ring.

3.5 Internal standards

Stable internal standards (ISs) were used to correct for lost analyte during sample preparation and variations during instrumental analysis. Deuterium (^{2}H) labelled ISs are commonly used in forensic toxicology to ensure qualitative and quantitative detections. However, for optimal correction for loss of analyte and possible MEs ^{13}C-labelled ISs are preferred over ^{2}H-labelled ISs (Berg and Strand, 2011). Both ^{13}C and ^{2}H-labelled ISs are routinely used at NIPH, and ^{13}C-labelled ISs were applied for this method. The calculations of the concentrations of IS-solutions are shown in Appendix I. All IS compounds were obtained from Chiron AS (Trondheim, Norway) and their structures are shown in Figure 25.

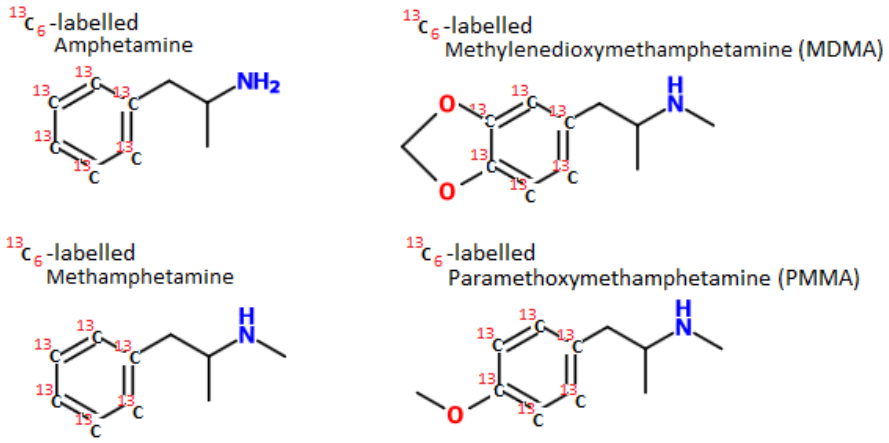

Figure 25. Structures of the $^{13}C_6$-labelled IS used for this thesis work (made at emolecules.com and modified in paint).

3.6 Solutions

3.6.1 Preparation of Stock Solutions, Standards and Control Samples

Table 9. Standard and control Samples

	Concentration (µM)
Blank	-
Standard 1	0.010
Standard 2	0.10
Standard 3	1.0
Standard 4	10
Standard 5	4.0
Control 1	0.0010
Control 2	0.0050
Control 3	0.010
Control 4	0.10
Control 5	1.0
Control 6	10
Control 7	5.0

The stock solutions were prepared by dissolving an appropriate quantity of the standard compound in pure methanol and were stored at -20 °C. Working solutions for calibrator and quality control samples were further eluted from these ready-to-use stock solutions and kept refrigerated at 4 °C. The solvent chosen for all working solutions was isopropanol.

The solutions were separated into two sets; A and B. The A solutions contained amphetamine, methamphetamine and MDMA, while the B solutions contained 2-, 3-, 4-fluoroamphetamine, 2-, 3-, 4-fluormethamphetamine and 2-, 3-, 4-methylmethcathinone. The analytes were separated due to their coelution and probable effect on ion detection either as ion suppressive or ion enhancing. The concentration levels are displayed in Table 9.

3.6.2 Blank blood

Whole blood containing 2 g sodium fluoride, 6 mL heparin and 10 mL water per 450 mL blood was used for development and validation of the method, and was purchased from the blood bank at Oslo University Hospital (Oslo, Norway). Different lots of human blood were used.

3.6.3 Preparation of modifier

The modifier solution consisting of 40 mM NH_3 in methanol used for the UHPSFC analysis was prepared by the addition of 5 mL NH_3 solution (2 M in methanol) in 250 mL methanol. The modifier solution consisting of 0.3 % NH_3 in methanol used for the UHPSFC analysis was prepared by the addition of 3 mL NH_3 solution (25 %, v/v) in 250 mL methanol. The calculations related to these solutions are displayed in Appendix II.

3.6.4 Preparation of buffer used for UHPLC analysis

The 50 mM ammonium formate stock solution used for the UHPLC analysis was prepared by dissolving 6.3 g of ammonium formate in 2000 mL MilliQ water. The 5 mM ammonium acetate buffer solution was prepared by dissolving the 50 mM stock solution by 1:10. The pH was adjusted by addition of aqueous ammonia (25 %) until pH 10.2.

3.7 Sample preparation

3.7.1 Sample preparation equipment

The SPE filters applied were Supelco Hybrid SPE from Sigma Aldrich (St. Louis, MO, USA), Oasis PRiME HLB from Waters corp. (Milford, MA, USA) and Phree from Phenomenex Aps (Torrance, CA, USA). All filters had a sample volume of 1 mL (30 mg).

3.7.2 Method procedure

All working solutions and blank blood from were set in room temperature to temperate. Blank blood of 100 µL was added to all blanks, standard and control samples. All working solutions were whirl mixed before use. To each standard and control samples (in accordance with Table 9) 100 µL of working solution was added, while 100 µL of distilled water (MilliQ H_2O) was added in the blank samples. Subsequently, 50 µL of internal standard was added in all samples and all samples were whirl mixed. Ammonium carbonate buffer (pH 9.3) was added in all samples at a volume of 100 µL and all samples were whirl mixed again. To extract the analytes from the blood matrix, 1000 µL of ethyl acetate and heptane (80:20, v/v) was added in all samples and then tilted for five minutes. Centrifugation was executed at 3500 min^{-1}, in 6 °C for five minutes. Transfer of 800 µL of the organic phase to new plastic tubes was followed by addition of 10 µL 0.1 % HNO_3 solution in all samples. All samples were evaporated until complete dryness in the N_2-evaporizer for 10 minutes, at 20 psi and 45 °C. The residues were reconstituted in 60 µL isopropanol and whirl mixed before transfer to autosampler vials and injection into the UHPSFC-MS/MS.

3.8 Data Analysis

MassLynx software version 4.1 obtained from Waters was used for instrument control, data acquisition and processing.

3.9 Method validation

Method validation was performed to confirm that this analytical procedure is suitable for its intended use. The data obtained can be used to evaluate the quality, reliability and consistency of the method. All validation parameters were executed in concordance with the validation regime of NIPH and by using blank blood spiked with the different analytes.

3.9.1 Interday precision and accuracy

Interday precision was defined as the measure of the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogenous sample. It is expressed numerically as imprecision. Interday accuracy is defined as the closeness of agreement between the mean of the results of measurements of a measurand and the true (or accepted true) value of an analyte. It is reported as a percent difference.

Precision and accuracy was determined for all analytes in the concentration range of 0.001 – 10 µM for the 2-, 3-, 4-isomers and in the range of 0.005 – 10 µM for the classical amphetamines. The calculation was based on eight series.

3.9.2 Intraday precision and accuracy

Intraday precision and accuracy of the method within a single series is a measure of the repeatability within one assay of analysis. Accuracy is calculated as percent deviation from the nominal value. The precision of the intraday (within-series) measurements was expressed as relative standard deviation (RSD). Repeatability was measured by ten parallels of each analyte at two different concentration levels; 0.25 and 1.0 µM.

3.9.3 Linearity

Linearity is defined as a measure of the range in which there will be a correlation between signal response (e.g. peak ratio of analyte and internal standard) and an analyte concentration in the sample. Linear weighted (1/x) calibration curves were chosen for this method. For the

2-, 3-, 4-isomers $^{13}C_6$-PMMA was used as IS, while amphetamine, methamphetamine and MDMA used corresponding $^{13}C_6$-labelled IS. Linearity was measured with extracted samples with a concentration of 0.001, 0.005, 0.010, 0.100, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00 µM.

3.9.4 Limit of detection and limit of quantification

Limit of detection (LOD) is the lowest concentration of analyte which can be confirmed to be present in the sample. Limit of quantification (LOQ) is the lowest concentration which can be quantified with satisfactory precision. Both have, in this thesis work, been determined by equation 4 and 5, respectively.

$$LOD = mean\ value\ of\ blank + 3\ x\ SD\ of\ 0.005\ \mu M\ control \qquad \text{Eq. 4}$$

$$LOQ = mean\ value\ of\ blank + 10\ x\ SD\ of\ 0.005\ \mu M\ control \qquad \text{Eq. 5}$$

3.9.5 Matrix effect

The matrix effect (ME) is a measure of the combined effect of the sample other than the analyte on the measurement of the quantity. In order to evaluate ME corrected with IS two concentration levels and injections volumes were chosen; 0.1 and 1.0 µM, 0.5 and 2.0 µL. Five different lots of blank human blood from the blood bank at Oslo University Hospital (Oslo, Norway) were used with two parallels of each.

Sample preparation was based on two sets of sample extracts. The first set consisted of ten extracts of the blank matrices spiked with the analytes of interest, added post extraction, using five lots of blank blood and two parallels of each lot. The second set consisted of four parallels with neat solutions containing equivalent amounts of compound of interest prepared in autosamplervials. The same procedure was performed for both concentration levels and all samples were extracted and analyzed the same day. After extraction and prior to evaporation, IS was added to the first set of samples. The calculation of ME was performed by dividing the mean peak height of the samples spiked after extraction (A) with the mean peak height found in the neat solutions (B) (eq. 6). The calculation of corrected ME was performed using the concentration of analyte (C) corrected with the peak height of an IS (D) (eq. 7).

$$ME = \frac{A}{B}\ x\ 100 \qquad \text{Eq. 6}$$

$$ME_{corrected} = \frac{C}{D}\ x\ 100 \qquad \text{Eq. 7}$$

A value above 100 % indicates ion enhancement, and a value below 100 % indicates ion suppression. RSDs were calculated based on the variation of the peak height s of the analytes in the five spiked extracts or the variation of the peak heights corrected with the peak heights of the IS. Acceptable values of ME are between 75 and 125 %, with RSDs ≤ 25 % when using deuterated ISs (Paul *et al*., 2009).

3.9.6 Extraction recovery

Extraction recovery (%) is a measure of the amount of analyte attainable from the extraction process chosen and was measured at two concentration levels with three parallels of each concentration and two different lots of blank blood (n=6).

The recovery was calculated by comparison of the calculated concentrations obtained for the validation samples with the analytes added before extraction and the IS were added after (n=6), with the samples obtained when both the analytes and the IS were added after the extraction step (n=4).

3.9.7 Carry-over

Carry-over was examined by injection of a samples with a concentration which the highest standard (10 µM), followed by two blanks. The samples were not extracted.

3.9.8 Specificity

The specificity of the method was investigated by analysis of a fortified sample with some commonly found drugs of abuse (n = 25). The chromatograms were evaluated for interfering peaks at the same retention time as the analytes of interest. The analytes and their respective concentrations are shown in Appendix VII, Table VII e. Additionally, eight blank samples

prepared with five different whole blood lots were also tested for possible interfering endogenic substances.

4 Results and discussion

In this thesis work a method was developed which could separate and distinguish a group of new synthetic amphetamines by a UHPSFC-MS/MS method. Traditionally, LC is applied for such compounds. However, this is intrinsically difficult due to the similarity between these isomers. The use of UHPSFC opens for additional ways to affect the elution and separation of analytes by adjusting more parameters attainable than in LC.

The group of analytes of interest in this thesis work are all new psychoactive substances and are attainable in three different positional isomers. These substances are listed in chapter 3.4, including their structural formulas in Figure 24. Since the abuse of these compounds is often accompanied with the abuse of the traditional amphetamines, intentionally or unintentionally due to impurities or dilution of illicit drugs, the validation performed included the classical phenethylamines amphetamine, methamphetamine and MDMA.

4.1 Sample preparation

All biological samples contain a complex mixture of molecules. In order to perform a robust and efficient chromatographic and mass spectrometric analysis of biological samples it is important to get rid of possible contaminants and also to retain as high a percentage of analyte as possible.

For the method in question whole blood was chosen as sample agent. Blood is generally considered to be the sample specimen that shows the best correlation between drug concentration and pharmacologic effect, as it reflects recent intake and can be used to assess impairment (Gjerde *et al.*, 2011). Phospholipid removal was considered to be an important evaluation point when considering different extraction methods, as removal of phospholipids from blood samples is crucial in order to avoid smudging and contamination of the MS. Additionally they could co-elute and ionize with the analytes of interest. A possible co-elution would result in ion-suppression of the MS signal that would cause variability and could lessen the accuracy of the methods result. There are also other sample matrix elements which could compromise the system, such as various proteins, which can precipitate in the SFC column (Wu *et al.*, 2008). Both SPE and LLE techniques were tested and are theoretically described in chapter 2.3.

4.1.1 Evaluation of extraction method

When choosing an appropriate extraction method, there were several factors to take into account. There should be adequate analyte yield, when extracting the analytes from the sample matrix. Meaning that the resulting extract should contain an amount of analyte molecules, which is representative for the primary sample. Furthermore, it is desirable to remove as much as possible of the redundant compounds of the extract, particularly troublesome components like blood proteins and phospholipids as they can cause contamination and compromise of the analysis result. The total cost must also be taken into account. Additionally, it is more cost efficient to apply one of the methods already used in the laboratory which will implement the technique. Among the routinely applied techniques in the sample preparation methods of the NIPH's forensic department, are SPE and LLE. Choosing an extraction method that is already in use, would be practical considering required appliances, purchase and storage of reagents. Time consumption is an important factor to take into consideration. There is a wish for developing more rapid sample preparation methods in order for results to be quickly dispatched to the requisitioner, but also time consumption is an extension of the cost evaluation. Sample preparation is often the most labor intensive part of the method procedure as the instrumental analysis is highly automated. The methods technical degree of difficulty will affect time consumption and a method which is technically advanced will more often be prone to error than more simplistic ones. Safety must also be taken into consideration, especially for the hazardous solvents used in LLE.

Three SPE filters and three LLE solutions were tested, see Table 10 and 11.

Table 10. SPE filters applied for the evaluation of extraction methods.

SPE filters
Supelco Hybrid SPE
HLB Prime
Phree

Table 11. LLE solutions tested for the evaluation of extraction methods

LLE solutions
Cyclohexane
Ethyl acetate and heptane (80:20)
Methyl ter-butyl ether (MTBE)

Protein precipitation by acetonitrile and methanol (85:15) was also tested and used as a frame of reference between the different methods as it removes less phospholipids compared to the SPE filters and LLE solutions chosen. All the sample preparation techniques were tested on their ability to remove phospholipids and appropriate analyte yield with UHPSFC-MS/MS analysis (data not shown). All extracts showed satisfactory peak height for all analytes, while only the chosen extraction technique was quantitatively evaluated upon its extraction yield.

The SPE cartridges Supelco Hybrid SPE and Oasis PRiME HLB, both showed satisfactory removal off phospholipids. The Phree plate however, showed a superior performance with a close to complete removal of phospholipids (see Figure 26 and 27). Nevertheless, the Phree plate demanded quite high vacuum in order for the fluid to flow through. This makes this method more difficult and time consuming to use in a routine application. Experiment design for the evaluation of extraction agent is shown in Appendix V.

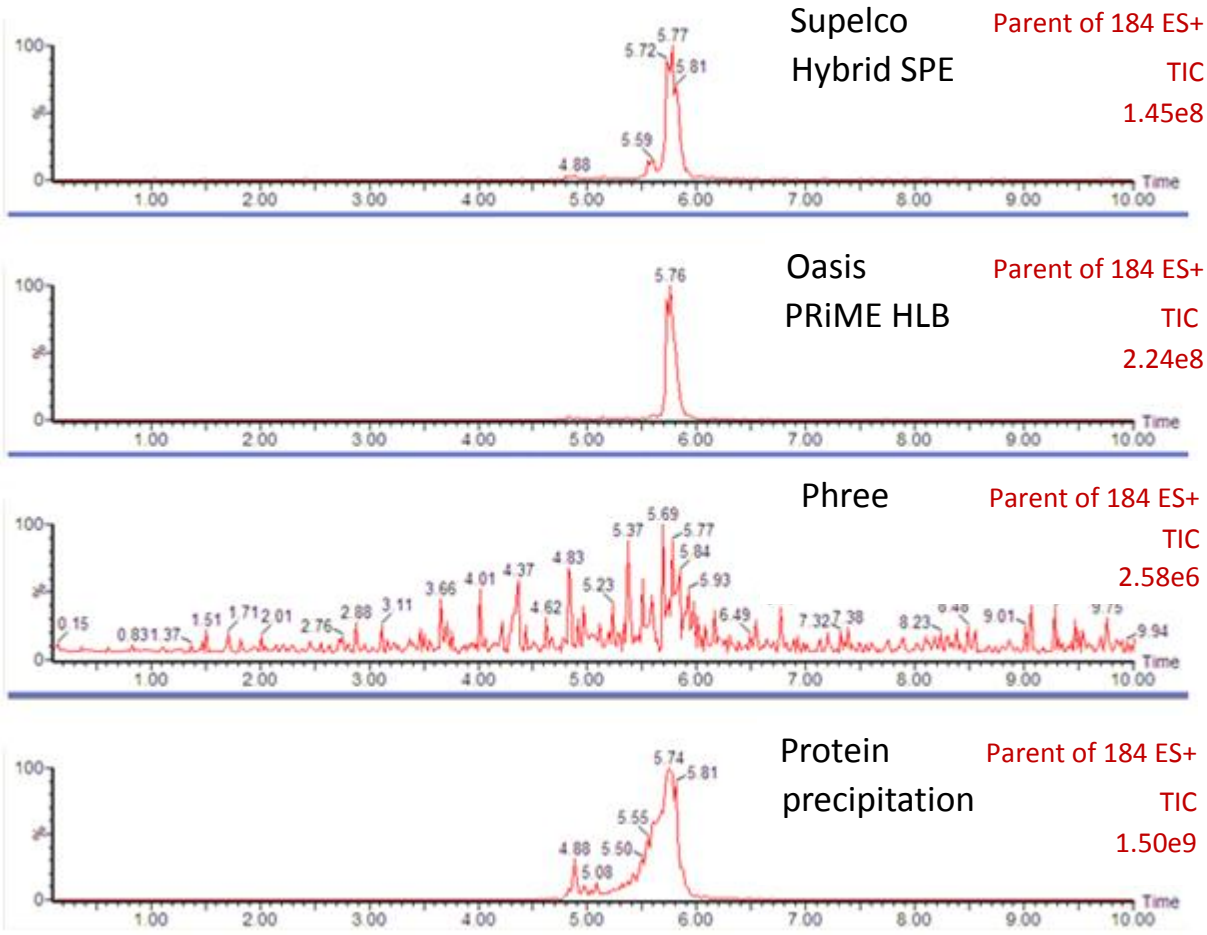

Figure 26. UHPLC-MS/MS analysis of extract after SPE and protein precipitation extraction techniques.

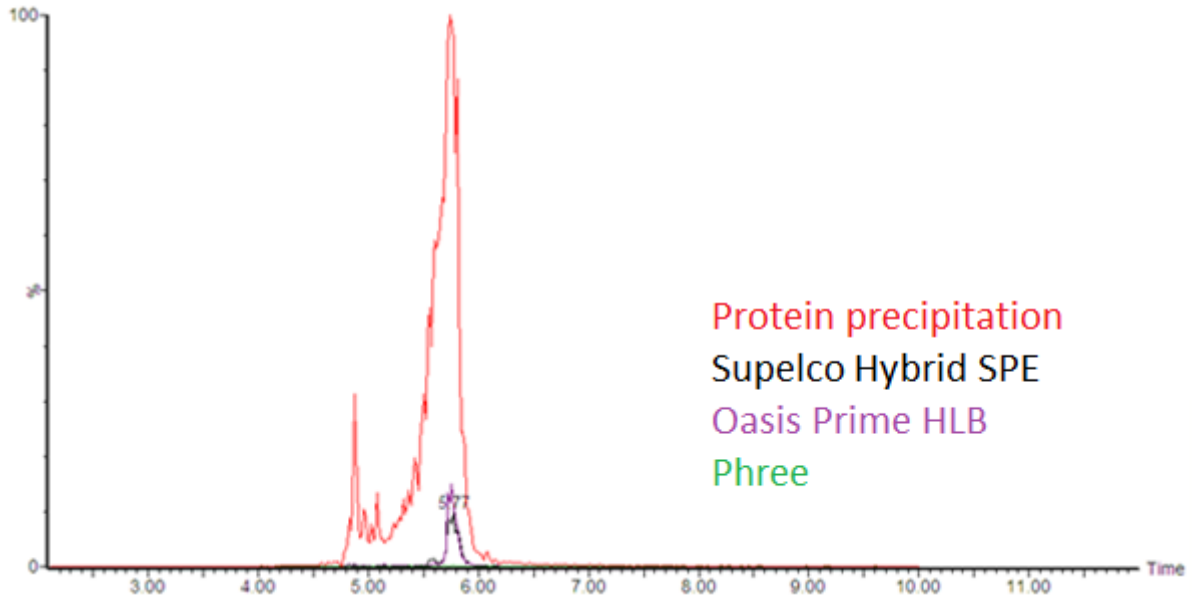

Figure 27. UHPLC-MS/MS analysis of blood extracts from SPE and from protein precipitation. UHPLC-MS/MS analysis were performed by parent ion scan of *m/z* 184. Protein precipitation (red), Supelco Hybrid SPE (black), Oasis PRiME HLB (purple) and Phree (green)

Another consideration to make for the appropriate sample extraction technique was the quantity of samples. As the analytes of interest are not as common as their phenethylamine cousins' amphetamine, methamphetamine and MDMA, the analysis will not produce more than a handful of samples per month in average. Not all SPE products are produced as single cartridges, but rather as 96-well plates. All the SPE techniques tested in this thesis work were attainable as single cartridges. Ostro SPE filter from Waters corp. was also considered, but was omitted as it is only attainable in 96-sample plates. As this method is a low sample volume method, there is not a large enough sample volume for the use of 96-sample plates.

The most phospholipids in whole blood were removed by LLE (Figure 28 and 29) and the recovery of analytes after extraction was considered satisfactory with cyclohexane and ethyl acetate and heptane (80:20) as a high analyte signal was produced (> 10^8, shown in Appendix V). Among the solvents used for LLE, cyclohexane is more volatile and hazardous than the other two options. As cyclohexane also did not provide any advantage over the others, it was not considered to be the best option. Ethyl acetate and heptane (80:20) was chosen as the best option for this method procedure, due to appropriate phospholipid removal (further details shown in Appendix V). Other mixtures of ethyl acetate and heptane were not tested as the solution mixture tested is already routinely used in several methods at NIPH.

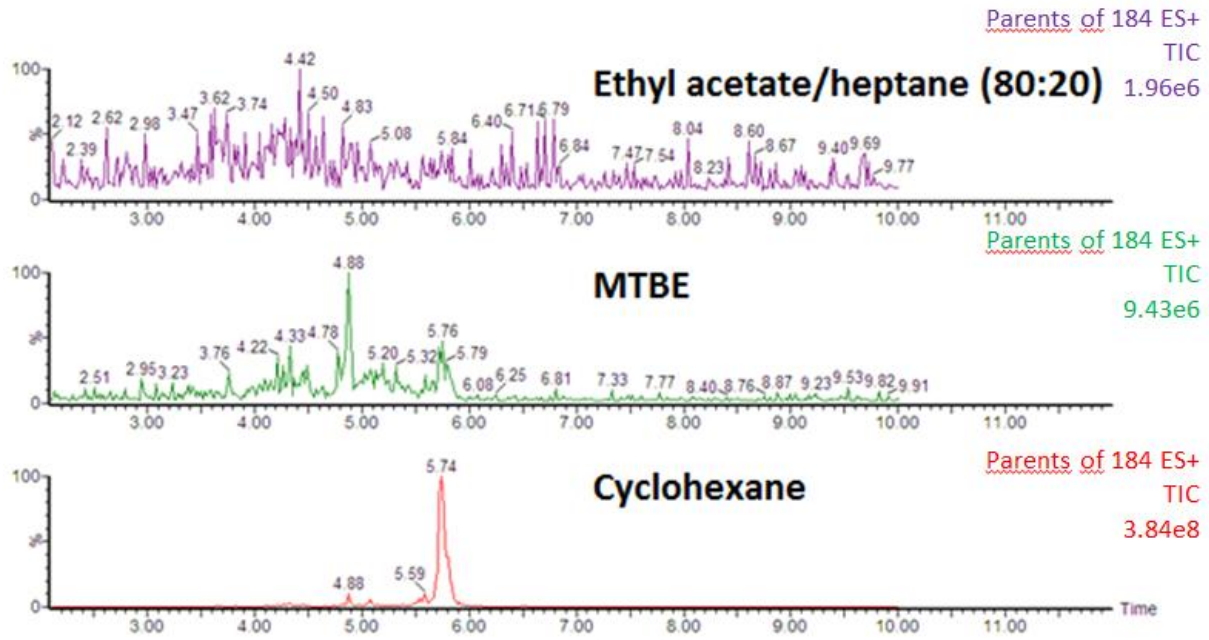

Figure 28. UHPLC-MS/MS analysis of extract showing residue phospholipids after LLE techniques. LLE by cyclohexane: Red; Ethyl acetate/heptane (80:20): Purple, MTBE: Green (chromatographic and MS-settings as described in chapter 3.1.1 and 3.2).

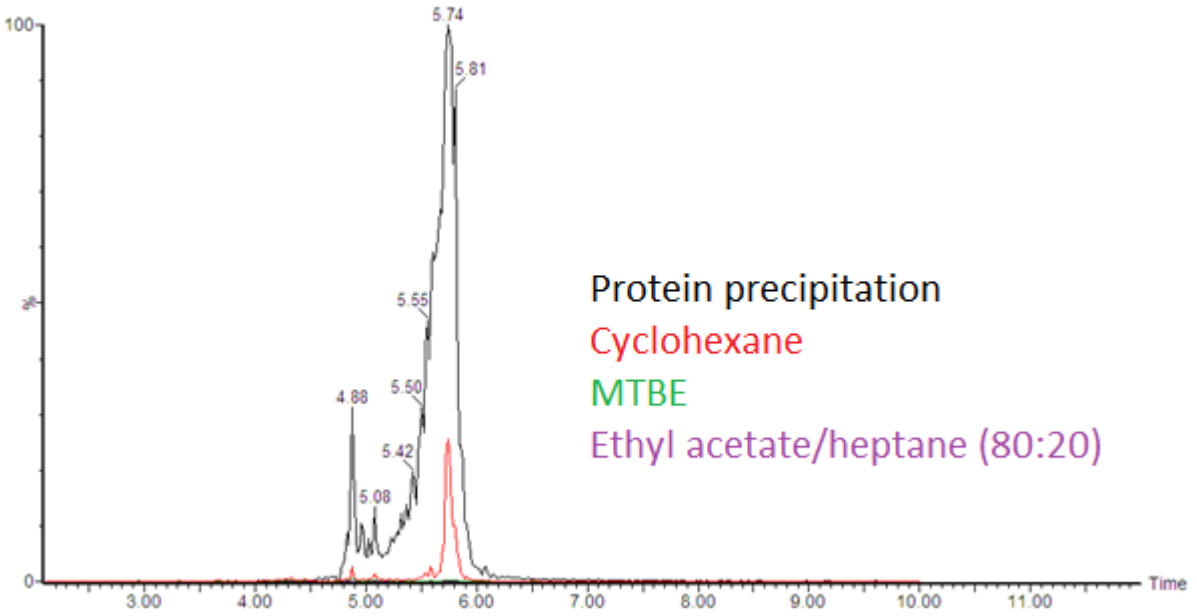

Figure 29. UHPLC-MS/MS analysis of extract showing a comparison of the residue phospholipids after LLE versus protein extraction techniques by overlaying graphs. Protein extraction: Black; LLE by cyclohexane: Red; Ethyl acetate/heptane (80:20): Purple, MTBE: Green (chromatographic and MS-settings as described in chapter 3.1.1 and 3.2).

4.2 Chromatographic separation by UHPSFC

In order to distinguish between the analytes of this thesis work, chromatographic separation by UHPSFC was utilized. To our knowledge, until now generally HPLC and GC have been utilized for the separation of some of the analytes (Davis *et al*., 2012; Mohr *et al*., 2012: Plotka *et al*., 2011; Plotka *et al*., 2012; Röhrich *et al*., 2012; Santali *et al*., 2011; Hegstad *et al*., 2014; Wang *et al*., 2015). Nevertheless, there seems to be a rising interest in UHPSFC for the analysis of such compounds (Grand-Guillaume Perrenoud *et al*., 2012a; Gourmel *et al*., 2013; Kalikova *et al*., 2014; Li and Lurie, 2014; Newmeyer *et al*., 2014; Pauk *et al*., 2015). In order to find the optimal conditions for the separation of the analytes a series of different experiments were performed. The UHPSFC technique can be varied by several parameters; pressure, density, co-solvent amount, additive amount, stationary phase, flow rate. The road to a complete method was a winding one and led to a series of additional questions and technical difficulties concerning the technique of supercritical fluid chromatography.

The supercritical fluid chosen for this thesis work was CO_2. Although there are other options, only CO_2 was considered as it is by far the most commonly applied supercritical fluid due to its beneficial properties as explained in chapter 2.4.2.

Although there is a high similarity between these analytes, they highly differ in their affinity and behavior in SFC for the conditions chosen as shown in Figure 30. The biggest difference is found in the functional group attached to the phenyl ring and its position. The ability to separate these closely related molecules can probably be accredited to these slight differences. Furthermore, the methyl group found on C1 of the common structure in methamphetamine, fluoromethamphetamine and methylmethcathinone appears to make the analytes somewhat more robust for chromatographic changes in the BEH stationary phase which are discussed in chapter 4.3.3.

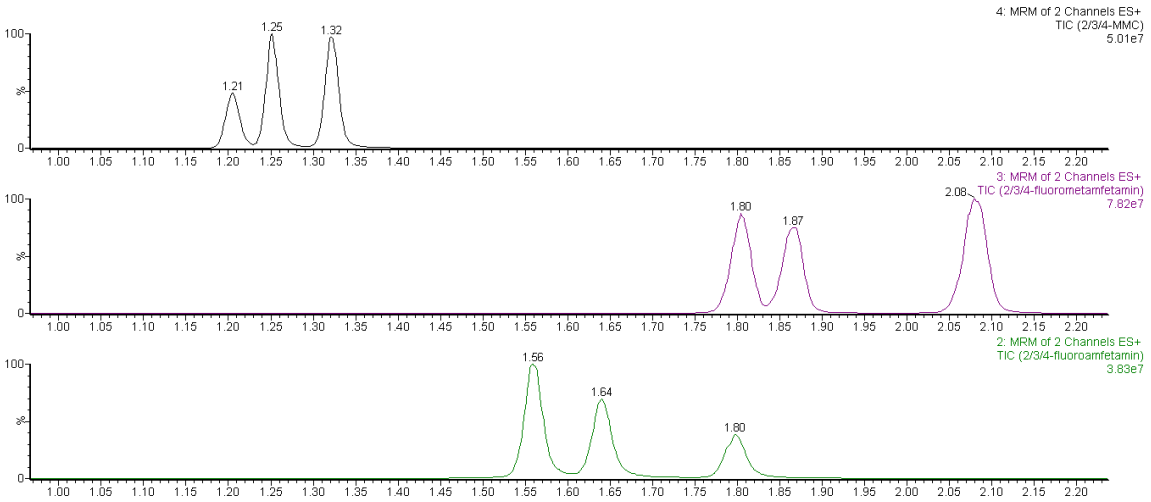

Figure 30. Optimal separation of the nine analytes of interest was found with a BEH-column, using a modifier solution of MeOH with 40 mM NH_3, 60 ° C and 1800 psi (chromatographic and MS settings as described in chapter 3.1.1 and 3.2.1). Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

The validation performed included the classical phenethylamines amphetamine, methamphetamine and MDMA. A chromatogram showing all the analytes is shown in Figure 31. However, due to the co-elution of the classical phenethylamines with the new psychoactive substances, the analytes were separated into two sets of solutions, referred to as A (the classical phenethylamines) and B (the 2-, 3-, 4-phenethylamines). This was done in an attempt to alleviate the possible ion suppression effects caused by the coeluting amphetamine. The ion suppression effect was observed for the analytes which co-eluted with the classical phenethylamines, but was not measured due to lack of time.

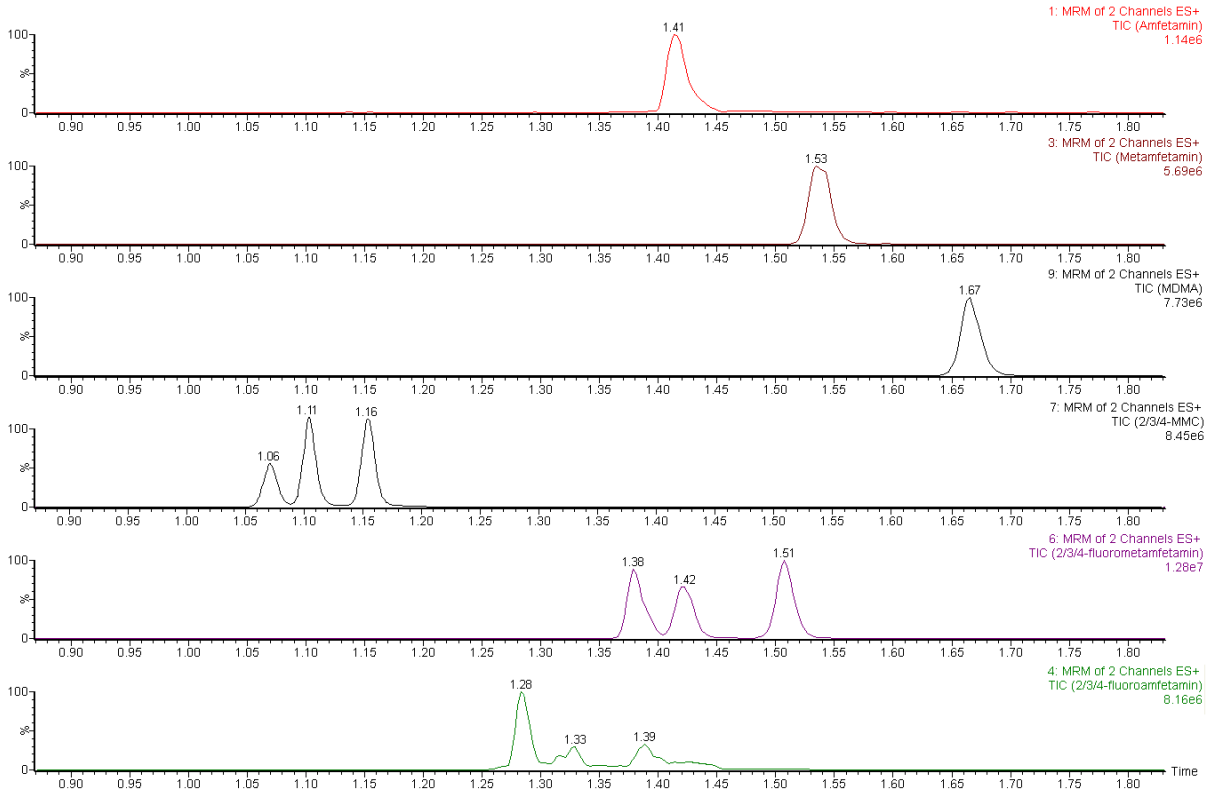

Figure 31. Chromatogram showing all the analytes of interest. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1. Analytes from top to bottom: Amphetamine (red), methamphetamine (brown), MDMA (black), methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

The 2-, 3-, 4-positional isomers of methylamphetamines were also initially included in the thesis work, but no conditions could be set to successfully separate these isomers (Figure 32). However, the methylamphetamines might be separated with a UHPLC (Figure 33). The chromatographic and mass spectrometric settings were comparable to the SFC settings, however with a slower and longer gradient with an aqueous 5 mM ammonium formate mobile phase and the column temperature set at 60 °C. The peaks are not baseline separated, because the separation was not optimized as this was beyond the scope of this thesis work. The analysis of the positional isomers of methylamphetamine was only tested on UHPLC to find an alternative way to analyze this set of isomers. The possible successful separation with UHPLC could suggest that a more polar solvent, such as water, is required to overcome the intermolecular interactions of these analytes.

Since baseline separation of the 2-, 3-, 4-methylamphetamines was not obtained by UHPSFC-MS/MS, the focus of this thesis work became aimed at the 2-, 3-, 4-fluoroamphetamine, 2-, 3-, 4-fluoromethamphetamine and 2-, 3-, 4-methylmethcathinone. These nine analytes are from here on collectively referred to as analytes of interest.

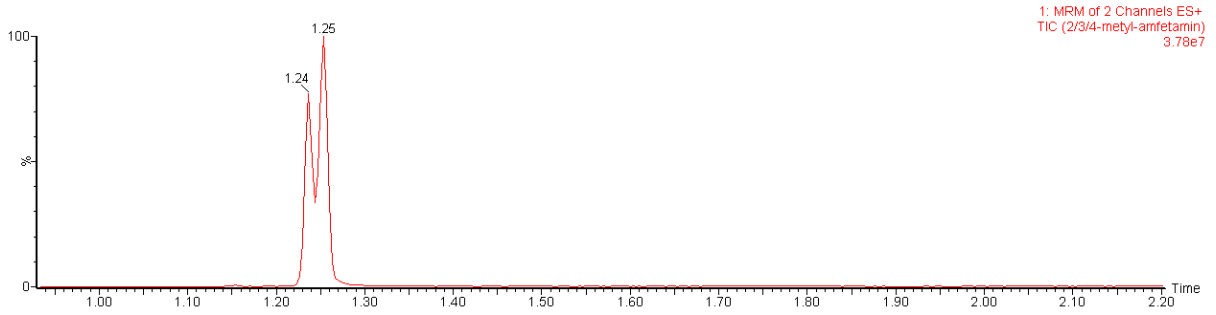

Figure 32. Poor chromatographic separation of 2-, 3-, 4-methylamphetamines with UHPSFC-MS/MS conditions. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively.

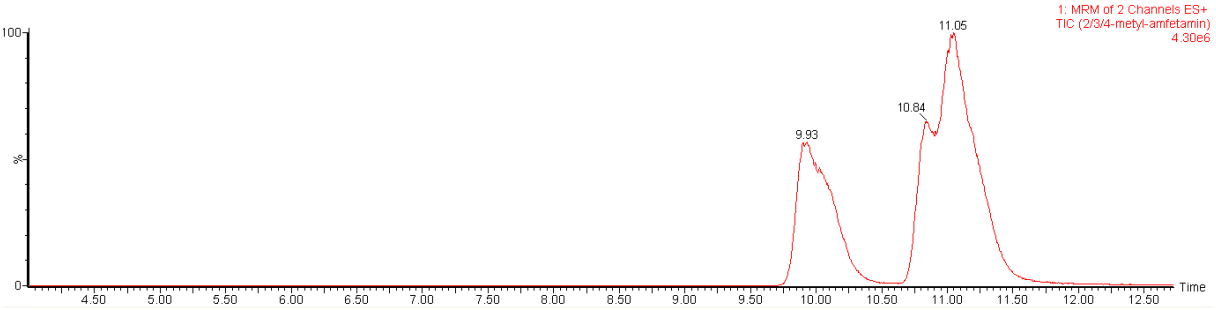

Figure 33. Possible separation of the positional isomers of methylamphetamine with UHPLC-MS/MS, using a 18 min gradient elution with a 5 mM ammonium formate and methanol mobile phase, 60 °C, and 0.5 mL/min flow rate (chromatographic and MS settings are further described in chapter 3.1.1 and 3.2.1, respectively)

4.2.1 Stationary phase chemistry

Five different column chemistries were tested for the separation of the chosen analytes;

- Ethylene bridged hybrid (BEH)
- Diethylamine (DEA)
- 2-picolylamine (2-PIC)
- 1-aminoanthracene (1-AA)
- Amylose tris-(3,5-dimethylphenyl)carbamate (AMY1)

The choice of stationary phases tested was solely based on evaluating the available UPC^2-columns from Waters, as they are compatible with the instrumentation nuts and grooves of the UPC^2 apparatus. There is an extensive selection of other columns from other suppliers, which are produced for SFC instrumentation. Among them, there might be several columns which can produce satisfactory separation of these analytes, but they were not considered in this thesis work, due to a restricted time schedule, funds and the possible detrimental effects to the

grooves and following leaking problems. However, the columns selected have provided us with a span of retention qualities. The columns selected gave a variety of results. In a recent paper by Lemasson *et al*. these columns were evaluated and rated as orthogonal to each other (Lemasson *et al*., 2015b).

All experiments are performed at a standard linear gradient (from 2 to 40 % modifier in 4 minutes) with a methanol modifier with 40 mM NH_3, at 60 °C and 1800 psi (described in chapter 3.1.1), except for the AMY1 column, which due to its completely different chemistry only showed similar separation with a different modifier.

The 2-PIC column produced satisfactory separation of the methylmethcathinone and fluoromethamphetamine isomers (Figure 34). The peaks of the fluoroamphetamine isomers however, experienced extensive tailing. The 2-PIC column was tested with different parameters (varying modifier, temperature, pressure, gradient etc., data not shown), but no condition could alleviate the poor chromatographic result for the fluoroamphetamine.

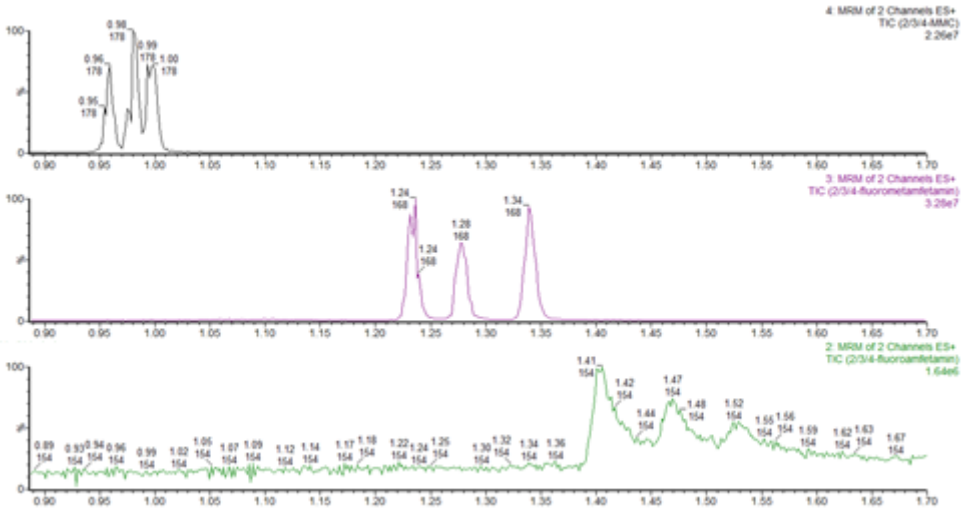

Figure 34. UHPSFC-MS/MS analysis of the 2-, 3-, 4-isomers with a 2-PIC stationary phase. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

The DEA column produced satisfactory baseline separation and peak shape for the isomers of fluoromethamphetamine, but no separation for the methylmethcathinone isomers and poor baseline separation of the isomers of fluoroamphetamine (Figure 35). The DEA column was also tested with different parameters (varying modifier, temperature, pressure, gradient etc.,

data not shown), but no condition could alleviate the poor chromatographic result for the isomers of methylmethcathinone and fluoroamphetamine.

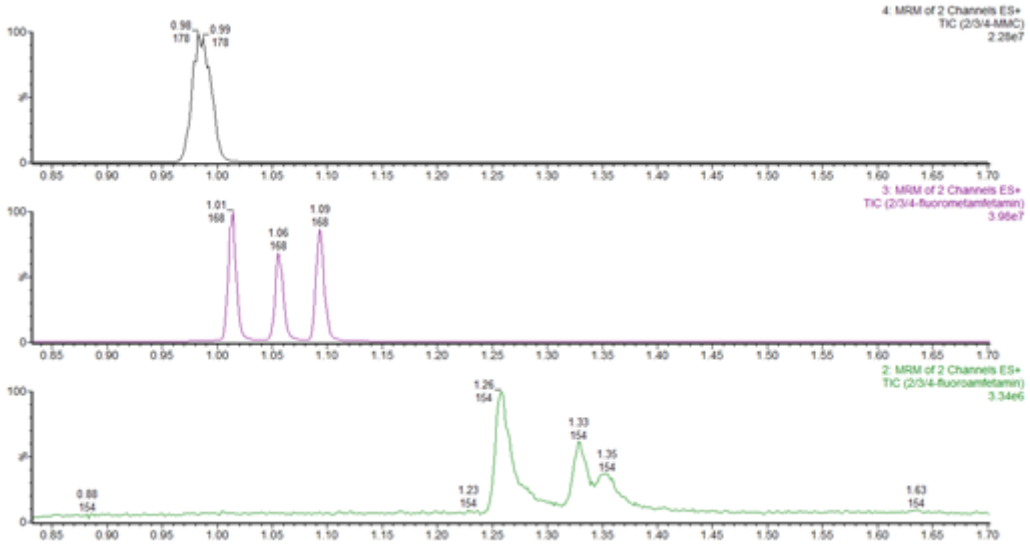

Figure 35. UHPSFC.MS/MS analysis of the 2-, 3-, 4-isomers with a DEA stationary phase. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

The chiral column, AMY1, was tested at several different conditions and it turned out to produce satisfactory separation of the analytes of interest at a different set of conditions than the other columns (Figure 36). The best separation was achieved using acetonitrile as a modifier, rather than methanol. This can probably be attributed to the chemistry of the AMY1 column being completely different, as it not made of particles that are part silica and part polymer, but is rather a complete polysaccharide-based stationary phase made to distinguish between compounds with high resemblance. The complexity of the structure has however obscured the mechanism of chiral recognition, which is largely unknown for most analytes and CSPs. The enantioselective environment of the AMY1 column might achieve higher interaction with the analytes in combination with a solvent with lower proton acceptor ability, which does not outcompete the active seats.

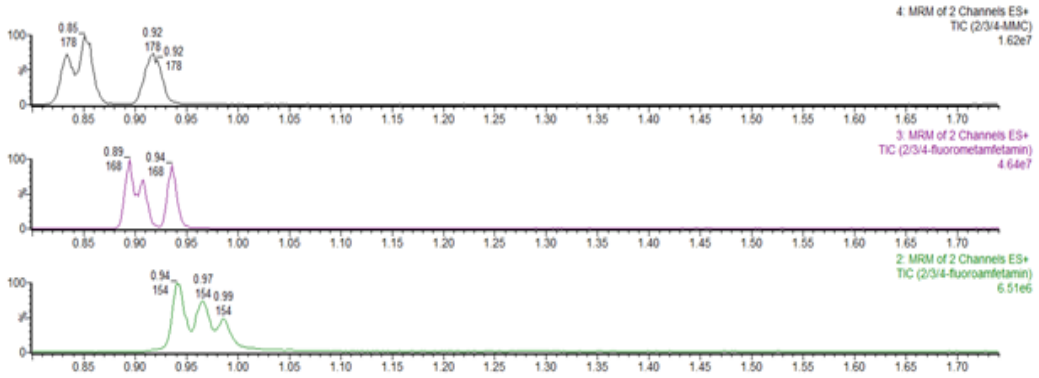

Figure 36. UHPSFC-MS/MS analysis of the 2-, 3-, 4-isomers with a AMY1 stationary phase. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

The 1-AA column was tested at an external laboratory, but did not produce satisfactory results for any of analytes (data not shown).

The BEH column produced satisfactory results for all the analytes of interest (Figure 37). Several parameters were tested and are more shown in the following part chapters. Most conditions produced satisfactory separation of most of the analytes, but for the optimal separation of all analytes a middle ground was chosen.

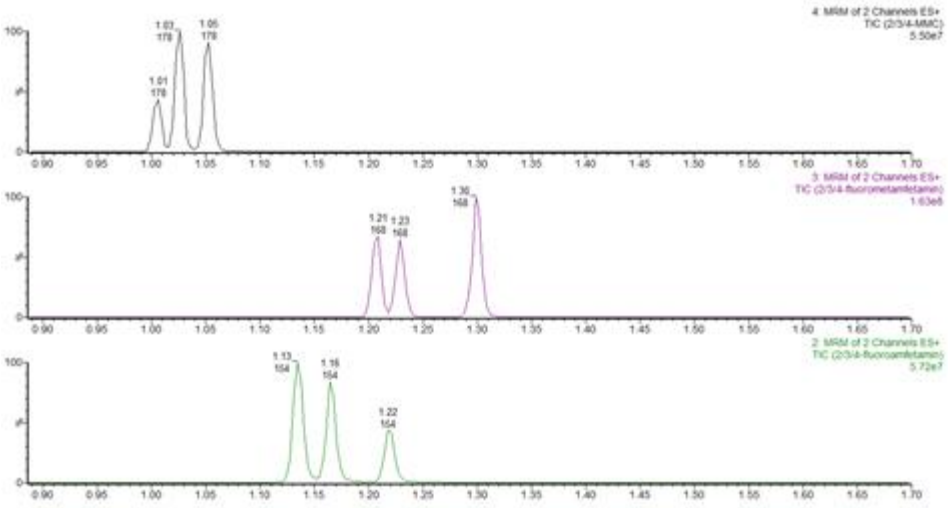

Figure 37. UHPSFC-MS/MS analysis of the 2-, 3-, 4-isomers with a BEH stationary phase. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

Out of the five columns, the BEH column showed a superior separation compared to the others: The chromatographic result with the BEH column was satisfactory for the analytes of interest. The result of the other columns displayed more tailing, broader peaks, lack of or less

separation of the isomers and so on. However, over time the BEH-column displayed a high degree of decrease in retention time and poor separation between the positional isomers. A possible shielding of silanol seats over time was proposed, which might explain the reduction in plate numbers of the column. The TORUS columns of Waters corp. are modified BEH particles are meant to withstand this effect by shielding the silanol seats from the direct or indirect bonding of modifier or additive. However, these modifications to the BEH particles also decreased the separation of the analytes of interest in this thesis, indicating that it is the silanol seats that are responsible for the effective separation at almost any condition. This is further discussed in chapter 4.3.3. The AMY1 column did however produce satisfactory separation of the analytes of interest. Nevertheless, the peaks were not as well baseline separated as with the BEH column. Additionally, these polysaccharide-based columns are not considered to be durable under the high pH conditions that are required for the separation of these analytes (Poole and Poole, 1991).

4.2.2 Gradient

The separation and behavior of the nine analytes of interest were investigated at different gradient regimes. It seems to be a non-important parameter for the relative separation of the analytes. Neither with nor without intermittent isocratic stages during the elution, the separation did not get affected in a significant way. However, the steepness of the gradient, thus the amount of modifier the analytes are exposed to before elution does affect the peak shape, retention time and the baseline separation between the isomers. These implications might be due to decreased interactions between the analytes and the silanol groups on the silica-based stationary phase or due to improved solubility. When increasing modifier percentage from 2 to 8 %, followed by a linear gradient, the peak shape and baseline separation are improved, while retention time is decreased. Retention time decrease is more pronounced for the later eluting compounds.

Gradient evaluation

Increased steepness of the gradient exposes the analytes to a higher percentage of modifier at an earlier stage, which causes them to elute earlier (Figure 38). Additionally, the baseline

separation is somewhat decreased with increased modifier. High amount of modifier probably causes less interaction between the amphetamine analytes and the silanol seats in the stationary phase, outcompeting these interactions.

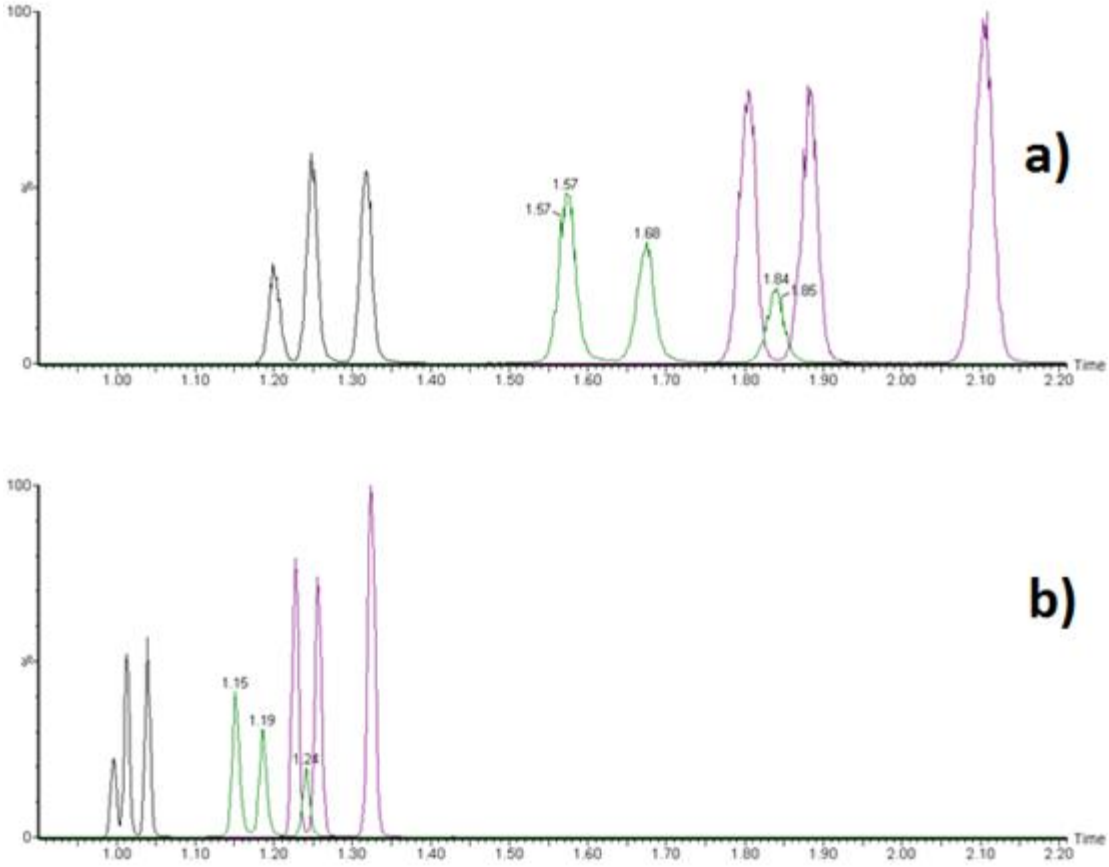

Figure 38. Low vs. high amount of modifier using a linear gradient on a BEH column with a mobile phase consisting of CO_2 and 40 mM NH_3 in methanol modifier. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green). a) Gradient elution using a linear gradient from 2-40 % modifier within 5.5 minutes. b) Gradient elution using a linear gradient from 2-40 % modifier within 4 minutes. Further chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively.

Isocratic elution experiment

In order to explore the behavior of the analytes at different modifier percentages, an isocratic elution experiment was designed (Figure 39). The analytes were subject to 1-20 % modifier (40 mM NH_3 in methanol) and the run time was set at 10 minutes. No analytes eluted at 1 % and only 3- and 4-methylmethcathinone eluted at 2 % modifier. All analytes eluted at 3 % modifier and above, but showed very broad peaks at low modifier percentages. The methylmethcathinones seems to be more sensitive to higher modifier percentages, as 2-

methylmethcathinone completely co-elutes with 3-methylmethcathinone at 10 % modifier and above. The general trend with respect to modifier amount is that increased amount of modifier decreases retention time and produces narrower peaks.

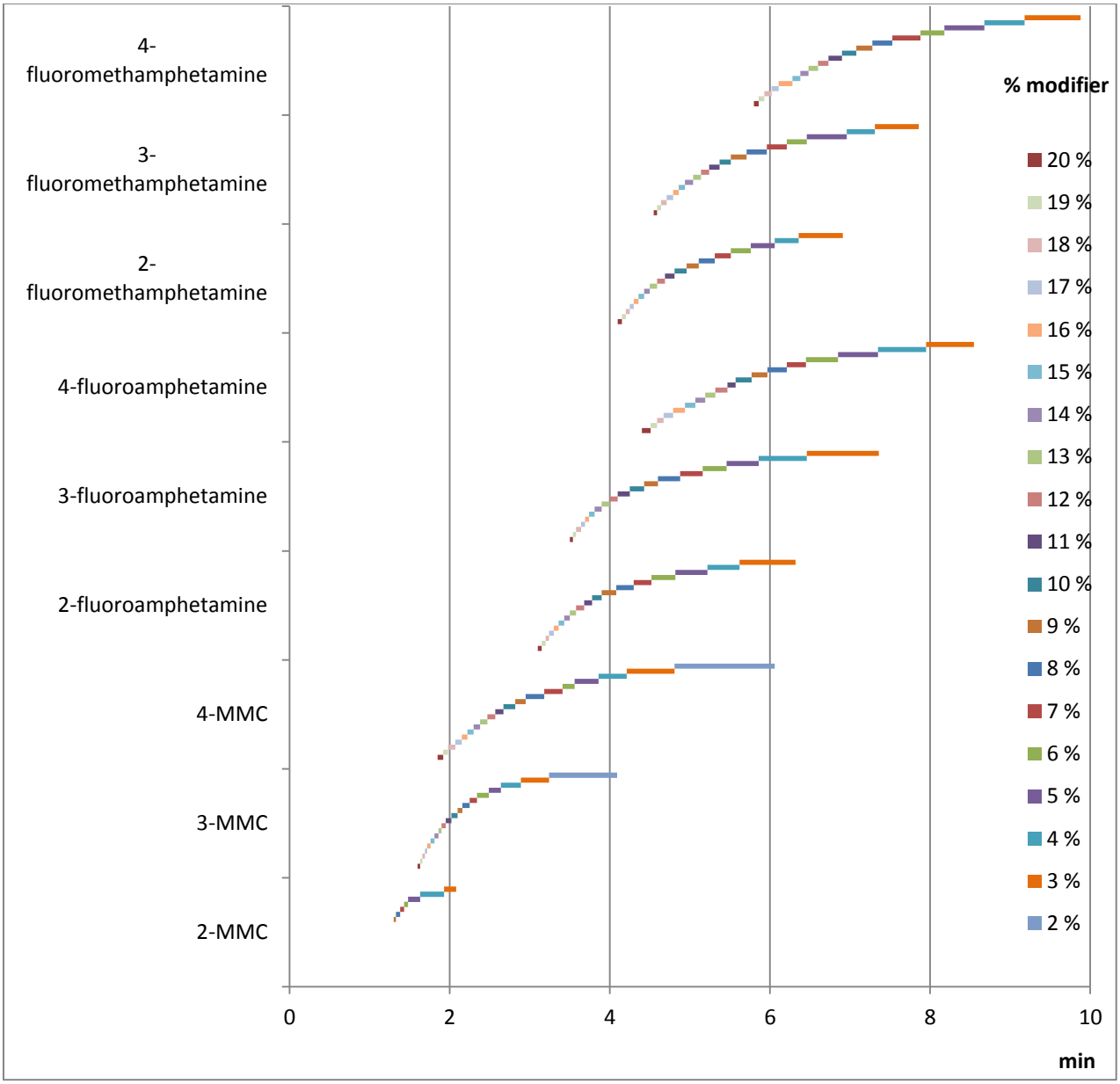

Figure 39. Gantt chart displaying the retention time for all the analytes of interest during isocratic elution on UHPSFC-MS/MS analysis. Long bars indicate broad peaks, short bars indicate narrow peaks.

4.2.3 Modifiers

As few analytes are soluble in supercritical CO_2 only, different organic solvents are often included. The organic solvent is often referred to as a modifier when added to the SFC mobile phase. Addition of modifier increases the solvating power of the mobile phase for better

solubility of polar analytes, by increasing the polarity of the mobile phase in SFC (see chapter 2.4.4). Different modifiers are compatible with SFC conditions and provide a range of possibilities in optimizing the separation of analytes. In this thesis work methanol, ethanol and acetonitrile were tested as modifers. All modifiers were tested both with and without NH_3 additive, here shown with 40 mM NH_3 and tested on the BEH column as it provided the best overall separation of the analytes of interest. Methanol was more extensively investigated with formic acid additive on the BEH column (data not shown) and different NH_3 additive amounts as well (as shown in chapter 4.1.4).

Methanol is by far the most commonly applied organic solvent used in SFC. It successfully makes the polar analytes solvable in the CO_2 dominated mobile phase and avoids sample precipitation when it is introduced at the column inlet. It created satisfactory separation in the least amount of time of the modifiers evaluated in this thesis work (Figure 40).

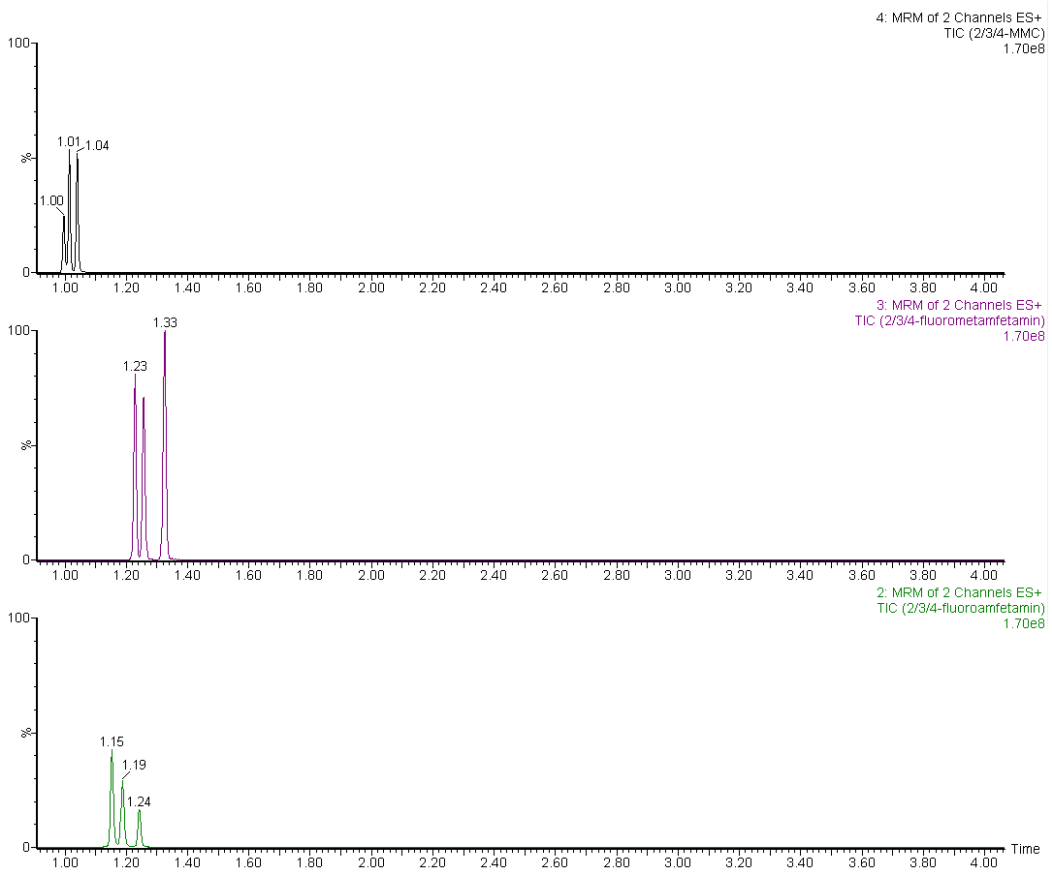

Figure 40. UHPSFC-MS/MS analysis of the analytes of interest on a UPC^2 BEH column using 40mM NH_3 in methanol as organic modifier. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

The use of ethanol also showed satisfactory separation of the analytes, but with longer retention time compared to methanol. However, the equilibrium of ethanol is shifted slightly towards the hydroxylation of water, producing a solution at equilibrium which contains 96 % ethanol and 4 % water (Figure 41). This effect is caused by the high vapor pressure of pure ethanol, and thus a low boiling point, which means that ethanol molecules can evaporate into a gaseous state producing an azeotrope. The implication of the low boiling point is that the mixture equilibrates at a state of minimum 95.6 % by mass of ethanol in the mixture. This is indicated by the composition curve of ethanol and water having a minimum value lower than the boiling points of either of the pure components, a positive deviation from Raoult's law commonly seen for such non-ideal mixtures of liquids. The effect of water in the mobile phase resulted in infrequent clogging of the system, which is further discussed in chapter 4.3.2.

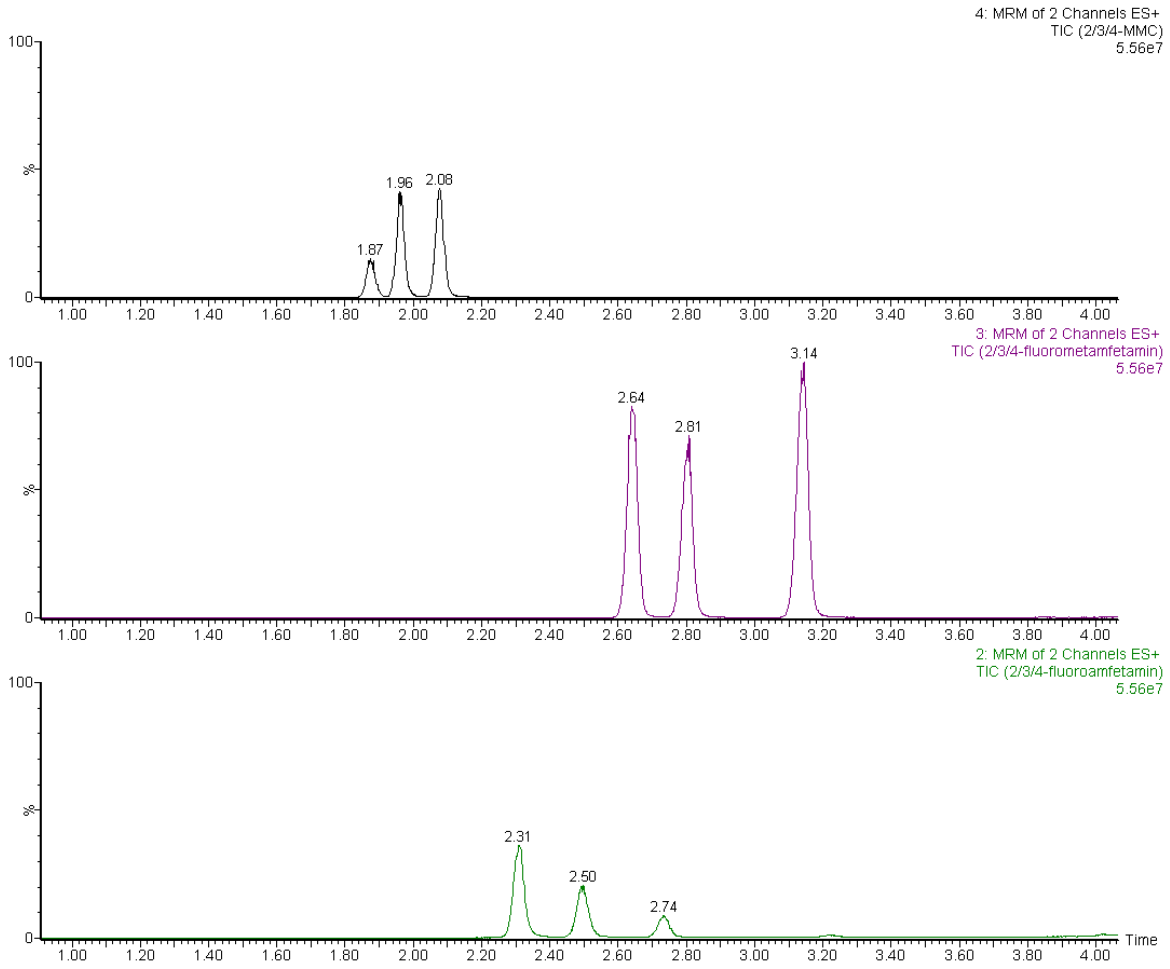

Figure 41. UHPSFC-MS/MS analysis of the analytes of interest on a UPC^2 BEH column using 40mM NH_3 in ethanol as organic modifier. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

Acetonitrile was also tested, but did not produce satisfactory results with the chosen stationary phase (Figure 42). It did, however, provide acceptable separation with the chiral column AMY1 (Figure 36). Acetonitrile was tested as an alternative to the alcohols, as it is classified as having different solvent selectivity according to Snyders triangle of solvents (Poole and Poole, 1991). The dissimilarity in selectivity behavior compared to the alcohols can be attributed to the higher dipole moment and less of proton acceptor ability of acetonitrile.

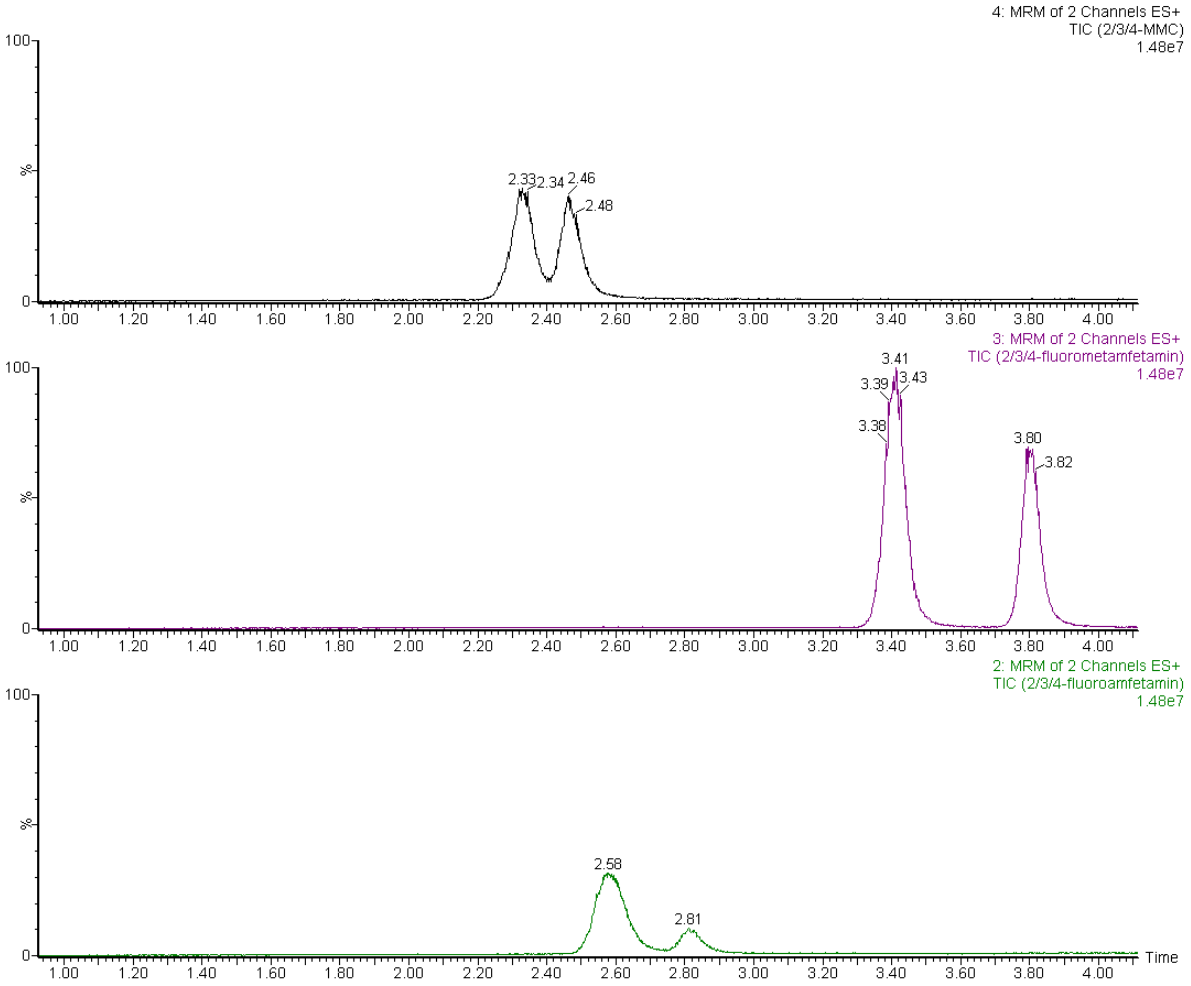

Figure 42. UHPSFC-MS/MS analysis of the analytes of interest on a UPC^2 BEH column using 40mM NH_3 in acetonitrile as organic modifier. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

Mixtures of the modifiers were also tested, at different proportion ratios, with all attainable stationary phases, gradients, isocratic elution, temperatures and pressures, but did not produce any improvement in chromatographic separation of the isomers of interest (data not shown).

Methanol did produce a satisfactory separation with a reasonably short retention time for all analytes and was fairly reproducible. Ethanol also produced good separation for the analytes

of interest, with a somewhat longer runtime, but was discharged due to its inherent hygroscopic capability. The use of ethanol for this purpose would thus be quite expensive as it would require absolute ethanol (water-free). Acetonitrile did not produce satisfactory separation of the analytes of interest.

4.2.4 Additive

In this thesis work, NH_3 was chosen as additive. NH_3 is compatible with MS detection and increases the pH of the modifier sufficiently to compensate for the high pK_a of the analytes. Formic acid was also tested in methanol modifier, but provided poor peak shapes as expected. An addition of 40 mM NH_3 was required for satisfactory peak shape of the analytes, as shown in Figure 43.

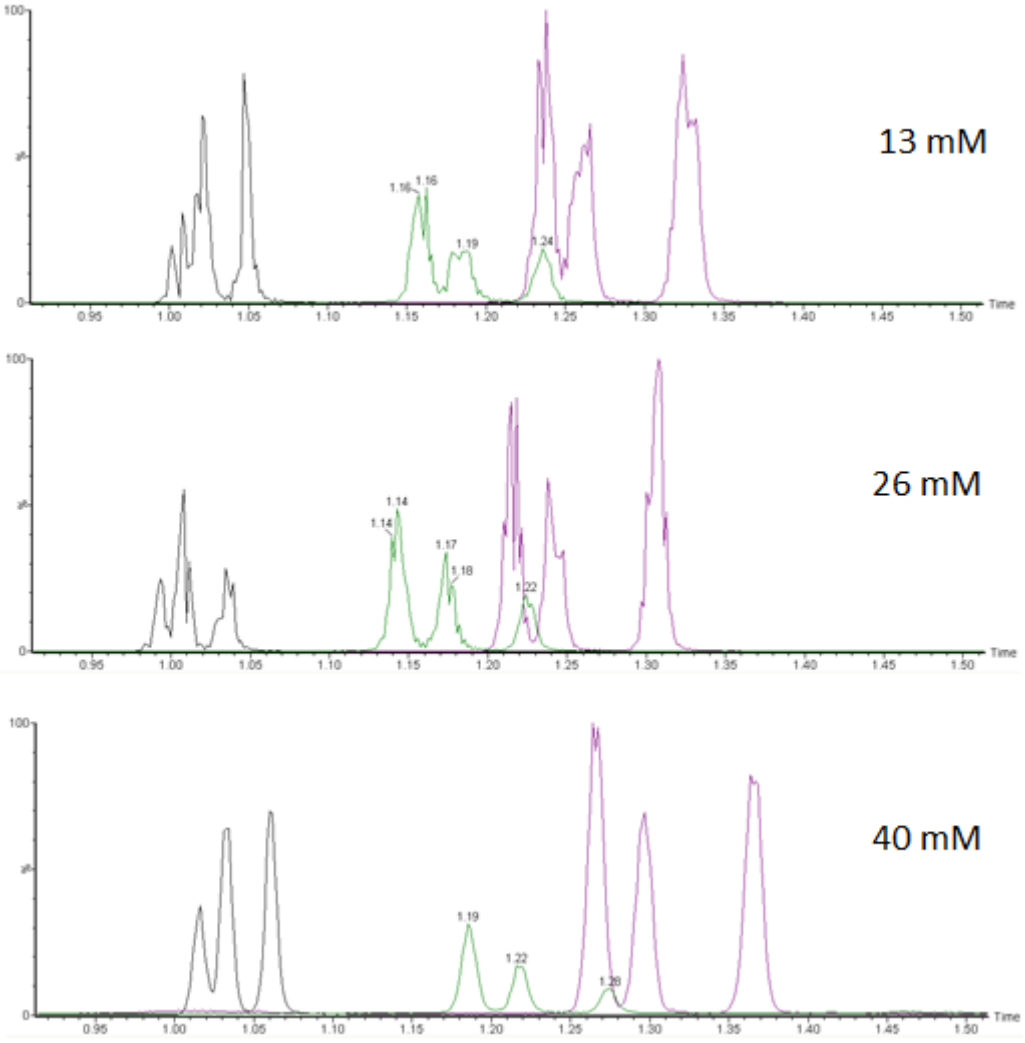

Figure 43. Aqueous NH_3 used as additive and tested at different concentrations for UHPSFC-MS/MS analysis of the analytes of interest. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

The first NH_3 additive tested was aqueous, meaning that water acted as a second additive in the mobile phase. Water has very low solubility in supercritical CO_2 (~0.1 %, w/w), due to its high dipolar nature in contrast with the apolar CO_2 (Tassaing *et al.*, 2004; Oparin *et al.*, 2005; Nováková *et al.*, 2014). Nevertheless, a small amount of water is miscible with supercritical

CO_2 when combined with an organic modifier. Water has twice the hydrogen bonding ability of methanol and becomes acidic in contact with CO_2 (Figure 44).

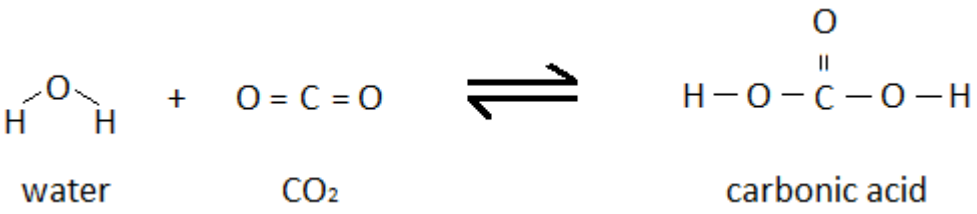

Figure 44. Equilibrium of water and CO_2, creating carbonic acid (adapted from Nováková *et al.*, 2014).

The addition of water could improve the elution of polar analytes and is increasingly used as an alternative additive in SFC (Taylor, 2012; Lemasson *et al.*, 2015a; Nováková *et al.*, 2015; Pauk *et al.*, 2015). However, the amount of aqueous NH_3 additive needed to produce satisfactory peak shape for the analytes of interest, created frequent clogging due to CO_2 and/or water freezing in the instrument. Thus, no amount above 40 mM aqueous NH_3 could be tested as it caused immediate clogging issues in the instrument. This occurrence was related to the amount of water added to the modifier and is discussed in chapter 4.3.2. Switching to a methanolic NH_3 completely alleviated the problems of clogging.

A methanolic NH_3 additive was tested, to exclude the use of water in the SFC system. The NH_3 additive was tested at concentrations greater than 40 mM as well. Nevertheless, concentrations greater than 40 mM did not produce improved peak shape, indicating that the system was saturated at 40 mM (Figure 45). Calculations of the additive proportions are shown in Appendix II.

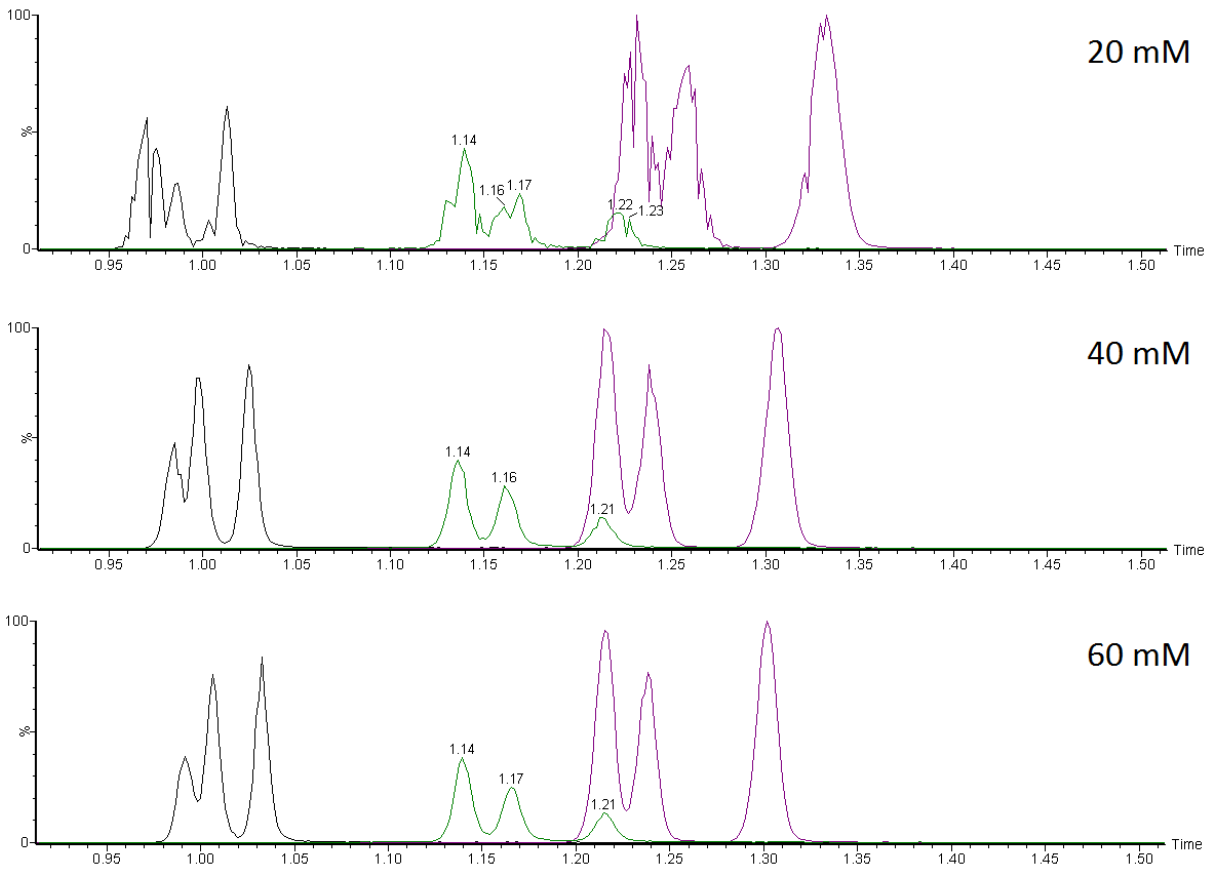

Figure 45. Methanolic NH_3 used as additive and tested at different concentrations for UHPSFC-MS/MS analysis of the analytes of interest. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

4.2.5 Column temperature

The separation and behavior of the nine analytes were measured at different column temperatures and is displayed in Figure 46. The results of the analytes behavior at different temperatures are shown for the BEH column, as it provided the best overall separation of the analytes.

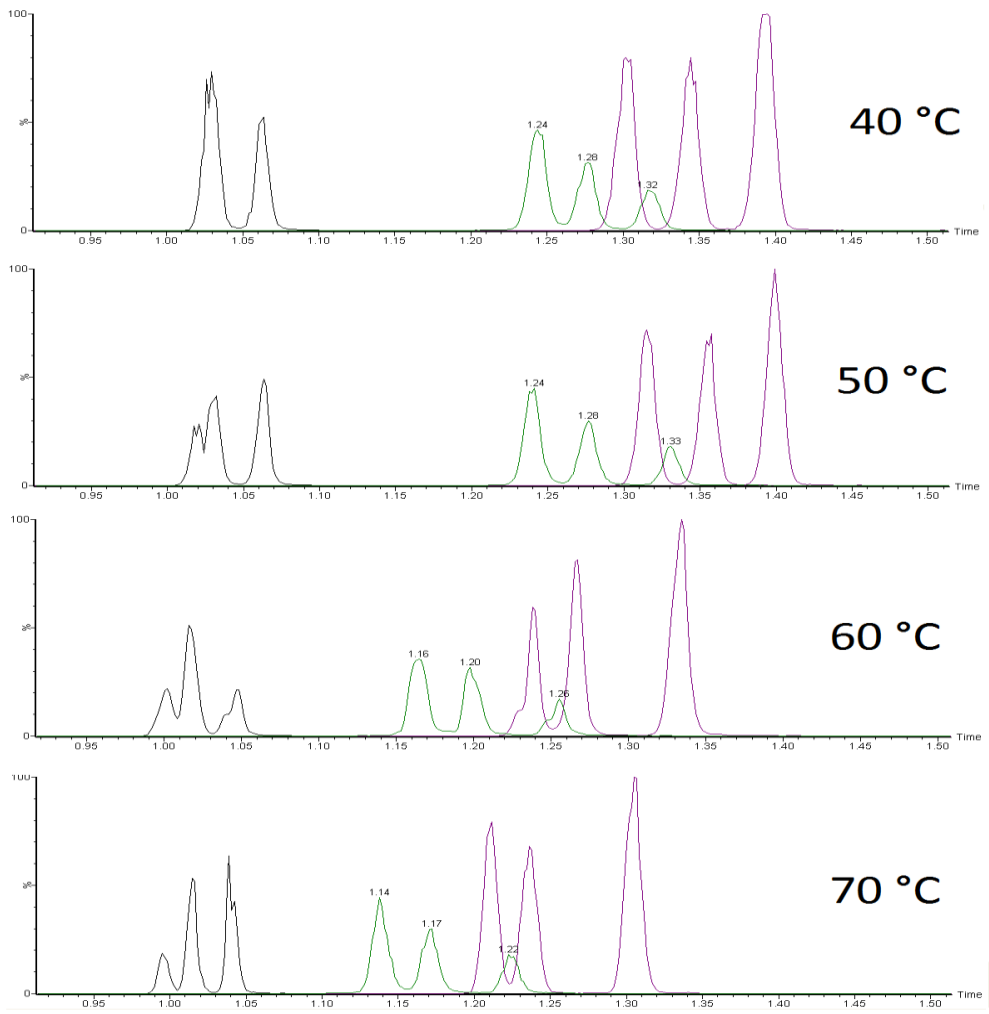

Figure 46. Temperature effect; 40 – 50 – 60 – 70 °C. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

On a later time 80 °C was tested but showed poor separation for the fluoromethamphetamines with only two eluting peaks for the three isomers (data not shown). The three isomers of methylmethcathinone produced only two peaks at 40 °C and shows increasing enhancement in separation with increasing temperature. The best baseline separated was achieved at 80 °C for the methylmethcathinone isomers. The three isomers of fluoromethamphetamine on the other hand show complete baseline separation at 40 °C, and become less separated with increased temperature. The three isomers of fluoroamphetamine display satisfactory separation at all the temperatures tested. The general trend seen in this thesis work is increasing temperature is related to a decrease in retention time, especially for the later eluting compounds. This is consistent with findings of other studies (West *et al*., 2012b). According to the group of Nováková, this initial retention decrease is due to a reduction in mobile phase density. After reaching a maximum, the retention decreases at very high temperatures. As

temperature affects the solubility parameters of both the solute and the mobile phase, it may also alter the affinity of the analyte towards the stationary phase (Nováková *et al*., 2014). Thus, the effect of temperature on the retention of analytes in SFC is complicated combination of various mechanisms, which can be difficult to explain and interpret. Another interesting aspect of SFC during gradient elution is that decompression cooling may occur due to increasing modifier proportion in the mobile phase (Pauw *et al*., 2014). This was not noted in the current thesis work.

The temperature chosen for this method was 60 °C, as it produced three symmetrical peaks for all the analytes of interest.

4.2.6 Back pressure

The separation and behavior of the nine analytes were measured at different back pressures and is displayed in Figure 47.

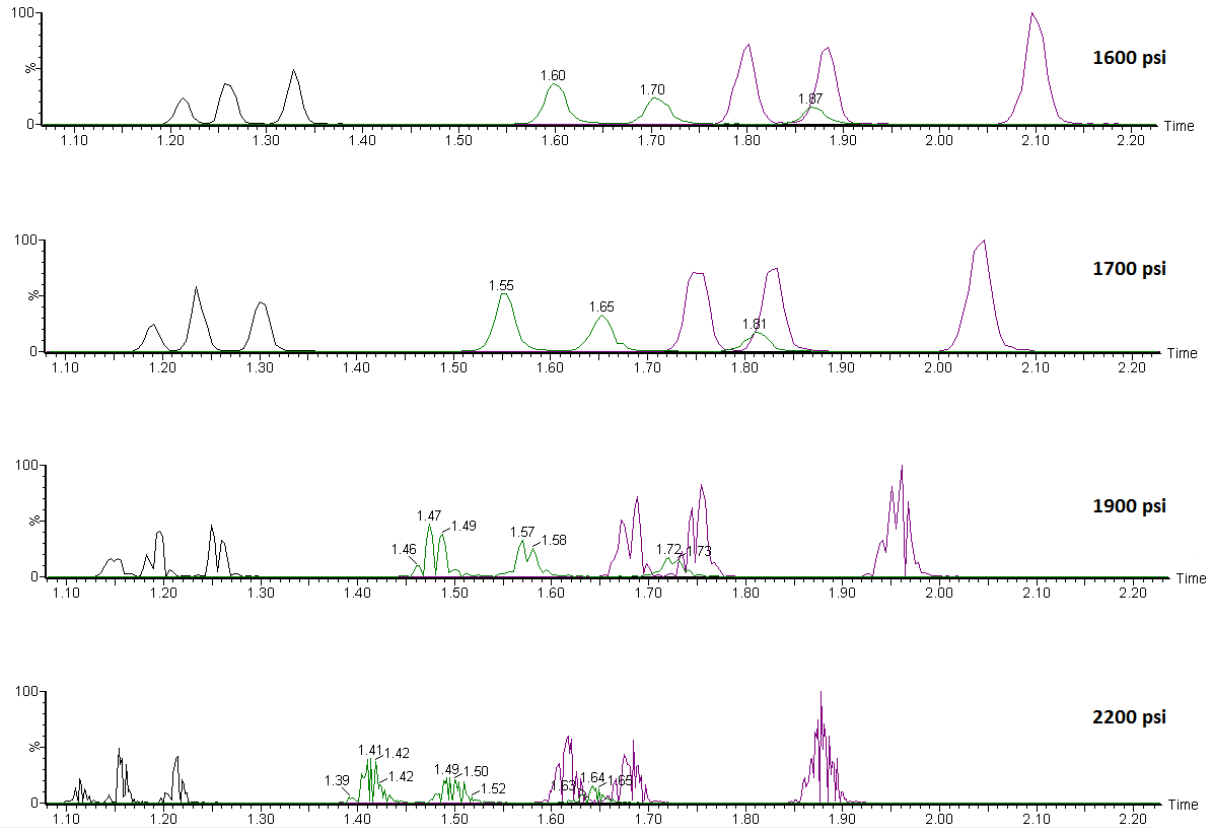

Figure 47. Effect of pressure measured for the analytes of interest; 1600, 1700, 1900, 2200 psi. The supplementary Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

Increasing the pressure resulted in decreased retention time for all analytes. This is a result of increased density of the mobile phase resulting in increased elution power. Nevertheless, an additional unexpected effect was seen with increased pressure above 1800 psi; the peaks became jagged and uneven. This phenomenon is described in chapter 4.3.1. In an attempt to avoid poor peak shape caused by too high back pressure, 1800 psi was chosen for this method. Lower back pressures were also considered, but were omitted in favor of the highest attainable pressure with satisfactory peak shape due to a wish for the mobile phase to be as homogeneous as possible.

Mixing the supercritical CO_2 with the methanolic modifier will change the physical properties of the fluid and require higher temperature and pressure to attain supercritical features of the fluid (described in chapter 2.3.4). Evaluating the homogeneity of the mobile phase in SFC is difficult, but as the analytes of interest elute at small modifier percentages there might be reasonable homogeneity at the chromatographic settings chosen for this method.

4.2.7 Sample solvents

For proper analysis, the sample solvent should dissolve in the SFC mobile phase without affecting sample retention or resolution and be compatible with the detection device. Ideally the sample solvent should be the same as the mobile phase, but in the case of SFC dissolving analytes in supercritical CO_2 in a vial would be challenging.

The six solvents tested (Figure 48) were chosen based on sample solubility and the elutropic properties on bare silica (Poole and Poole, 1991; Fountain *et al*., 2014). The ideal sample solvent is a trade-off between solubility in the relatively apolar mobile phase of SFC and the solubility of the polar analytes to achieve optimal peak shape. It should also be noted that volatile solvents can evaporate in the vials, leading to a continuous concentration of the sample over time. Nonpolar solvents such as hexane and heptane are best compatible with the mobile phase, but the analytes in this thesis work are quite polar and thus more soluble in polar solvents (Iraneta *et al*., 2013). Regarding the MS detection, the sample solvent should be properly volatile in order for the complete evaporation in the ionization step.

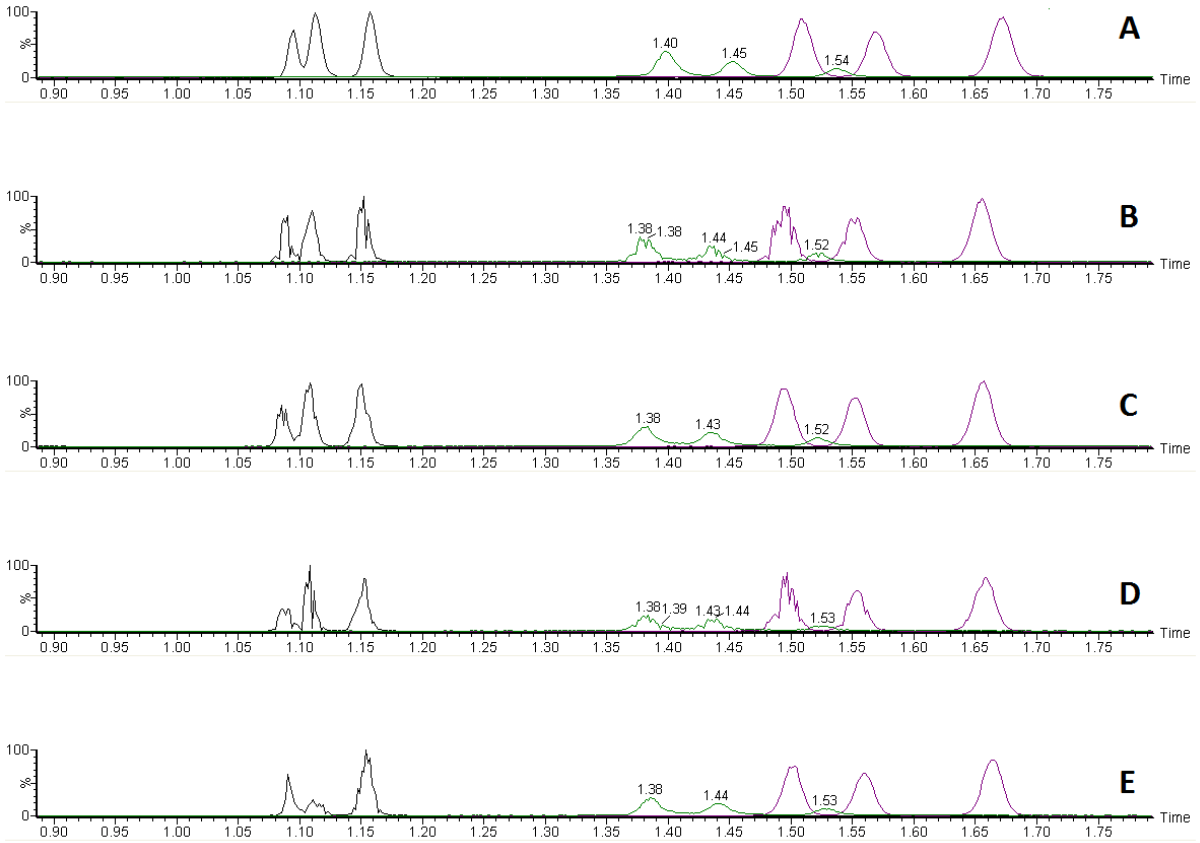

Figure 48. A: H_2O; B 50:50 H_2O and methanol C: Ethanol; D: Methanol E: Isopropanol. Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

Isopropanol produced satisfactory peaks for all the analytes of interest (Figure 48E).

Methanol showed poorer peak shape (Figure 48D), probably due to its higher eluotropic value, while the slightly weaker eluent ethanol produced quite good peak shape (Figure 48C). However, it should be noted that the ethanol mixture probably contained about 4 % water as well. Although ethanol provided excellent peak shape, it was not chosen due to the wish for exclusion of any water from the instrument.

Mixing water and methanol provided quite poor peak shape (Figure 48B), whereas water varyingly produced good peak shape, but also highly inadequate chromatograms (Figure 48A). This is probably a result of the inherent immiscibility of water in the CO_2-dominant mobile phase, even at as small amounts as 0.5 µL (Figure 49).

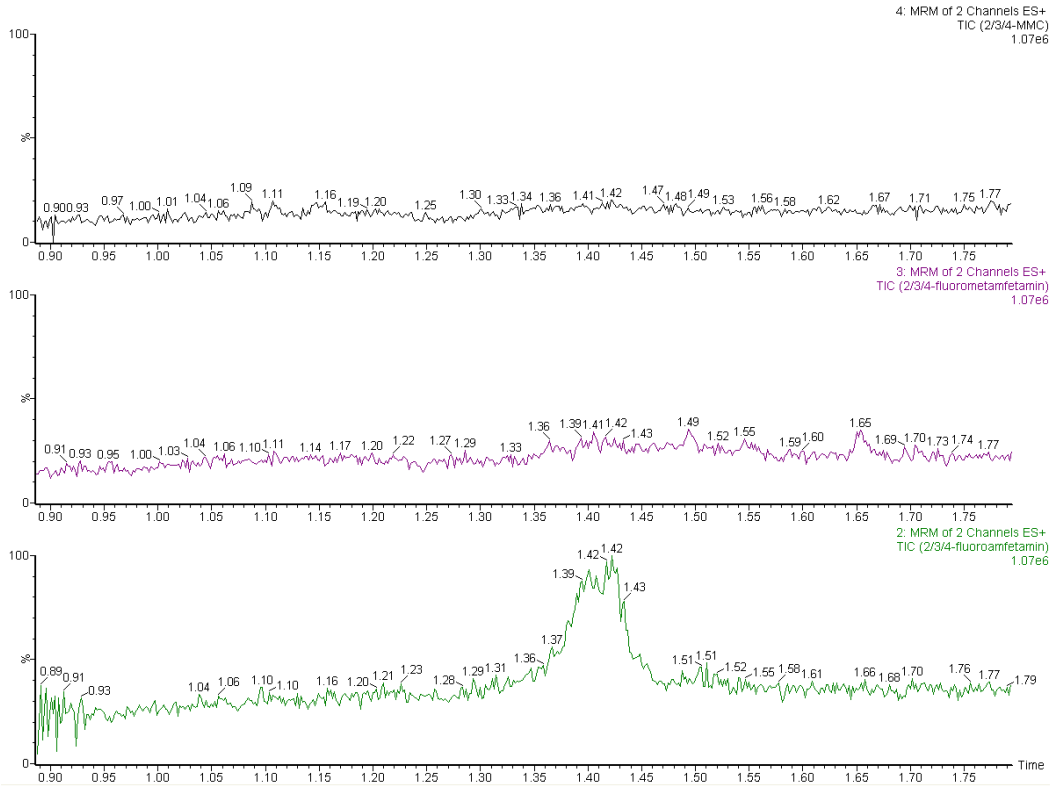

Figure 49. Poor chromatogram as a result of using water as sample solvent Chromatographic and MS conditions as described in chapter 3.1.1 and 3.2.1, respectively. Analytes from top to bottom: Methylmethcathinones (black), fluoromethamphetamines (purple) and fluoroamphetamines (green).

A mix of heptane and isopropanol was also tested (90:10), but did not provide any improvement in the chromatographic peak shape compared to isopropanol (data not shown). As it offered no advantage, it was excluded due to a somewhat more time consuming sample work up.

The differences in the results of the tested sample solvents could be caused by the contrasting elution strength of the solvents and viscous fingering effects (Abrahamson and Sandahl, 2013; Enmark *et al*., 2015). Isopropanol was chosen as a sample solvent as it provided satisfactory peak shape and provided easy sample dissolution.

4.2.8 Summary

The chosen settings for this method became a sample preparation consisted of LLE using ethylacetate:heptane (80:20, v/v). After evaporation, the samples were reconstituted in isopropanol before injection. Four $^{13}C_6$-labelled analogs were used as ISs (chapter 3.5, Figure 25). The preferred stationary phase was an ethylene-bridged hybrid (BEH) column (3 mm x 100 mm, 1.7 µm), using a flow rate of 2 mL/min, 1800 psi in back pressure, and gradient elution with 40 mM ammonia in methanol and supercritical CO_2. The injected volume was 0.5 µL. Quantification was performed by tandem MS using multiple reaction monitoring (MRM) in positive mode, applying two transitions for the compounds and the IS. The run time for the method was 4 min.

Although the chosen conditions initially produced satisfactory separation for all nine analytes of interest, there was an alteration in the stationary phase chemistry which became apparent over time. This was seen as a retention time and the baseline separation decreased for all the analytes. This effect is more extensively discussed in chapter 4.3.3.

Nevertheless, this effect became especially apparent for the methylmethcathinone analytes which became impossible to separate on the degraded stationary phase. A regeneration regime was designed and produced only temporarily satisfactory results for the methylmethcathinone analytes. As it was not possible to validate the method for the methylmethcathinone analytes, they were excluded from the validation procedure.

4.3 Technical challenges

Although many of the former difficulties of applying SFC have been reasonably resolved, there also appeared a couple of new challenges. The challenges encountered in this thesis work will be somewhat elaborated on in this chapter.

4.3.1 Spray pulsing

During the work of evaluating the optimal pressure for this method, a surprising phenomenon occurred in the chromatograms. As the pressure was increased the peaks became increasingly degraded as shown in Figure 47, chapter 4.2.6, and Figure 50. During the troubleshooting process, it was discovered that the spray flowing into the ESI appeared to be pulsating.

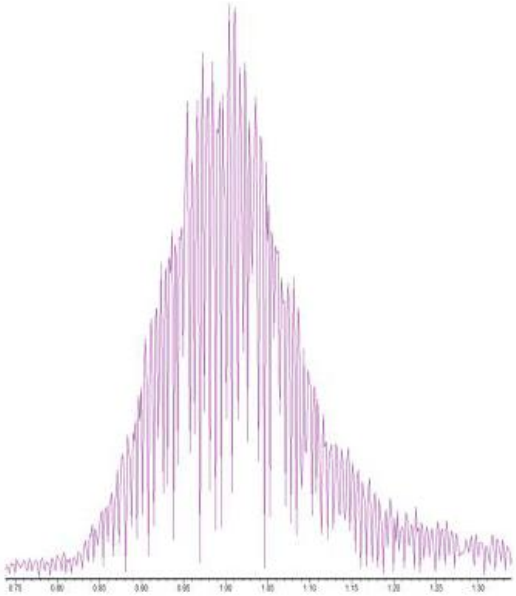

Figure 50. Degraded peak due to spray pulsing (Fogwill, 2015).

Spray pulsing or poor spray stability is a phenomenon that occurs with SFC-MS, likely a result of the transition between high and atmospheric pressure at the interface of the UHPSFC and MS detector. The SFC-MS interface consists of a split restrictor and a makeup pump, as shown in Figure 20 and 21 (chapter 3.1). The split restrictor (Figure 51) has the function of splitting the flow between going to the MS and the back pressure regulator. The split ratio is the ratio of mobile phase flow directed to the MS and to the BPR. According to Waters co., a large split ratio (i.e. 10:1) means that a small portion of the total flow rate is directed to the MS, whereas a small split ratio (i.e. 2:1) entails that a larger portion of the mobile phase flow is directed to the MS. The size of the split ratio is governed by the flow speed and BPR. The makeup pump functions in

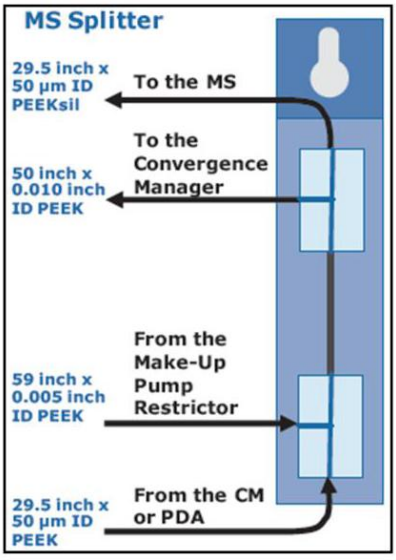

Figure 51. Schematic diagram of the split restrictor (Fogwill, 2015).

accordance with the split restrictor and has a multitude of functions. The makeup flow aids the transport of analytes trough the split restrictor, restrictor and probe, provides flow to the back pressure regulator in order to appropriately adjust the back pressure, and introduces ionization-enhancing compounds to the MS to increase ionization efficiency.

High pressure in the system, allows alcohols and other fluids to be at least partially solved in the supercritical CO_2. However, when the pressure decreases the solvation power of the CO_2 also decreases as it transitions towards gaseous phase.

The spray pulsing occurs as a result of the more or less homogenous mobile phase being decompressed along the length of the split restrictor as the pressure decreases towards atmospheric pressure (Figure 52). As the density drops, the CO_2 gradually loses its miscibility with the co-solvent. At the outlet of the split restrictor, there is complete phase separation between the liquid co—solvent and the gaseous CO_2, where the co-solvent coalesce into/forms droplets. These droplets are pushed through the probe by gaseous CO_2, producing the pulsing spray into the MS (Fogwill, Waters).

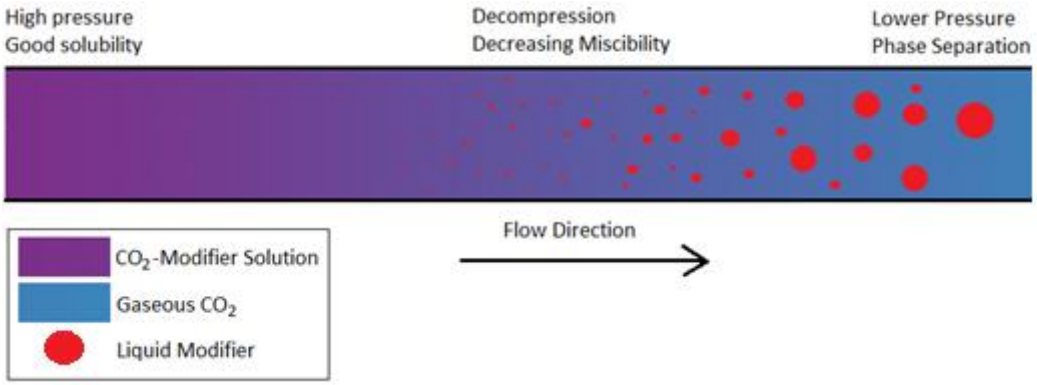

Figure 52. The phase separation that occurs in the split restrictor (adapted from figure of Michael Fogwill, Waters)

There are no published studies so far on the phenomenon of spray pulsing at the interface of SFC and MS. The phenomenon was presented on a Waters conference for UPC^2 users in Gothenburg, Sweden, in May of 2015. However, the group of Lesellier *et al*. recently published a study on the optimization of the mobile phase composition of SFC for drug analysis. When comparing of the effect of different additives, an unexpected poor result was obtained for ammonium formate. As a volatile salt, it has often been recommended in SFC-MS studies. The group concluded that the surprising result was most likely a result of clogging at the interface of the SFC and MS (Lemasson *et al*., 2015a). As the mobile phase is decompressed from sample inlet throughout the column to the split restrictor, an adiabatic

expansion of the CO_2 thus occurs at the split restrictor and beyond, which could result in the formation of CO_2 ice particles at the interface of the SFC instrument and the MS.

4.3.2 Effect of water in the UHPSFC system

During this thesis work, both aqueous and methanolic NH_3 was tested as additives, and the use of aqueous NH_3 lead to extensive problems with clogging of the system, probably due to the formation of CO_2 ice particles. Apparent CO_2 ice formation was distinguished by that the clogged instrumental parts felt cold and flow passage was found to be strenuous or impossible at the particular site during troubleshooting. A possible explanation of why this occurs more frequently when using aqueous NH_3could be related to the fact that the system has difficulties with producing a miscible and homogenous mobile phase when a fourth compound is added. Additionally, the difference in polarity between CO_2 and water might also be the cause of clogging within the system as it affects the miscibility in the apolar CO_2.The clogging apparently occurred at several locations in the instrument (as tested by unscrewing the instrument and running flow through it, feeling for cold patches etc. in collaboration with a Waters co. technician).

However, when the NH_3 solution used was replaced with an NH_3 solution in methanol clogging was no longer an issue. The clues as to how this could occur were provided when testing different percentages of NH_3 on the system. 13 and 26 mM (0.1 and 0.2 %) was unproblematic. Mostly nice chromatograms were produced with 40 mM (0.3 %), but also regular “downtime” due to clogging and subsequent pressure problems. A concentration of 53 mM (0.4 %) of aqueous NH_3 additive was never possible to achieve, only a single run could sometimes be performed with such a solution. The pressure would suddenly increase rapidly and cause the system to stop the flow as it passed the maximum pressure of 6000 psi. This effect occurred most frequently at high modifier percentage, which made a new hypothesis occur; that this effect was caused by the NH_3 additive, water or the combination of both?

In an attempt to answer the hypothesis, an NH_3 additive dissolved in methanol was tested, to alleviate water from the system. Any NH_3 amount tested with the methanolic NH_3 solution, even at concentrations higher than 53 mM (0.4 %) could be used without any clogging occurring. This lead to the conclusion that it was probably the water in the modifier solution that caused the infrequent clogging problems. Water was also added to the modifier solution

with the NH_3 solved in methanol, at the same amount as in the aqueous NH_3modifier, which produced the same clogging problems as seen with the aqueous NH_3 (calculations shown in Appendix II, data not shown).

Water did not appear to have significant effect on the separation of the analytes of interest, as the water-free modifier produced comparable chromatograms as shown in Figure 53. However, the retention time of the analytes was affected by the removal of water, which is further discussed in chapter 4.3.3.

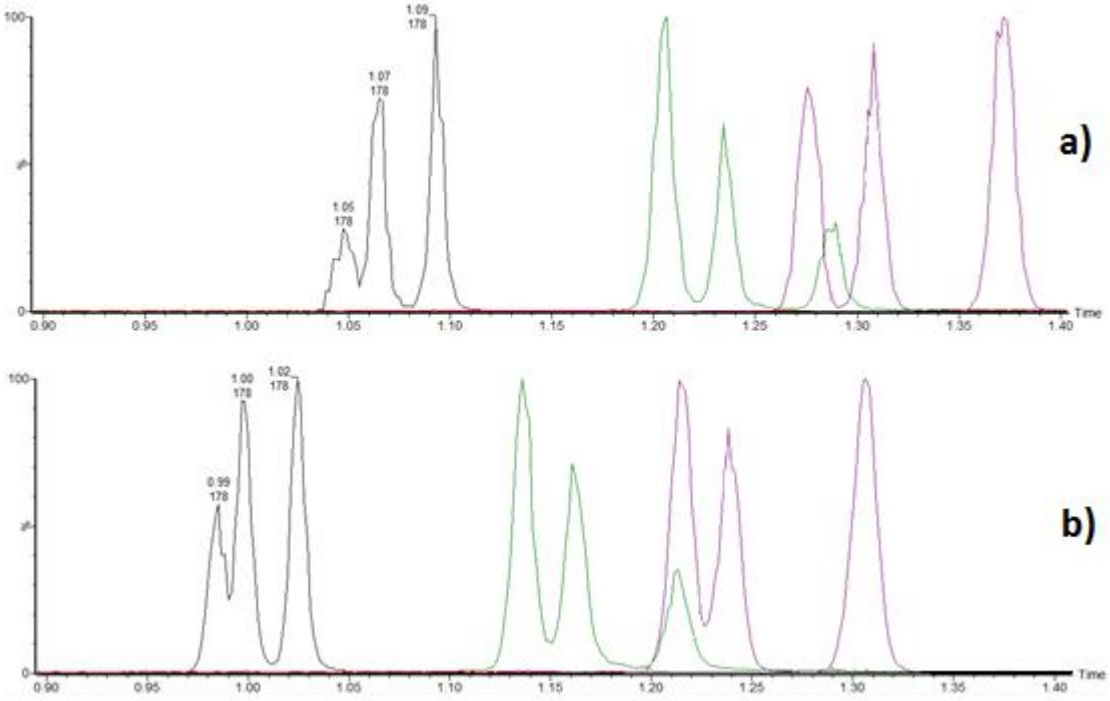

Figure 53. UHPSFC-MS/MS analysis of the nine analytes of interest using a) 40 mM aqueous NH_3 in methanol and b) 40 mM NH_3 in methanol.

The study of Tassaing *et al.*, 2004 concerning the miscibility of water in supercritical CO_2 gives good insight into the reasons why even small amounts of water in this SFC instrument causes clogging and pressure problems. Considering that supercritical CO_2 is not miscible with water at nearly any amount at the conditions attainable with the SFC instrument from Waters co., it is reasonable to assume that even small amounts of water causes inconsistent pressure due to clogging problems. The SFC instrument from Waters can attain a maximum temperature and pressure of 90 °C and 5800 psi (400 bar). However, due to gradient elution being the most optimal method of choice a lower BPR pressure must be chosen as higher modifier percentage causes a proportional increase in pressure (modifier gradient causes additionally a pressure gradient). A typical attainable program for the analytes of interest

would have settings of maximum 80 °C and 2600 psi (180 bar). Nevertheless, in order for a small percentage water (up to 5 %, w/w) to exist in a dimer, creating a partially mixed phase with supercritical CO_2, the minimum conditions would have to be at least 100 °C and 2900 psi (200 bar). In order to create a total miscibility, where there are only dimers detected in CO_2, conditions of minimum 360 °C and 2900 psi (200 bar) must be attained (Figure 54).

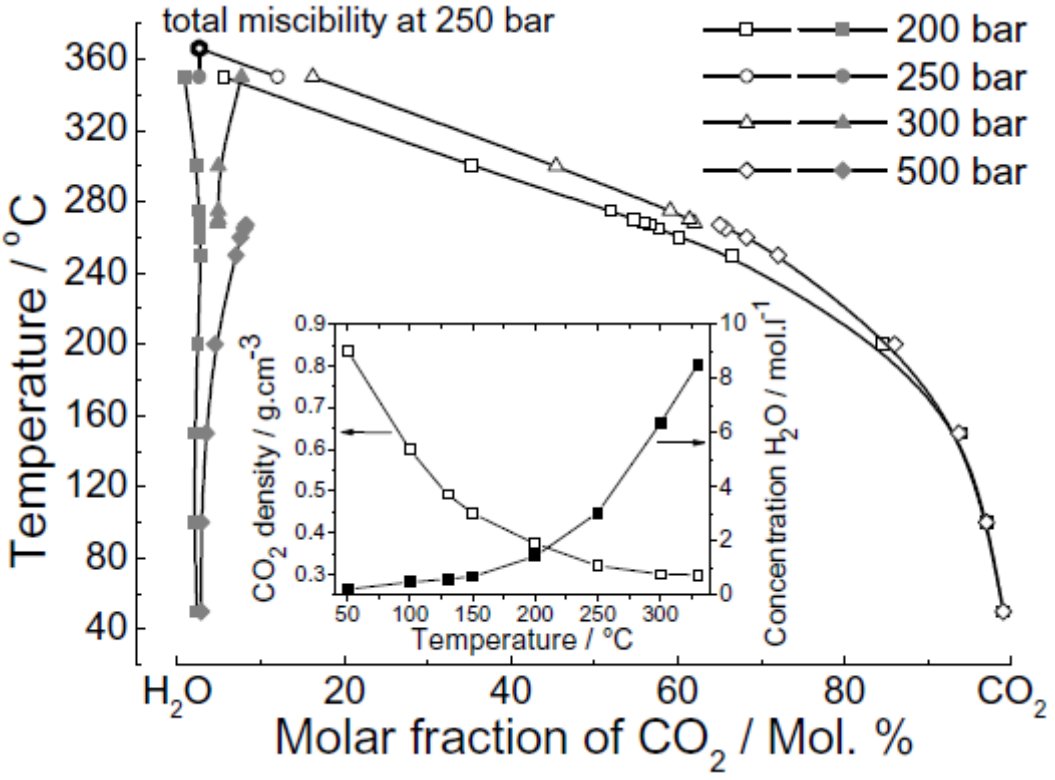

Figure 54. Phase diagram of the H_2O/CO_2 mixture at constant pressure. Insert: Concentration of water dissolved in CO_2 and density of the CO_2 rich phase as a function of the temperature at constant pressure, P=250 bar (Tassaing *et al.*, 2004).

Additionally, even with an instrument which could attain high enough temperature and pressure other issues would occur. Supercritical water is highly corrosive and would demand corrosion resistant alloys like austenitic stainless steel, made of alloys with high amounts of chromium and nickel (Kritzer, 2004; Marrone and Hong, 2009). Additionally, the compounds to be analysed must withstand the oxidative properties of supercritical water.

What happens inside the SFC instrument is difficult to elaborate on, but as water is immiscible with the CO_2 rich mobile phase it forms droplets which could obstruct the passage of the supercritical CO_2. The unsteady flow could cause occasional expansion of CO_2, and as the system is well isolated the process would be adiabatic. The expanding fluid would thus cool and sublimation of CO_2 and possibly water could occur.

4.3.3 Evaluation of retention drift

The BEH column showed a superior separation compared to the other stationary phase chemistries tested in this thesis work. However, after repeated injections the BEH-column displayed a high degree of decrease in retention time and poor separation between the positional isomers. This retention drift was also observed in two of the Torus columns; 2-PIC and DEA. Nevertheless, as these columns where not chosen for this method they were not investigated as meticulous as the BEH column and no certain conclusion can be drawn. No conclusion can be made about the robustness of the 1-AA or AMY1 columns, as they were tested only once at an external laboratory and due to a restricted time schedule, respectively.

The problem was not apparent at first. It was recently discovered to happen very quickly under the water-free conditions chosen for this method. At first an aqueous NH_3 solution was used as an additive. Although the small amount of water in this solution caused clogging issues, it probably has somewhat inhibited the effect of retention drift. Switching to a methanolic NH_3 solution completely solved the problems of clogging. Nevertheless, it also resulted in a pronounced retention drift in the system with use; the retention time decreased significantly, especially for the later eluting compounds. Additionally, the separation and in particular the baseline separation between the analytes were significantly decreased after a few hundred injections, in particular for the methylmethcathinones eluting at lower modifier percentages as shown in Figure 55.

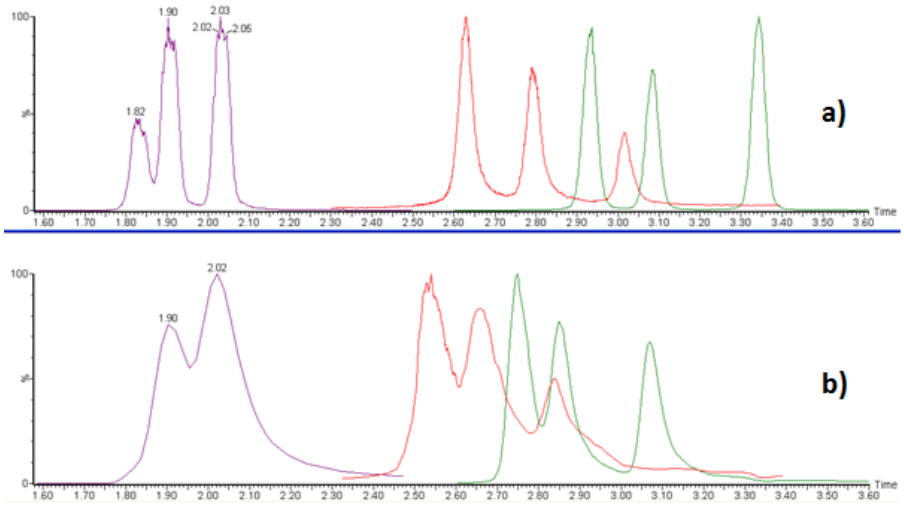

Figure 55. Using a methanolic NH_3 additive lead to a rapid retention drift and decreased baseline separation of the nine analytes of interest using the BEH column in the UHPSFC-MS/MS system. a) After 2 injections on a new BEH column. b): After 400 injections on the same BEH column. (Purple: 2-, 3-, 4-methylmethcathinone; Red: 2-, 3-, 4-fluoroamphetamine; Green: 2-, 3-, 4-fluoromethamphetamine).

Explaining the cause of this effect is risky, as no specific experiments were made to further investigate this effect. Rather, the effect was observed and possible causes are considered. Considering the chemistry of the BEH column as consisting of particles with mainly unbounded silanol seats on its surface, there is a single source of retention on the stationary phase. When assuming no change in other chromatographic conditions, any retention change can be correlated to a modification of the silica surface (Fairchild *et al*., 2015).

Many papers have been published on the adsorption of modifier onto stationary phases in chromatographic systems, especially in SFC, possibly explaining the chromatographic changes (Jinno and Niimi, 1988; Janssen *et al*., 1989; Strubinger *et al*., 1991b; Heaton *et al*., 1994; Gurdale *et al*., 2000; Lesellier *et al*., 2002; Smuts *et al*., 2011; Vajda and Guiochon, 2013; Lesellier and West, 2015). Nonetheless, Fairchild and colleagues have suggested that this retention drift is caused by a silyl ether formation when using methanol as a modifier in an SFC system on a silica-based stationary phase (Figure 56) (Fairchild *et al*., 2015). This has also been proposed in earlier years by other groups (Hirata and Nakata, 1984; Schmitz *et al*., 1987).

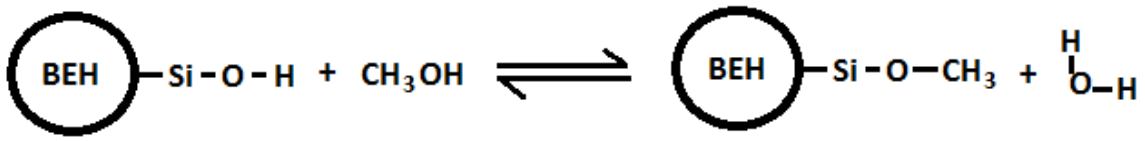

Figure 56. Silyl ether formation as proposed by Fairchild *et al*. to occur in typical SFC conditions when using silica-based stationary phase like the BEH column and methanol as a modifier (adapted from Fairchild *et al*., 2015).

Both of these effects are indicative of a higher modifier proportion. It is already known that modifier adsorption occurs in chromatographic systems using a Lewis acid (electron pair acceptor, electrophile) mobile phase in combination with a Lewis base (electron pair donor, nucleophile) stationary phase, such as methanol on a silica support. This effect has been proven in several studies, observed in both LC and SFC systems (Scott and Kucera, 1978; Janssen *et al*., 1989; Lesellier and West, 2015). However, it is more pronounced in SFC systems due to the apolar nature of CO_2, which probably accelerates the retention drift when combined with the polar methanol modifier and NH_3 additive. The apolar nature of CO_2 makes it repulse the polar counterparts. The inherent immiscibility of water in supercritical CO_2 under the conditions applied in this system adds to the retention drift, as only miniscule amounts of water can be added to mitigate this effect. In SFC systems both CO_2 and methanol can be prone to adsorption onto the stationary phase. However, adsorption of CO_2 is favored on octadecylsilane (ODS) stationary phases, while the adsorption of methanol is higher onto

silica stationary phases (Janssen *et al*., 1989; Strubinger *et al*., 1991a and b; Heaton *et al*., 1994; Gurdale *et al*., 2000; Lesellier *et al*., 2002; Lesellier and West, 2015). The mobile phase in this thesis work is also added a basic NH_3 additive. The NH_3 molecule having a significant dipolar moment makes it another possible influence on the chemistry of the stationary phase, by hydrogen bonding to the silanol seats of the BEH particles and/or by acting as a catalyst of adsorption of modifier or of silyl ether formation.

A study by West and Lesellier (2013) showed that the mobile phase components adsorb on the stationary phase surface in such a manner that the modifier proportion in this layer of adsorbed mobile phase components is much larger than in the bulk mobile phase. A study indicated that the adsorbed layer was nearly 25 % methanol when the mobile phase only contained 2 % methanol (Strubinger *et al*., 1991). The modifier is likely to adsorb onto the stationary phase by alcohol condensation. Two factors accelerates this process in the UHPSFC-system; 1) The apolar nature of the super-/subcritical CO_2 drives the polar solutes to interact more with the polar seats at the stationary phase. 2) The silyl ether formation is base catalyzed. The conditions chosen as the best for the separation of the analytes in question entails a relatively high dose of alkaline additive of 40 mM NH_3 in the modifier flask. In comparison; the recommended maximum dose from the supplier is 20 mM NH_3 added in the modifier bottle.

Some studies have compared the behavior of the most commonly applied modifiers; methanol, ethanol and isopropanol (Strubinger *et al*., 1991b; Vajda and Guiochon, 2013; West and Lesellier, 2013). They have indicated that the slower adsorption of ethanol versus methanol might be a result of the reaction kinetics expected to be significantly lower for the formation of longer alkyl chain silyl ethers. Longer hydrocarbon chain makes the ethanol molecule less polar compared to the methanol molecule, making it a poorer nucleophile and possibly causing steric hindrance. Therefore the modifier adsorption or silyl ether formation of ethanol is proposed to be many times slower than with methanol. Based on the indications made by Strubinger *et al*. (1991b)and their own observations, West and Lesellier (2013) suggested that the observed surprising behavior of methanol versus ethanol might result from different aggregation of alcohol molecules on the stationary phase, which might induce different surface chemistry. The conformational arrangement of the solvated selector might be different, but also the overall composition of the layer of adsorbed mobile phase components resulting from different proportions of alcohol and CO_2.

However, when ethanol was applied as a modifier clogging problems eventually occurred with a solution older than a few hours due to the equilibria of ethanol being shifted slightly towards the hydroxylation of water, producing a solution which contains about 4 % water. Fairchild proposed to use 5 % water in the modifier solution to compensate for the use of NH_3 (Fairchild *et al*., 2015). However, the use of smaller amounts of water has been problematical in this thesis work especially in repeated injections.

Furthermore, Fairchild *et al*. (2015) demonstrated that a washing regime using pure distilled water could mitigate the proposed silyl ether formation according to proposed equilibrium (Figure 54) and the principle of Le Chatelier. A similar washing regime was tested to attempt to regenerate the BEH column. The result was a partially restored BEH column, but no complete regeneration was attained (data not shown/details in Appendix VI). The degradation of the column occurred more rapidly with the regenerated columns than with unused columns.

So, although the chosen conditions initially produced satisfactory separation for all nine analytes of interest, there was an alteration in the stationary phase chemistry which became apparent over time. This phenomenon affected the analytes differently; the methyl group found on C1 of the common structure in methamphetamine, fluoromethamphetamine and methylmethcathinone appeared to make the analytes somewhat more robust for these chromatographic changes, but the reason for this effect is not known. Conversely, this effect became especially apparent for the methylmethcathinone analytes, which became impossible to separate on the degraded stationary phase as shown in Figure 53. The regeneration regime tested produced only temporarily satisfactory results for the methylmethcathinone analytes, only attainable for the first few injections. As a result it was not possible to validate the method for the methylmethcathinone analytes, and they were excluded from the validation procedure.

A possible reason as to why the methylmethcathinones analytes are more sensitive for the chromatographic alterations in the column could lie in the molecular structure. Structural studies have indicated that methylmethcathinones are considerably more hydrophilic than methylamphetamines and that the ketone group in the side chain contributes to far more planar structure (Gibbons and Zloh, 2010; Kelly *et al*., 2011). The other 2-, 3-, 4-positional isomers have in common that they have a fluor atom on the phenyl ring. Fluor being the most electronegative atom could give the analytes containing such a nucleophile a higher affinity for the polar seats in the BEH stationary phase compared to methylmethcathinone. Thus, the methylmethcathinone molecules are successfully separated on a BEH stationary phase, but

they are more sensitive to the covering of the silanol seats by modifier or the NH_3 additive after repeated injections.

4.4 Method validation

Method validation was performed to confirm that this analytical procedure is suitable for its intended use. The data obtained is used to evaluate the quality, reliability and consistency of the method. All validation parameters were executed in concordance with the validation regime of NIPH and by using spiked blood samples. All calculations were performed using Microsoft Excel software.

4.4.1 Interday precision and accuracy

The results for interday precision and accuracy (Table 12 and 13) were considered to be satisfactory for this method, as relative stand deviation (RSD) was below 15 % for all analytes above their respective LOQ. The classical phenethylamines displayed a precision below 10 % for all the levels above the LOQ. Accuracy was below 20 % for all the 2-, 3-, 4-phenethylamines for the levels above LOQ, while the classical phenethylamines demonstrated an accuracy below 10 % at all levels. However, the accuracy was below 3 % for most of the levels.

For the concentration levels below LOQ there was poor precision and accuracy, as expected. The complete data set for the between-series measurements of precision and accuracy can be seen in Appendix VII.

Table 12. Interday precision and accuracy of the 2-, 3-, 4-isomers.*

n=8

Analyte	Conc. (µM)	Mean value (µM)	Accuracy (%)	Precision (RSD, %)
2-fluoroamphetamine				
	0.0050	0.0044	-12	32
	0.010	0.0094	-6.2	24
	0.10	0.11	9.3	8.2
	1.0	1.1	7.8	8.5
	5.0	5.4	7.4	7.4
	10	10	1.8	8.3
3-fluoroamphetamine				
	0.0050	0.0042	-17	35
	0.010	0.01	0.61	22
	0.10	0.11	5.7	16
	1.0	1.2	16	6.4
	5.0	5.7	12	12
	10	10	4.4	7.1
4-fluoroamphetamine				
	0.0050	0.0046	-9.5	40
	0.010	0.01	-4.5	46
	0.10	0.11	13	14
	1.0	1.1	13	6.6
	5.0	5.6	11	12
	10	9.8	-2.8	2.7
2-fluoromethamphetamine				
	0.0050	0.0053	3.8	49
	0.010	0.01	1.2	17
	0.10	0.12	22	15
	1.0	1.2	18	7.5
	5.0	5.6	11	8.2
	10	11	5.2	6.4
3-fluoromethamphetamine				
	0.0050	0.0067	31	71
	0.010	0.011	12	32
	0.10	0.12	19	15
	1.0	1.2	18	8.5
	5.0	5.6	10	5.4
	10	11	9	5.8
4-fluoromethamphetamine				
	0.0050	0.012	140	44
	0.010	0.014	45	30
	0.10	0.11	12	8.9
	1.0	1.1	11	6.1
	5.0	5.2	3.1	12
	10	10	0.36	4.2

***The dotted line indicates LOQ. Conc. = Concentration**

Table 13. Interday precision and accuracy of the classical phenethylamines.*

n=8

Analyte	Concentration (µM)	Mean value (µM)	Accuracy (%)	Precision (RSD, %)
Amphetamine				
	0.0050	0.0070	41	46
	0.010	0.011	8.4	19
	0.10	0.10	2.3	5.4
	1.0	1.0	-0.76	9.2
	5.0	5.0	-0.76	5.6
	10	10	0.69	3.7
Methamphetamine				
	0.0050	0.0060	20	34
	0.010	0.010	-2.1	13
	0.10	0.10	2.2	5.4
	1.0	1.1	9.1	17
	5.0	5.1	2.3	5.1
	10	10	3.1	3.5
MDMA				
	0.0050	0.0074	45	50
	0.010	0.010	-0.35	24
	0.10	0.10	-0.91	6.5
	1.0	1.0	-0.84	6.2
	5.0	4.9	-2.2	5.6
	10	9.9	-2.4	5.9

*The dotted line indicates LOQ.

4.4.2 Intraday precision and accuracy

Precision was below 15 % for all analytes at the levels above LOQ. Accuracy given as bias was calculated as the percent deviation of the measured mean of the QC samples from the nominal concentration. Accuracy was +/- 20 % from the true value for most analytes, given as a bias where 0 % indicates perfect accuracy. For the lowest concentrations of 4-fluormethamphetamine and amphetamine, poor accuracy was obtained with a calculated concentration that was close to three times as large as the theoretical value. However, as this is below LOQ it will not affect the reliability of the results provided to the consignors. The

accuracy and precision of the classical phenethylamines was generally higher than that of the 2-, 3-, 4-phenethylamines, probably due to the use of analogue isotope labelled ISs. All accuracy and precision data are summarized in Table 14 and 15. The complete dataset for the intraday precision and accuracy experiment is shown in Appendix VII.

Table 14. Intraday precision and accuracy for the 2-, 3-, 4-phenethylamines.*

2-fluoroamphetamine				n=10
Concentration	**Calculated mean**	**SD**	**Precision (RSD)**	**Accuracy**
µM	µM	µM	%	%
0.01	0.0082	0.0019	23	-18
0.1	0.11	0.015	14	10
1.0	1.2	0.15	12	20
5.0	6.1	0.51	8.3	21
10	11	0.88	7.6	16

3-fluoroamphetamine				
Concentration	**Calculated mean**	**SD**	**Precision (RSD)**	**Accuracy**
µM	µM	µM	%	%
0.01	0.0091	0.00085	9.4	-9
0.1	0.11	0.012	10	15
1.0	1.2	0.073	5.9	23
5.0	6.6	0.39	6.0	30
10	11	0.63	5.3	18

4-fluoroamphetamine				
Concentration	**Calculated mean**	**SD**	**Precision (RSD)**	**Accuracy**
µM	µM	µM	%	%
0.01	0.0078	0.00089	11.5	-22
0.1	0.12	0.0078	6.6	17
1.0	1.29	0.056	4.3	27
5.0	6.2	0.57	9.3	22
10	11	0.62	5.8	6

2-fluoromethamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Accuracy
µM	µM	µM	%	%
0.01	0.012	0.0015	12.3	25
0.1	0.13	0.0171	13.2	29
1.0	1.2	0.074	6.1	21
5.0	5.8	0.42	7.2	16
10	11	0.57	5.1	11

3-fluoromethamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Accuracy
µM	µM	µM	%	%
0.01	0.011	0.0013	11.7	11
0.1	0.13	0.0034	2.6	31
1.0	1.2	0.065	5.5	17
5.0	5.9	0.34	5.7	17
10	11	0.73	6.6	10

4-fluoromethamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Accuracy
µM	µM	µM	%	%
0.01	0.029	0.0011	3.8	187
0.1	0.13	0.0072	5.5	30
1.0	1.2	0.060	5.2	16
5.0	5.9	0.29	5	16
10	11	0.36	3.2	11

***The dotted line indicates LOQ.**

Table 15. Intraday precision and accuracy for the classical phenethylamines.*

Amphetamine				n=10
Concentration	**Calculated mean**	**SD**	**Precision (RSD)**	**Accuracy**
µM	µM	µM	%	%
0.01	0.031	0.0085	28	210
0.1	0.11	0.0063	5.8	6.4
1.0	1.0	0.050	5.0	2.3
5.0	4.9	0.18	3.7	-2.3
10	10	0.37	3.6	1.0

Methamphetamine				
Concentration	**Calculated mean**	**SD**	**Precision (RSD)**	**Accuracy**
µM	µM	µM	%	%
0.01	0.0092	0.0013	14	-8.3
0.1	0.095	0.0055	5.8	-4.9
1.0	1.0	0.037	3.6	3.4
5.0	5.1	0.14	2.7	1.7
10	10	0.18	1.8	2.7

MDMA				
Concentration	**Calculated mean**	**SD**	**Precision (RSD)**	**Accuracy**
µM	µM	µM	%	%
0.01	0.0094	0.00053	5.7	-6.2
0.1	0.099	0.0024	2.4	-0.83
1.0	1.0	0.030	2.9	2.4
5.0	5.0	0.099	1.5	-1.0
10	10	0.18	1.8	1.6

***The dotted line indicates LOQ.**

4.4.3 Linearity

In order to evaluate the linearity of the method the calibration curves were produced, based on one parallel of each of the eight calibrators. Linear calibration curves with $R^2 \geq 0.99$ were achieved for all analytes using a weighted (1/x) linear calibration curve and including the origin. Eight calibrators were used to measure linearity of the method, but on a routine basis only five calibrators are used. Linearity was measured with extracted samples with a concentration of 0.001, 0.005, 0.010, 0.100, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00 µM. The results are shown in Table 16, while the linearity graphs are shown in Appendix VII.

The standard curves for the 2-, 3-, 4-isomers were slightly flattening at high concentrations, indicating that an incomplete ionization occurs in the ESI. This effect could be alleviated with the use of specific ISs and/or by the use of a quadratic standard curve. Quadratic standard curves were tested and produced good correlation of the lowest concentrations, but poorly described the concentration of the high samples.

Table 16. Data from the linearity experiment.

Substance	Internal Standard	Calibration	Measuring range (µM)	r^2
2-fluoroamphetamine	$^{13}C_6$-PMMA	1/x-weighting	0.001 – 10	0.9939
3-fluoroamphetamine	$^{13}C_6$-PMMA	1/x-weighting	0.001 – 10	0.9971
4-fluoroamphetamine	$^{13}C_6$-PMMA	1/x-weighting	0.001 – 10	0.9959
2-fluoromethamphetamine	$^{13}C_6$-PMMA	1/x-weighting	0.001 – 10	0.9917
3-fluoromethamphetamine	$^{13}C_6$-PMMA	1/x-weighting	0.001 – 10	0.9940
4-fluoromethamphetamine	$^{13}C_6$-PMMA	1/x-weighting	0.001 – 10	0.9929
Amphetamine	$^{13}C_6$-amphetamine	1/x-weighting	0.001 – 10	0.9976
Methamphetamine	$^{13}C_6$-methamphetamine	1/x-weighting	0.001 – 10	0.9867
MDMA	$^{13}C_6$-MDMA	1/x-weighting	0.001 – 10	0.9937

4.4.4 Limit of detection and limit of quantification

The calculated LOD and LOQ are within expected values for similar compounds analysed with UHPLC at NIPH. The LOD varied from 0.007 to 0.02 µM for all analytes. The LOQ varied from 0.02 to 0.06 µM for all analytes. The results for LOD and LOQ are shown in Table 17.

Table 17. LOD* and LOQ for the 2-, 3-, 4-isomers.**

	LOD (µM)	LOQ (µM)
2-fluoroamphetamine	0.0067	0.017
3-fluoroamphetamine	0.0068	0.017
4-fluoroamphetamine	0.011	0.024
2-fluoromethamphetamine	0.010	0.029
3-fluoromethamphetamine	0.019	0.052
4-fluoromethamphetamine	0.024	0.061
Amphetamine	0.013	0.036
Methamphetamine	0.0076	0.022
MDMA	0.014	0.039

* LOD is calculated based on the formula: LOD = mean blank + 3SD control 0.005 µM

** LOQ is calculated based on the formula: LOQ = mean blank + 10SD control 0.005 µM

4.4.5 Matrix effect

ME was measured at two different concentration levels and with two different injection volumes, as displayed in Table 18 – 21. The contribution of ME is considered acceptable between 75 and 125 %, with RSDs ≤ 25 % when using deuterated ISs (Paul *et al*., 2009). MEs were between 67 and 81 % for all the 2-, 3-, 4-isomers with RSDs between 15 and 22 %, while the classical phenethylamines experienced between 88 and 93 % ME with RSDs between 3.8 and 8.2 %. This suggests that the analytes experiences ion suppression. However, it should be noted that MEs were overall low for most analytes. The poor correction of ME by the ISs for some of the analytes could be due to the calculations being based on concentration, rather than on peak height. The 2-, 3-, 4-isomers had high RSDs which might have been possible to correct with an isotope-labelled analogue IS, but no commercial $^{13}C_6$-labelled or deuterated analogue IS was currently available for all the isomers. ME was also tested at 2.0 µL injection with the same concentration levels. ME ranged between 85 and 102 % for all the 2-, 3-, 4-isomers with RSDs between 10 and 20 %, while the classical phenethylamines experienced between 70 and 76 % ME with RSDs between 3 and 10 %.

Table 18. Data for matrix effect experiment of 0.1 µM samples with 0.5 µL injection.*

0.1µM, n=10

Substance	ME corrected	RSD	ME	RSD
2-fluoroamphetamine	93 %	14 %	112 %	7.3 %
3-fluoroamphetamine	85 %	13 %	103 %	7.0 %
4-fluoroamphetamine	102 %	20 %	123 %	15 %
2-fluoromethamphetamine	99 %	13 %	119 %	5.0 %
3-fluoromethamphetamine	93 %	10 %	112 %	6.6 %
4-fluoromethamphetamine	94 %	16 %	113 %	5.6 %
Amphetamine	76 %	10 %	98 %	6.5 %
Methamphetamine	73 %	3.0 %	106 %	6.3 %
MDMA	70 %	4.9 %	106 %	5.5 %

*Matrix effect was measured with two parallels and five different lots of blank blood.

Table 19. Data for matrix effect experiment of 1.0 µM samples with 0.5 µL injection.*

1µM, n=10

Substance	ME corrected	RSD	ME	RSD
2-fluoroamphetamine	81 %	22 %	95 %	16 %
3-fluoroamphetamine	67 %	17 %	79 %	14 %
4-fluoroamphetamine	77 %	19 %	90 %	16 %
2-fluoromethamphetamine	78 %	17 %	92 %	14 %
3-fluoromethamphetamine	70 %	19 %	82 %	15 %
4-fluoromethamphetamine	72 %	15 %	85 %	9.2 %
Amphetamine	89 %	8.2 %	84 %	4.4 %
Methamphetamine	93 %	5.6 %	96 %	2.7 %
MDMA	91 %	3.8 %	105 %	3.1 %

*Matrix effect was measured with two parallels and five different lots of blank blood.

Table 20. Data for matrix effect experiment of 0.1 µM samples with 2.0 µL injection.*

(0.1µM, n=10)

Substance	ME corrected	RSD	ME	RSD
2-fluoroamphetamine	98 %	7.9 %	94 %	7.5 %
3-fluoroamphetamine	97 %	9.2 %	93 %	6.9 %
4-fluoroamphetamine	111 %	6.5 %	107 %	2.9 %
2-fluoromethamphetamine	101 %	8.1 %	96 %	5.8 %
3-fluoromethamphetamine	104 %	7.2 %	99 %	3.6 %
4-fluoromethamphetamine	97 %	7.6 %	93 %	3.7 %
Amphetamine	79 %	8.4 %	60 %	13 %
Methamphetamine	75 %	4.3 %	68 %	8.4 %
MDMA	73 %	3.8 %	77 %	4.8 %

*Matrix effect was measured with two parallels and five different lots of blank blood.

Table 21. Data for matrix effect experiment of 1.0 µM samples with 2.0 µL injection.*

(1.0 µM, n=10)

Substance	ME corrected	RSD	ME	RSD
2-fluoroamphetamine	81 %	22 %	95 %	16 %
3-fluoroamphetamine	67 %	17 %	79 %	14 %
4-fluoroamphetamine	77 %	19 %	90 %	13 %
2-fluoromethamphetamine	78 %	17 %	92 %	13 %
3-fluoromethamphetamine	70 %	19 %	82 %	16 %
4-fluoromethamphetamine	72 %	15 %	85 %	9.7 %
Amphetamine	95 %	4.3 %	60 %	4.9 %
Methamphetamine	93 %	4.9 %	70 %	4.7 %
MDMA	87 %	4.7 %	80 %	4.2 %

*Matrix effect was measured with two parallels and five different lots of blank blood.

4.4.6 Extraction recovery

Recoveries were above 80 % for all the 2-, 3-, 4-isomers (Table 22) and above 50 % for the classical phenethylamines (Table 23). The recoveries for the 2-, 3-, 4-phenethylamines are generally high at both concentration levels. Recovery of the classical phenethylamines is lower with low concentration samples, but it is considered to be adequate as the interday and intraday precision and accuracy is satisfactory even at lower concentration levels.

Table 22. Data from extraction yield of the 2-, 3-, 4-isomers.

n=6

Compound	Calculated mean	Recovery %	Corrected recovery* %
2-fluoroamphetamine	0.25 µM	68 %	85 %
	1.0 µM	76 %	95 %
3-fluoroamphetamine	0.25 µM	69 %	87 %
	1.0 µM	74 %	92 %
4-fluoroamphetamine	0.25 µM	67 %	84 %
	1.0 µM	67 %	84 %
2-fluoromethamphetamine	0.24 µM	77 %	96 %
	1.0 µM	64 %	80 %
3-fluoromethamphetamine	0.28 µM	72 %	90 %
	0.99 µM	78 %	98 %
4-fluoromethamphetamine	0.27 µM	73 %	91 %
	0.99 µM	78 %	98 %

***Corrected recovery refers to the correction for 800 µL volume taken after LLE during sample preparation. Described in chapter 3.7.2 and in AppendixV, Table V a.**

Table 23. Data from extraction yield experiment of the classical phenethylamines.

n=6

Compound	Calculated mean	Recovery %	Corrected recovery* %
Amphetamine	0.27 µM	40 %	50 %
	0.99 µM	68 %	84 %
Methamphetamine	0.25 µM	57 %	71 %
	1.0 µM	65 %	81 %
MDMA	0.27 µM	48 %	60 %
	0.98 µM	73 %	91 %

***Corrected recovery refers to the correction for 800 µL volume taken after LLE during sample preparation. Described in chapter 3.7.2 and in AppendixV, Table V a.**

4.4.7 Carry-over

Analysis of concentrations up to 1 µM was performed without carry-over for most analytes, but analysis of 10 µM samples could produce false positives with concentrations around LOQ, especially for the fluoromethamphetamines. This carry-over could affect the identification and quantification of the subsequent/the following samples. After high concentrations, it should be considered to inject a blank sample or inject to parallels of the following sample. This may be considered for routine analysis, where high concentration values can be expected from autopsy and intoxication patients, by e.g. blanks being injected 1-2 times after every real sample.

Carry-over under analysis was examined by injection of three consecutive blanks after one injection of a high concentration sample. Calculated concentrations of the chromatograms for the blanks injected was given as in µM and as percentage carry-over of the calculated concentration of the high concentration sample (10 µM), given in Table 24 and 25.

Table 24. Percentage carry-over for the 2-, 3-, 4-isomers.

n=3

	Blank 1		Blank 2		Blank 3	
Analyte	Calc. conc. µM	Average carry-over %	Calc. conc. µM	Average carry-over %	Calc. conc. µM	Average carry-over %
2-fluoroamphetamine	0.049	**0.44** %	0.015	**0.13** %	0.0077	**0.07** %
3-fluoroamphetamine	0.070	**0.66** %	0.019	**0.18** %	0.0073	**0.06** %
4-fluoroamphetamine	0.087	**0.82** %	0.031	**0.29** %	0.017	**0.16** %
2-fluoromethamphetamine	0.049	**0.46** %	0.012	**0.11** %	0.0053	**0.05** %
3-fluormethamphetamine	0.050	**0.47** %	0.012	**0.11** %	0.0057	**0.05** %
4-fluormethamphetamine	0.069	**0.65** %	0.039	**0.37** %	0.032	**0.29** %

Abbreviations: Calc. = calculated, conc. = concentration

Table 25. Percentage carry-over for the classical phenethylamines.

Carry-over

n=3

Analyte	Blank 1		Blank 2		Blank 3	
	Calc. conc. µM	Average carry-over %	Calc. conc. µM	Average carry-over %	Calc. conc. µM	Average carry-over %
Amphetamine	0.040	**0.44** %	0.025	**0.28** %	0.018	**0.19** %
Methamphetamine	0.030	**0.34** %	0.0079	**0.09** %	0.0049	**0.06** %
MDMA	0.043	**0.50** %	0.011	**0.12** %	0.0058	**0.07** %

Abbreviations: Calc. = calculated, conc. = concentration

4.4.8 Specificity

No interfering peaks were found for the MRM transitions of the analytes of this method when testing 25 common drugs of abuse (Appendix VII). However, due to human error very low therapeutic concentrations were tested. Although the resulting absence of interfering peaks could indicate high specificity, the experiment should be repeated at higher concentrations. The eight blank samples prepared from blank blood indicated no interfering endogenic substances, which further ads to the specificity of this method.

5 Final remarks and future experiments

This thesis work only scratches the surface of the possibilities and constraints of using SFC. Still there are questions left unanswered and effects left undiscovered as there are several experiments to do in order to further elaborate on these questions. Most challenges and unanswered questions are mentioned in the thesis/discussion so far, but they are also summed up below;

5.1 Linearity

Too high concentration of standards and controls lead to a non-linear standard curve. A second order curve fitted the standard curve somewhat better for concentrations up to 5-6 µM, as the highest concentrations lead to a possible saturation during ESI and thus it is probable that these concentrations are outside the linear area.

5.2 Internal standards

The 2-, 3-, 4-isomers where analysed with isotope-labelled PMMA. It would be interesting to also run the analysis and validation with isotope-labelled analogue ISs, in order to reduce variation due to ion suppression (Berg and Strand, 2011).

5.3 Stability

The stability of the samples where not tested. Analysis methods of similar compounds at NIPH indicates that the analytes tested in this thesis work might be stable over longer periods of time under different storage conditions. Still, differences in stability might exists and should be tested in future experiments.

5.4 Evaluation of nitric acid as a keeper during extraction

Adding a proton donor such as a strong acid proved to be necessary in order to avoid the escape of analyte during evaporation. The proton donor is often referred to as a "keeper", due to its ability to keep the analyte in the vial by protonation making it less capable of escaping the vial during evaporation.

The sample preparation was performed both with and without using the nitric acid as a keeper. Adding a small amount (10 µL) of 0.1 % nitric acid in methanol ensured escape of the somewhat volatile analytes. Without addition of nitric acid, much of the analytes evaporated and the samples were no longer suitable for analysis (data not shown).

Nitric acid is an effective keeper; however it caused some clogging problems downstream of the MS splitter, especially in the capillary of the MS. This might be caused by out crystallization of nitric acid salts in the transition to atmospheric pressure. Adding other acids as keepers or different dilutions of nitric acid added should be further evaluated in order to avoid deterioration of the capillary of the MS.

5.5 Evaluation of retention drift

The deteriorating effect of basic methanol on the silica-based stationary phases could, as mentioned, be due to effects such as modifier adsorption or silyl ether formation. However, the distinction between these two was not made in this thesis work. Rather, the deteriorating effect has been observed and no experiments have been done to elaborate on how the deterioration occurs.

5.6 Evaluation of sample solvents

Only a few sample solvents were tested and all were quite polar solvents. It would be interesting to also test solvents with weaker eluotropic value to better match the super- or subcritical mobile phase mainly consisting of the apolar CO_2.

5.7 Supercritical or subcritical conditions

To determine if the experiments are performed under supercritical conditions or not is a difficult matter (Page et al., 1991). However, the conditions might be or close supercritical when a small amount of modifier is added (Saito, 2013). Considering that the mixtures used in SFC only are monophasic and homogeneous under supercritical conditions, it calls for further research. It would be especially interesting to investigate the precision and accuracy of experiments under subcritical conditions compared to supercritical conditions.

6 Conclusion of the thesis

New synthetic amphetamines are being synthesized at high speed to circumvent current legislation. These synthetic amphetamines are often methylated or halogenated versions of the originals. This produces a range of possible products of synthesis, giving 2-, 3- and 4-positional isomers. These isomers have been challenging to distinguish by the laboratory of forensic analysis at NIPH by LC-MS/SMS. These new synthetic amphetamines and constitutional isomers of these amphetamines call for new and improved analytical tools. The interest of SFC has been renewed lately due to new and improved instrumentation. Amphetamine and methamphetamine have been analysed by SFC, but no effort has yet been made on employing this method for separation of constitutional isomers of "designed" amphetamines.

The utilization of UHPSFC-MS/MS proved to be a good tool for the detection and quantification of nine out of twelve of the constitutional isomers included in this thesis work. However, there were a few challenges to the method. Utilization of an aqueous NH_3 mobile phase additive caused occasional problems of clogging and was substituted for a methanolic NH_3 additive. By using a water-free mobile phase this clogging problem was reduced. However, over time the analytes which initially could be separated by UHPSFC experienced a retention time drift, peak broadening and reduced separation with the water-free mobile phase corresponding to the number of injections of the column. This degeneration of the column could be a result of modifier and/or NH_3 adsorption to the silanol seats of the stationary phase, and could be partially reversed by washing the column with water. Furthermore, spray pulsing was occasionally observed. This problem occurred at the interface of the UHPSFC and the tandem MS, caused by the transition of the compressed CO_2-dominated mobile phase to atmospheric pressure. As CO_2 is only soluble with solvents in supercritical state, the transition of CO_2 to gas phase separates the mobile phase into two phases of liquid and gas, causing a pulsating spray through the MS injection device.

The 2-, 3-, 4-methylamphetamines were not separated by UHPSFC-MS/MS. The 2-, 3-, 4-methylmethcathinones were initially successfully separated with UHPSFC, but their separation was irreversibly affected by the degradation of the column and could not be quantified over time. However, an UHPSFC-MS/MS method for analysis of 2-, 3-, 4-fluoroamphetamines, 2-, 3-, 4-fluormethamphetamines, amphetamine, methamphetamine and MDMA was successfully developed and validated. However, the 2-, 3-, 4-

methylamphetamines could not be successfully separated with UHPSFC, but could potentially be separated with UHPLC.

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APPENDIX

Appendix I Preparation of Standard, Control and Internal Standard Solutions

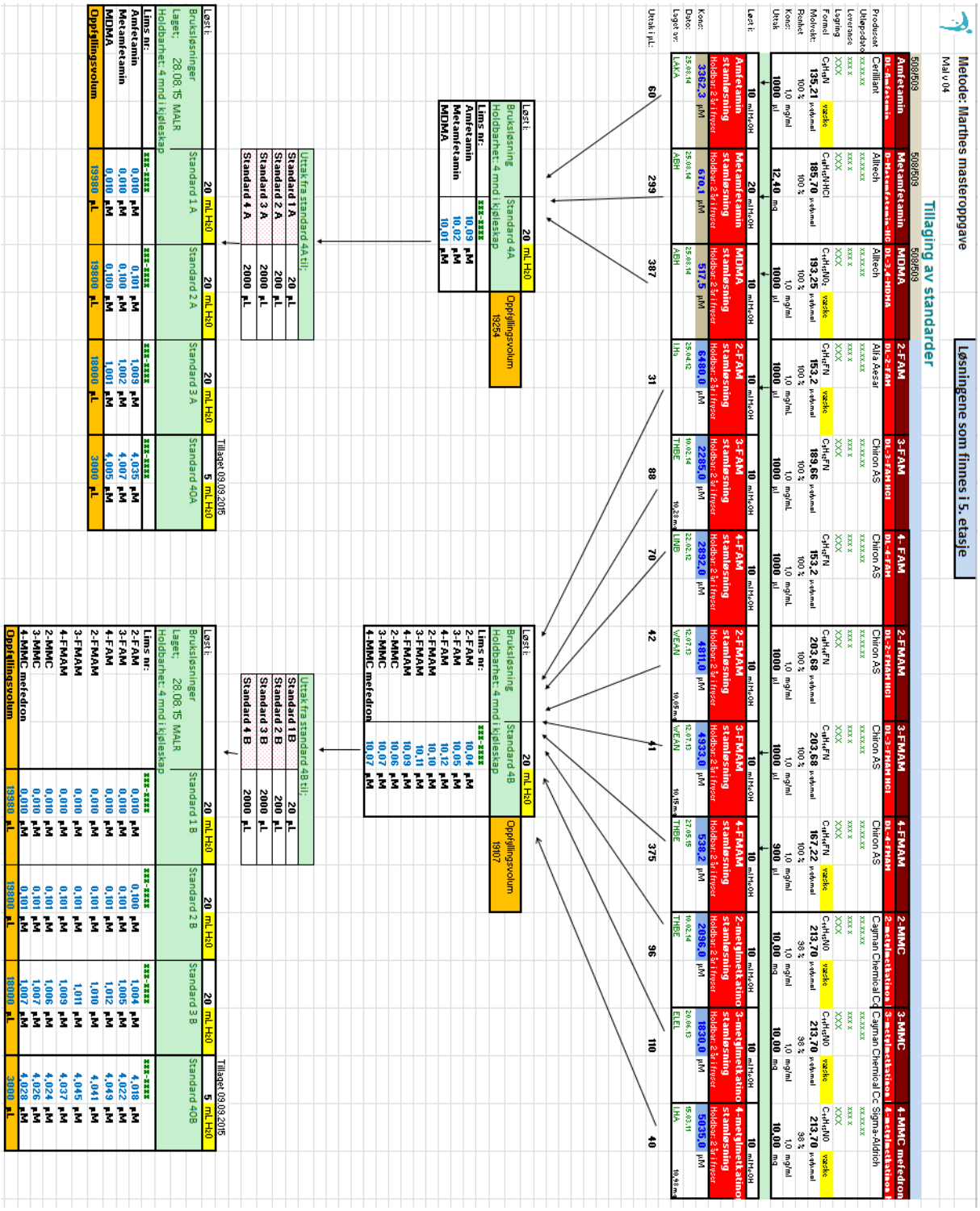


Figure I a. Preparation of standard solutions (in Norwegian).

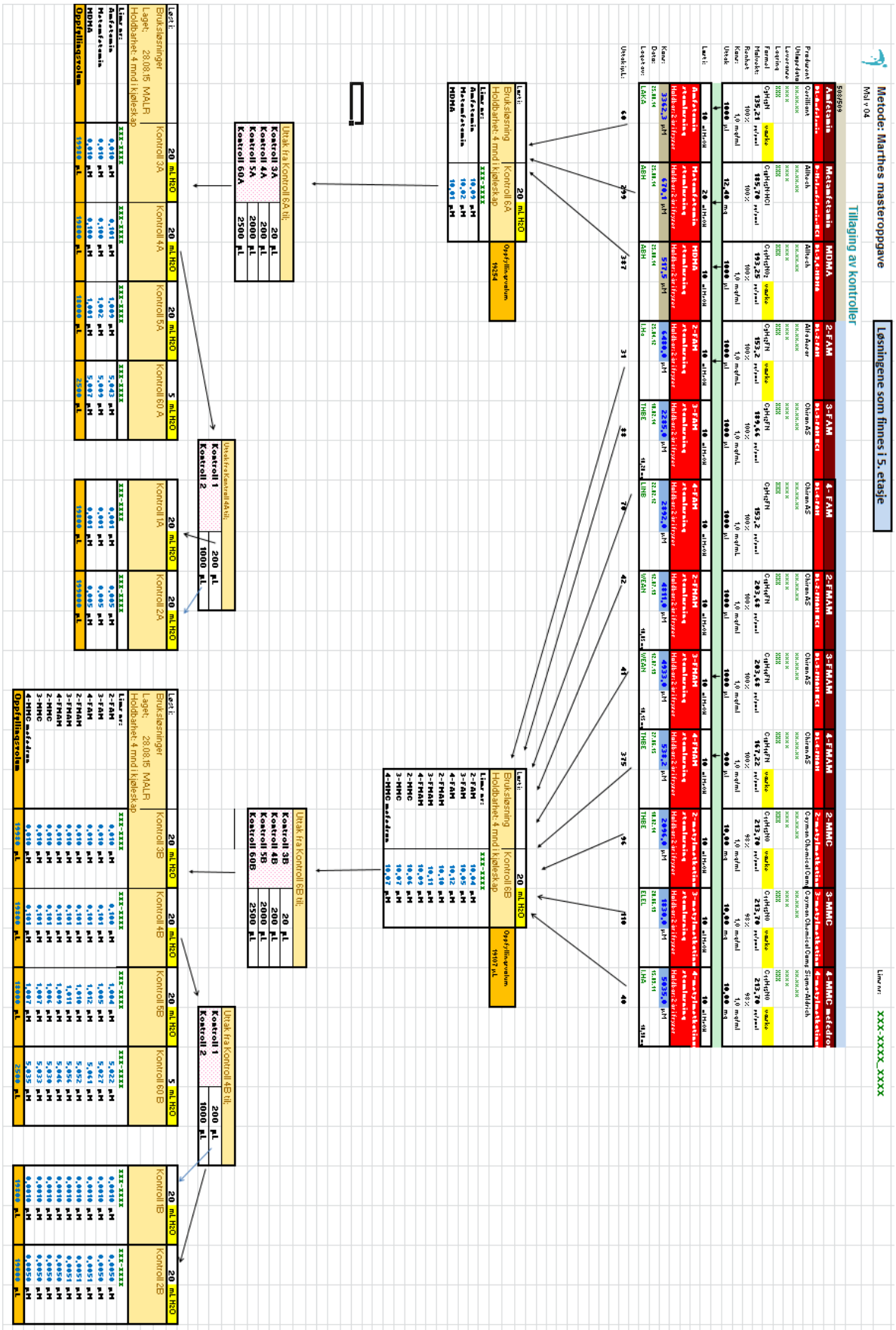


Figure I b. Preparation of control solutions (in Norwegian).

	Metode: Marthe 2015 2/3/4 - Amfetaminer Kva Bl SFC-MS/MS				Tillaging av internstandard
Renstoff:	**$^{13}C_6$-Amfetamin sulfat**	**$^{13}C_6$-Metamf. HCL**	**$^{13}C_6$ - MDMA HCL**	**$^{13}C_6$ MDEA HCl**	**$^{13}C_6$-PMMA HCl**
Produsent:	Chiron	Chiron	Chiron	Chiron	Chiron
Leveranse/lagring:					
Molekylformel:					
Molvekt:	374.45 g/mol	191.65 g/mol	235.66 g/mol	249.69 g/mol	220.67 g/mol
Renhet:	200.0 % pga.sulfat	100.0 %	100.0 %	100.0 %	100.0 %
Kons. v/ Ampulle:					
Uttak v/ampulle (µL):					
Uttak v/tørrstoff (mg):	5.0 mg	5.0 mg	2.14 mg	2.10 mg	mg
Stamløsning:	**$^{13}C_6$-Amfetamin stamløsning**	**$^{13}C_6$-Metamf. Stamløsning**	**$^{13}C_6$ - MDMA stamløsning**	**$^{13}C_6$ MDEA stamløsning**	**$^{13}C_6$-PMMA stamløsning**
Kolbe (mL/løsn.):	20 MeOH	20 MeOH	5 MeOH	5 MeOH	5 MeOH
Laget	25.06.2014 ABH	25.06.2014 ABH	10.10.2013 THBE	10.10.2013 THBE	10.10.2013 THBE
Holdbarhet	2 år fryser	2 år fryser	2 år fryser	2 år fryser	2 år fryser
Kons (µM)	1335 µM	1304 µM	1816 µM	1682 µM	2057 µM
Uttak (µL):	150	155	110	120	100

Bruksløsning:	**Bruksløsning**	Konsentrasjoner i bruksløsning		...Konsentrasjoner i bruksløsning	
Kolbe (mL/løsn.):	**50 mL H_2O**	$^{13}C_6$-Amfetamin	4.01 µM	$^{13}C_6$-MDMA	4.00 µM
Laget	17.08.2015 MALR	$^{13}C_6$-Metamfet.	4.04 µM	$^{13}C_6$-MDEA	4.04 µM
Holdbarhet	6 mnd kjøleskap			$^{13}C_6$-PMMA	4.11 µM
Uttak til hver prøve (µL):	50				

Blodprøve:	Konsentrasjoner**	Konsentrasjoner i blodprøve		...Konsentrasjoner i blodprøve	
Mengde blod (µL)	**100 µL**	$^{13}C_6$-Amfetamin	2.00 µM	$^{13}C_6$-MDMA	2.00 µM
		$^{13}C_6$-Metamfet.	2.02 µM	$^{13}C_6$-MDEA	2.02 µM
				$^{13}C_6$-PMMA	2.06 µM

** Konsentrasjonene er regnet i forhold til mengde blod, og ikke mengde blod + internstandard, da det er mengde internstandard per blodprøve som brukes for beregning av stoffkonsentrasjoner

Figure I c. Preparation of IS solution (in Norwegian).

Dissolved in:	isopropanol	20 mL H_2O	20 mL H_2O	20 mL H_2O	5 mL H_2O	20 mL H_2O	20 mL H_2O
Working solutions Prepared: Durability: 4 months in refrigerator		Control 3 A 28.08.15	Control 4 A 28.08.15	Control 5 A 28.08.15	Control 60 A 09.09.2015	Control 1 A 28.08.15	Control 2 A 28.08.15
Amphetamine		0,010 µM	0,101 µM	1,009 µM	5,043 µM	0,001 µM	0,005 µM
Methamphetamine		0,010 µM	0,100 µM	1,002 µM	5,009 µM	0,001 µM	0,005 µM
MDMA		0,010 µM	0,100 µM	1,001 µM	5,007 µM	0,001 µM	0,005 µM
Fill volume		19980 µL	19800 µL	18000 µL	2500 µL	19800 µL	19900 0 µL

Figure I d. Concentrations of the different control solutions, group A.

Dissolved in:	isopropanol	20 mL H_2O	20 mL H_2O	20 mL H_2O	5 mL H_2O	20 mL H_2O	20 mL H_2O
Working solutions Prepared: Durability: 4 months in refrigerator		Control 3B	Control 4B	Control 5B	Control 60 B	Control 1B	Control 2B
2-FAM		0,010 µM	0,100 µM	1,004 µM	5,022 µM	0,0010 µM	0,0050 µM
3-FAM		0,010 µM	0,101 µM	1,005 µM	5,027 µM	0,0010 µM	0,0050 µM
4-FAM		0,010 µM	0,101 µM	1,012 µM	5,061 µM	0,0010 µM	0,0051 µM
2-FMAM		0,010 µM	0,101 µM	1,010 µM	5,052 µM	0,0010 µM	0,0051 µM
3-FMAM		0,010 µM	0,101 µM	1,011 µM	5,056 µM	0,0010 µM	0,0051 µM
4-FMAM		0,010 µM	0,101 µM	1,009 µM	5,046 µM	0,0010 µM	0,0050 µM
2-MMC		0,010 µM	0,101 µM	1,006 µM	5,030 µM	0,0010 µM	0,0050 µM
3-MMC		0,010 µM	0,101 µM	1,007 µM	5,033 µM	0,0010 µM	0,0050 µM
4-MMC		0,010 µM	0,101 µM	1,007 µM	5,035 µM	0,0010 µM	0,0050 µM
Fill volume		19980 µL	19800 µL	18000 µL	2500 µL	19800 µL	19000 µL

Figure I e. Concentrations of the different control solutions, group B.

Appendix II Calculations of molar volumes

Volume required to make 0.3 % NH_3 250 mL prepared solution from a 25 % NH_3 solution:

$$c_1V_1 = c_2V_2$$

$$V_1 = \frac{c_2V_2}{c_1}$$

$$V_1 = \frac{0.3\ \% * 250\ mL}{25\ \%}$$

$$\underline{V_1 = 3\ mL}$$

Molar quantity of NH_3 in 0.3 % NH_3:

Using 25 % aqueous NH_3, with a density of 0.903 g/mL

Volume of prepared solution: 250 mL

Molar mass NH_3: 17.031 g/mol

Finding the volume of pure NH_3 in the 0.3 % NH_3 250 mL prepared solution:

$$\frac{250\ mL * 0.3\ \%}{100\ \%} = 0.75\ mL$$

Finds the mass of this volume:

$$V * d = m$$

$$0.75\ mL * 0.903\frac{g}{mL} = 0.67725g$$

Finds the mass of pure NH_3 by correcting for using 25 % NH_3:

$$\frac{0.67725\ g * 25\%}{100\ \%} = 0.1693125\ g$$

Finds the number of moles in the prepared solution:

$$n = \frac{m}{M}$$

$$n = \frac{0.1693125\ g}{17.031\ g/mol}$$

$$n = 0.009941430333\ mol$$

Finds the molar concentration of NH_3 in the prepared solution:

$$c = \frac{n}{V}$$

$$c = \frac{0.009941430333\ mol}{0.250\ L}$$

$$c = 0.039765721\ mol/L$$

$$\underline{c \approx 40\ mM}$$

The solution of 0.3 % aqueous $NH_3$is equivalent to 40 mM NH_3 prepared from NH_3 dissolved in methanol. The molar concentrations of the 0.1, 0.2 and 0.4 % solutions are calculated in the same manner.

<u>The volume required to produce a 250 mL 40 mM NH_3 in methanol solution from a 2 M NH_3 in solution:</u>

$$c_1V_1 = c_2V_2$$

$$V_1 = \frac{c_2V_2}{c_1}$$

$$V_1 = \frac{0.040\ M * 250\ mL}{2\ M}$$

$$c_2 = 5\ mL$$

The concentration of the 250 mL 40 mM NH_3 in methanol solution produced from a 2 M NH_3 in solution:

$$c_1V_1 = c_2V_2$$

$$c_2 = \frac{c_1V_1}{V_2}$$

$$c_2 = \frac{2\ M * 5\ mL}{250\ mL}$$

$$c_2 = 0.04\ M$$

$$c_2 = 40\ mM$$

Appendix III Name of the Field of Supercritical Fluid Chromatography

Supercritical fluid is defined as an element or a compound above its critical pressure and critical temperature. It is a misleading name, which implies enhanced properties that in reality are unjustified. Initially, supercritical fluid chromatography was categorized as high-pressure or dense gas chromatography (HPGC or DGC) (Giddings *et al*. 1969; Saito, 2013). Supercritical fluid chromatography may not be a good name, as most of the present day analyses are performed in subcritical conditions due to addition of organic modifier. Jim Lovelock suggested the name "critical state chromatography" at a GC conference in 1957. In 1962, Klesper called the technique "high pressure GC above critical temperatures". This is not a good name, since it is not a GC technique, considering that the mobile phase acts like a solvent and is not inert. Giddings, probably the most influential chromatographic theorist in the 1960's, used several names, including: turbulent flow chromatography, ultra high pressure gas chromatography (to 2000 atmospheres) and dense gas chromatography. However, none of these names captured the solvating nature of the technique. Sie and Rijnders first used the name supercritical fluid chromatography. This name is generally accepted/currently most used, but implies that the fluid must always be in a supercritical state, meaning above critical point to display the desirable characteristics, which also is not true. Caude was the first to use the term 'subcritical' to denote the fact that some modified CO_2 based fluids were of high density, while still highly compressible, but acted as a solvent even below their critical temperature (Berger, 2014). So in fact, SFC is almost always carried out below supercritical conditions, in or below the subcritical area when the mobile phase contains significant concentration of an organic modifier or some other additive, which is most frequent in preparative applications due to the relatively low solubility of polar compounds in liquid CO_2.

The name supercritical fluid chromatography is thus often inadequate as the conditions often are below critical value for the mobile phase fluid. The term subcritical fluid chromatography is alternatively used, but is equally inadequate as a subcritical fluid (with $P>P_c$ and $T>T_c$) is actually a liquid. The term "enhanced fluidity liquid chromatography" (EFLC) has also been used, mainly be the group of Susan Olesik, when adding CO_2 to methanol/water mobile phases in RPLC (Cui and Olesik, 1995; Lee and Olesik, 1995). The purpose of using CO_2 in EFLC is to reduce the fluid viscosity, thereby improving efficiency, and reducing the analyses duration both by the flow rate enhancements and the change of solvent strength. The increase in solutes diffusivity may be related to the ability of CO_2 to break the hydrogen bonds

between methanol and water. It was also shown that isocratic elution EFLC compared favorably to gradient elution HPLC, allowing the separation of compounds with a large range of retention factors.

The approach of EFLC differs from that of SFC in that the former consists of introducing a portion of CO_2 (up to 50 %) into an aqueous liquid mobile phase, while the latter consists of introducing a portion of a liquid solvent (up to 50 %) into a CO_2 supercritical fluid. The difference between the two is thus subtle or nonexistent. Consequently, EFLC and SFC can be considered as two ends of a unique method. A recent paper from the group of Takeshi Bamba showed a gradient elution moving from 2 to 100 % modifier (methanol-water 95:5 with 0.2 % ammonium formate) to achieve the separation of water- and fat-soluble vitamins in a single run (Taguchi *et al.*, 2014). This chromatogram really bridges the gap between SFC and HPLC, and may be representative of the future trends in operating SFC.

More recently, the name "convergence chromatography" was proposed by Waters inc., to suggest the intermediate position of the mobile phase between gases and liquids. This idea was based on a comment from Giddings (1969), saying that "one of the most interesting features of ultra-high pressure gas chromatography would be the convergence with classical chromatography." While it makes sense, it may be confusing to inexperienced chromatographers wondering what the difference between "convergence chromatography" and "SFC" is. Since no consensus has yet been reached, in the present thesis work, SFC is used as an acronym. SFC here defines a chromatography technique with CO_2 in the mobile phase, rather than a fluid state.

Appendix IV Prevalence

In recent years, methamphetamines have experienced a larger increase in consumption relative to amphetamine. Bramness studied this increase and emphasized the unusual tendency in Norway in respect to the lack of user distinction between methamphetamine and amphetamine and attributed this to the fact that both amphetamine and methamphetamine are sold as white powder in Norway, while it is more often sold in its crystalline form in other countries. Bramness indicated that the perceived ability of some users to evaluate the purity of the drug could in fact be recognition of methamphetamine rather than amphetamine. Due to the lack of distinction between the two among users, amphetamine and methamphetamine are most often reported collectively as amphetamines in surveys in Norway (SIRUS - The drug situation in Norway, 2009-2014; Bramness *et al*., 2015).

Table IV a. Annual number of samples received at NIPH and %-positive detections. Data from the NIPH-report, 2014.

Annual number of cases received at NIPH and %-positive detections

Case category	2010		2011		2012		2013		2014		Annual average	
	n	%-pos	n	%-pos	n	%-pos	n	%-pos	n	%-pos	n	%-pos
Drug abuse among prison inmates	20922	30 %	12183	42 %	12188	42 %	12367	44 %	14225	43 %	14377	40 %
Driving under the influence of drugs (police)	4727	93 %	4570	93 %	5107	94 %	5311	94 %	5947	92 %	5132	93 %
Driving under the influence of alcohol (police)	3204	97 %	3341	97 %	3494	97 %	3187	98 %	2349	98 %	3115	97 %
Social medicine	2227	29 %	2496	29 %	2216	27 %	1892	21 %	1489	31 %	2064	27 %
Medical cases	2030	78 %	2374	69 %	1911	59 %	1674	78 %	2107	85 %	2019	74 %
Forensic autopsies	1603	76 %	1599	78 %	1528	77 %	1731	80 %	1692	77 %	1631	78 %
Workplace drug testing	1844	3 %	1736	2 %	1464	5 %	1508	4 %	1149	5 %	1540	4 %
Impairment due to drugs (police)	1195	84 %	1162	85 %	1306	84 %	1496	84 %	1747	84 %	1381	84 %
Medical-related offences /Offences handled by the healthcare	413	65 %	492	65 %	510	61 %	565	59 %	670	60 %	530	62 %
Abuse of drugs other than alcohol	342	65 %	333	86 %	386	82 %	479	80 %	462	79 %	400	83 %
Impairment due to alcohol (police)	203	70 %	190	70 %	237	17 %	207	61 %	219	64 %	211	67 %
Abuse of drugs among military recruits	8	13 %	10	0 %	1	0 %	7	14 %	8	100 %	7	25 %
Annual number of cases and %-pos.*:	38718	49 %	30486	59 %	30348	60 %	30424	62 %	32064	62 %	32408	58 %

* %-pos. = %-positive samples

Appendix V Evaluation of Extraction Agent

Table V a. Overview of the extraction agent experiment.

Sample	Blank blood	Std. 4 (in H_2O, r) and c=10µM)	IS (before/afte volume		Phospholipid-extraction	Solvent	Amount	Time of solvent addition	Centrifugation (3500 omdr/min, 5min)	Transfer (in new tubes)	Format	Buffer, pH 9.3
1+2	0,1 mL	0,1 mL	50 µL	PP on filter	Supelco	ACN/MeOH (85:15)	600 µL	before filtration	Yes	500 µL	Single filter	-
3+4	"	"	"	PP on filter	Oasis Prime HLB	ACN/MeOH (85:15)	600 µL	before filtration	Yes	500 µL	Single filter	-
5+6	"	"	"	PP on filter	I,Phree	ACN/MeOH (85:15)	600 µL	before filtration	Yes	500 µL	Single filter	-
7+8	"	"	"	PP on filter	Ostro (NOT TESTED*	ACN/MeOH (85:15)	600 µL	before filtration	Yes	-	96	-
9+10	0,1 mL	"	"	PP without filter	Manuell	ACN/MeOH (85:15)	600 µL					-
11+12	0,1 mL	"	"	LLE		MTBE	600 µL		Ja	500 µL	-	-
13+14	"	"	"	LLE		MTBE	600 µL		Ja	500 µL	250 µL	
15+16	"	"	"	LLE		EA/Hp (4+1)	600 µL		Ja	500 µL	-	
17+18	"	"	"	LLE		EA/Hp (4+1)	600 µL		Ja	500 µL	250 µL	
19+20	"	"	"	LLE		cyklohexane	600 µL		Ja	500 µL	-	
21+22	"	"	"	LLE		cyklohexane	600 µL		Ja	500 µL	250 µL	

* Ostro not tested as it was only attainable as 96-well plates, which is not optimal for this low-volume method

PP = Protein precipitation

ACN/MeOH = Acetonitrile and methanol

MTBE = Methyl ter-butyl ether

EA/Hp = Ethyl acetate and heptane

Table V b. Evaluation of agent properties and recoveries.

Compound	Properties	Toxicity	Availability at NIPH	Evaporationstime, min (20 psi, 60 °C)	Recovery of analyte*	Residue of phospholipids after extraction**
MTBE	Highly flammable Irritating skin	Some		7	Good, 10^8	Poor, 10^8
Ethyl acetate	Highly flammable Causes serious eye irritation	High		15	Good, 10^8	Good, 10^5
Heptane	Highly flammable Fatal if swallowed, especially to airways Irritant to skin	"	"	"	"	"
Cyklohexane	Highly flammable Fatal if swallowed, especially to airways Irritant to skin	Some		30	OK, 10^7	Good, 10^5

*= MS-signal analytt

**= MS-signal fosfolipid

Appendix VI Regeneration of the BEH-Column

BEH columns were washed at different regimes, but the best results were obtained at a flow of 0.5 mL/min with H_2O (Milli-Q water with 0.003 % formic acid) for 6 hours and then 1 hour of acetonitrile on a HPLC instrument.

Appendix VII Complete Data Set of Method Validation

Table VII a. Interday precision and accuracy

	n=8			
Analyte	**Concentration (µM)**	**Mean value (µM)**	**Accuracy (%)**	**RSD (%)**
2-fluoroamphetamine	0.000	0.0025	-0.010	120
	0.001	0.0024	140	75
	0.005	0.0044	-12	32
	0.010	0.0094	-6.2	24
	0.100	0.11	9.3	8.2
	1.004	1.1	7.8	8.5
	5.022	5.4	7.4	7.4
	10.04	10	1.8	8.3
3-fluoroamphetamine	0.000	0.0024	-0.010	130
	0.001	0.0033	230	61
	0.005	0.0042	-17	35
	0.010	0.010	0.61	22
	0.101	0.11	5.7	16
	1.005	1.2	16	6.4
	5.027	5.7	12	12
	10.05	10	4.4	7.1
4-fluoroamphetamine	0.000	0.0051	0.01	110
	0.001	0.0034	240	77
	0.005	0.0046	-9.5	40
	0.010	0.010	-4.5	46
	0.101	0.11	13	14
	1.012	1.1	13	6.6
	5.061	5.6	11	12
	10.12	9.8	-2.8	2.7
2-fluoromethamphetamine	0.000	0.0025	-0.010	120
	0.001	0.0023	130	55
	0.005	0.0053	3.8	49
	0.010	0.010	1.2	17
	0.101	0.12	22	15
	1.010	1.2	18	7.5
	5.052	5.6	11	8.2
	10.10	11	5.2	6.4
3-fluoromethamphetamine	0.000	0.0048	-0.010	140
	0.001	0.0049	390	120
	0.005	0.0067	31	71
	0.010	0.011	12	32
	0.101	0.12	19	15
	1.011	1.2	18	8.5
	5.056	5.6	10	5.4
	10.11	11	9.0	5.8
4-fluoromethamphetamine	0.000	0.0079	-0.010	68
	0.001	0.0087	770	66
	0.005	0.012	140	44
	0.010	0.014	45	30
	0.101	0.11	12	8.9
	1.009	1.1	11	6.1
	5.046	5.2	3.1	12
	10.09	10	0.36	4.2

Table VII b. Precision and accuracy

		Precision and accuracy measured in between series (n=8)		
Analyte	**Concentration (µM)**	**Mean value (µM)**	**Accuracy (%)**	**RSD (%)**
Amphetamine	0.000	0.0037	-0.010	64
	0.005	0.0070	41	46
	0.010	0.011	8.4	19
	0.100	0.10	2.3	5.4
	1.004	1.0	-0.76	9.2
	5.022	5.0	-0.76	5.6
	10.04	10	0.69	3.7
Methamphetamine	0.000	0.0014	-0.010	130
	0.005	0.0060	20	34
	0.010	0.010	-2.1	13
	0.101	0.10	2.2	5.4
	1.005	1.1	9.1	17
	5.027	5.1	2.3	5.1
	10.05	10	3.1	3.5
MDMA	0.000	0.0026	-0.010	110
	0.005	0.0074	45	50
	0.010	0.010	-0.35	24
	0.101	0.10	-0.91	6.5
	1.012	1.0	-0.84	6.2
	5.061	4.9	-2.2	5.6
	10.12	9.9	-2.4	5.9

Table VII c. Intraday precision and accuracy

Intraday precision and accuracy for the 2-, 3-, 4-phenethylamines

2-fluoroamphetamine n=10

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0050	0.0030	0.0034	113	61
0.010	0.0082	0.0019	23	82
0.10	0.11	0.015	14	110
1.0	1.2	0.15	12	120
5.0	6.1	0.51	8.3	121
10	11	0.88	7.6	116

3-fluoroamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0050	0.0045	0.0012	28	90
0.010	0.0091	0.00085	9.4	91
0.10	0.11	0.012	10	115
1.0	1.2	0.073	5.9	123
5.0	6.6	0.39	6	130
10	11	0.63	5.3	118

4-fluoroamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0051	0.0029	0.0010	35	57
0.010	0.0078	0.00089	11	78
0.10	0.12	0.0078	6.6	117
1.0	1.29	0.056	4.3	127
5.0	6.2	0.57	9.3	122
10	11	0.62	5.8	106

2-fluoromethamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0050	0.0072	0.0019	26	140
0.010	0.012	0.0015	12.3	125
0.10	0.13	0.0171	13.2	129
1.0	1.2	0.074	6.1	121
5.0	5.8	0.42	7.2	116
10	11	0.57	5.1	111

3-fluoromethamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0050	0.0057	0.00073	13	112
0.010	0.011	0.0013	12	111
0.10	0.13	0.0034	2.6	131
1.0	1.2	0.065	5.5	117
5.0	5.9	0.34	5.7	117
10	11	0.73	6.6	110

4-fluoromethamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0050	0.0225	0.0010	4.4	450
0.010	0.029	0.0011	3.8	287
0.10	0.13	0.0072	5.5	130
1.0	1.2	0.06	5.2	116
5.0	5.9	0.29	5.0	116
10	11	0.36	3.2	111

Table VII d. Intraday precision and accuracy for the classical phenethylamines

Intraday precision and accuracy for the classical phenethylamines

n=10

Amphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0050	0.016	0.0057	35	330
0.010	0.031	0.0085	28	308
0.10	0.11	0.0063	5.8	106
1.0	1	0.05	5	100
5.0	4.9	0.18	3.7	98
10	10	0.37	3.6	101

Methamphetamine

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0050	0.0060	0.0021	34	120
0.010	0.0092	0.0013	14	92
0.10	0.095	0.0055	5.8	95
1.0	1.0	0.037	3.6	103
5.0	5.1	0.14	2.7	101
10	10	0.18	1.8	103

MDMA

Concentration	Calculated mean	SD	Precision (RSD)	Trueness
µM	µM	µM	%	%
0.0050	0.0071	0.0028	39	142
0.010	0.0094	0.00053	5.7	94
0.10	0.099	0.0024	2.4	99
1.0	1.0	0.030	2.9	102
5.0	5.0	0.099	1.5	100
10	10	0.18	1.8	102

Linearity graphs of the 2-, 3-, 4-isomers:

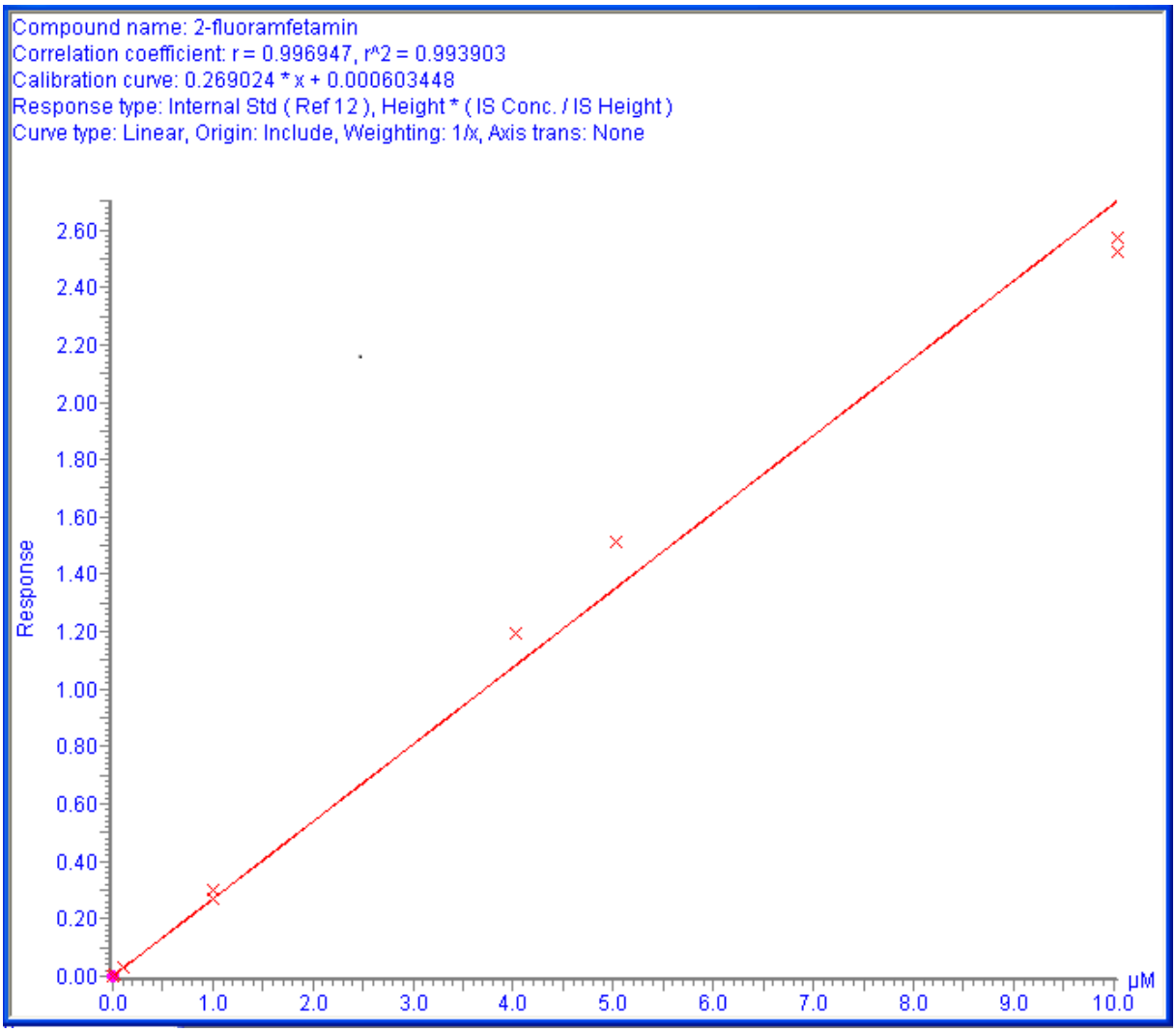


Figure VII a. Linearity graph for 2-fluoroamphetamine.

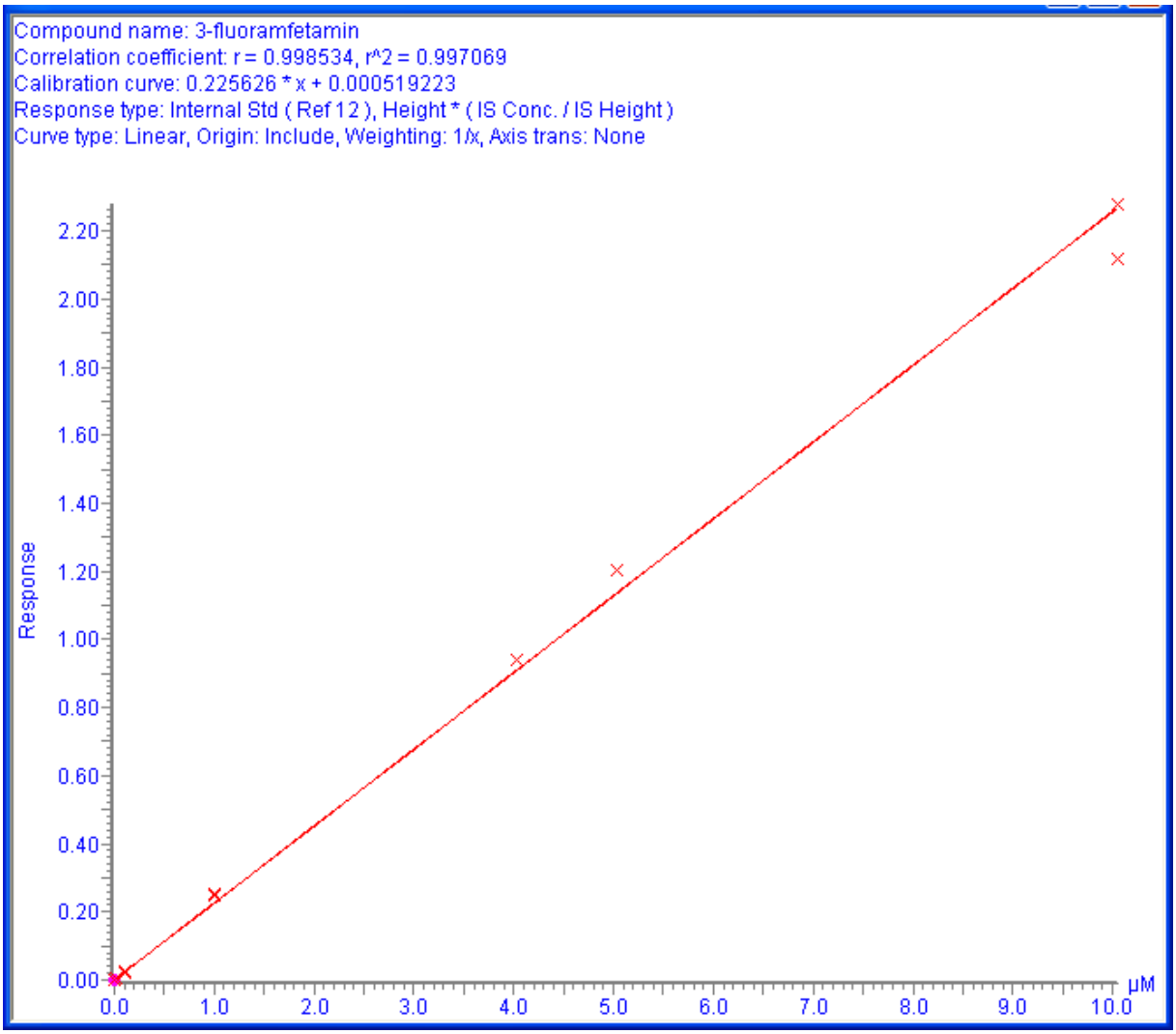


Figure VII b. Linearity graph for 3-fluoroamphetamine.

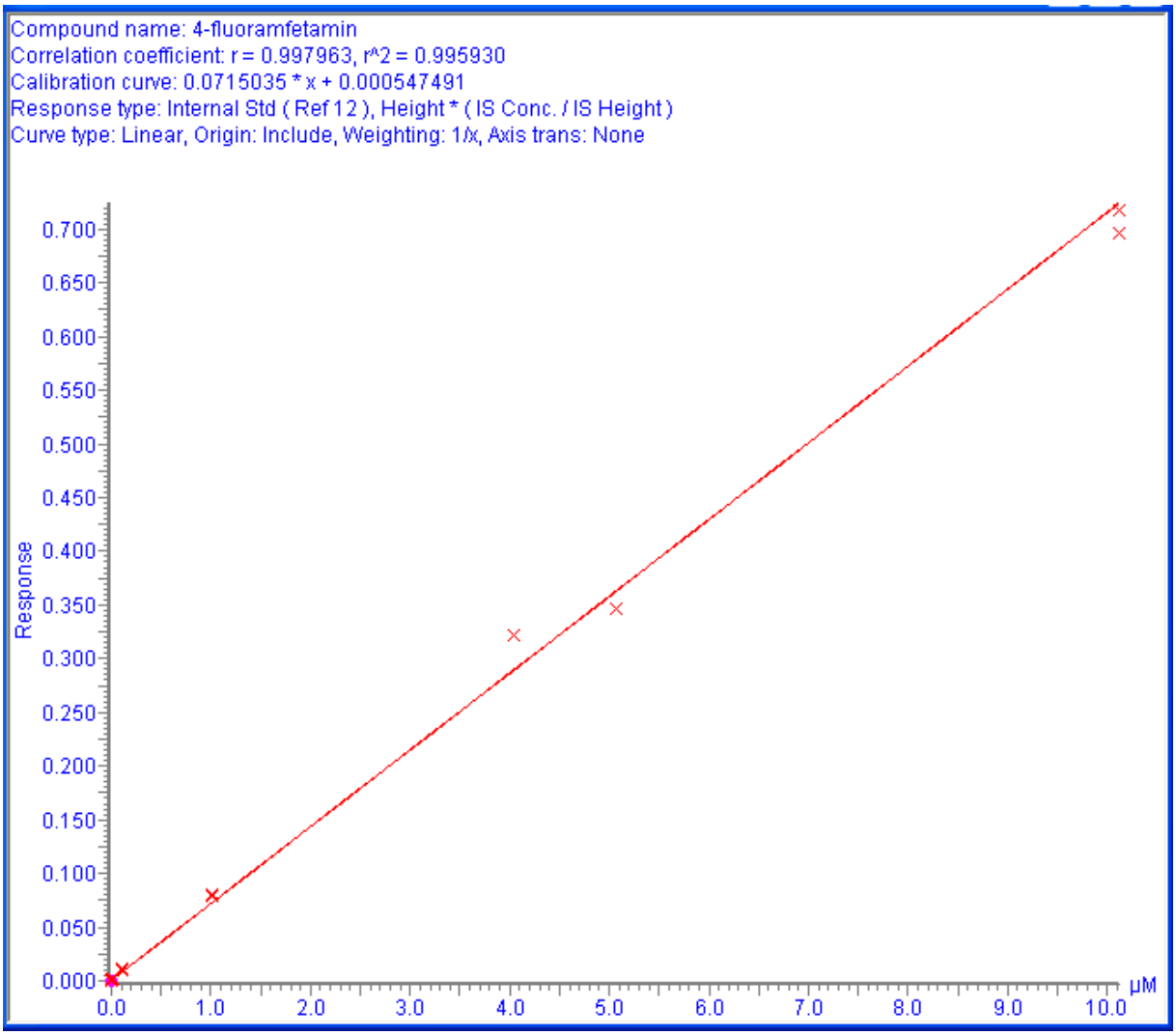


Figure VII c. Linearity graph for 4-fluoroamphetamine.

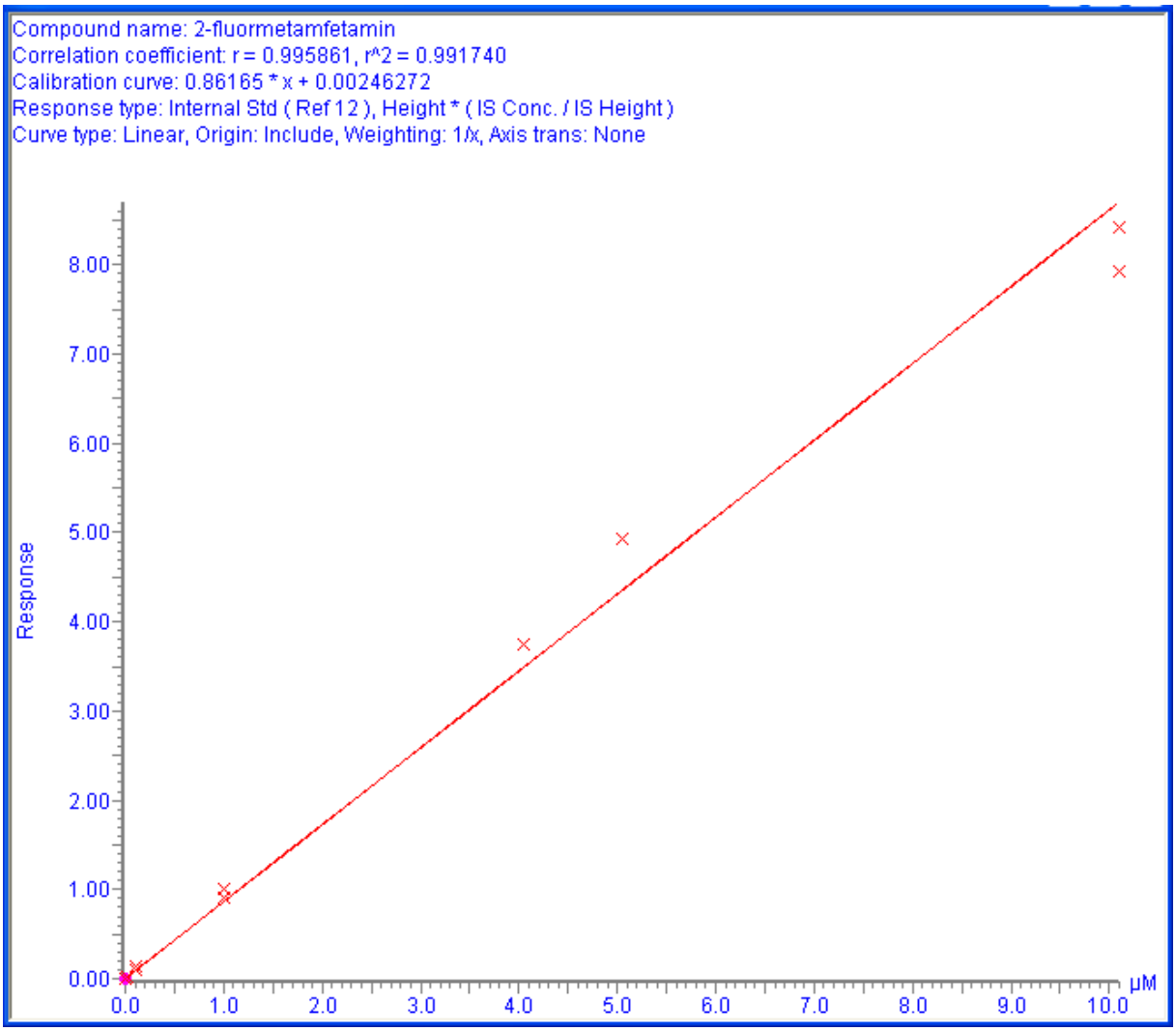


Figure VII d. Linearity graph for 2-fluoromethamphetamine.

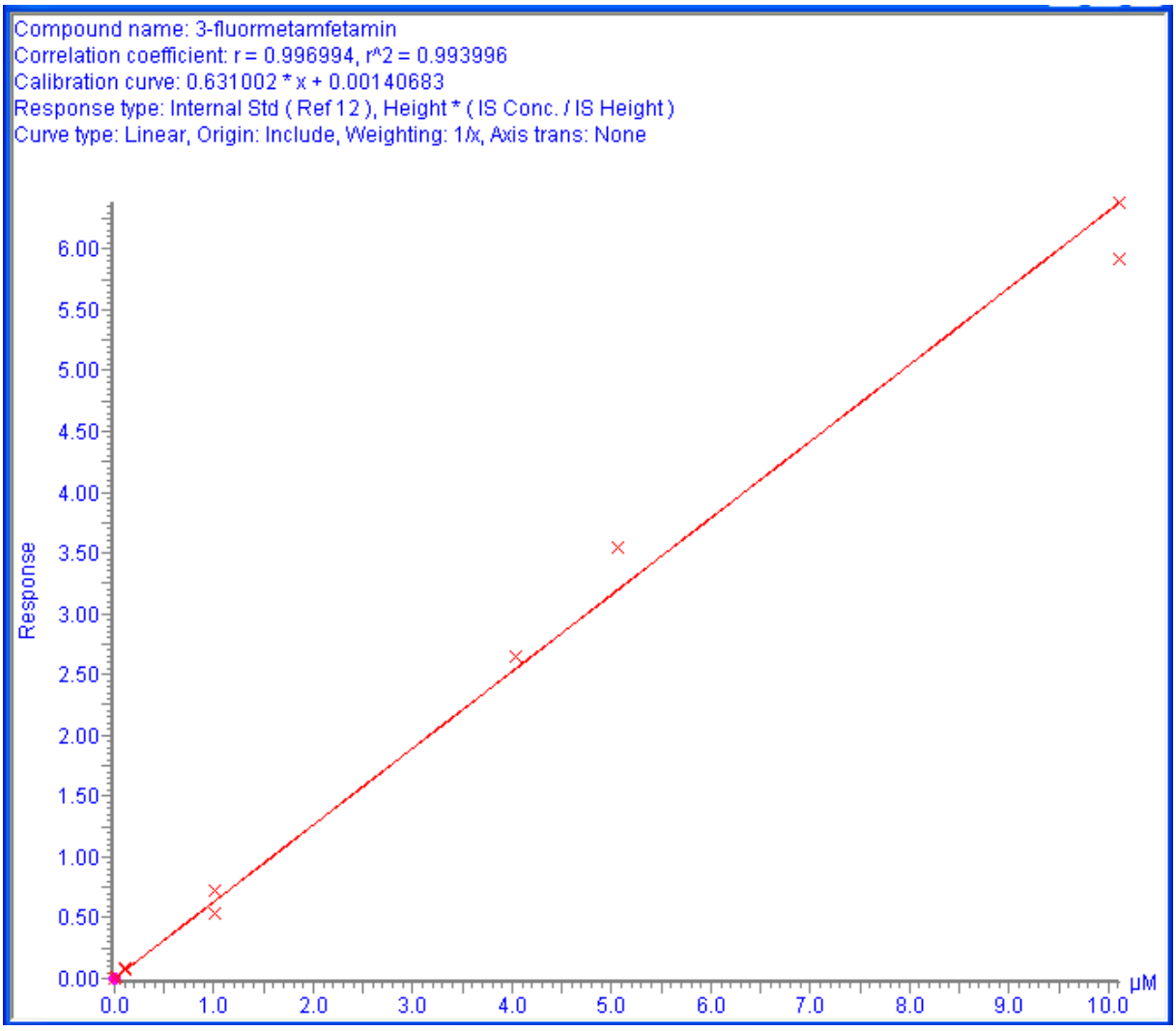
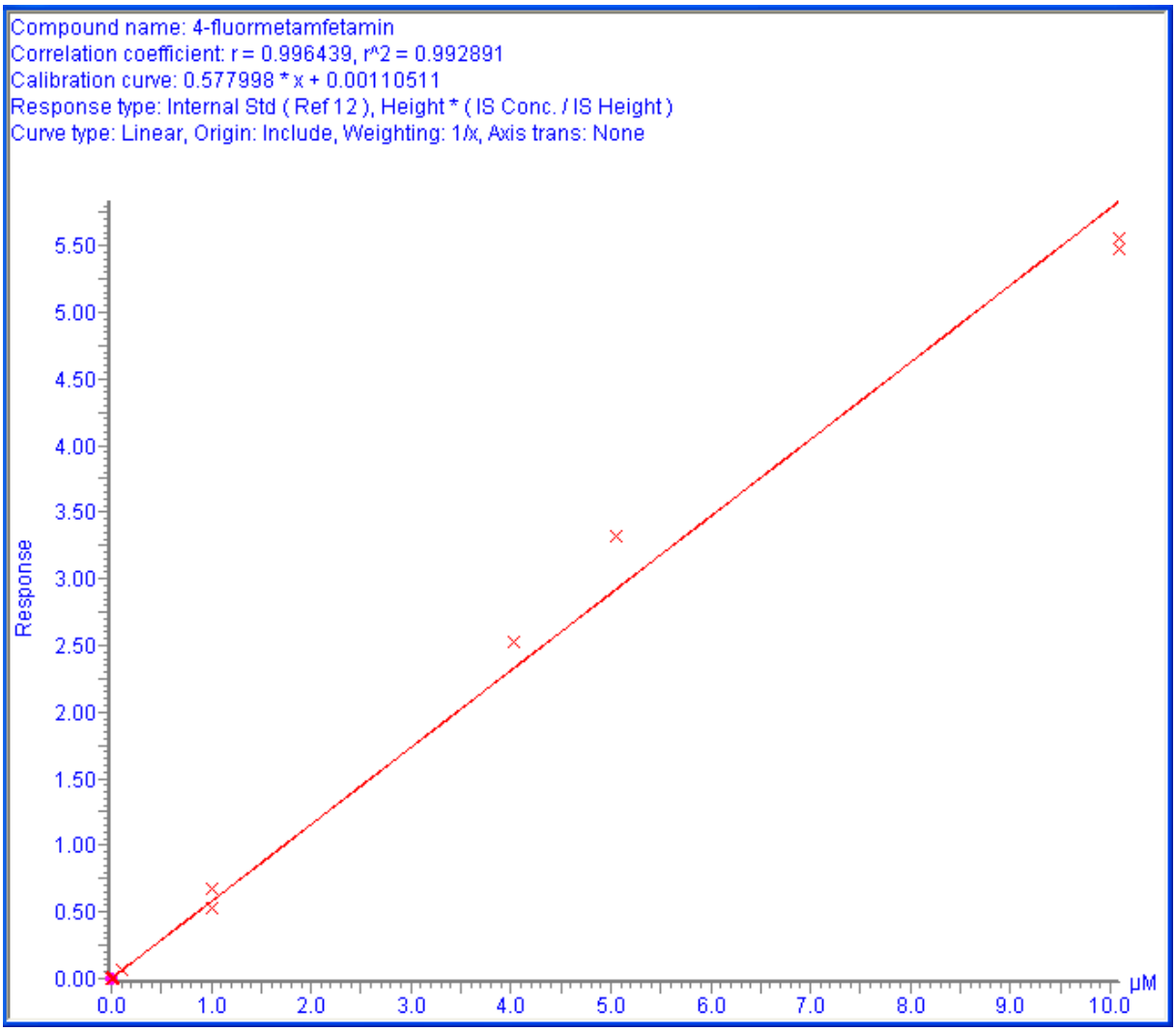


Figure VII e. Linearity graph for 3-fluoromethamphetamine.



FigurVII f. Linearity graph for 4-fluoromethamphetamine.

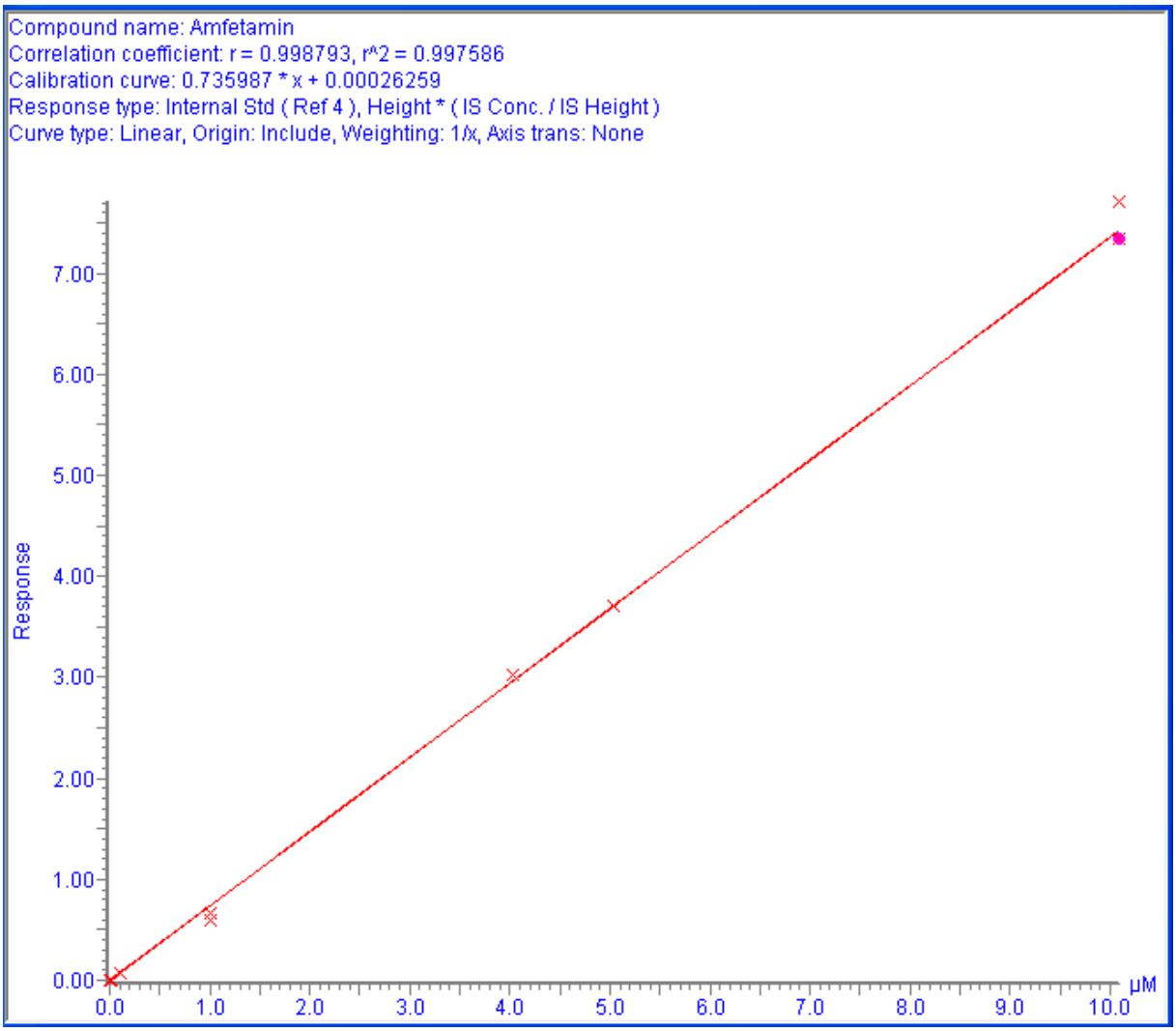


Figure VII g. Linearity graph for amphetamine.

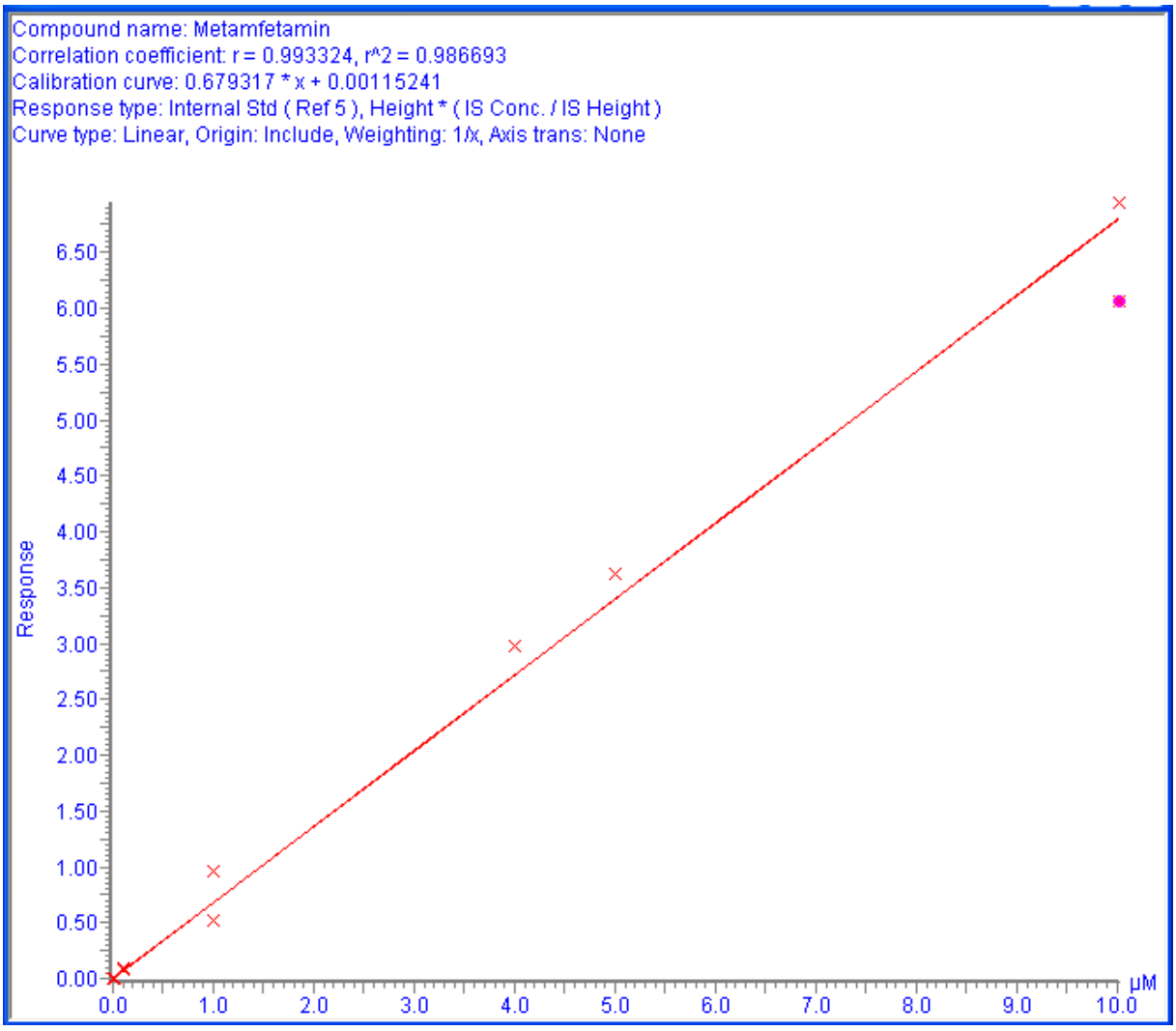


Figure VII h. Linearity graph for methamphetamine

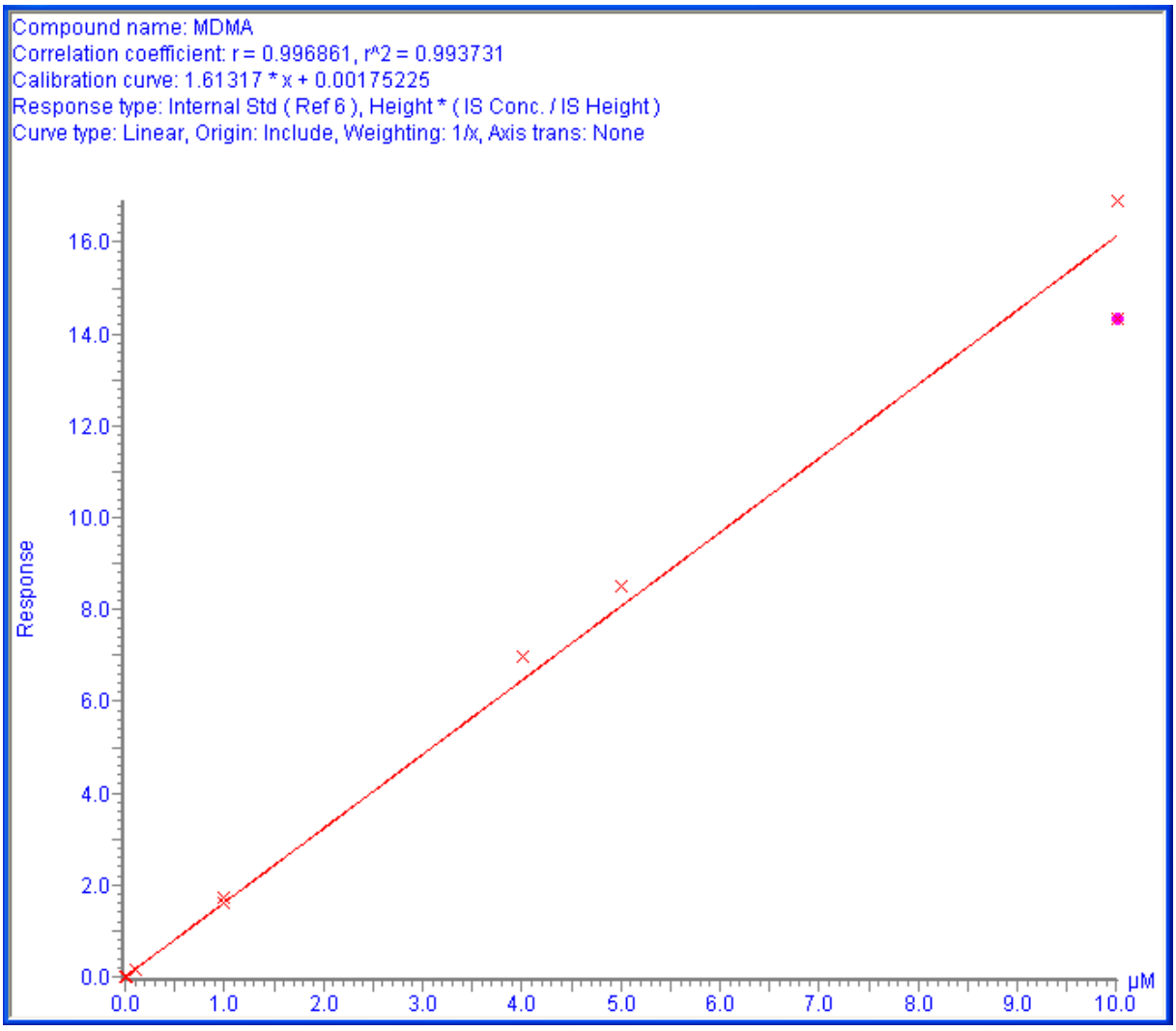


Figure VII i. Linearity graph for MDMA

Table VII e. Table showing the drugs and their respective concentrations used in measuring specificity for the method.

Substance	Concentration (µM)
Morphine	0.005625
Codein	0.005625
Ethylmorphine	0.0375
Oxycodone	0.009375
Fentanyl	0.0005625
Amphetamine	0.028125
Metamphetamine	0.028125
MDMA	0.028125
Tramadol	0.046875
Zolpidem	0.009375
Alprazolam	0.00425
Flunitrazepam	0.0009375
Klonazepam	0.00225
Nitrazepam	0.009375
Fenazepam	0.001875
Bromazepam	0.009375
Lorazepam	0.005625
Buprenorfine	0.000475
Diazepam	0.03125
N-desmethyldiazepam	0.03125
Midazolam	0.01625
Oxazepam	0.15625
Methadone	0.0225
Meprobamate	0.625
Carisoprodol	0.625

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