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Development of a Liquid Chromatography Mass Spectrometry Method for the Determination of Tryptamines in Whole Blood

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Chemistry

Abstract

The recent development and distribution of new psychoactive tryptamines has caused increased use in Norway. This has exposed a need to develop sensitive and robust determination methods for forensic cases involving tryptamines. Therefore, the aim was to develop a sensitive determination method for 21 tryptamines in whole blood. Three different separation columns were compared with different chromatographic parameters. Five different sample preparation methods were tested, Protein precipitation with Captiva ND filter plates and Captiva EMR filter plates, liquid-liquid extraction, solid phase extraction and electromembrane extraction. Protein precipitation with Captiva EMR lipid filter plates gave the best recoveries and precision for all the tryptamines except those with a hydroxy substituent, these could only be extracted after addition of ascorbic acid prior to protein precipitation, lipid plate filtration, and analysis by ultra-high-performance liquid chromatography and tandem mass spectrometry. The final method was evaluated using three analysis series and could determine 19 tryptamines with LODs between 0.14 and 0.6 ng/mL and linear ranges between 0.4 and 1512 ng/mL with R^2 values above 0.99. The results satisfy international guidelines and are promising with respect to a full validation.

Preface

The work and writing of this master`s thesis was carried out at the Section of Drug Abuse Research at Oslo University Hospital between September 2020 and august 2021. The project was supervised by Professor Dag Ekeberg at NMBU, senior researcher Åse Marit Leere Øiestad, senior researcher Elisabeth Leere Øiestad and researcher Thomas Berg. You have all been extremely supportive and generous with your time and knowledge. Professor Dag Ekeberg provided a much needed helping hand in the beginning of this project, for which I am truly grateful. Senior researcher Åse Marit Leere Øiestad was instrumental in helping me getting over the first hurdles of this project. Senior researcher Elisabeth Leere Øiestad has provided invaluable help, support, and guidance far beyond what can be expected. Researcher Thomas Berg has been a great support during the whole project with small and big talk.

During the project I also had the opportunity to learn about cutting edge sample preparation from Professor Stig Pedersen-Bjerregaard and PhD-student Fredrik André Hansen at the Faculty of Pharmaceutical Chemistry at the University of Oslo. They provided generous help with both theoretical and practical understanding of novel extraction techniques and the scientific process.

All the people at Section of Drug Abuse Research have made my stay truly enjoyable, Vigdis for responding so positively to my request to do a master`s thesis. Elisabeth, Håvard, Benedicte, Marissa, Marianne, Galina, Synne, Hilde, Stig Tore, Ragnhild you are all very cool.

And last but not least, my family deserves a big, big thank you! I would not have been able to finish without your generous help. A big thanks to my kids Einar and Per for reality checks and sweet times. And lastly, the love of my life, thank you Guro for holding on!

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Tao Angell-Petersen McQuade

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Abbrevations

4-ACO-DET	4-acetoxy-diethyltryptamine
4-ACO-DIPT	4-acetoxy-diisopropyltryptamine
4-ACO-DMT	4-acetoxy-dimethyltryptamine
4-ACO-MET	4-acetoxy-methylethyltryptamine
4-MEO-DMT	4-methoxy-dimethyltryptamine
4-OH-DET	4-hydroxy-diethyltryptamine
4-OH-EPT	4-hydroxy-ethylpropyltryptamine
4-OH-MET	4-hydroxy-methylethyltryptamine
4-OH-MIPT	4-hydroxy-methylisopropyltryptamine
5-HT_{2A}	Serotonine, 5-hydroxytryptamine receptor 2A
5-MEO-DIPT	5-methoxy-diisopropyltryptamine
5-MEO-DMT	5-methoxy-dimethyltryptamine
5-MEO-DPT	5-methoxy-dipropyltryptamine
5-MEO-EPT	5-methoxy-ethylpropyltryptamine
5-OH-DMT	5-hydroxy-dimethyltryptamine
ACN	Acetonitrile
DEHPi	bis(2-ethylhexyl) phosphite
DET	Diethyltryptamine

DMT	Dimethyltryptamine
DMT-N-OXIDE	Dimethyl-N-Oxide
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EPT	Ethylpropyltryptamine
EME	Electromembrane extraction
ESI	Electrospray Ionisation
H	Plate Height
HILIC	Hydrophilic Interaction Liquid Chromatography
LC	Liquid Chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LSD	Lysergic Acid diethylamide
MAO	Monoamine oxidase
MDMA	Methylenedioxymethamphetamine
MeOH	Methanol
MET	Methyl-Ethyltryptamine
MIPT	Methyl-Isopropyltryptamine
MS	Mass Spectrometry
MS/MS	Tanden Mass Spectrometry
NMBU	Norwegian University of Life Sciences

NPS	New Psychoactive Substances
OUH	Oslo University Hospital
PALME	Parallel Artificial Liquid Membrane Extraction
PL	Phospholipids
PPT	Protein Precipitation
REAN	Section of Forensic Toxicological Analytics
REFU	Section of Drug Abuse Research
RSD	Relative standard deviation
SPE	Solid Phase Extraction
SRM	Single reaction monitoring
UiO	University of Oslo

1 Introduction

1.1 Forensic toxicology

In forensic toxicology the aim is to deliver precise and accurate determinations of toxic or psychoactive substances in biological samples such as urine, blood, saliva, post-mortem blood, hair, or tissue samples [1-3]. The analytes are medicinal and recreational drugs that might lead to intoxication or death, and many are restricted or prohibited by law. Samples are taken by policing authorities or medical examiners upon suspicion of use. They are then analysed by different screening methods depending on matrix and/or suspected drug to be found. If a positive result is found for a particular substance, the sample can be analysed by a confirmation method, which preferably should be different from the screening method. Using different analytical methods for screening and confirmation is an important measure taken to minimize the chances of false results [4]. As the samples in many cases are considered evidence in criminal investigations, analytical precision and accuracy is of utmost importance, potentially acquitting or convicting a defendant [5].

The department of forensic toxicology at the Oslo University Hospital (OUH) analyses approximately 35 000 cases a year. Cases come from different government agencies. The Norwegian Correctional Service and the Traffic Police account for approximately two thirds of the cases. This high sample-throughput yields high demands on the analysis time, specificity, and the robustness of the method in question. Especially analysis time is an important factor because each case can generate many injections. Throughout method development choices between different techniques and methods are made with these factors in mind [6].

1.1.1 Background

In the mid-eighties and nineties many new psychoactive substances (NPSs) started to appear on the illegal drug market. These were fentanyl derivatives and amphetamine derivatives in the beginning, and later piperazines, cathinones and cannabinoids followed [7]. NPSs are made synthetically by clandestine laboratories and are sold in different forms such as herbal mixtures, incense, bath soaps, party pills etc [8], and they can easily be obtained via the internet. To avoid legal ramifications, the compounds are chemically modified, which gives a similar and/or stronger pharmacological effect compared to its molecular ancestor [9, 10]. Because of their often undefined legal status they can be sold with less risk, higher purity, and lower cost [11].

New, unregistered NPSs have been reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) at a rate of approximately 50 new NPSs per year between 2008 and 2014 [12], as Figure 1.1 shows. This signifies a great interest in these compounds, and explains the increase in attention NPSs has had from researchers and other drug affiliated authorities [13]. After 2014, reports of new NPSs started to decrease, probably because of the drugs being scheduled by national authorities, which often leads to a decrease in interest [14]. However, the number of reported NPSs, new or old, has increased from 2008 to 2017 (2018 saw a small reduction of reported cases), possibly indicating a stable demand [12].

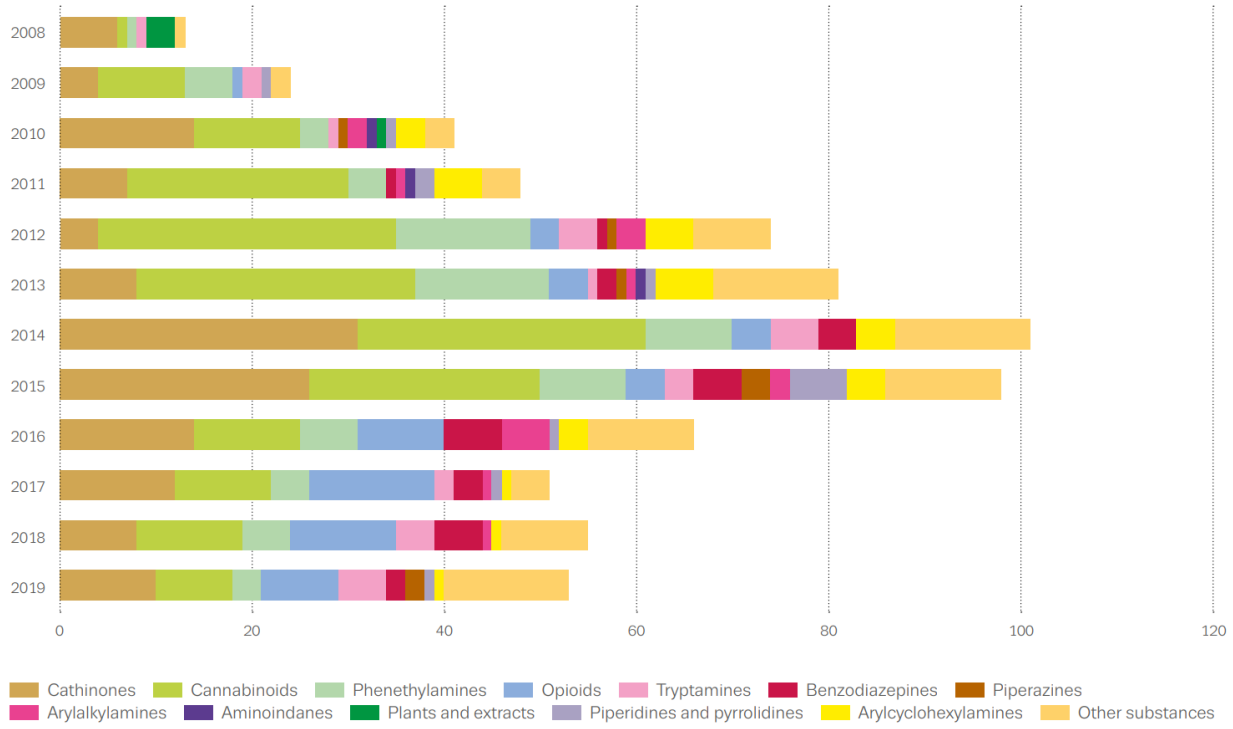


Figure 1.1. Numbers and categories for different categories of New Psychoactive substances reported to the EU early warning system between 2008 and 2019 [12].

In Norway the number of times NPSs have been found on people by the police peaked at 900 confiscations in 2015 and then decreased again similar to the rest the EU. On average NPSs has stood for one percent of total police confiscations the last ten years [15]. However, NPSs were discovered in eight percent of cases in a study from 2017, involving patients from an emergency clinic and from Oslo University Hospital (OUS) [16]. This indicates that NPS use was quite low. Nonetheless, these are potent drugs with low toxic concentrations, and they have frequently caused death and intoxications [17].

1.2 Tryptamines

Tryptamines are a class of drugs under the NPS umbrella. Reports of tryptamines to EMCDDA has followed the same pattern as NPSs, with a rapid increase in the emergence

of new compounds from 2008 to 2012, and a decrease from 2013 until now. However, reports of tryptamines in the EU, new and old, have been relatively stable between 2013 and 2018 [18]. Table 1.1 shows the tryptamines that have been confiscated by the Norwegian customs in 2018, 2019, and 2020 including October 1 [19]. These numbers display the importance of having analytical methods for tryptamines, especially since customs only confiscate a small portion of the amounts that are illegally brought into the country.

Table 1.1. Tryptamine confiscations made by the Norwegian customs between 2018 and October 2020

Compound	**Confiscations**	**Grams of powder**	**User dose** (mg)	**User doses** (estimate)
4-OH-MET	5	1.5	15 - 25	82
4-OH-MIPT	2	0	15 - 25	6 tablets
5-OH-DMT	1	253.7	20 - 40	8457
5-MeO-DIPT	1	1.5	10 - 15	120
5-MeO-DMT	8	20	7 - 10	2351
4-AcO-DMT	3	5	10 - 30	252
AMT	10	6.8	20 - 40	206
DIPT	5	8.4	30 - 75	157
DMT	18	54.4	20 - 40	1974
NMT	2	20.2	50 - 100	270
DPT	2	1	150 - 250	12 (7 tablets)

Although tryptamines are categorized as NPSs, the most famous ones have been used for centuries. Throughout Central- and South America psychotropic mushrooms have been used in shamanistic rituals [20]. In South America indigenous tribes have used the drink Ayahuasca to induce spiritual experiences since ancient times [21]. The active ingredients in both the psychotropic mushrooms and Ayahuasca, are tryptamines.

Ayahuasca is a world-famous psychotropic beverage, and its hallucinogenic effect comes from dimethyltryptamine (DMT) [6]. DMT is extracted from the leaves of the shrub *psychotria viridis*. For the drink to have an effect, *P. viridis* must be accompanied by a plant containing monoamineoxidase (MAO) inhibitors, otherwise MAO will cause DMT degradation after entering the body [22]. This is accomplished by adding the plant

Banisteriopsis caapi, which contains MAO inhibitors such as harmine and harmaline [23]. Ayahuasca has been used by indigenous tribes of the Amazonian basin for centuries for various spiritual and social ceremonies, this has caused certain neo-religious groups to claim that Ayahuasca should be legally permitted for them to use in religious practices in United States and elsewhere, advertising with legal highs and claiming they are protected by religious freedom laws [24, 25].

The hallucinogenic effect of psychotropic mushrooms comes from psilocybin and psilocin. Once psilocybin enters the gut and liver, it is turned into psilocin, which then enters the central nervous system and induces its hallucinogenic effect [26]. The mushrooms which contain these compounds belong to the genus of *psilocybe,* and can be found all over the world, where its psychotropic effects have been utilized in most cultures where it has been found [27].

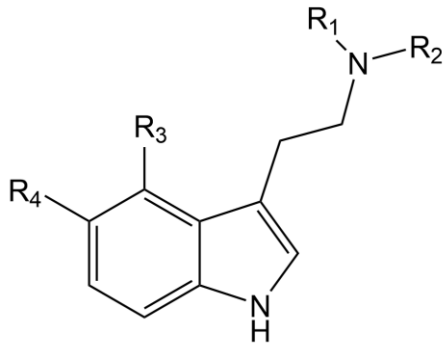
Long term usage of tryptamines has not been reported to cause addiction or any serious, long lasting effects [15]. However, an overdose, if not fatal, can cause serious conditions such as hyperthermia, tachycardia, tremor, extreme sweating, visual hallucinations, and other symptoms [28]. On the other hand, users have also reported that using tryptamines can lead to states such as euphoria, laughter, visual and audial hallucinations, increased tactile awareness, increased libido, ease of concentration, warmth, and inner peace [29]. These positive states and the potential lack of addictive properties connected to tryptamine use are the reasons that some tryptamines are and have been candidates for treatment of depression and anxiety [30]

1.2.1 Tryptamine chemistry

The basic structure of tryptamines is the indole ring, which consist of a combined benzene and pyrrole ring. In the body the most important tryptamine is the neurotransmitter serotonin which is synthesized from the amino acid tryptophan [6]. Tryptamine itself also exists in the body, in small amounts, and has no psychoactive effects [31, 32]. The effect comes only when different substituents are added to the tryptamine structure [33]. As Table 1.2 indicates there are four different positions where adding a functional group generates psychoactive effects, position four and five on the indole ring, the alpha position

of the ethylamine group, and on the nitrogen atom of the ethylamine group [33]. Although tryptamines mainly get their hallucinogenic properties from the indole ring, the structural modifications give different chemical properties and consequently induce different hallucinogenic states [34]. When either the four or five positions of the indole nucleus are given a hydroxy, methoxy or acetoxy group, the hallucinogenic effect is said to increase, adding alkyl groups to the nitrogen atom of the ethyl amin group also increases psychoactive intensity. The maximum effect is achieved when position four or five of the indole structure, and the ethylamine group, both are substituted [33]. Adding substituents at the six or seven position of the indole structure, or alkyl groups longer than a propyl group, yields little or no psychoactive effect [33].

Table 1.2. Molecular structures of the tryptamines studied, in order of increasing mass.



Analyte	**R_1**	**R_2**	**R_3**	**R_4**
Tryptamine	H	H	H	H
DMT	CH_3	CH_3	H	H
MET	CH_3	CH_2CH_3	H	H
5-OH-DMT	CH_3	CH_3	H	OH
4-OH-DMT (psilocin)	CH_3	CH_3	OH	H
DMT-N-Oxide*	CH_3	CH_3	H	H
MIPT	CH_3	$CHCH_3CH_3$	H	H
5-MeO-DMT	CH_3	CH_3	H	OCH_3

Analyte	R_1	R_2	R_3	R_4
4-OH-MET	CH_3	CH_2CH_3	OH	H
4-MeO-DMT	CH_3	CH_3	OCH_3	H
EPT	CH_2CH_3	$CH_2CH_2CH_3$	H	H
4-OH-MIPT	CH_3	$CHCH_3CH_3$	OH	H
4-OH-DET	CH_2CH_3	CH_2CH_3	OH	H
4-AcO-DMT	CH_3	CH_3	$OCOCH_3$	H
4-OH-EPT	CH_2CH_3	$CH_2CH_2CH_3$	OH	H
5-MeO-EPT	CH_2CH_3	$CH_2CH_2CH_3$	H	OCH_3
4-AcO-MET	CH_3	CH_2CH_3	$OCOCH_3$	H
4-AcO-DET	CH_2CH_3	CH_2CH_3	$OCOCH_3$	H
5-MeO-DPT	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	H	OCH_3
5-MeO-DIPT	$CHCH_3CH_3$	$CHCH_3CH_3$	H	OCH_3
4-AcO-DIPT	$CHCH_3CH_3$	$CHCH_3CH_3$	$OCOCH_3$	H

Depending on administration route, the amounts required to attain psychotropic effects are usually from 10 to 200 mg, or below 10 mg if administered intravenously [6]. This means that the potential for overdose is high if the user is unaware of the concentrations he or she is dealing with. For instance, if DMT is consumed through drinking Ayahuasca, the effect comes after approximately one hour, and lasts for about 4 hours [17]. However, if DMT is smoked the effect comes almost instantly, escaping degradation by MAO in the gut and liver, but this way the effect lasts less than 30 minutes [17]. Other N-alkyl substituted tryptamines such as methyl-ethyltryptamine (MET), di-ethyltryptamine (DET), methyl-isopropyltryptamine (MIPT), or ethyl-propyltryptamine (EPT) are not consumed by MAO, making oral administration viable [35]. Ignorance concerning administration route, amount, time of uptake in the body and which type of tryptamine one is dealing with, can be critical. The toxic doses are quite low and delayed onset of effects can occur. For this reason users might repeat the intake, which can lead to a lethal overdose [35].

Tryptamines act on many of the same receptors as serotonin, and it is believed that the hallucinogenic properties which tryptamines induce mainly comes from their action on the

5-HT_{2A} receptor [36]. The many 5-HT_{2A} receptors in the visual cortex explains this feature [37]. Phenalkylamines such as mescaline, or ergolines such as LSD, act on the same receptors as tryptamines and produces cross-tolerance [38]. The hallucinogenic properties distinguishes them from other drug classes like cannabinoids and amphetamines [33].

1.2.2 Aqueous and biphasic behaviour of tryptamines

Most of the tryptamines included in this study are typical drugs in the sense that they are basic [39], and that the log P values are between one and four, which is comparable to other drugs of abuse such as amphetamines, cocaine, and MDMA. As pH goes down, their hydrophilic affinity goes up, as can be seen from the log D values in Table 1.3. The data in table 1.3 is important in that it can be used to predict the state which the tryptamines will be in when they are in the blood stream or in different solvents, and how pH, organic solvents, or stationary sorbents can be used to extract them from their matrix.

Table 1.3. Chemical properties of the 21 tryptamines studied. pKa and log D values were acquired from [40], and log P values from [41].

Analyte	**pKa 1** $_{amino}$	**pKa 2** $_{phenol}$	**Log P**	**Log D** $_{pH\ 10}$	**Log D** $_{pH\ 6}$	**Log D** $_{pH\ 2}$
Tryptamine	9,73	-	1.6	1,29	-1,47	-1,55
DMT	9,55	-	2.5	2,17	-0,92	-1,19
MET	9,81	-	2.9	2,44	-0,67	-0,84
5-OH-DMT	9,91	9,23	1.2	1,29	-1,23	-1,50
PSILOCIN	9,78	8,97	2.1	1,04	-1,20	-1,50
DMT-N-OXIDE	-	4,82*	2.0	1,77	1,17	1,09
MIPT	10,09	-	3.3	2,73	-0,33	-0,43
5-MEO-DMT	9,58	-	1.5	2,00	-1,10	-1,40
4-OH-MET	9,99	9,03	2.4	1,34	-0,40	-1,15
4-MEO-DMT	9,54	-	2.3	2,01	-1,08	-1,40
EPT	10,32	-	3.8	3,05	0,10	0,04
4-OH-MIPT	10,23	9,07	2.8	1,67	-0,60	-0,73
4-OH-DET	10,22	9,01	2.7	1,61	-0,65	-0,79

Analyte	pKa 1 $_{amino}$	pKa 2 $_{phenol}$	Log P	Log D $_{pH\ 10}$	Log D $_{pH\ 6}$	Log D $_{pH\ 2}$
4-ACO-DMT	9,54	-	2.1	1,78	-1,30	-1,59
4-OH-EPT	10,43	9,08	3.3	2,06	-0,17	-0,27
5-MEO-EPT	10,33	-	2.8	2,90	-0,06	-0,12
4-ACO-MET	9,54	-	2.5	2,06	-1,05	-1,23
4-ACO-DET	9,52	-	2.8	2,29	-0,77	-0,88
5-MEO-DPT	10,56	-	3.3	1,99	-0,58	-0,60
5-MEO-DIPT	10,64	-	3.1	1,92	-0,68	-0,71
4-ACO-DIPT	10,62	-	3.7	2,74	-0,01	-0,04

Those tryptamines with an amine functional group only can be made neutral through pH adjustment, which can be utilized in different sample preparation techniques such as liquid-liquid extraction (LLE), solid phase extraction (SPE), or parallel artificial liquid membrane extraction (PALME). However, the tryptamines with a phenol group and an amine group will mostly be charged throughout the pH range, as Figure 1.2 shows. Between pH eight and 12, four different species of the hydroxy tryptamines are present, which can make them difficult to extract using partition-based sample preparation procedures such as LLE or PALME. There are other sample preparation techniques that can extract charged analytes such as SPE or electromembrane extraction (EME). These are discussed in later chapters.

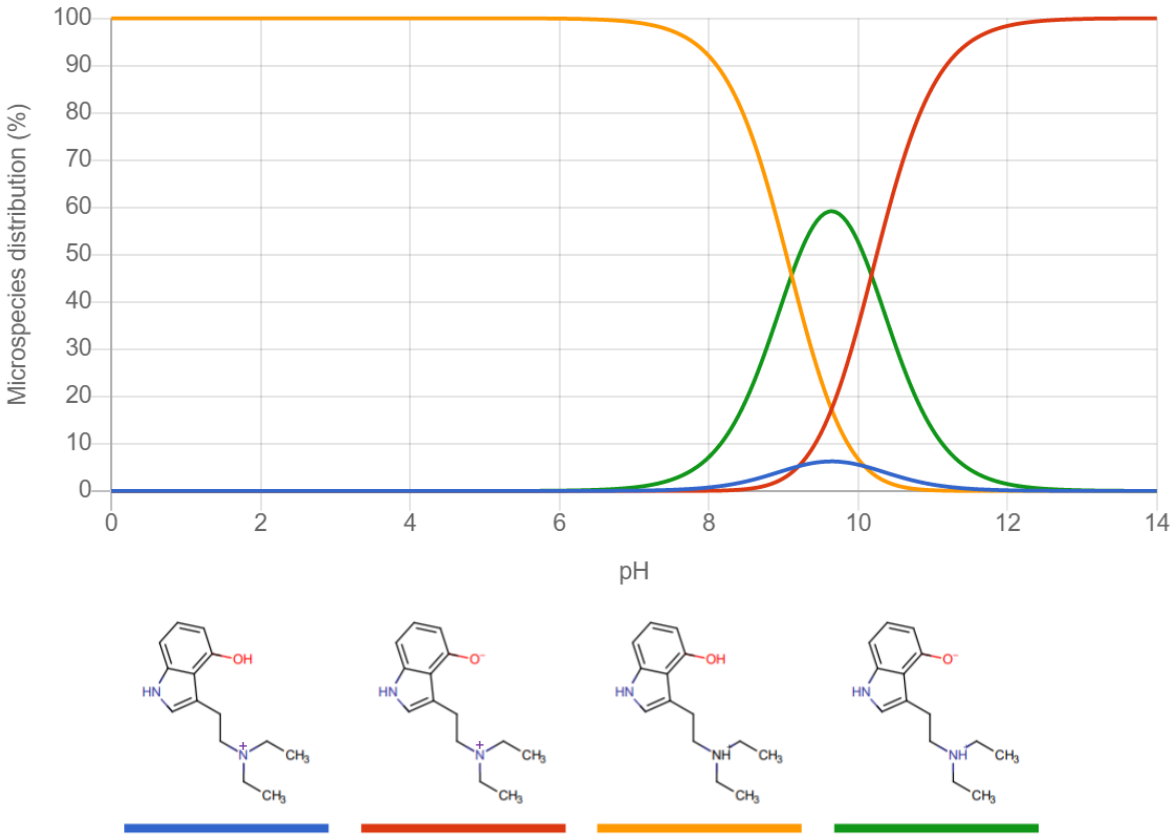


Figure 1.2. Bjerrum plot of the distribution of different species of 4-OH-DET at pH 0-14. Figure adapted from [40].

1.3 Liquid Chromatography

Liquid chromatography (LC) is a technique that is used to separate and determine different chemical compounds in a mixture. The basic principle is that a mobile phase is pumped through a column which contains an immobilized stationary phase. Compounds in the sample will be retained and separated based on their degree of affinity to the stationary and mobile phases. The varying affinities towards the stationary phase is based on differences in physical and/or chemical properties such as size, shape, charge, acidity, basicity, polarity, etc [42, 43]. After separation in the column, the compounds reach a detector which produces a signal in the form of a peak, which again is plotted against time to make a chromatogram.

To separate the different compounds, the separation columns are filled with low µm-size particles to which the stationary phase is covalently bonded. If the analytes are polar to medium polar (log P <1), a polar stationary phase is suitable e.g. hydrophilic interaction liquid chromatography (HILIC) or normal phase chromatography [44]. If they are non-polar, a reversed phase column can be used. Although there are many different liquid chromatography principles to choose from, reversed phase chromatography is usually chosen because of better applicability and flexibility than others [45]. Additionally, reversed phase columns can usually separate most biologically relevant compounds in an acceptable way.

How efficiently a column can retain a given analyte is dependent on three main variables. Firstly, eddy diffusion, which is a result of the multiple paths the analytes can take through a particle-packed separation column [46]. The second is longitudinal diffusion, which is dependent on how easily the analyte spreads in the mobile phase, this term is inversely proportional to the mobile phase velocity [46]. The third is how the analyte band broadens as a result of its resistance to transfer between the stationary and the mobile phase [46]. These variables are depicted in the van Deemter equation, which describes the band broadening (H) in a simplified form, to suit this context, Equation 1.

$$H = Cd_p + \frac{c_1 D_m}{u} + \frac{f_1(k) * {d_p}^2 * u}{D_m} + \frac{f_1(k) * {d_p}^2 * u}{D_s} \quad (1)$$

Where C is a constant, d_p is the particle diameter, D_m is the diffusion coefficient in the mobile phase, $f_1(k)$ and $f_1(k)$ are functions of the retention factor, D_s is the diffusion coefficient in the stationary phase and u is the mobile phase flow velocity. The two first terms are usually named A and B while the two last terms are put together as C for simplicity.

As can be seen from Equation 1, an important variable is the particle diameter. Plate height (*H*) is decreased by a large factor when decreasing the particle diameter [47]. This has been possible as the equipment around the column such as pumps, nuts and ferrules

have been developed to handle the high backpressures resulting from the use of smaller particles [48]. At the department of forensic toxicology columns with particle sizes down to 1.7 µm in diameter are used, which gives high separation efficiency [49].

Mobile phase velocity, u, is another variable which can be optimized to improve separation efficiency once the column chemistry has been chosen. As can be seen from Figure 1.3, where height equivalent to theoretical plates (HETP), which is *H* from Equation 1, has been plotted against mobile phase velocity, the plate height reaches a minimum at a certain velocity. However, with the 1.7 µm particles the curve is flat at the minimum plate height for a long range of mobile phase velocities, which means that faster analysis can be achieved at lower plate heights [50]. In routine settings this can be of great value as the instruments often run day and night to deliver results to the different customers.

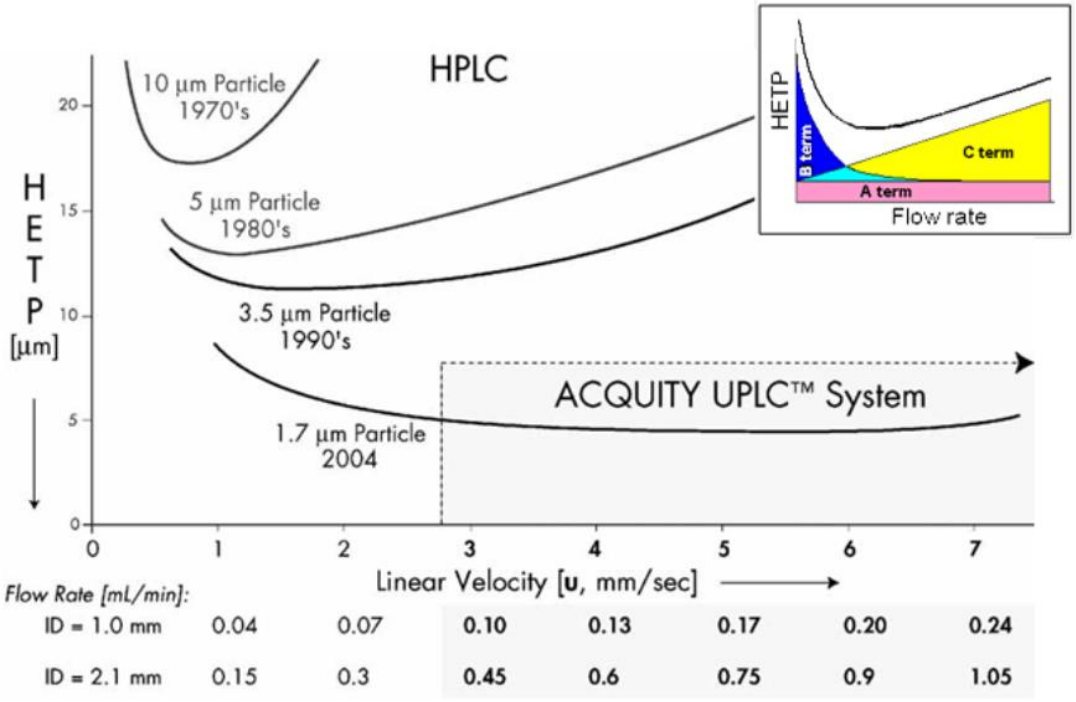


Figure 1.3. van Deemter plots of different particles sizes and their efficiency (HETP). Columns used in this project has internal diameters of 2.1 mm. Figure adapted from Swartz [51]. The small image shows how each term in the van Deemter equation contributes to the resultant curve [39].

As the particles in the separation column has become smaller and smaller throughout the last 60 years, the backpressure has increased with it [51]. Equation 2 shows that as the particles size is halved, the backpressure increases by four.

$$\Delta P = \frac{\theta * \eta * L * u}{{d_p}^2} \qquad (2)$$

Where θ is a flow resistance parameter, η is the mobile phase viscosity, L is column length, u is mobile phase velocity, and d_p represents the particle diameter. This means that by reducing the column length, backpressure will be reduced proportionally. If high resolution is needed, a 10 or 15 cm column can be used, if fewer analytes are to be determined a five cm column will be a good starting point. Because of lower backpressure a higher mobile phase velocity can be used, and analysis time will be shorter compared to a 10 cm column.

Changing mobile phase viscosity η is also possible in order to reduce backpressure. This is usually done by changing the organic phase from, for instance, methanol to acetonitrile. As can be seen from Figure 1.4**,** when the percent methanol is between 30 and 50 %, the viscosity is almost doubled. For acetonitrile, the increase is much smaller. However, acetonitrile and methanol have other differences as mobile phase constituents.

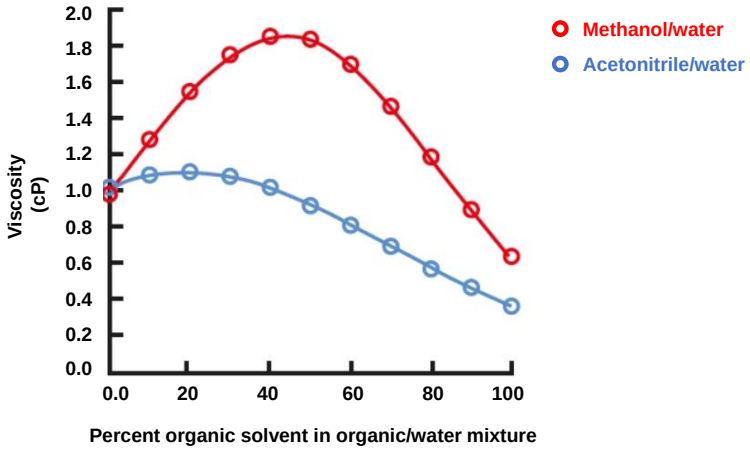


Figure 1.4. Viscosity of methanol and acetonitrile as a function of volume fraction of water. Figure adapted from [52, 53].

Acetonitrile has a stronger elution strength than methanol in reversed phase chromatography, this can cause a different selectivity [54]. Additionally, acetonitrile is more expensive, has a higher potential toxicity, and have had availability issues as a consequence of global economic market fluctuations [55, 56]. Nevertheless, acetonitrile is a much-used solvent in routine analysis.

In the section of drug abuse research (REFU) at OUH, methanol and acetonitrile are both used as organic modifiers, their low boiling points make them evaporate easily in the evaporation zone between the ESI-needle and the entrance cone of the MS. And their polarities make them water miscible. However, their differences can contribute to different ionisation efficiencies. Acetonitrile has a higher boiling point, a higher log P value and a lower viscosity compared to methanol. Higher boiling point suggests a lower ionization efficiency because of the droplet evaporation process [57]. Low viscosity promotes ionization in the ESI, due to faster droplet formation [57]. The differences between acetonitrile and methanol will cause different ionization efficiency for each analyte. In a study performed to evaluate acetonitrile and methanol as organic modifiers in analysing common pharmaceuticals with LC-ESI-MS/MS, methanol gave the best ionization efficiencies [58]. This does not mean that methanol can be used in every assay without considering acetonitrile, it has many advantages over methanol.

In the section of forensic toxicological analytics (REAN) three different types of reversed phase columns are in regular use. These are HSS T3, Biphenyl and BEH C18. HSS T3 has C18 alkyl chains as its stationary phase and is made such that it retains polar compounds more than other separation columns [59]. However, these columns are made with silica particles which contain silanol groups on the surface, this can cause peak tailing [60].

The Kinetex biphenyl column has a solid core and a porous outer layer where the biphenyl groups are attached. This feature provides less band broadening and reduced retention times due to shorter migration paths through the column [60]. Although these properties certainly are wanted, the sample capacity and retention is not as good as with fully porous particles [61] . With biphenyl groups as the stationary phase the affinity for aromatic functional groups can change the retention order compared to alkyl chained stationary phases [62]

Since the BEH C18 column has an ethyl bridging group at 20 mol% of the siloxane bonding sites, it behaves differently compared to columns with bare silica or organic polymers as support for the stationary phase [63]. This almost eliminates peak tailing of basic compounds caused by the residual silanol groups. Additionally, the pH range is widened so it can work under both acidic and basic conditions [63]. The BEH columns also provide faster analysis compared to columns with the same expected performance [64].

Finding the right separation column for a specific method is done through comparing their performance in the different categories that the method demands. These can be for instance selectivity, resolution, peak shape, and/or total run time. The mobile phase constituents and gradient elution can improve a columns performance and compensate for its drawbacks in many of these categories, but testing is still necessary to optimize the methods performance.

Mobile phase composition is a parameter which can affect both sensitivity and selectivity in an analytical assay, it is therefore of great interest to find out which organic phase and which aqueous modifiers to use [58]. A binary pumping system is most common. Normally part A is an aqueous buffer or a suitable acidic or basic solution, and part B is an organic

modifier. There are many options when considering what to use in the different mobile phase reservoirs, but when using electrospray ionization as interface between the separation column and the MS, certain conditions have to be met in order to get a sufficient amount of the analytes into the MS as ions. These are low viscosity, low boiling point and, in the case of basic analytes such as tryptamines, an acid or a low pH buffer for pH control. Using mobile phases that conform to these requirements increases the ionisation efficiency and the sensitivity of the analysis [58]. Toxicity, cost, and eco-friendliness are also important factors when choosing mobile phase, especially in a high throughput lab such as REAN.

Gradient elution, where the mobile phase composition is changed during an analysis, is an alternative to isocratic elution, which employs a constant mobile phase composition throughout the analysis. Although, gradient elution requires less analysis time compared to isocratic elution; it has some disadvantages, and selectivity issues between two or more peaks can arise when transferring a method to other instruments [65]. Two of the important variables causing this are, (i) dwell time, which causes a time delay between the wanted mobile phase composition and the actual mobile phase composition. This is caused by the dwell volume and (ii) how fast/well the mobile phase delivery system mixes the contents of the different mobile phase reservoirs [65]. However, the advantages of shortened analysis time and similar or improved resolution compared to isocratic elution far outweighs the disadvantages.

1.4 Sample preparation

Because biological samples often contain a multitude of different types of structures, from large cells to small molecules, some sort of sample pre-treatment is necessary to be able to separate and measure them. In forensic toxicology the matrices used can be quite diverse, and in addition to blood and urine which are the most commonly used matrices, hair, nails, muscle, saliva and others are used [66].

Drugs have different concentration profiles in different matrices. The detection window for blood is about 2 – 12 hours after administration for most drugs [67]. For the information of what may have caused intoxication at the sampling time, or what may have caused

death minutes or a few hours before, blood is best suited [68]. In urine, the detection window is much longer. Furthermore, in blood the analyte exists more unaltered, as opposed to urine where more water soluble metabolites will usually be the main species [69].

Sample preparation is also done to increase the longevity of the LC-MS/MS instruments. The separation column with its small particles, will easily be clogged by larger particles such as red blood cells, white blood cells or blood platelets. Precipitated proteins will also clog the columns. Precipitation of salts in the tubing can cause problems with back pressure. These are all reasons why what is injected into the LC-MS/MS instruments must be relatively clean.

1.4.1 Sample preparation and matrix effects

Another reason for the importance of sample clean-up is the removal of compounds that cause matrix effects. Matrix effects are caused by compounds that co-elute with the analytes and either cause an increase (ion-enhancement) or a decrease (ion suppression) in the ionization of the analyte [5]. They can originate from a variety of sources, the matrix itself, containers used throughout the assay, different kinds of anti-coagulants added to the blood sample, and more [70]. These effects can be detrimental to a quantitative determination method possibly causing sensitivity issues and false results [71].

Typically, the substances that cause most matrix effects are polar plasma components, which will elute quite early from the separation column if reversed phase chromatography is used [72]. However, the most problematic substances are phospholipids (PL) [73]. These are compounds found cell membranes, tissues and biological fluids which are strongly retained in reversed phase chromatography. PL come as a wide variety of compounds which are present in different ratios in different individuals [74]. As they cause serious ionization decrease or increase for co-eluting analytes they must be minimized, either prior to analysis or by chromatographic means.

Minimizing the effect of co-elution with PL can be solved in many ways, by simple dilution with an organic solvent [75], gradient adjustment [71], column switching [76], adding

isotopically labelled internal standards [77], to name a few. However, *Trufelli et al.* has proposed that the most efficient PL removal is done before analysis, during sample preparation [78].

There are three main sample preparation techniques used prior to LC-MS/MS at REAN, LLE, solid phase extraction (SPE) and protein precipitation (PPT). Used by itself PPT removes proteins to a large extent and not phospholipids. PPT can be used as a first sample preparation step either before filtration by specialized PL removal filtration plates, or prior to SPE. Matrix effects from PL were recorded with PPT only and with PPT-SPE, the results showed a decrease from 34.8 to 5.1 % of PL content [79]. LLE removes PL to a great extent, and is considered a simple technique, but can have problems with extracting compounds that are amphoteric or very polar. SPE can be a very selective technique and produce clean extracts, especially if mixed mode SPE is used [80]. But it is often a time-consuming method with many steps involved. When choosing a sample preparation method recovery, precision and time are the most important parameters.

Extraction of most tryptamines should in theory be similar to extracting many basic drugs, as they have many structural similarities. Amphetamines, cathinones, LSD and many more, all have a phenyl group and an amine group, with various other attachments. And all the three above mentioned sample preparation techniques have been able to extract tryptamines satisfyingly. In 2016, *Vaiano et Al* extracted 64 NPSs using a PPT extraction method with acetonitrile as precipitant [81]. Two of them were tryptamines, 4-OH-DIPT and 5-MeO-DIPT, and recoveries were 91 and 84%, respectively. Another study that used the same PPT procedure for the extraction of 143 NPSs included 18 different tryptamines of which 11 are included in this study [82]. However, recovery data is only included for DMT (17.1%) but the other tryptamines had LODs between 0.1 and 0.5 ng/mL. In 2014, *Meyer et al* extracted 37 different tryptamines with an LLE method using a 50/50 mixture of butyl- and ethyl acetate as organic phase, the recoveries are not listed but the LODs for most of the tryptamines were 100 ng/mL [83]. In 2010, *Wohlfart et al* used mixed mode SPE for the analysis of 35 different designer drugs in serum, of which 6 were tryptamines, recovery results are not given but LODs were from 1.0 to 2.5 ng/mL [84]. This shows that

all the three sample preparation principles are applicable to tryptamines and worth testing to be able to compare the results and optimize the analysis method.

1.4.2 Protein precipitation

To remove as much as possible of red and white blood cells, blood platelets and proteins from whole blood, protein precipitation is often used. It is done by adding a precipitant which alters the conditions around and on the proteins, so they precipitate. There are three different kinds of precipitants that are used, a salt, an acid, or a miscible organic solvent, each with a unique precipitation mechanism.

When salting out the proteins from blood the ions of the dissolved salts compete with the proteins for the water molecules thereby removing the hydration layer around the proteins. This exposes the hydrophobic parts of the proteins which causes them to aggregate and precipitate [85]. However, care has to be taken because salts can precipitate in the mobile phase or in the ionization/evaporation process in the ESI-interface, and damage both the LC-system and the inlet cone of the MS.

Adding an acid such as trichloroacetic acid (TCA), works through lowering the pH of the blood so that the acidic moieties of the proteins are protonated. This allows the anions of the added acid to bind to the protonated bases of the proteins, which makes the proteins neutral. Thus, the hydration layers of the proteins no longer have a repulsive force and the neutral proteins can interact and precipitate [86]. Because of low pH caused by adding this acid, pH adjustment might have to be done to avoid damage to the LC-column [62].

Using an organic phase that is miscible with blood will also precipitate the proteins. Through decreasing the dielectric constant (ε^0) of the blood (or an aqueous solution), the rigidity of the hydration layers decreases, and the proteins will interact more closely and precipitate [86]. If reversed phase LC is used, the analytes will be dissolved in an organic solution after precipitation, injected untreated this can cause severe band broadening [62].

Even though over 90 % of sample proteins can be removed through these PPT-techniques [86], an additional step such as dilution, filtration, or evaporation and reconstitution is usually required to remove remaining unwanted residues [87]. Therefore, PPT is often used as a precursor to other preparative methods

1.4.3 Liquid liquid Extraction

LLE is a sample preparation technique that has been used for many years. It can produce clean extracts and is especially useful when screening for a wide range of analytes in a sample [88]. It works by the partitioning of the analytes between the aqueous phase (usually the sample) and an immiscible organic phase. This can be described with Equation 3 for the partition coefficient (K):

$$K_{LLE} = \frac{[analyte]_{organic\ phase}}{[analyte]_{aqueous\ phase}} \quad (3)$$

Where $[analyte]_{organic\ phase}$ and $[analyte]_{aqueous\ phase}$ are the concentrations of the analyte in the organic and aqueous phases, respectively. When the aqueous phase is water, and the organic phase is octanol K is called P, whose logarithm (log P) is a common measure of a substance's lipophilic nature.

To make LLE more selective there are a few tools available, in the aqueous phase adjusting the pH by adding a buffer can make the analyte neutral, increasing its affinity toward the organic phase. Using an organic phase that suits the analyte in question will increase the K-value and lead to higher recoveries [62]. For acidic analytes, proton accepting organic solvents (e.g., methyl *t*-butyl ether or iso-propyl alcohol) will increase the K-value. For basic analytes, such as tryptamines, the addition of a proton donating solvent (e.g., chloroform) will increase its affinity to the organic phase [89]. Hydrogen bonding is a strong bonding type, but other bonding types can be utilized in LLE as well. Dipole, induced dipole, and lipophilic bonding are three other bonding mechanisms that can give additional selectivity towards an analyte, or a specific kind of functional group [62]. Solvents that can give strong interactions of these kinds are for instance dichloromethane, ethyl acetate and heptane, respectively [90].

1.4.4 Solid-Phase Extraction

Solid-phase extraction offers many different types of extraction principles and can offer sample preparations with high selectivity. Sorbents include normal phase, reversed phase, ion exchangers, and many more [91]. In addition, mixed-mode sorbents are available, for instance, a cation exchanger can be mixed with a reversed phase sorbent. Mixed-mode SPE with RP and cation or anion exchangers, can give exceptionally clean extracts as both unwanted polar and hydrophobic substances can be washed out, while the analytes are retained [92]. A disadvantage with mixed-mode SPE is its many different steps, making it a complex and time-consuming technique. A general procedure with extraction from whole blood typically looks like this:

- **Conditioning**. To activate the sorbent, it needs to be conditioned with an organic solvent and then water, or a buffer. There are two different kinds of ion-exchangers, weak and strong. The weak exchangers consist of bonded anions or cations which are charged over a smaller pH window [62]. These can be used for instance, if the analytes are unstable in a certain pH-area. Whereas the strong ion exchangers are charged over the whole relevant pH range [62].
- **Sample application**. The sample must be added slowly because of slower transfer kinetics in ion exchangers compared to reversed phase sorbents [93].
- **Wash**. First, the polar matrix components are washed out with an aqueous solution while analytes are retained by the sorbent. The pH of the washing solution cannot be such that it removes the charge from the analytes. Hydrophobic matrix components are then washed out by an organic solvent while the analytes are retained by the ion-exchange moieties, if the organic washing solution is too strong, analytes can be lost.
- **Elution**. Analytes are eluted by adjusting the pH to remove the charge on the analytes. This is done by adding a base, usually 2 – 5 % ammonia in 50 – 100 % methanol, if the analytes are basic, and an acid, usually 2 – 5 % acetic acid in 50 – 100 % methanol if analytes are acids [94]. Methanol is there to ensure that analytes are released from the secondary reversed phase interactions.

Because of all these steps, SPE is a laborious sample preparation method, and with all the different solutions that are used it takes time to optimize. However, if matrix effects turn out to be impossible with easier sample preparation methods, mixed mode SPE can be a solution [95].

1.4.5 Electromembrane extraction

Electromembrane extraction (EME) is a fairly new sample preparation technique, first introduced in 2006 by *Bjerregaard et al* [96]. It employs an external electric field to move charged analytes from an aqueous sample, through an immobilized, water-immiscible organic solvent, and into an aqueous acceptor solution [97]. The supported liquid membrane (SLM) separating the donor- and acceptor solution is typically a porous filter with a diameter of approximately 1 cm and 100 or 200 µm thickness. A generic setup is shown in figure 1.5.

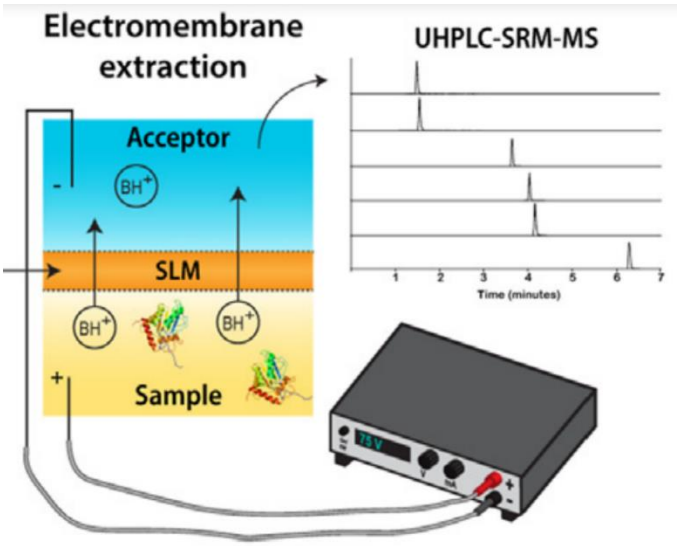


Figure 1.5. EME setup for charged basic analytes traversing the SLM followed by direct analysis of the acceptor solution by UHPLC-SRM-MS. Figure reproduced with permission from [98].

To extract basic analytes, the anode (+) is placed in the sample and the cathode (-) in the acceptor solution. For the extraction of acidic analytes, the electrodes are placed opposite [97]. The pH is adjusted in both donor and acceptor solutions to keep analytes charged.

Selectivity in EME is mainly controlled by three parameters: the magnitude of the applied voltage, the type of SLM, and the pH in the donor- and acceptor solution [98, 99]. It has been shown that a high voltage will extract a wider range of basic compounds, from polar to non-polar and that lowering the voltage reduces the extraction efficiency of the more polar analytes [100]. The voltage should, however, not exceed a certain threshold, where the current is too high (> 50 µA per well) as this can cause water hydrolysis at the electrodes and subsequent pH alterations [100].

The optimal SLM should be immiscible with water to avoid bleeding of the SLM into the surrounding solutions. Additionally, it should keep a low current at higher voltages and be free from impurities [97]. To achieve a selective extraction, the chemical interactions between the analytes and the SLM should be considered, these are hydrogen bonding, polar interactions, and hydrophobic interactions. For instance, bis(2-ethylhexyl) phosphite (DEHPi), previously used as an SLM in EME, has hydrogen bond accepting sites enabling hydrogen bonding with basic analytes (hydrogen bond donor) and sites for dipole interactions [101]. More recently, natural deep eutectic solvents, composed of mixtures of naturally abundant compounds such as thymol, coumarin, or menthol have gained interest as SLMs in EME [98]. In addition to being green alternatives to traditional organic solvents, they have relatively low hydrophobicity, hydrogen bonding properties, and often aromatic properties. For the extraction of tryptamines, these two examples are in theory promising candidates and have also proven to be efficient for the extraction of polar bases from biological matrices before [98, 101].

1.5 Mass Spectrometry

In the eighties and nineties gas chromatography – mass spectrometry (GC-MS) was the gold standard in forensic toxicology, as it had high sensitivity and separation power. Laborious sample preparation procedures due to the basic nature of most of the relevant analytes [39, 102] was however a drawback. The coupling between LC and MS was invented by Fenn and Yamashita in 1984 [103], but it took many years to overcome the major drawbacks to this technique and make it an established procedure in forensic

toxicological assays [104]. The drawbacks were mainly poor ionization reproducibility and matrix effects [105].

After the different compounds have been separated in the separation column, they are ionized by the ESI-interface and emitted into the mass spectrometer as gas-phase ions. Here, they are separated by their mass-to-charge-ratio (*m/z*) and detected by their *m/z* and abundance [106]. Computer software then transforms the signal from the detector into a mass spectrum. Figure 1.6 shows the setup of a generic mass spectrometer.

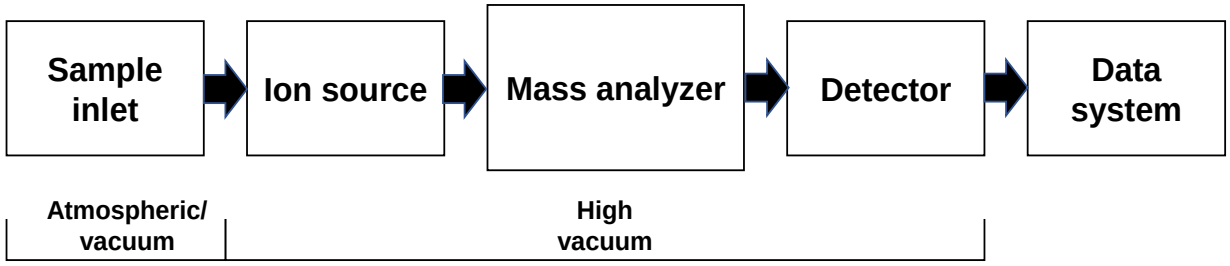


Figure 1.6. Scheme of a general mass spectrometer setup. Figure adapted from [106].

1.5.1 Electrospray ionization

When analytes have been separated in the separation column the eluent enters a capillary which has a high voltage coupled to it, typically between 2 and 5 kV [57]. The inlet of the MS has the opposite polarity to the outlet capillary and as the eluent leaves the capillary it enters a strong electric field [107]. As can be seen in Figure 1.7, this electric field causes the positive ions (if positive mode is applied) to form a so-called Taylor cone at the end of the capillary which subsequently produces a burst of small droplets containing the analyte ions [108]. To help remove the solvent a high temperature desolvation gas, usually N_2, is introduced into the ionization chamber, a drying gas directed in the opposite direction to the ions is also utilized in this respect [39].

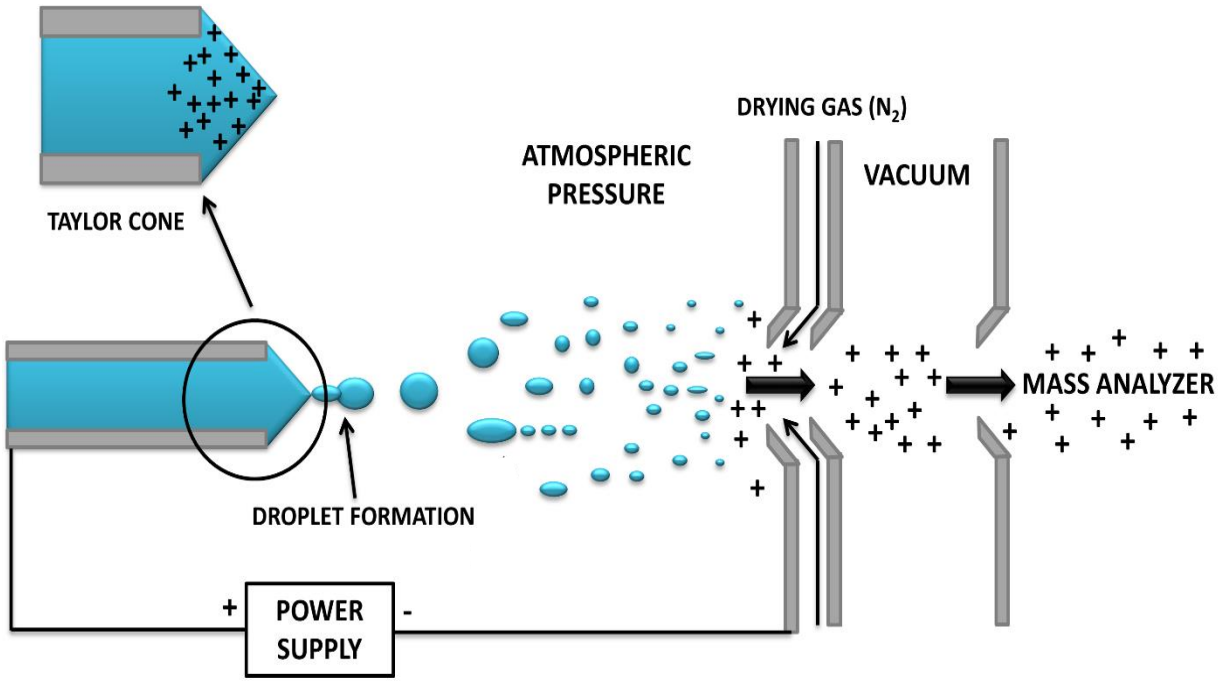


Figure 1.7. Schematic drawing of the electrospray ionization process. Ions in a solvent are brought to the gas-phase by desolvation and expulsion from the droplets by repulsion. Figure used with permission from [109].

As the ions have burst into a spray of droplets from the Taylor cone [110], they keep bursting into smaller and smaller droplets. As the repulsion increases among ions in the droplet, they reach a certain threshold where the droplet bursts into even smaller droplets [110]. There are two different theories as to how the gas-phase ions are created after this. The charge residue model suggests that as the solvent evaporates the repulsive charges cause formation of ever smaller droplets until there is only one ion left in a droplet which becomes a gas-phase ion when the solvent evaporates [111]. According to the ion evaporation model the electric field on the surface of the small droplets is high enough for direct transfer from solvated ions into gas-phase ions [78].

These above-mentioned mechanisms explain why it is important with compound separation prior to mass spectrometric analysis. If many compounds other than the analyte of interest is present in the droplets, they can supress or enhance the ionization

of the analyte. This is the major pitfall of LC-ESI-MS [71], and leads to poor quantitation, reproducibility and accuracy [78]. According to *Trufelli et al,* the four most relevant matrix effects mechanisms are the following [78]:

- Competition between matrix components and analytes for the available charges.
- Matrix components in high concentration can affect the viscosity and surface tension of the droplets. This can alter droplet formation and evaporation and will affect the transfer of the analytes to the gas-phase.
- Non-volatile additives can create solid particles with analytes
- Mobile phase additives or matrix components can create ion pairs with analytes thus making the analyte part of a neutral compound.

In forensic toxicology analytical methods are usually made with one specific matrix in mind, such as whole blood. Concentrations of different endogenous constituents in whole blood can vary between individuals [95]. PL are well known to cause of matrix effects and if they co-elute with the analyte of interest, some of the above-mentioned mechanisms might take place. Measures to overcome the problems PL and other matrix constituents can create are the following, (i) using internal standards, preferably isotopically labelled analyte analogues [112], (ii) Change gradient or column chemistry to keep analytes out of the retention time window where the phospholipids elute, (iii) If possible, another sample preparation method can be used. A fully validated determination method in forensic toxicology contains an analysis of matrix effects and demands that they don't disturb the quantitation of the analytes [5].

1.5.2 Mass analysis

To separate gas-phase ions many different types of mass analysers can be used. The oldest MS is the sector-field instruments which separated the different *m/z* using a magnetic or electric field, compared to today's instruments these are slow and difficult to operate [106]. Today the analysers that are used the most in forensic toxicology is the quadrupole, Time of flight and orbitrap. The quadrupole will be explained later in this

chapter. Time of flight (TOF) is a very fast analyser with high resolution measuring the ions by their speed over a certain length [106]. The orbitrap uses a mathematical process to deconvolute ion oscillations around a spindle-shaped electrode [106], and is one of the analysers that offers the highest resolution. The reason quadrupoles are used is low price, fast scan rate and usefulness for tandem-MS [106].

The quadrupole works by passing the ions through four cylindrical rods, as shown in Figure 1.8, who together make an oscillating electric field which will stabilize an ion based on how strong the two pairs vary between exerting attractive and repulsive forces [106]. A low mass ion with a +1 charge will "feel" the push or pull from the attractive and repulsive forces faster than a high mass ion. Therefore, the low mass ions have stable oscillations thorough the four rods when the radio frequency is fast and the high mass ions have it when they are slower [106]. After the ions are out of the rods they hit a detector, usually a so-called secondary electron multiplier or a photomultiplier, which amplifies the signal proportional to the number of ions hitting it [113].

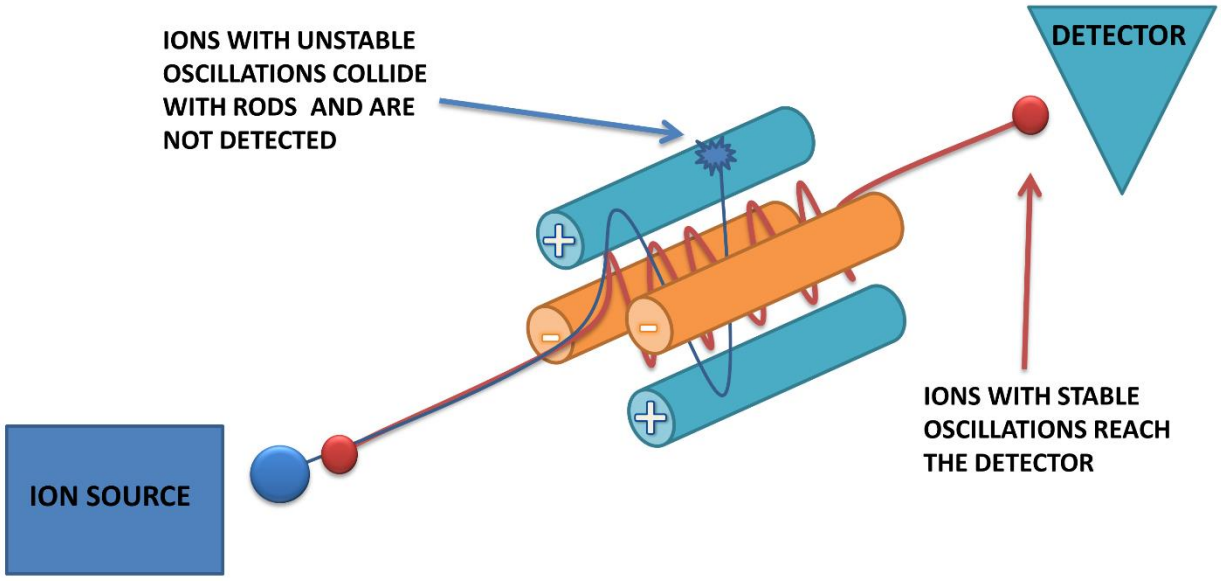


Figure 1.8. A quadrupole making a stable trajectory for an ion with a certain *m/z* value, leaving ions with other *m/z* values to collide with the rods. Figure used with permission from [109].

When three mass analysers, such as quadrupoles, are assembled after one another, or two quadrupoles combined with a collision cell, it is called tandem MS. The first analyser either scans a mass range or selects one or more ions which in the next analyser/collision cell collides with neutral gas molecules (or atoms) and dissociates into smaller fragments [106]. In the last analyser scans or selected ion monitoring can be done as in the first analyser. There are mainly four different modes in which tandem MS is operated. In a *product ion scan* a chosen molecule and its most abundant fragments can be found [109]. In *precursor ion scans* the last analyser is set to a specific *m/z* value and the first analyser is set to scan, in this way the compounds which give the specific fragment will give a signal in the detector [106]. *Neutral loss scan* scans a chosen mass range in both analysers but with the second analyser set at a certain mass below the first [109]. In a *selected reaction monitoring* (SRM) scan the first analyser selects a molecular ions mass and in the second analyser selects one or more known fragment ions [114]. This mode is used in quantitative analysis because it increases selectivity and sensitivity of a method, since a scan over a range of ions takes time, the analysers might have missed some ions after moving to the next *m/z* [115]. In forensic toxicology at least two of these modes are used often, namely *product ion scan* and SIM. However, SIM is used with many molecular ions selected at the same time, which gives it the name *multiple reaction monitoring* (MRM).

As part of method development and optimization on the mass spectrometer, there are many parameters that can be optimized, with time constraints on the method development these three are the most important: cone voltage, collision energy and finding the most suitable fragment ions.

Just after the inlet of the mass spectrometer there is a space between a nozzle and an orifice which has an intermediate vacuum between the atmospheric pressure of the ESI interface and the high vacuum in which the analysers are contained [106]. Due to possibilities of analyte collisions with residual gas molecules, or the formation of ion-clusters, the voltage between the nozzle and the inlet orifice is used to affect the number of ions entering the analysers [116]. In Waters UHPLC instruments, which are used in

this study, this is called cone voltage, other names such as skimmer, declustering potential, or orifice potential are also used [116].

The most suitable product ions, often called daughter ions or fragments, must also be found. These daughter ions are often specific for each analyte. Retention time and the ratio between these ions (ion ratio) are used for identification, while peak heights or areas are used for quantitative determination of the analytes [117]. Through scanning the fragments, a molecular ion gives in the collision cell, the fragment ions that are most suited can be found. The fragments with the highest abundance are often used. As each fragment ion requires a different amount of energy, optimizing the abundance of each fragment means trying different collision energies in the collision cell [118]. Since the sensitivity of an analysis method ultimately is determined by the number of ions that hit the detector, this is an important part of method development.

1.6 Aim of study

Tryptamines are a class of psychoactive compounds whose presence in the illicit drug market has increased over the last two decades. To prevent that the use of these substances goes under the radar, analysis methods for their determination must be made. With a sensitive determination method for a wide range of different tryptamines, judicial consequences for illegal use can be implemented, and understanding of tryptamine use and effects in society can improve.

The overall aim of the study was to develop and validate an LC-MS/MS method for the determination of 21 tryptamines in whole blood. This was divided into a subset of four aims:

- To find a good compromise between separation of the analytes, peak shapes and run times during chromatographic separation by UHPLC.
- To optimize MS/MS settings and find the best MRM transitions.

- To develop a sample preparation method with a combination of satisfying recovery, pure extracts, good precision, and short operating time.

- Validate the developed LC-MS/MS method according to current guidelines with regards to precision, accuracy, LOD, LOQ, and linearity.

2 Experimental

2.1 Chemicals, materials, and procedures

2.1.1 Chemicals

All water used in this project was type 1 (18,2 MΩ), purified by a Milli-Q® Advantage A10 water purification system with a Q-POD® (0.22 µm filter) remote dispenser, from Merck (Kenilworth, NJ, USA). Methanol (MeOH, Chromasolv™ ≥ 99.9%) was obtained from Honeywell/Riedel-de Haën™ (Seelze, Germany), Acetonitrile (HPLC grade) was purchased from J.T.Baker (Center Valley, PA, USA). Isopropanol (for analysis) and Heptane (analysis grade) was obtained from Merck (Darmstadt, Germany). Ethanol (absolute) and HNO_3 (65 % in methanol) was obtained from Merck Millipore (Billerica, MA, USA). Coumarin, thymol and bis(2-ethylhexyl) phosphite (DEHPi) were all obtained from Sigma Aldrich (St. Louis, MO, USA).

Formic acid (FA, GPR RECTAPUR 98%) was acquired from VWR (Radnor, PA, USA), and Ammonia (NH_3, 28%, for analysis) from Sigma-Aldrich (Saint-Louis, MO, USA). Ascorbic acid was obtained from Sigma Aldrich (Steinheim, Germany). For carbonate buffer, di-sodium tetra borate decahydrate (GR) was obtained from

Whole blood was obtained from the blood bank at Oslo University Hospital, kept at – 20 °C and thawed before use.

2.1.2 Analytes and internal standards

Tryptamine (≥ 97%) was obtained from Sigma-Aldrich. N,N-dimethyltryptamine (DMT, 1.000 ± 0.006 mg/mL in methanol) from Cerilliant Corporation (Round Rock, TX, USA). N-methyl-N-ethyl-tryptamine (MET, ≥ 98% as oxalate), 4-methoxy-N,N-dimethyltryptamine (4-MEO-DMT, 98%), 4-acetoxy-N,N-dimethyltryptamine (4-AcO-

DMT, 95%), N-methyl-N-isopropyltryptamine (MIPT, 98.6%), N-ethyl-N-propyltryptamine (98.0%), 5-methoxy-N-ethyl-N-propyltryptamine (5-MeO-EPT, 99.4%), 5-methoxy-N,N-dipropyltryptamine (5-MeO-DPT, 99.2%), 5-methoxy-N,N-diisopropyltryptamine (5-MeO-DIPT 100.0%), 4-acetoxy-N,N-diisopropyltryptamine (4-AcO-DIPT 100.0%), 4-hydroxy-N,N-ethylpropyltryptamine (4-OH-EPT, 98.1%), 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT, 98.0%) and 5-hydroxy-N,N-dimethyltryptamine (5-OH-DMT, ≥ 98% as hydrochloride) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). N,N-dimethyltryptamine-N-oxide (DMT-N-oxide, 97.8%), 4-hydroxy-N,N-methyl-ethyltryptamine (4-OH-MET, 97,3%), 4-acetoxy-N,N-methyl-ethyltryptamine (4-AcO-MET, 97.4%), 4-acetoxy-N,N-dimethyltryptamine (4-AcO-DMT, 100µg/mL as hemifumarate in acetonitrile), 4-acetoxy-N,N-diethyltryptamine (4-AcO-DET, 96.5% as hemifumarate) and 4-hydroxy-N,N-diethyltryptamine (4-OH-DET, 97,9%) was acquired from Chiron AS (Trondheim, Norway). 4-hydroxy-N,N-dimethyltryptamine (Psilocin, 99.9%) was purchased from Lipomed AG (Arlesheim, Switzerland).

Serotonin 13C6-15N (5-HT-13C6-15N) (98.1%) and 5-methoxytryptamine-d4 (5-MeO-trypt-d4) (98.0%) were acquired from Chiron AS. N,N-dimethyltryptamine-d6 (DMT-d6) was purchased from Toronto Research Chemicals Inc (Toronto, ON, Canada). Psilocin-d10 was bought from Cerilliant Corporation.

2.1.3 Solutions

The analytes' recovery in the initial Captiva ND, LLE, and SPE sample preparation methods was tested with the standard solution having a concentration of 0.16 µM in MeOH:H_2O (1:1). For the initial Captiva EMR and the subsequent comparisons of Captiva ND and Captiva EMR, and the EME experiments, the hydroxy tryptamines concentration was 5.0 µM and the rest of the tryptamines were 0.5 µM, both in MeOH:H_2O (1:1).

Standard and quality control samples were made from a stock solution where all analytes were 20 mM in MeOH:H_2O (1:1). Table 2.1 shows the standard and quality control concentrations used.

Table 2.1. Concentrations of standards used for the calibration curve and quality control samples.

Standard	Concentration (nM)
1	2
2	5
3	10
4	40
5	200
6	1000
7	5000

Quality control	Concentration (nM)
1	2
2	5
3	30
4	500
5	4000

2.2 Materials and equipment

2.2.1 Lab materials and sample preparation equipment

The Captiva ND 96-well lipid plates, the Captiva EMR 96-well lipid plates and the Plexa PCX mixed mode SPE filters were all purchased from Agilent Technologies (Santa Clara, CA, USA).

The multipipette used for liquid transfer was a multipipette® E3x and the tips were combitips advanced, both from Eppendorf (Hamburg, Germany). Single pipetting was performed with pipettes of the brand Finnpipette® F1 and tips from Thermo Scientific (Waltham, MA, USA). When working with blood samples single pipettes were from Bioheat (Helsingfors, Finland), with tips from Sartorius AG (Göttingen, Germany). The tubes were vortexed either on a classic advanced whirlmixer from VELP SCIENTIFICA (Usmate Velate, MB, Italy), or a VX-2500 Multitube vortexer from VWR (Radner, PA, USA). To elute liquids through the different sample preparation filters a Positive Pressure 96 processor from Waters (Milford, MA, USA). Centrifugation of the samples was performed on a Centrifuge from Beckman Coulter (Brea, CA, USA). 96-well collection plates were evaporated on a SPE Dry™ 96 from Biotage (Uppsala, Sweden). The collection plates were Nunc U96 PP 2 mL from Thermo Scientific (Waltham, MA, USA).

For the EME experiments an in-house 96-well set-up, shown in Figure 2.1, developed at the section for pharmaceutical chemistry at the University of Oslo was used.

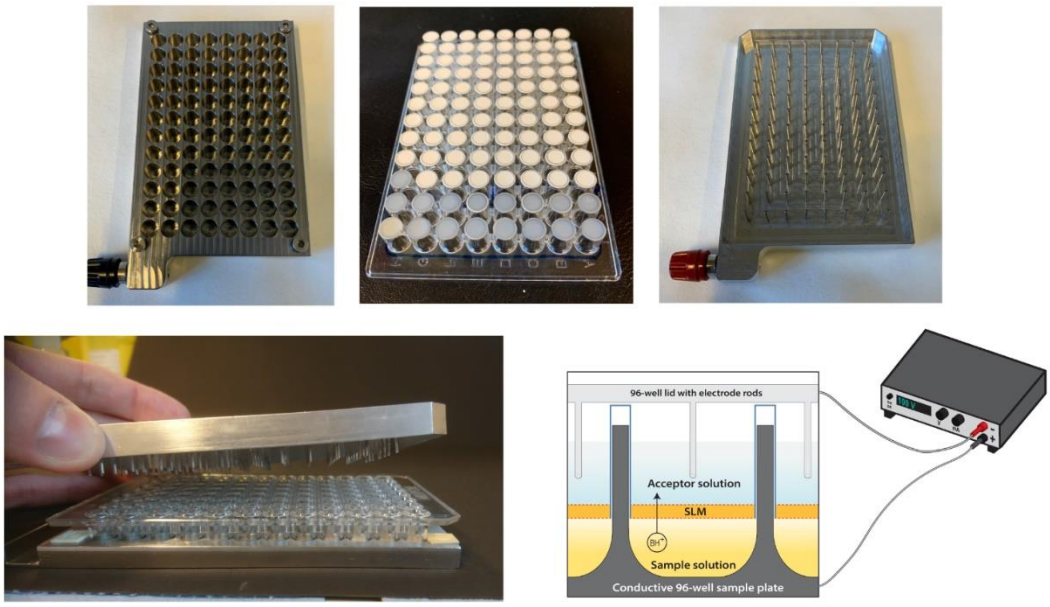


Figure 2.1. Set-up of the 96-well format for EME. Made in-house at section for pharmaceutical chemistry at the University of Oslo. Reproduced with permission from [98].

2.2.2 Gradients

Tables 2.2 and 2.3 shows the gradients used for column comparisons, and for sample preparation testing and validation series whose results are shown in chapter 3.2, 3.3 and 3.4. The given percentage is organic modifier (methanol).

Table 2.2. Gradient used for column comparisons.

time	%B
initial	2.5
0.2	2.5
8.0	40.0
8.1	100.0
9.0	100.0
9.1	2.5

Table 2.3. Gradient used for sample preparation testing and evaluation series

time	%B
initial	2.5
0.2	2.5
0.3	6.0
2.5	10.0
3.75	10.0
5.70	50.0
8.00	100.0
10.0	100.0
10.1	2.5

2.2.3 Liquid chromatography columns and mass spectrometry equipment

Four different column types were used for this study. HSS T3 1.8 mm 2.1 x 100 mm, BEH C18 1.7 mm, 2.1 x 100 mm, BEH C18 1.7 mm, 2.1 x 50 mm all Acquity UPLC® LC Columns from Waters. And a 1.7 mm 100 Å Biphenyl 100 x 2.1 mm LC Column from Kinetex ® from Phenomenex (Torrance, CA, USA).

All columns were kept at 60 °C during analysis.

The mass spectrometer used in this study was a Xevo® TQ-S with a Zspray™ ionization source coupled to a Acquity I class UPLC® sample and binary solvent manager and an Acquity UPLC® sample organizer from Waters. Mass spectrometric data treatment was done by MassLynx V4.1 software, also from Waters. The main mass spectrometric parameters used for each analyte in this study can be seen in Table 3.1.

2.3 Sample Preparation

2.3.1 Captiva ND lipid plates procedure

To see how the 21 tryptamines behaved being extracted with Captiva ND lipid plates, 100 µL whole blood was spiked with 100 µL of analytes. In set A, analytes were added before extraction, and for set B, the analytes were added after extraction to provide reference.

100 µL of MeOH/H_2O (1:1) was then added to set B for volume control. The samples were then vortexed and 750 µL of -20 °C ACN:MeOH (85:15) mixture was added to each sample before they were shaken on a Multi-Tube Wortexer. The tubes were then centrifugated for 2 min at 4500 rpm and 4 °C. Subsequently 600 µL of the supernatant was transferred to the Captiva ND lipid 96-well filter plate which was then filtered through by applying positive pressure. After filtration, 50 µL of internal standard was added to all samples, 100 µL of analyte solution was added to set B, and 100 µL of neat MeOH/H_2O (1:1) was added to set A for volume control. The samples were then focused with nitrogen gas at 45 °C until 200 – 300 µL was left in the wells. Then samples were analysed by LC-MS/MS.

When the Captive ND lipid plates were used to test ascorbic acid as an additive with or without methanol as precipitant, 2 µL of 25 % ascorbic acid dissolved in EtOH:H_2O (30:70) was added before the whole blood was added. Methanol just replaced the other precipitant ACN:MeOH (85:15).

2.3.2 Captiva EMR lipid plate procedure

For the recovery studies the Captiva EMR procedure was as follows:

100 µL of whole blood was added to each 5 mL plastic tube. 50 µL of standard (dissolved in MeOH:H_2O, 1:1) was added to set A, set B was added 50 µL MeOH:H_2O (1:1). Then 50 µL of EtOH:MeOH (1:2) was added to all tubes and vortex mixed for approximately one minute. Subsequently 100 µL of EtOH:H_2O (30:70) is added to all tubes which are vortexed approximately 30 seconds. Then the precipitation solution with 400 µL ACN:MeOH (85:15) straight from a – 20 °C freezer is added. The tubes are capped and put on a vortex mixer for 20 seconds. Then the tubes are centrifuged at 4 °C, with 3000 rpm for 10 minutes. While the tubes are in the centrifuge 200 µL of a ACN:H_2O (4:1) with 1% FA mixture is added to all the filter plate wells. 420 µL of the supernatant from the centrifuged tubes are transferred with a pipette/Multipipette and added to the Captiva EMR filter plates wells and there for 2 minutes. The samples are filtered slowly under mild positive pressure onto the collection plate and 50 µL internal standard solution is added to each plate. Then set A is added 50 µL of MeOH:H_2O (1:1), and set B is added 50 µL

of the standard solution. After this the plate is covered and put on a multitube wortexer and mixed for 30 seconds before UHPLC-MS analysis.

When this method was tested again for a comparison between methanol and ACN:MeOH (85:15) as precipitation agents and with or without addition of ascorbic acid, the same procedure as above was followed, but with methanol as precipitating agent and the addition of 2 µL of 25 % ascorbic acid dissolved in EtOH:H_2O (30:70) in the tubes before whole blood was added.

When the Captive EMR plates were used for method validation the procedure was as follows:

100 µL whole blood, 100 µL standard solutions and 50 µL internal standards (which contained 4% (v/v) of a EtOH:H_2O (30:70) mixture with 25% ascorbic acid) was added to all the standard and control samples. Then, the samples were vortexed for 5 – 6 seconds. After this 100 µL EtOH:H_2O (30:70) was added before the samples were vortexed again for 5 – 6 seconds. Subsequently, 400 µL of freezer cold ACN:MeOH (85:15) was added to all samples, which are capped, vortexed for 30 seconds and put on a centrifuge at 4 °C, 3000 rpm for 10 minutes. During centrifugation 200 µL of an ACN:H_2O (4:1) with 1% formic acid mixture was added to the filtration plate wells. After centrifugation 420 µL of the supernatant of each sample was added to the filtration plates and left for two minutes before a mild positive pressure was applied to elute the filtrate. The collection plate was put under the evaporator and the samples were evaporated for 50 – 60 minutes at 43 °C. Subsequently 50 µL of 10 mM ammonium formate buffer, pH 3.1, with MeOH 5% (v/v). The collection plate was sealed and vortexed and ready for UHPLC-MS/MS analysis.

2.3.3 LLE procedure

Four different conditions were tested with this LLE procedure, these are described in chapter 3.3.3. Analyte solution was added to set A before extraction and to set B after extraction. In addition, a zero-concentration sample and a blank sample was put through the same process as the extraction samples, both without analyte solution and the blank sample without internal standard. First 100 µL of blood was added to each 5 mL plastic

tube. 50 µL of analyte solution was added to set A. MeOH/H_2O (1:1) was added to set B and the blank and zero samples. Blank samples did not contain analytes and zero samples did not contain analytes or internal standards. After this 75 µL of buffer was added, followed by mixing. Then 1,2 mL organic phase was added. The samples were then “turned” for 10 minutes, then centrifuged for 10 minutes at 4500 rpm and 4 °C. After which 750 µL of the organic phase was transferred over to glass tubes for vaporization to dryness at 40 °C and 1,5 L/minute gas flow for approximately 30 minutes. After evaporation 50 µL internal standard was added to set A, set B and the zero-concentration sample, but not the blank sample. 69 µL of 10 mM, pH 3,1 ammonium formate buffer (mobile phase buffer) was added to all samples. And 31 µL of MeOH/H_2O (1:1) was added to set A, the zero-concentration and blank samples, while 31 µL internal standard solution was added to set B. After mixing, they were ready for LC-MS/MS analysis.

2.3.4 SPE procedure

The mixed mode PCX-plexa filter plates and the procedure used to test the extraction of the 21 tryptamines was done as follows:

100 µL of whole blood was added to each sample, set A was added 20 µL of the standard, set B was added 20 µL of MeOH:H_2O (1:1). 500 µL of freezer cold ACN:MeOH (85:15) was added to each sample and vortexed a few seconds after addition. After this the samples were centrifuged for 2 minutes, at 4 °C, with 4500 rpm. When finished, the samples were put in a freezer, at - 20 °C for 10 minutes. After this, 450 µL of the supernatant was transferred to new tubes where 500 µL of 2% FA was added and the tubes were vortexed for 2 – 5 seconds. The Plexa PCX extraction was first conditioned with 500 µL of MeOH, then 500 µL of 0.1 % FA. After the conditioning the samples were transferred to the Plexa PCX filtration plates and mild pressure was applied to let the analytes adsorb to the stationary phase. Subsequently, the plates were washed with 500 µL 0.1 M HCl and then 500 µL MeOH:H_2O (1:1). The analytes were then eluted into a collection plate with twice with 500 µL Ethylacetate:MeOH (1:1) with 2% NH_3 solution (25% NH_3 in H_2O). After elution 25 µL of internal standards were added to all sample wells, set B was added the standard solution and set A was added MeOH:H_2O (1:1).

Before evaporation all samples were added 10 µL of 0.01 % HNO_3 in methanol. The samples were then evaporated at 45 °C for approximately 50 minutes before reconstitution in 100 µL of mobile phase, 10 mM ammonium formate pH 3.1 with 10% methanol (v/v), prior to LC-MS/MS analysis.

2.3.5 EME procedures

The 96-wellplate EME set-up contained a donor phase (sample), an organic SLM and an acceptor phase. Five different EME experiments were conducted. In all experiments 100 µL of 100 mM formic acid (pH 2.4) in the acceptor phase was used, while donor and SLM was varied as described in Table 2.4. The experiments were all conducted at 30 V, except for the coumarin/thymol with 2% DEHPi as SLM experiment with spiked whole blood in the donor phase, which was conducted at 2 V. For experiment numbers one and three, the reference solutions were made in the same way as the donor solutions. For experiment numbers two, four and five 8.3 µL of standard was added to 91.7 µL of 100 mM formic acid. In the lab these solutions were scaled up 20 times.

Table 2.4. EME parameters for the five different conditions for the recovery of the 21 tryptamines.

EME experiment no.	**SLM**	**Donor phase**	**Acceptor phase**	**Voltage (V)**	**Extraction time (min)**
1	Coumarin/thymol (2:1 w/w) with 2% DEHPi	12.5 µL std. 87.5 µL 100 mM formic acid	100 µL 100 mM formic acid	30	15
2	Coumarin/thymol (2:1 w/w) with 2% DEHPi	8.3 µL std. 33.3 µL whole blood. 58.4 µL 100 mM formic acid	100 µL 100 mM formic acid	2	15
3	DEHPi	12.5 µL std. 87.5 µL 100 mM formic acid	100 µL 100 mM formic acid	30	15

4	DEHPi	8.3 µL std. 33.3 µL whole blood. 58.4 µL 100 mM formic acid	100 µL 100 mM formic acid	30	15
5	DEHPi	8.3 µL std. 33.3 µL whole blood. 58.4 µL 100 mM formic acid	100 µL 100 mM formic acid	30	30

2.4 Calculations

Recovery was calculated by dividing the response of the analyte that was added to the samples before the extraction procedure, by the response of the analyte added after the extraction procedure and then the ratio was multiplied with 100 to find percent recovery, as Equation 4 shows.

$$Recovery = \frac{response\ of analyte\ added\ before\ extraction}{response\ of\ analyte\ added\ after\ extraction} \times 100 \quad (4)$$

3 Results and discussion

3.1 Framework of study

In the present study, a LC-MS/MS method for the determination of 21 tryptamines in whole blood was developed and evaluated. The sample preparation procedure, chromatographic separation by UHPLC and MS/MS-parameters were all optimized to find the best compromise to achieve a sensitive, robust, and reliable method. Five different sample preparation methods, applying four different sample preparation principles, were tested.

Figure 3.1 shows a graphical presentation of the workflow. First, a mass spectrometric method had to be developed. Optimization of cone-voltages (CV) and collision energies (CE) were done for each of the 21 analytes, to find the fragments with the highest intensity. Subsequently, to find the most suitable LC-conditions for fast and selective separation, different UHPLC columns and mobile phases were tested. To extract the tryptamines from the whole blood matrix, aiming for high recoveries and clean extracts, five different methods were tested, of which electromembrane extraction (EME) is the only method not used routinely by the Department of Forensic Sciences at OUH. Finally, the developed method was evaluated with three evaluation series.

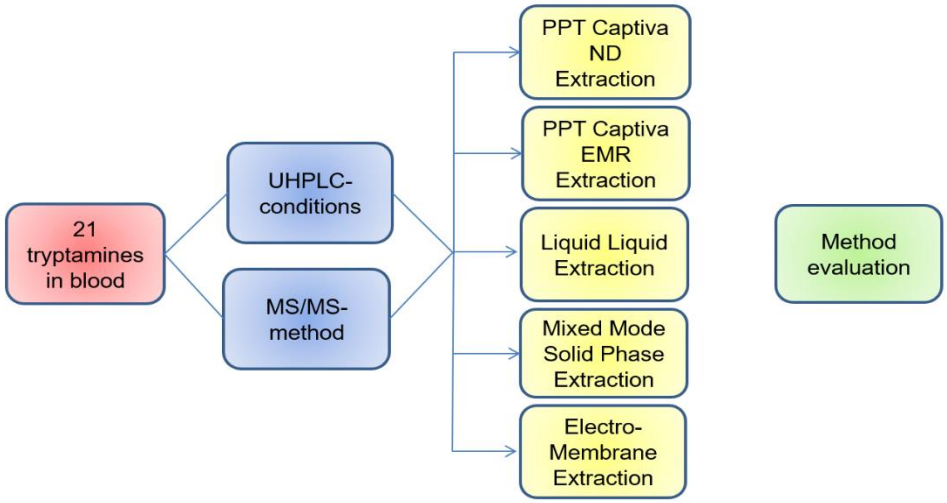


Figure 3.1. Graphical presentation of the workflow of this study.

3.2 Mass spectrometric method

To make a good MS-method certain parameters must be individually adjusted to each analyte, this is not only because the analytes have different behaviours in the electrospray ion source and collision cell, but also because MS methods are not as robust and transferable as, for instance, nuclear magnetic resonance methods. This means that MS methods from other labs applying the same conditions will not necessarily produce the same results.

Each analyte was put into a syringe and infused by a syringe pump which made a continuous stream into the ESI and MS. Here different cone voltages were tested to find which gave the highest response, and different collision energies were tested for the highest responses from fragment ions. Table 3.1 shows the results of the MS parameter optimisation.

As can be noted from Table 3.1, many of the tryptamines share the same fragment ion, especially tryptamines with the same functional group, or with the same number of alkyl substituents. This comes from the fragmentation pattern of tryptamines which favours the breaking of certain bonds, especially alpha cleavage [119]. This creates fragment ions of the indole structure and the amine group [119]. Separating the compounds chromatographically before MS/MS analysis therefore becomes essential for determination of structural isomers.

Table 3.1. MS parameters, retention times and peak numbers for the tryptamines and internal standards used in this study.

Analyte	Internal standard	Peak numbers Chapter 3.3 chromatograms	Cone voltage (V)	Q1 m/z	Q3 m/z	Collision potential Q3 (eV)	Q3.2 m/z	Collision potential Q3.2(eV)	Retention time (min) in validation series
Tryptamine	-	4	15	161.11	91.10	35	144.10	10	2.67
DMT	DMT-d6	5	20	189.14	117.10	30	144.10	15	3.06
MET	DMT-d6	9	25	203.15	72.10	15	144.10	30	4.27
5-OH-DMT	5-HT-13C6-15N	1	25	205.13	58.10	15	77.10	45	1.20
PSILOCIN	5-HT-13C6-15N	2	20	205.13	132.10	25	142.10	30	1.74
DMT-N-OXIDE	DMT-d6	11	20	205.13	117.10	30	144.10	20	4.48
MIPT	DMT-d6	14	20	217.16	86.10	20	144.10	20	5.10
4-OH-MET	5-HT-13C6-15N	3	30	219.15	72.10	15	172.10	30	2.35
5-MeO-DMT	5-MeO-trypt-d4	8	20	219.15	58.10	15	130.10	40	3.62
4-MeO-DMT	5-MeO-trypt-d4	15	20	219.15	130.10	40	142.10	35	5.14
EPT	DMT-d6	18	20	231.17	100.10	15	144.10	15	5.51
4-OH-MIPT	5-HT-13C6-15N	7	20	233.15	86.10	20	160.10	20	3.42
4-OH-DET	5-HT-13C6-15N	6	25	233.17	132.10	30	160.10	20	3.25
4-AcO-DMT	5-MeO-trypt-d4	10	25	247.14	115.10	15	202.10	40	4.55
4-OH-EPT	5-HT-13C6-15N	12	20	247.18	100.20	20	132.20	30	4.90
5-MeO-EPT	5-MeO-trypt-d4	17	15	261.18	100.10	20	174.10	20	5.48

Analyte	Internal standard	Peak numbers Chapter 3.3 chromatograms	Cone voltage (V)	Q1 m/z	Q3 m/z	Collision potential Q3 (eV)	Q3.2 m/z	Collision potential Q3.2(eV)	Retention time (min) in validation series
4-AcO-MET	5-MeO-trypt-d4	13	25	261.15	72.10	15	160.10	25	5.03
4-AcO-DET	5-MeO-trypt-d4	16	25	275.18	86.10	30	160.10	30	5.28
5-MeO-DPT	5-MeO-trypt-d4	19	20	275.20	114.10	20	174.00	20	5.74
5-MeO-DIPT	5-MeO-trypt-d4	21	25	275.20	114.10	15	174.10	30	5.56
4-AcO-DIPT	5-MeO-trypt-d4	20	25	303.41	114.10	30	160.10	30	5.65
DMT-d6	-	-	20	195.14	64.10	25	144.10	15	3.03
5-HT-13C6-15N (13C6-15N serotonin)	-	-	20	180.10	162.10	18	132.10	20	1.06
5-MeO-trypt-d4	-	-	20	195.30	178.10	43	134.10	43	3.21

3.3 Chromatographic separation

Only reversed phase (RP) LC was considered for separation of the tryptamines. Other separation principles could have been tested, such as supercritical fluid chromatography and HILIC, however, as RP-LC offered satisfactory robustness and efficiency for the tryptamines, this was chosen to save time. Also, in a high throughput laboratory, such as the Section of Forensic toxicological Analytics, where most LC analysis is done with reversed phase columns, adding a different separation principle would break with the effort to streamline the routine analysis laboratory as much as possible.

In the first couple of months of this project only 14 tryptamines were available as reference compounds. Therefore, parts of the chromatographic comparison tests were done with fewer tryptamines than what was available later. However, the techniques chosen for the tryptamine determinations are versatile enough for inclusion of more tryptamines. The studied compounds cover the whole range of relevant molecular modifications to tryptamines except alpha substitutions. Adding more tryptamines to the assay after validation is likely to be unproblematic, although some gradient adjustment might be necessary.

3.3.1 Choice of mobile phase composition and gradient elution

Different options were tested for the aqueous part of the mobile phase namely, ammonium formate buffer (pH 3.1 and 10.3) and formic acid (pH 2.7). These are standard additives to the aqueous solvent phase. Results of these tests are discussed in chapter 3.3.4 and 3.3.5.

Previous studies that have included tryptamines have used either acetonitrile or methanol as organic modifier [83, 84]. Methanol is used in the routine laboratory because of its relatively low toxicity [55]. Due to its lower elution strength, using methanol can additionally contribute to higher retention of polar compounds that are not retained when acetonitrile is used [120]. Acetonitrile/water mixtures with lower viscosity compared to methanol/water mixtures have been used in columns with smaller particle sizes due to lower backpressure [121], as this offers the opportunity for higher mobile phase velocity.

However, on account of the potentially higher health hazards, higher price, and possible availability issues [55, 56], and the fact that methanol gave satisfactory results, acetonitrile was not tested as organic modifier in this study.

Gradient elution compared to isocratic elution shortens analysis time and gives narrow peaks throughout the retention time window [14].

3.3.2 Column length

Because high mobile phase velocity often gives better chromatographic efficiency and shorter analysis time [62], a high velocity is desirable. However, higher velocity gives higher back-pressure, and the pumping system has an upper limit of 1000 bars on the pumps used in this project. An important factor in this respect is column length. As Equation 2 in chapter 1.3 points out the back pressure is proportional to the column length, hence a 5 cm column yields half the backpressure of a 10 cm column.

Both five and ten cm long BEH C_{18} columns were tested to find out which column length gave the best separation. As Figure 3.2 shows, and as expected theoretically since the columns have the same particle sizes and stationary phase, the 5 cm column yields quite similar separation efficiency as the 10 cm column, but with a few exceptions [122]. The separation window is approximately the same, but the analytes elute about 30 seconds earlier, which speaks to its advantage as fast separations are sought after. Separation between compounds 3 and 4. 6 and 7, 9 and 10 and 13 and 14 were better in the 10 cm column (**Figure 3.2b**). In contrast, the resolution of compounds 5 and 6 is better in the five cm column (**Figure 3.2a**).

If analysis time is considered the most important factor, the five cm column would probably be the best choice. However, since seven more tryptamines were ordered and had to be added to the method later, peak capacity and chromatographic resolution, which longer columns provide [123, 124], were considered more important when analysis time reduction was only 30 seconds. It was therefore decided to use the 10 cm long columns for further investigations.

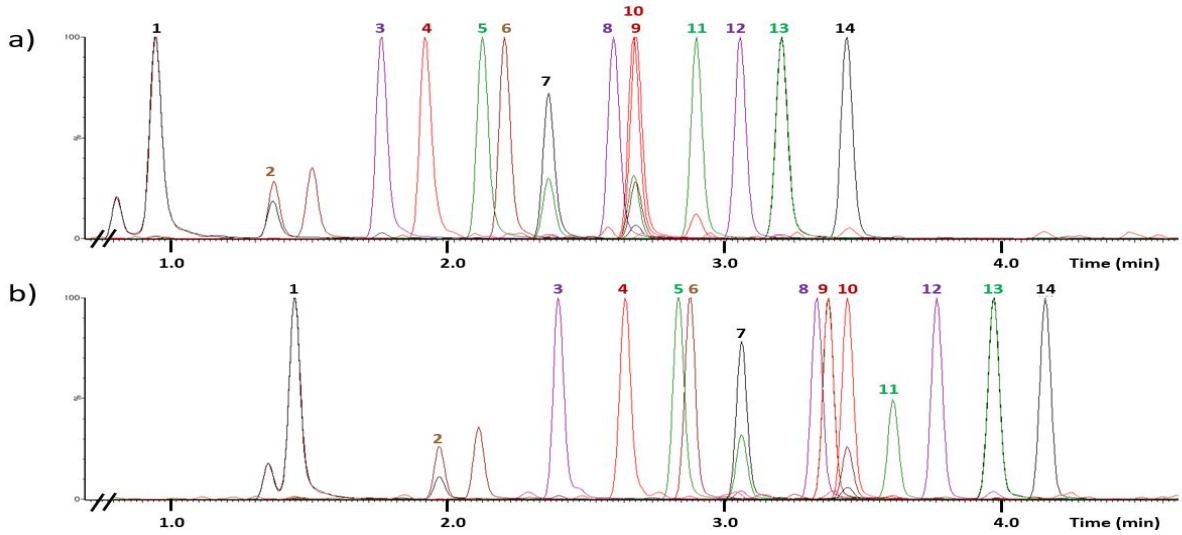


Figure 3.2. Chromatographic separation of the 14 tryptamines using a 5 cm column (a) and a 10 cm column (b). The peak numbers are 1. 5-OH-DMT, 2. Psilocin, 3. 4-OH-MET, 4. DMT, 5. 5-MeO-DMT, 6. MET, 7. 4-AcO-DMT, 8. DMT-N-Oxide, 9. 4-OH-MET, 10. 4-AcO-MET, 11. 4-MeO-DMT, 12. 4-AcO-DET. 13. 4-AcO-MET, 14. MIPT.

3.3.3 Mobile phase velocity

As mentioned in Chapter 3.3.2, a high mobile phase velocity can increase the chromatographic efficiency. Van Deemter plots of plate height versus velocity show this, especially for columns containing particles with a diameter below 2 µm [125]. A high flow rate does however increase back-pressure, which can cause problems for the pumping system, and if the pressure limit is exceeded, the system shuts down. In a high-throughput routine setting this can be critical as it results in reanalysis and queues. For compounds such as tryptamines, delayed analysis can in addition be a problem due to limited compound stability.

Analysis was performed with a flow of 0.4, 0.5 and 0.6 mL/min, respectively. As shown in Figure 3.3, a 10 cm long column with a velocity of 0.6 mL/min gave the best chromatographic separation and time of analysis. Comparing peaks five, six, eight and nine from this run (a), with the run with 0.4 mL/min mobile phase velocity (c), this becomes evident. According to the van Deemter equation (Equation 1, Chapter 1.3), the reason for

this would be that the B-term (longitudinal diffusion) is smaller for higher velocities [62]. Although studies of tryptamine analysis have reported LC-methods where the mobile phase velocities have been lower [16], in this study 0.4 mL/min separated the peaks poorly and increased analysis time considerably.

Figure 3.3b shows the chromatogram with 0.5 mL/min, this velocity displayed a performance in between 0.4 and 0.6 mL/min with regards to analysis time, but the separation efficiency is closer to the 0.6 mL/min velocity performance. This could indicate that the velocities 0.5 and 0.6 mL/min are close to the minimum of the van Deemter curve.

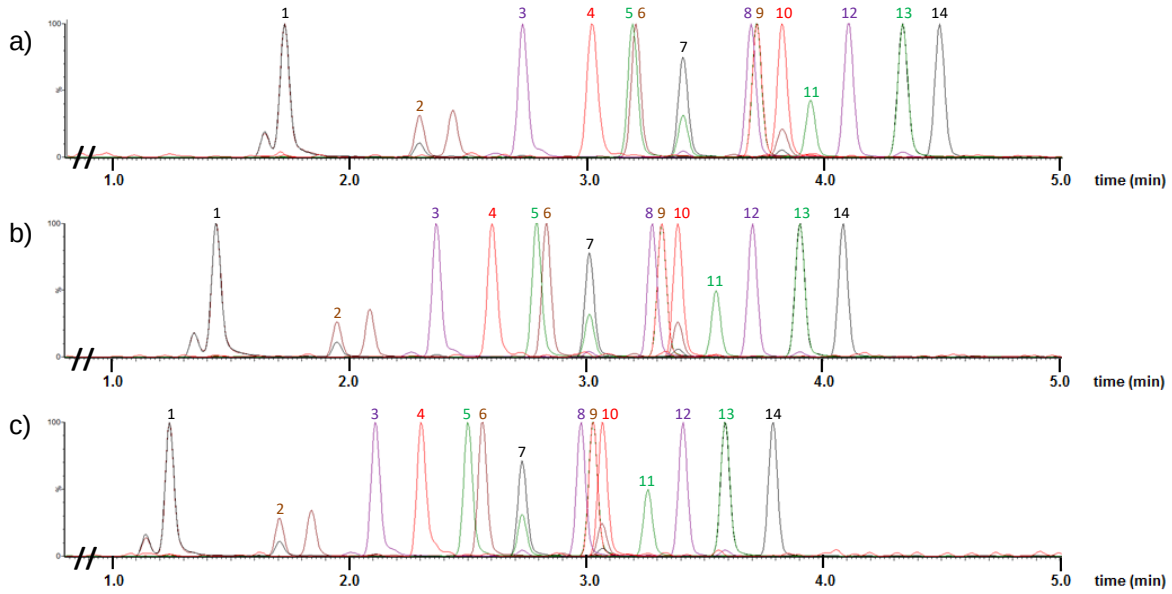


Figure 3.3. Chromatographic separation of 14 tryptamines with 0.4 mL/min (a), 0.5 mL/min (b) and 0.6 mL/min (c). Using a linear gradient from 2.5% - 40 % B over 5.8 minutes, the column temperature was 60 °C. Peak numbers are listed in table 3.1.

While a flow rate of 0.6 mL/min gave the best performance, the backpressure it produced was too close to the upper limit of 1000 bar/15 000 psi. As Figure 3.4 demonstrates, the methanol/water mixture back pressure reaches a maximum at approximately 1300 psi (896 bar) at about 50 % B, and the risk of system pressure overload was deemed too high with this velocity [126]. Correspondingly a flow rate of 0.5 ml/min was chosen as method robustness was prioritized over separation efficiency.

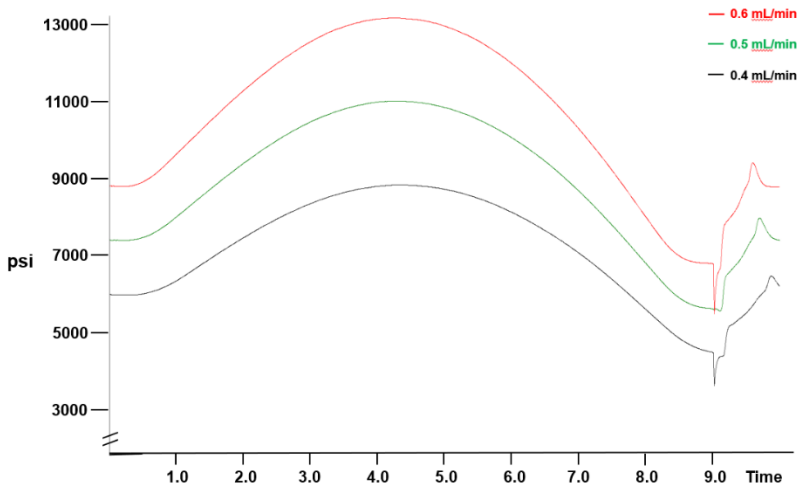


Figure 3.4. Backpressure from a 10 cm BEH C18 column at three different mobile phase velocities. See Table 2.1 for the gradient used.

3.3.4 Buffer pH

The HSS T3 and Biphenyl columns can only be operated with neutral or acidic mobile phases. In contrast the BEH C18 column can be operated within a pH range of 1-12.

Using high pH buffers in the mobile phase is an effective tool when an orthogonal analysis method is needed, for instance for confirmation methods in routine settings [127]. Additionally, high pH mobile phases compared to acidic mobile phases will often give higher ESI-MS/MS responses for bases [128]. This phenomena can probably be explained by the fact that increased retention causes the bases to elute with a higher organic solvent content, which will enhance ESI efficiency [129].

To see if the high pH mobile phase would yield better chromatographic results compared to the acidic mobile phase (pH 3.1), the tryptamines were analysed with a BEH-C18 column using 5 mM ammonium formate, pH 10.3, as aqueous mobile phase additive. The high pH (10.3) of the mobile phase approximately matches the pKa of the basic tryptamines (see Table 1.3 and Figure 1.2, chapter 1.2.2), a bigger portion of the basic tryptamines are neutral, making them more hydrophobic. For this reason, the gradient used (see figure text of Figure 3.3), with the acidic mobile phase could not be transferred

to the basic mobile phase without getting very long retention times. Therefore, a gradient starting at 30 %B was used.

As Figure 3.5 shows, the retention order is rearranged when using the basic buffer. The amphoteric tryptamines (with phenol and amine groups) have changed into four different species in the basic mobile phase, of which 10 – 20 % are neutral (see Figure 1.2, in Chapter 1.2.2). This seems to have added to their hydrophobicity in that they are retained for one minute with the gradient used for the basic mobile phase.

The use of a high pH buffer is associated with a heightened risk of causing damage to the mass spectrometer, especially if columns such as HSS T3 and Kinetex biphenyl are used on the same instrument. A high pH mobile phase will dissolve the support material on these columns, and if it is washed into the MS/MS it might pollute the instrument, cause instrument shutdown, and cost a lot of money. In addition, if buffers are changed from acidic to basic without proper washing of all the tubing, pumps, and columns, precipitation can take place and cause pressure overload. Therefore, an acidic mobile phase was preferred.

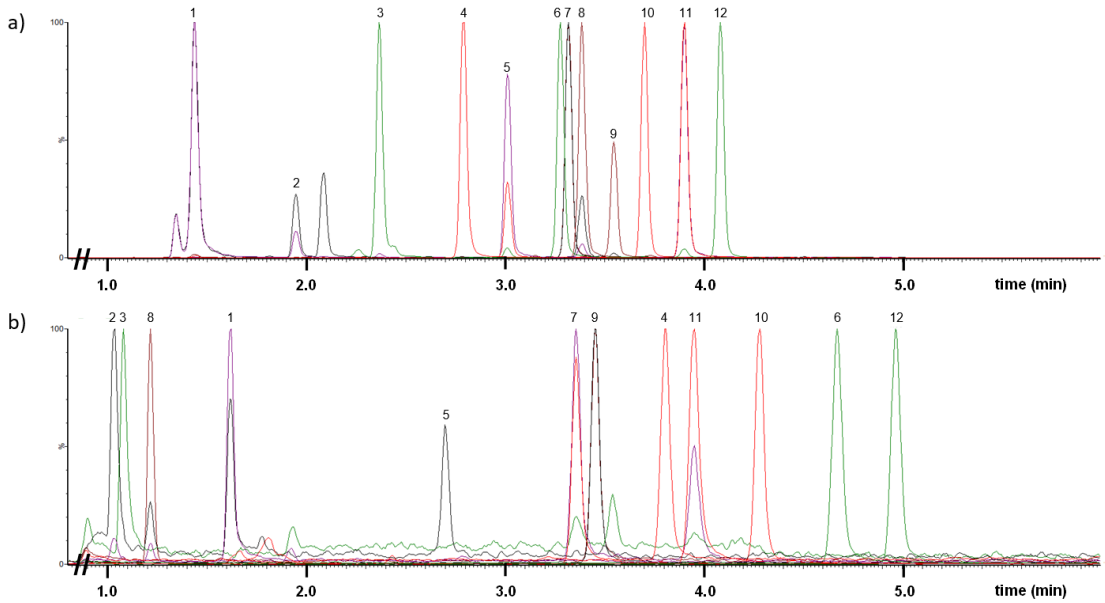


Figure 3.5. Chromatographic separation of 12 tryptamines with acidic buffer (10mM ammonium formate, pH 3.1) (a). And basic buffer (5 mM ammonium formate, pH 10.3) (b). The gradient started at 2.5 – 40 % B in 5.8 minutes with the acidic buffer (a), and 30 – 55 % B in 5.8 minutes with basic buffer (b).

3.3.5 Column comparison

At the Department of Forensic sciences nowadays only reversed phase LC-separations are used, and only three different column chemistries are used for routine analysis of biological samples: Acquity HSS T3, Acquity BEH C18 and Kinetex Biphenyl, all 10 cm long. Testing column types outside the repertoire of the routine department did not seem prudent on account of the necessity to minimize operating options, as there are numerous different operators, instruments, and methods to handle. In a UHPLC-MS/MS method choosing the right separation column is an important task. As shown in Table 1.2 the tryptamines have quite similar structures, and many are structural and/or positional isomers, which makes good LC-separation crucial. Acquity HSS T3 is a C18 column capable of retaining polar compounds well [59]. Phenomenex`s Biphenyl column separates based on hydrophobicity, but with a special affinity for aromatic compounds [130]. It also uses core-shell particles. The BEH C18 is a column with a wide pH-range and low column bleed [131]. To compare the columns separation performance each were tested with the two above mentioned acidic mobile phase additives (formic acid and ammonium formate), 0.5 mL/min flow velocity and methanol as organic phase.

The three columns and their performance separating the 21 tryptamines are shown in Figure 3.6. The biphenyl column differs from the BEH and HSS T3 columns in the retention order of the compounds, and in the retention window. As previous studies have shown, the cause of the retention order being different for the biphenyl column as compared to the C18 columns (BEH and HSS T3), probably lies in the aromatic properties of the indole structure of the tryptamines, which opens up for π-π interactions between analytes and stationary phase [130]. This effect also holds the tryptamines longer in the column, which generates a larger peak capacity, but also longer analysis time.

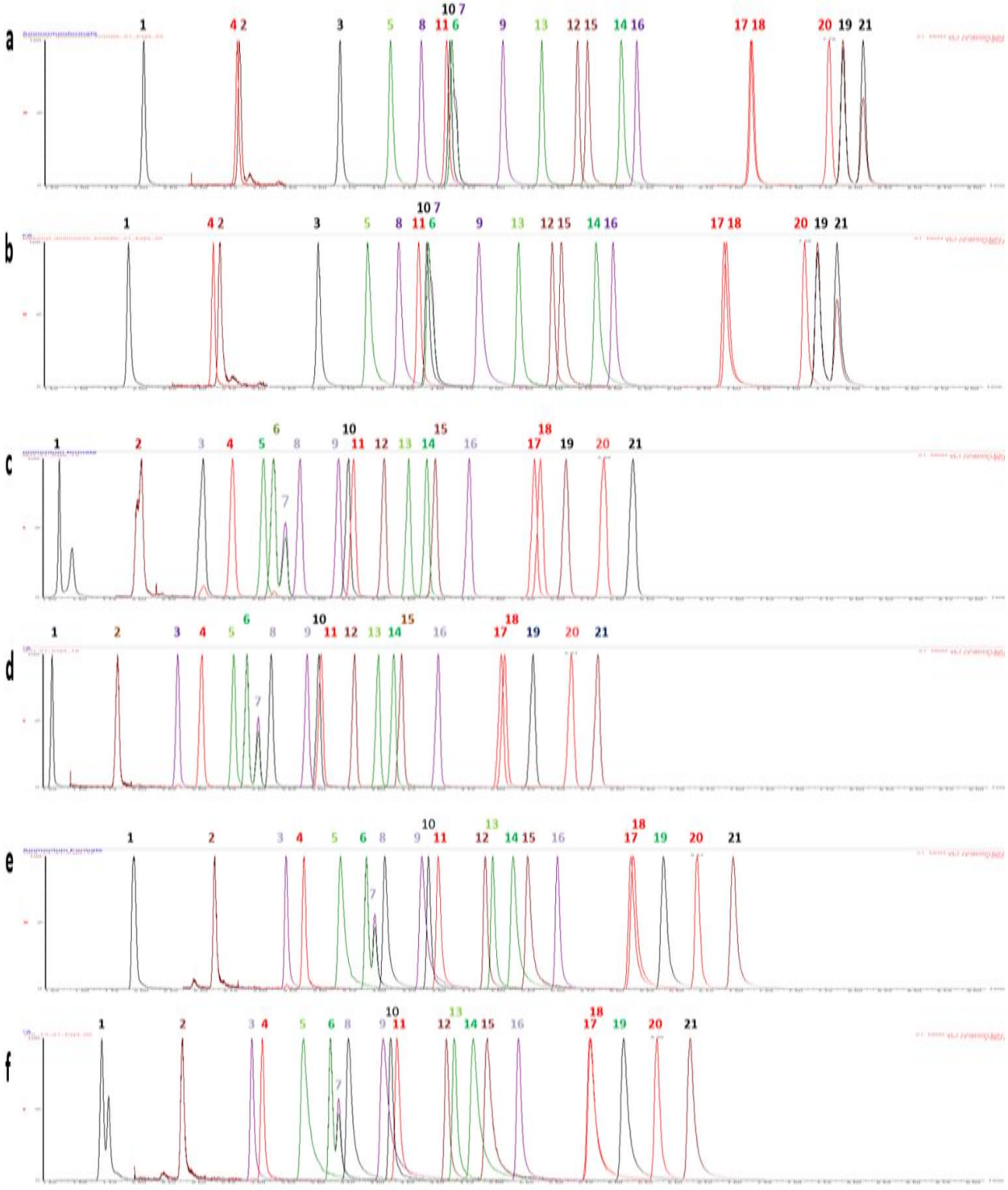
Compared to the two C18-columns, there are four compounds which the biphenyl column does not separate properly, namely 5-OH-DET and 5-OH-MIPT, and 5-MeO-EPT and EPT which have the numbering six, seven, 17 and 18 in the chromatograms of Figure 3.6, respectively. Co-elution of the latter two compounds does not represent a big problem since the molecules have different mass. This makes them distinguishable by the first

quadrupole (Q1), making a false result unlikely. However, the former compounds have identical mass and fragmentation patterns which make them difficult to distinguish if they are co-eluting. It might have been possible with a gradient with low elution strength, but that would increase the run time too much.

When comparing the performances of the HSS T3 and BEH columns, they gave the same retention order to the tryptamines, which is due to both having C18 stationary phases, although HSS T3 is known for retaining polar compounds longer than other reversed phase columns [59]. The observed difference in retention times between BEH and HSS T3 probably lies in the stationary phase support structure, which for the HSS T3 column is bare silica [59], as opposed to the BEH columns which has ethyl groups mixed with the support structure, making it less polar [131]. Additionally, the pKa (3.5 – 4.5) of the residual silanol groups in silica support structure, which are more plentiful in HSS T3, can cause unwanted peak tailing, especially for basic analytes such as tryptamines.

The columns that were used to separate the tryptamines were not new, they had been used before by other operators, analysing other analytes, for other projects. Column performances tend to decrease after a certain number of injections. Each mobile phase velocity was tested at least three times in a row, but their performance over several days was not tested. Using new columns and looking at performance over several days would have given a more solid basis to decide which column to use in the method. However, these are robust columns used in routine analysis every day, which means that their performance has been tested and confirmed over time by institutions that demand repeatable performance.

In all cases 0.1% formic acid (pH 2.7) in the aqueous phase provided faster analysis time compared to 10 mM ammonium formate (pH 3.1). Since the log D values of the



tryptamines decreases with lower pH (see Table 1.3, Chapter 1.2.2), they become more

Figure 3.6. Chromatograms of the 21 tryptamines injected into three different separation columns, Biphenyl (a and b), BEH (c and d) and HSS T3 (e and f). In chromatograms a, c and e 10 mM ammonium formate (pH 3,1) was used as aqueous phase. In chromatograms b, d and f, 0.1% formic acid (pH 2.7) was used. All the chromatograms show the retention time window from 1.25 minutes to 9.0 minutes. The peaks are normalized to the same height by the MassLynx software. The gradient used can be found in figure 2.2. Peak numbers are listed in table 3.1.

hydrophilic, making their affinity towards the stationary phase decrease, which could be what is causing the decreased retention times. Minimizing the retention window causes some peaks to coelute and others to move apart. However, as the retention window decreases, the general trend is that the peaks are more crammed together.

Based on these results it is obvious that the biphenyl stationary phase yields the highest peak capacity, and that the BEH column yields the shortest retention time window. HSS T3 shows substantial amounts of peak-tailing, which causes the peaks to overlap. Therefore, the choice stood between biphenyl and BEH. With the linear gradient used in this comparison test, BEH has both shorter retention time and better selectivity, BEH manages to spread out the peak cluster that the biphenyl column has with compounds six, seven, 10 and 11. Compounds six and seven are especially important to separate chromatographically because they have identical monoisotopic mass, and they produce the same daughter ions upon fragmentation . Several gradients were tested to separate these compounds on the biphenyl column, but it proved to be difficult. Although separating the two compounds on the biphenyl column could have been possible, it would have been at the expense of analysis time since a low elution strength mobile phase composition would have to be used in order to separate them.

To summarize, gradient elution with ammonium formate (pH 3.1) and methanol as aqueous and organic mobile phase solvents was found to be most suitable for this assay. A mobile phase velocity of 0,5 mL/min was considered the best choice, mainly to avoid pressure overload. The columns performances suggested that the BEH-column gave the most appropriate combination of speed of analysis and peak separation.

3.4 Sample preparation

In the evaluation of the different extraction methods, recovery, and precision (RSD) were the essential parameters to consider. International and national accreditation institutions have guidelines on these performance parameters and acceptable limits. Therefore, when considering and comparing the different sample preparation methods during testing, a recovery of 10 to 20% was considered a minimum, and an RSD lower than 15% was considered acceptable for each of the 21 tryptamines. This is in accordance with the

demands set up by relevant accreditation authorities [132]. If the sample preparation method gave results which went outside these limits it was considered less desirable.

All the samples were analysed right after sample preparation was finished, except for the PCX extracted samples which was analysed approximately 17-18 hours after extraction. This could have had an effect on the recovery of certain unstable analytes. Stability studies on the 21 tryptamines was performed in house by master student Monica Eidhammer during time frame of this project [133]. The conclusions were that the acetoxy substituted tryptamines were the most unstable, with 4-AcO-DIPT being stable only for a day in a fridge, and two weeks in a -20 °C freezer [133].

In the following discussion about sample preparation, six compounds were chosen as representatives for their substitution category, or as singulars. In order to make the presentation easier to follow MET represents the unsubstituted tryptamines, 4-AcO-DIPT represents acetoxy substituted tryptamines and 5-MeO-EPT represents the methoxy tryptamines. Psilocin and 5-OH-DMT did not behave in line with the rest of the hydroxy-tryptamines, psilocin therefore represents itself and 5-OH-DMT. DMT-N-oxide was the only compound of its kind in the method and thus represents itself only. Where necessary, all analytes are included.

3.4.1 Captiva ND Lipid extraction plates

The Captiva ND lipid filter plates are used by the routine department as a sample preparation method in the quantitative determination of amphetamines in blood. They work by removing particles, lipids, and proteins from blood samples through a polypropylene filter and has shown good results with extraction of compounds of varying polarity and hydrolytic behaviour from blood [134, 135].

In Figure 3.7 recovery and RSD for the 6 representative tryptamines are shown. This sample preparation method was based on protein precipitation with freezer cold (-20 °C) ACN:MeOH (85:15) mixture, and then filtration through the Captiva plates, followed by a partial evaporation step. The recovery of the different tryptamines seemed to follow a trend based on substituent types. The

tryptamines without a substituent in position 4 or 5 on the indole ring all show recoveries between 55 and 62%. Those having a methoxy group on position 4 or 5 had recoveries between 53 and 59%, and those with an acetoxy substituent on the mentioned positions gave between 37 and 47% yield. However, the tryptamines with hydroxy substituents were divided into two groups, one with 5-OH-DMT and 4-OH-DMT (Psilocin), with 51 and 50 % recovery, respectively, and the three other hydroxy tryptamines with recoveries around 10 %. The total sample preparation process with protein precipitation, filtration through the Captiva ND lipid plates, and evaporation resulted in RSD values between 4 and 18%.

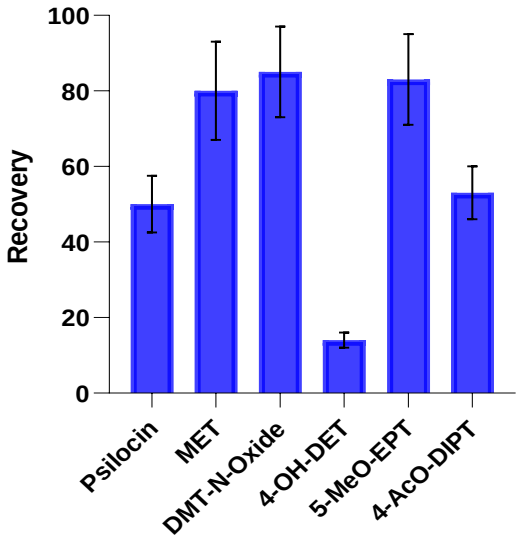


Figure 3.7. Percent recovery and standard deviation (error bars) of the six tryptamine representatives extracted with Captiva ND lipid filter plates. Extraction was performed with 6 replicates.

3.4.2 Captiva EMR plates

Captiva EMR lipid plates are the second-generation lipid removal plates from Captiva. They offer high lipid/matrix removal properties and very little analyte loss and work by combining size-exclusion and reversed phase principles [136]. In the routine laboratory they are used in a screening method for a wide range of drugs and prescription drugs, for both whole- and post-mortem blood. The properties demanded for sample clean-up when screening for such a wide range of analytes should also work well for the tryptamines, which have similarities with many of the compounds screened for in this method. Additionally, being quite similar to the Captiva ND plates, but without evaporation, this method could confirm if the evaporation step at the end of the Captiva ND method caused the low recovery for the hydroxy tryptamines.

As with the Captiva ND method, extraction started with a protein precipitation and centrifugation step to remove the red blood cells, followed by sample dilution with ethanol/water (30:70) and filtration through the EMR-plates. A difference from the Captiva ND lipid plate procedure was that the EMR-plates were conditioned with ACN/H_2O (4:1) with 1% formic acid. After being filtered through the EMR-plates, the samples were analysed directly without evaporation or dilution.

From Figure 3.8 it is clear that the Captiva EMR plates gave both high recovery and low RSDs for most of the compounds, whereas the hydroxy tryptamines showed low recovery and relatively high RSDs. This indicates that the above-mentioned evaporation step is most likely not the cause of the poor recovery for the hydroxy tryptamines.

Captiva EMR would have been an obvious choice as the most suited sample preparation method. However, since the hydroxy tryptamines have such a low recovery and the Captiva ND plates have acceptable recoveries and RSDs for the other tryptamines, and higher recovery for the hydroxy tryptamines, Captiva ND seems best suited if as many tryptamines as possible are to be included in the method.

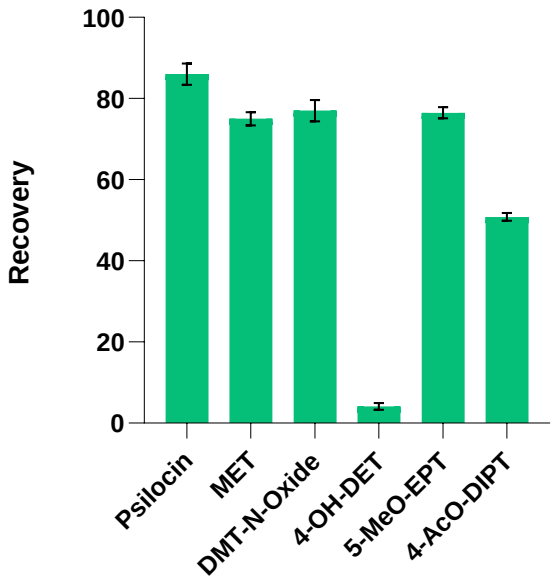


Figure 3.8. Recovery and RSD for the representative tryptamines using Captiva EMR lipid plates, with n = 6.

3.4.3 Liquid-Liquid Extraction

The tryptamines were extracted from blood with four different LLE conditions, to see if LLE in general was a sample preparation technique worth pursuing. According to the data in Table 1.3, Chapter 1.2.2 high pH is needed to have the analytes as uncharged species, and an increase or decrease in pH would shift the distribution of the different species of each compound to favour a higher or lower organic phase solubility. For the analytes with a phenol functional group, however, only limited amount of the substance will be uncharged at high pH.

Out of the four conditions two were with carbonate buffer (pH 10.3) and two with borate buffer (pH 11.3). Each of the two buffers were tested with one organic phase consisting of ethyl acetate and heptane (80:20 v/v), and another acceptor phase consisting of ethyl acetate and heptane mixed with isopropanol (64:16:20 v/v), to make the acceptor phase more polar. This has proven to increase extraction yields for polar compounds, making use of both hydrogen bonding and dipole interactions [137]. Additionally, LLE removes

much of the most abundant lipids found in full blood, such as phosphatidyl-choline and sphingomyelins, which protein precipitation by itself does not remove [95]. After adding the two phases and mixing, the samples were evaporated to dryness, reconstituted, and analysed.

As can be seen from Figure 3.9(a), when carbonate buffer was used with IPA added to the organic phase, the extraction results improved. Recovery for the unsubstituted and the methoxy tryptamines went down from above 100% to more logical values around 80 %, and the variation (precision) also improved. The other tryptamines improved as well, but to different degrees. For psilocin and 5-OH-DMT the results declined, with less recovery and unchanged variation.

Figure 3.9(b) shows the extraction of the representative tryptamines using borate- instead of carbonate buffer. The results were quite similar, but the precision when IPA was not used in the organic phase was better. The most relevant differences are the variations between the two non-IPA conditions, and higher RSDs for borate buffer with IPA compared to those of carbonate with IPA. Hence, the carbonate buffer with IPA gave the best results.

The effect of adding IPA to the acceptor phase of the borate buffer extraction was similar to adding it to the carbonate extraction. For the NN-alkylated- and the methoxy-tryptamines, the recovery went down from above 100% to between 67 and 92%. The acetoxy-tryptamines increased recovery and decreased the RSDs upon addition of IPA, which could be due to the increased polarity.

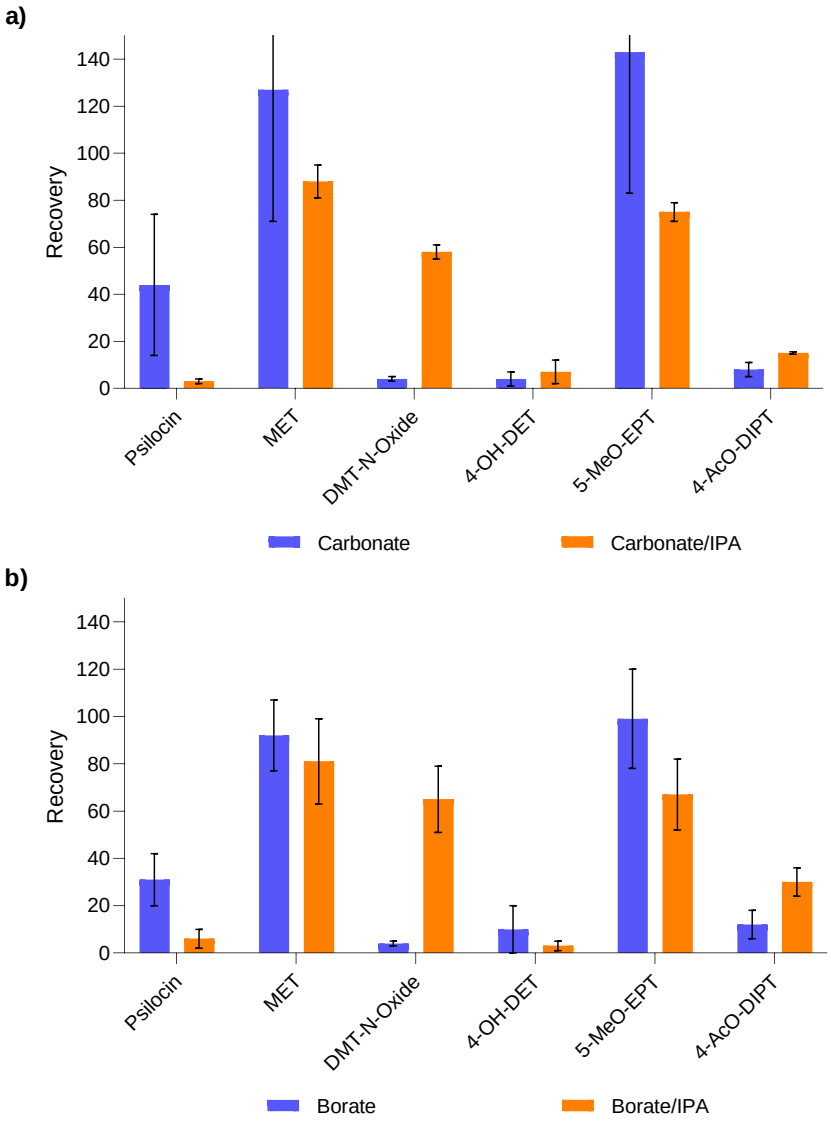


Figure 3.9. (a) Percent recovery and standard deviation for the six representative tryptamines. LLE with (orange) and without (blue) IPA in the organic phase and carbonate buffer (pH 10.3) in the aqueous phase. (b) Percent recovery and standard deviation for the six representative tryptamines. LLE with (orange) and without (blue) IPA in the organic phase and borate buffer (pH 11.3) in the aqueous phase.

As with the Captiva plates, the hydroxy tryptamines had poor recovery and precision (RSD) with these LLE conditions. At the pH of both the buffers approximately 10 to 20% of the hydroxy tryptamines are in a neutral form, the rest is divided between the different ionic species that arise from having both an amine and a phenol functional group. This could be part of the explanation for their low recovery. The evaporation step could in addition cause some tryptamines to evaporate or decompose. For example, psilocin is known to be a light sensitive and temperature labile compound [138], and although no previous stability studies have been found concerning the other hydroxy-tryptamines, instability might be a reason for their poor recovery and high variation.

3.4.4 Mixed mode cation exchanger

When considering the Bjerrum-plots, Chapter 1.2.2, a strong mixed-mode cation exchanger would likely give good extraction yields for the 21 tryptamines, and a modified procedure from a method the routine laboratory used for the screening of benzodiazepines and z-hypnotica in urine was therefore tested. Both psilocin and bufotenine (5-OH-DMT) have been extracted successfully from plasma with mixed mode cation exchange previously [139], and theoretically, this approach suites tryptamines well with its lipophilic moieties and amine functional group.

The PCX plates demonstrated quite good extraction recoveries and RSDs for the methoxy- and acetoxy tryptamines, as shown in Figure 3.10. While the other tryptamines had RSD values exceeding the desired limits of precision. The recovery of psilocin is particularly high, possibly due to signal enhancement. Additionally, the RSDs for psilocin, MET and DMT-N-Oxide are above the RSD limits.

Since the method starts with a protein precipitation step similar to that of the Captiva ND lipid plate procedure, and the recoveries for the hydroxy tryptamines are as low. This could indicate that the hydroxy tryptamines have a higher affinity to the polar aqueous phase. Drug-protein binding might also be a factor.

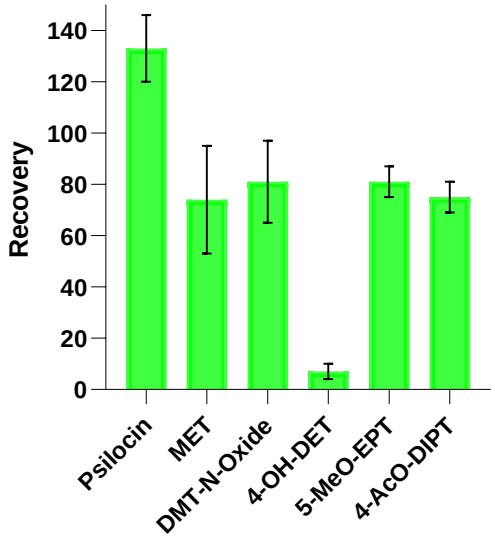


Figure 3.10. Recovery and standard deviation for the representative tryptamines, with n=4, Using a mixed mode strong cation exchange method.

Mixed mode ion exchange is supposed to be a selective sample preparation method, but in this case the results gave too high RSDs. There are many steps to this procedure, and they all have optimization potentials for the tryptamines. For example, the second washing step with MeOH/H_2O (1:1) might have eluted some of the hydroxy analytes, because of the solution being too polar or too lipophilic. Secondly, for unstable compounds the exposure to a strong basic solution in the elution step, might also have caused some degradation of the compounds.

Even though the mixed mode cation exchanger would have given satisfying results for all the compounds, it would still be considered a complicated and time-consuming preparation method. The number of different steps and relatively high solvent consumption speaks against choosing it as part of the analysis method.

3.4.5 Electromembrane extraction

Since the hydroxy tryptamines are charged over the whole aqueous pH spectrum EME is theoretically an ideal match, as it uses transport of charged species across an organic membrane as extraction pinciple. EME is a simple and fast extraction technique as it extracts the analytes into an aqueous solution that can be injected into the LC-MS/MS analyser without time consuming evaporation or clean-up procedures [97]. Most published works on EME extractions from biological matrices use plasma [98, 101]. One paper from 2008 by *Gjelstad et al* extracted basic drugs from whole blood with recoveries between 19 and 50% [140]. Therefore, testing EME for the 21 tryptamines seemed reasonable.

Since the two SLMs, DEHPi and the deep eutectic solvent coumarin:thymol (2:1) had shown promising results extracting polar bases [98, 101], they were also used in these experiments. The analytes were extracted both from neat solutions and when spiked in whole blood. For the DEHPi SLM both 15- and 30-minutes extraction time was tested. In both the donor and acceptor solutions 100 mM formic acid was added to maintain charged analytes. Tests were made before the extractions to make sure that the buffer capacity of whole blood was surpassed, and pH maintained low enough for the tryptamines to keep their charge.

As Figure 3.11 shows the deep eutectic solvents extracted the tryptamines poorly. Only MET and DMT-N-Oxide showed any consistency, but with quite low recovery. None of these have any substituents on the phenyl group, this could have opened up for strong π-type interactions between these analytes and coumarin, which has a high aromatic affinity [98]. The many matrix-ions and large charged particles in whole blood could also be the reason for the unstable current seen in Figure 3.11 top panel, even at a voltage down to 2 V,

The current profiles of EME with DEHPi as the SLM were good, and from the buffer-to-buffer extraction (Figure 3.12 lower panel) it was obvious that the tryptamines easily traversed the SLM with the conditions used (see chapter 2.3). However, the results with whole blood as matrix had slightly low recovery and precision. The complexity of the

whole blood matrix could be an important cause of this [140], as well as slower extraction kinetics due to higher viscosity in whole blood.

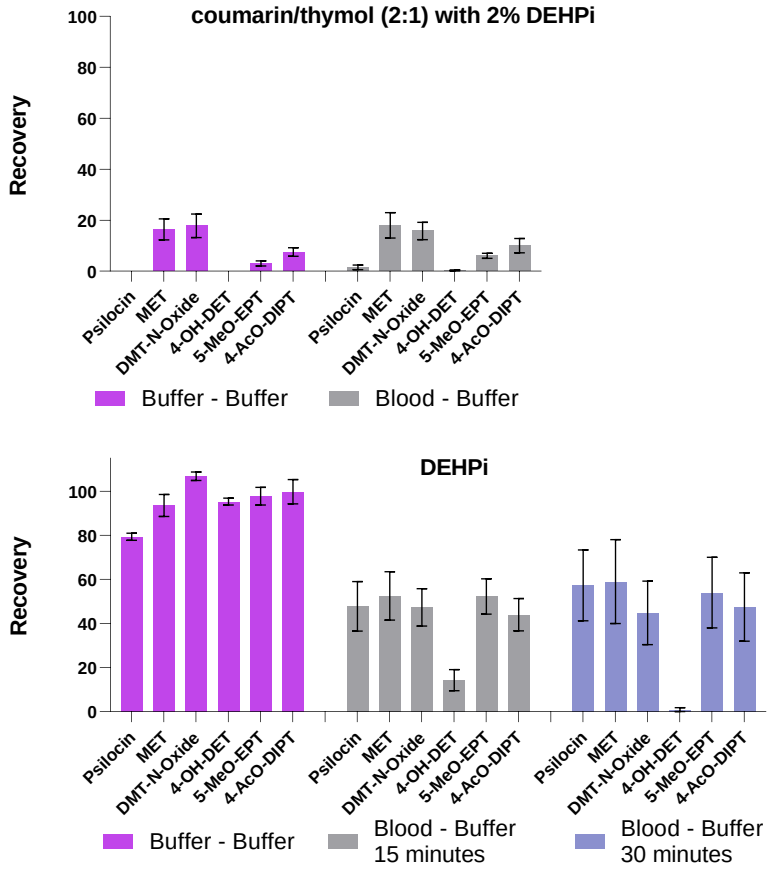


Figure 3.11. Extraction recoveries from EME of 6 representative tryptamines. Upper panel, coumarin/thymol with 2% DEHPi as SLM. Lower panel, DEHPi as SLM. Donor and acceptor phase separated by hyphen in the figures.

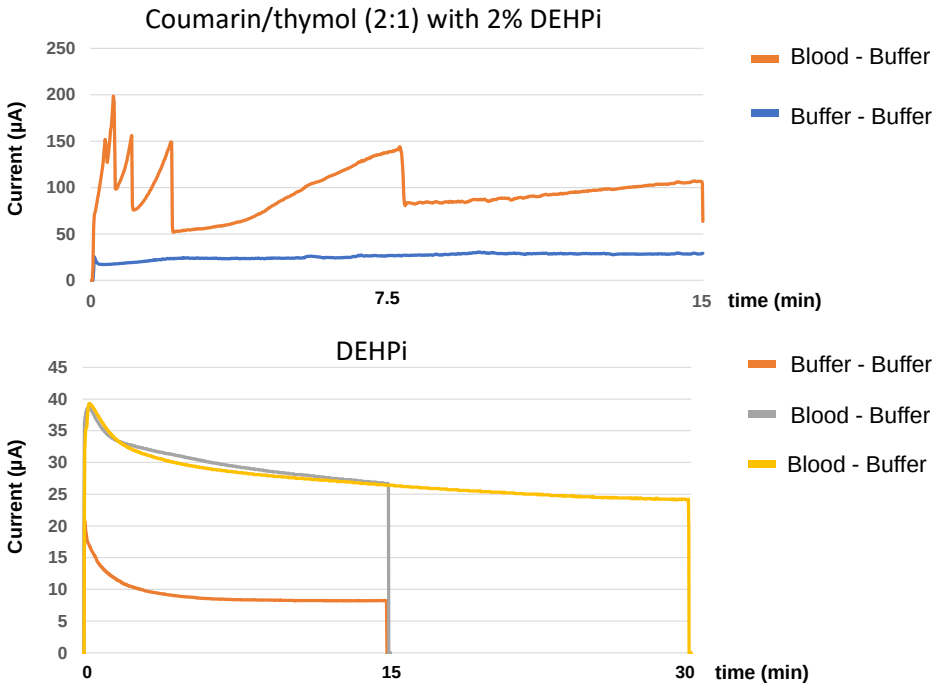


Figure 3.12. Current profiles of EME of 21 tryptamines. Top, coumarin/thymol (2:1) with 2% DEHPi as SLM. Lower, DEHPi as SLM. Donor and acceptor phase separated by hyphen in the figures.

3.4.6 Addition of ascorbic acid

Since the neurotransmitter serotonin (5-hydroxy-tryptamine) is quite similar to the hydroxy tryptamines, and has a high level of protein binding in the blood [141], it was suspected that an extraction method for this compound could be used to extract the hydroxy tryptamines and the other tryptamines. Furthermore, many of the articles concerning serotonin determination in blood has used ascorbic acid as a preservative [142, 143]. Indeed, ascorbic acid is a compound commonly employed to preserve oxygen sensitive analytes, such as psilocin and bufotenine [139], and it seemed sensible to investigate if it would help extract the hydroxy- and the other tryptamines.

The investigated serotonin determination methods used perchloric acid as precipitating agent [144, 145], and PPT of the tryptamines with low pH had been tried, resulting in dirty

extracts. Therefore, certain critical steps of a PPT method used to extract olanzapine from whole blood, containing an addition of ascorbic acid, and using methanol as the precipitating agent [146], was transposed to the Captiva ND-method already used. This resulted in recoveries in line with the previous Captiva ND results, however, the hydroxy tryptamines were extracted to the same degree as the other tryptamines. This indicated that the addition of ascorbic acid, or the use of methanol as precipitant and organic layer, caused the hydroxy tryptamines to be extracted. To examine this, a series of four different extraction conditions were set up as shown in Table 3.2. As the Captiva EMR method provided the best results for the other tryptamines, the conditions were also tested on that method.

Table 3.2. Conditions for comparison of two different extraction methods with or without ascorbic acid with two different precipitants.

Extraction method	Condition	Additive	Precipitant
Captiva ND	1	-	ACN/MeOH (85:15)
Captiva ND	2	Ascorbic acid	ACN:MeOH (85:15)
Captiva ND	3	-	MeOH
Captiva ND	4	Ascorbic acid	MeOH
Captiva EMR	1	-	ACN:MeOH (85:15)
Captiva EMR	2	Ascorbic acid	ACN:MeOH (85:15)
Captiva EMR	3	-	MeOH
Captiva EMR	4	Ascorbic acid	MeOH

As presented in Figure 3.11, condition 1 for both the Captiva ND and Captiva EMR methods represented the original conditions (see Chapters 3.3.1 and 3.3.2), which gave acceptable recoveries for all the tryptamines except for the hydroxy tryptamines. Adding ascorbic acid, condition 2, dramatically improved the recovery for the hydroxyl tryptamines for both methods. Replacing ACN:MeOH (85:15) with methanol as the precipitant, condition 3 and 4, varied in the same way as condition 1 and 2, respectively,

although the recoveries were reduced. These results indicate that ascorbic acid causes the increased recovery of the hydroxy tryptamines regardless of precipitation solvent.

Because of serotonins role in coagulation and vasoconstriction its presence in blood must be controlled [147]. In the blood this is done primarily by Human Serotonin Transporter (hSERT) [148]. According to a study on the hSERT protein, the hydroxy substituent on the serotonin molecule is instrumental in hSERTs selectivity towards serotonin [149]. Because of the similarities between the hydroxy tryptamines and serotonin, the hydroxy tryptamines might also bind to hSERT. A possible cause of the effect ascorbic acid had on the hydroxy tryptamine extraction is that the polyphenol group of the added ascorbic acid molecule could occupy this binding site. Thereby preventing the hydroxy tryptamines binding to it.

The results of the eight different extraction procedures (Figure 3.11) show that the ACN:MeOH precipitation mixture gave slightly higher recoveries compared to the pure MeOH precipitant. The reason for this could be that the high proton-accepting properties from MeOH, and dipole-interacting properties from ACN were combined in the ACN/MeOH mixture. As precipitants ACN is known to give slightly more clean extracts than MeOH, but with a lower range of extracted compounds [150]. With the tryptamines, having both protic and electron-rich substituents, it is no surprise that the ACN/MeOH precipitation solvent would yield higher recoveries compared to MeOH alone, since acetonitrile and MeOH complement each other with hydrophobic and proton-accepting properties, respectively [151].

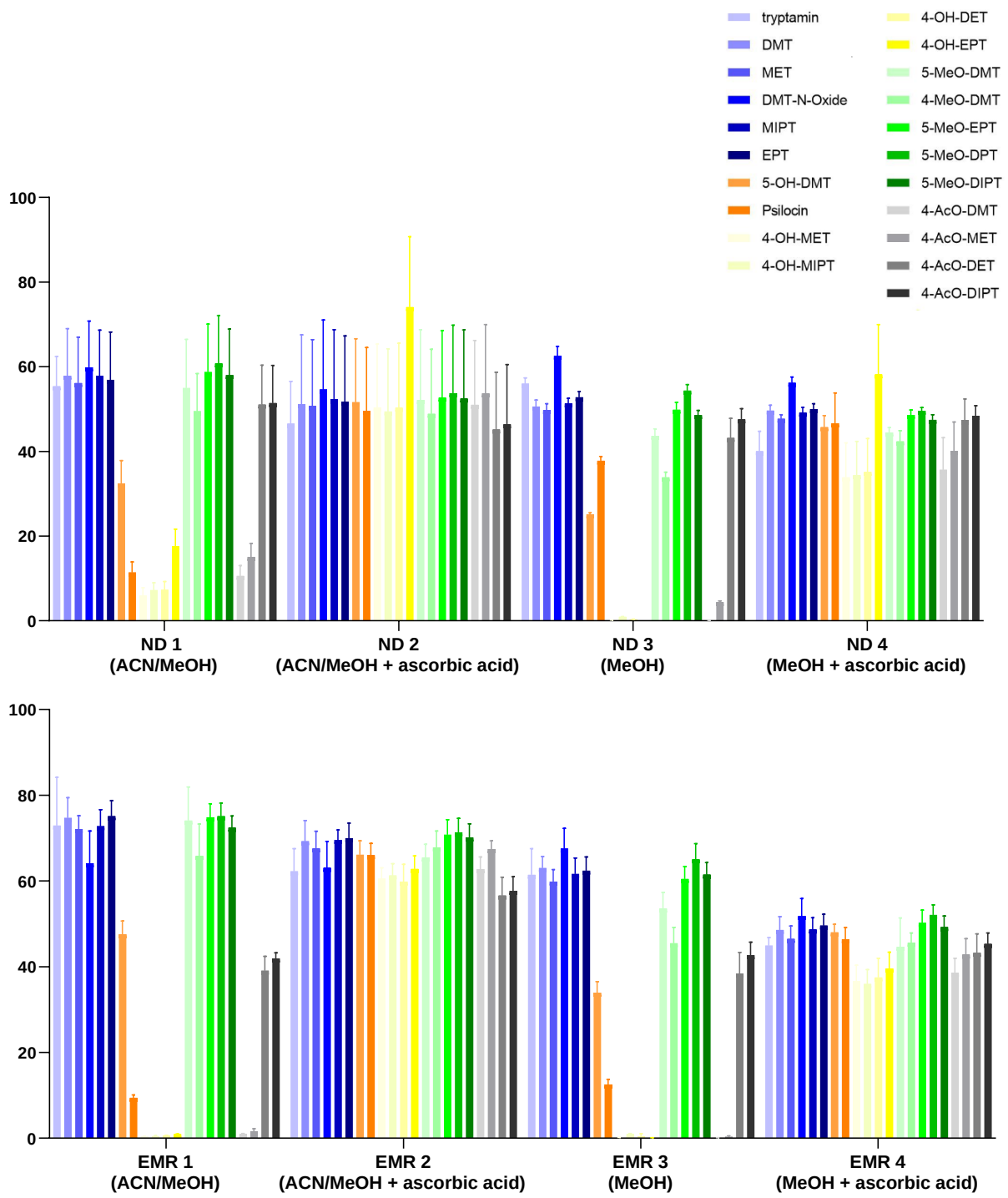


Figure 3.11. The recoveries for the 21 tryptamines using Captiva ND filter plates (upper panel) and Captiva EMR (lower panel) with and without addition of ascorbic acid before precipitation, and different precipitation solvents. Each extraction method was performed using 4 replicates. Precipitation solvent and additive are given in parentheses.

The supernatant from the MeOH precipitations had a slight yellow colouring compared to the ACN/MeOH solvent. Colouration in the extracts is undesirable because it most likely indicates the presence of compounds that could degrade the separation columns and contaminate the inlet cone of the MS. This favours the acetonitrile and methanol mixture.

High extraction recovery can give lower LOQs. Therefore, the sample preparation method with highest recoveries is usually prioritized, unless it has other severe drawbacks. Because of higher recovery and better precision, the Captiva EMR method with ascorbic acid added and with acetonitrile/MeOH (85:15) as precipitant, was chosen as the sample preparation method in the determination of the 21 tryptamines.

3.5 Method validation

To prove the functionality of the method, a validation is needed. This is normally composed of at least 5 – 7 series with additional selectivity and matrix effect studies. However, because of time limitations only three complete series were analysed. Therefore, this is only a method evaluation.

Determination of matrix effects is an important part of method validation, due to time constraints this was however not covered in this method evaluation. The recovery from the extraction method was included in Table 3.3, but this is the recovery from the initial extraction experiments given in Figure 3.11 lower panel. Usually, recovery is reported at two different concentration levels.

Although tryptamine had been part of the validation process from the beginning, it was decided to exclude it from the method because it is endogenous to humans. The psilocin-d10 internal standard was prepared from an old reference material and deemed to have decomposed giving other species present in the solution. As a consequence, 4-OH-MIPT had an interfering background peak and could only be detected qualitatively.

3.5.1 Pre-validation optimization

When testing the validation method, it was found that the earliest eluting compounds (5-OH-DMT and psilocin) had somewhat distorted peaks due to the filtrate being injected directly, without any evaporation or dilution with mobile phase. This especially affected the lower concentrations of these compounds. Since both the compounds are in circulation in society, especially psilocin, the method needed to be able to determine concentrations down to at least 5 nM.

Initially 1 µL was injected into the UHPLC-MS/MS system, but this caused the mentioned distorted peaks. Partial evaporation and dilution with 50 µL ammonium formate buffer 10 mM, pH 3.1 with 5 % methanol was tested in order to be able to inject more than 1 µL without getting distorted peaks.

3.5.2 Precision and Accuracy

Precision was determined by extraction and analysis of five QC samples at five different concentration levels from low to high concentration within the range 2 to 5000 nM, each level with three replicates. At the low end, if precision was poor for one of the analytes, the cut-off was raised to the QC sample above.

Accuracy (bias) was determined by calculating the percentage by which the mean of the QC samples deviated from the theoretical value. Table 3.3 shows the precision and accuracy for the 19 tryptamines. Precision between the QC samples at the different concentration levels varied between 2 and 19%, while accuracy varied between -9 and 62%. If the QC samples at 2 nM having values outside 20% is removed, as stated above, the precision varied between 2 and 18%, and accuracy varied between – 4 and 18%, which is within criteria set by international guidelines [152], while having a LOQ of minimum 5 nM. However, for a full validation, more series have to be analysed.

3.5.3 Limit of detection and limit of quantification

The LOD for each compound was determined by multiplying the standard deviation of the lowest QC with precision (RSD) and bias ≤20%, with three and adding the average of the blank samples. The lower limit of quantitation was determined the same way but multiplying with 10 instead of three. As for the upper limit of quantitation the concentration of the highest calibrator was used.

The goal with respect to LOD and LOQ was that the cut-off (LOQ) should be the same or better than 5 nM. This was achieved. Although the sample preparation method was relatively comprehensive, with both PPT and the use of filter plates, matrix effects could still be the cause of the hydroxy tryptamines all (except 5-OH-DMT) having cut-off at 5 nM and not 2 nM which was the lowest calibrator and quality control concentration. The cause could also be their behaviour in the ESI. The capillary voltage used in this study was 1 kV. In 2016 Vaiano et al made a screening method which included 4-OH-DIPT and 5-MeO-DIPT, both had LOQs of 0.3 ng/mL [81]. In that method 4 kV was used, but with a different instrument. Furthermore, their sample preparation method with 91 and 85% recovery could also have contributed to the lower LOQs.

Table 3.3. Sensitivity, precision and accuracy for the determination method of 19 tryptamines in whole blood using PPT, Captiva EMR filter plates and UHPLC-MS/MS, n=3.

Compound	LOD (ng/mL)	LOQ (ng/mL)	QC sample conc. mean. nM	ng/mL	Precision (RSD) (%)	Bias (%)
5-OH-DMT	0.15	0.50	4269	872	9	7
			513	104.8	4	3
			31	6.3	11	3
			5	1	6	0
			2.2	0.45	11	8
Psilocin	0.23	0.76	4165	850.8	8	4
			527	107.7	6	5
			32	6.6	12	8
			5.1	1	7	2
			2.4	0.49	25	18
4-OH-MET	0.25	0.84	4233	924.1	15	6
			517	112.9	6	3
			31	6.7	13	2
			4.8	1	8	-4
			2.4	0.52	27	18
DMT	0.22	0.72	4155	782.2	4	4
			511	96.1	2	2
			30	5.7	5	1
			5.3	1	9	5
			2.3	0.4	17	16
4-OH-DET	0.28	0.92	4149	964	12	4
			518	120.2	4	4
			32	7.4	11	6
			5	1.2	8	0
			2.5	0.58	26	27
5-MeO-DMT	0.14	0.46	4166	909.4	12	4
			521	113.7	4	4
			31	6.7	7	3
			5.2	1.1	9	4
			2.1	0.5	10	7
MET	0.22	0.72	4260	861.7	5	6
			506	102.4	4	1
			31	6.2	4	3
			5.1	1	4	2
			2.3	0.5	16	14
5-MeO-EPT	0.23	0.76	4260	1109.2	10	7

Compound	LOD (ng/mL)	LOQ (ng/mL)	QC sample conc. mean. nM	ng/mL	Precision (RSD) (%)	Bias (%)
			522	135.8	4	4
			32	8.3	12	7
			5.3	1.4	8	6
			2.1	0.5	14	4
EPT	0.38	1.26	4195	966.3	5	5
			515	118.7	4	3
			32	7.4	6	7
			5.5	1.3	10	10
			2.5	0.58	29	23
5-MeO-DIPT	0.23	0.76	4325	1186.9	9	8
			520	142.6	3	4
			31	8.4	12	2
			5.2	1.4	12	4
			2.2	0.6	13	9
4-ACO-DIPT	0.46	1.52	4573	1383	10	14
			504	152.5	6	1
			27	8	14	12
			5.7	1.7	9	13
			3.2	0.97	12	62
5-MeO-DPT	0.34	1.13	4227	1159.9	12	6
			495	135.9	11	-1
			28	7.6	18	-7
			4.9	1.3	20	-3
			2.3	0.6	18	15
DMT-N-oxide	0.28	0.92	4168	851.3	7	4
			528	107.8	5	6
			32	6.5	8	7
			5.3	1.1	8	5
			2.4	0.5	19	18
4-AcO-DMT	0.46	1.53	4100	1009.7	12	2
			456	112.3	4	-9
			30	7.5	10	1
			5.2	1.3	12	3
			2.4	0.59	20	19
4-OH-EPT	0.4	1.4	4204	1035.6	7	5
			514	126.6	6	3
			29	7.2	9	-3
			5.2	1.3	11	4
			2.6	0.64	8	32

Compound	LOD (ng/mL)	LOQ (ng/mL)	QC sample conc. mean. nM	ng/mL	Precision (RSD) (%)	Bias (%)
4-AcO-MET	0.6	2.1	4331	1127.5	9	8
			475	123.7	13	-5
			28	7.3	14	-7
			5.8	1.5	14	16
			2.6	0.68	7	32
MIPT	0.21	0.7	4267	923.1	7	7
			511	110.5	4	2
			30	6.6	5	1
			5.1	1.1	8	2
			2.3	0.5	14	16
4-MeO-DMT	0.37	1.25	4214	919.8	9	5
			514	112.2	4	3
			31	6.7	9	2
			5.5	1.2	10	10
			2.5	0.55	18	25
4-AcO-DET	0.48	1.59	4614	1266	7	15
			492	135.1	3	-2
			28	7.6	11	-8
			5.6	1.5	10	12
			3.2	0.88	17	60

3.5.4 Calibration model and range

Seven calibrators with two replicates were prepared in the range of 2 to 5000 nM, and calibration curves were made for each of the tryptamines, based on the ratio of the peak height of the analytes versus the peak height of an internal standard. When deciding on a calibration model, a weighted (1/x) second-order regression line, including the origin, was found to give the best fit to the data points for all the tryptamines based on inspection of residuals and calibration curve fit. Calibration range was set between LOQ and the highest calibration standard. To determine curve fit the regression coefficient R^2 calculated by the MassLynx software was used, and had a value above 0.99 for all compounds, Table 3.4.

Table 3.4. Calibration range, Calibration curve and regression coefficients for the 20. Tryptamines.

Compound	Calibration range		Calibration curve	R^2
	nM	ng/mL		
5-OH-DMT	2 - 5000	0.4 - 1021	Quadratic	>0.99
Psilocin	5 - 5000	1.0 - 1021	Quadratic	>0.99
4-OH-MET	5 - 5000	1.1 - 1091	Quadratic	>0.99
DMT	2 - 5000	0.4 - 941	Quadratic	>0.99
4-OH-DET	5 - 5000	1.2 - 1161	Quadratic	>0.99
5-MeO-DMT	2 - 5000	0.4 - 1091	Quadratic	>0.99
MET	2 - 5000	0.4 - 1011	Quadratic	>0.99
DMT-N-Oxide	2 - 5000	0.4 - 1021	Quadratic	>0.99
4-AcO-DMT	2 - 5000	0.5 - 1231	Quadratic	>0.99
4-OH-EPT	5 - 5000	1.2 - 1231	Quadratic	>0.99
4-AcO-MET	5 - 5000	1.3 - 1301	Quadratic	>0.99
MIPT	2 - 5000	0.4 - 1081	Quadratic	>0.99
4-MeO-DMT	5 - 5000	1.1 - 1091	Quadratic	>0.99
4-AcO-DET	5 - 5000	1.4 - 1371	Quadratic	>0.99
5-MeO-EPT	2 - 5000	0.5 - 1301	Quadratic	>0.99
EPT	2 - 5000	0.5 - 1371	Quadratic	>0.99
5-MeO-DIPT	2 - 5000	0.5 - 1372	Quadratic	>0.99
4-AcO-DIPT	2 - 5000	0.6 - 1512	Quadratic	>0.99
5-MeO-DPT	2 - 5000	0.5 - 1372	Quadratic	>0.99

4 Conclusion and further work

In the present study a determination method for 19 tryptamines have been developed, not 21 as planned. Tryptamine was taken out due to it being an endogenous compound and 4-OH-MIPT could only be detected qualitatively because of coelution with psilocin-d10 residues.

All three column chemistries tested provided acceptable peak shapes and separation. However, the BEH-C18 column gave the most suitable compound separation, the best peak shapes, and the fastest separation times. Together with the other parameters that were optimized and used in the final method the chromatographic separation was satisfactory in that it could separate the 21 tryptamines rapidly and effectively. A MS/MS method with MRM-transitions that distinguished co-eluting compounds, and that could determine the tryptamines quantitatively was successfully developed.

Of the five sample preparation techniques tested, Captiva EMR and Captiva ND provided the best recovery and precision, whereas extract cleanliness was sufficient for all methods tested. Operation time was shortest for EME. However, none of the techniques could extract the hydroxy tryptamines above 20% recovery in the original set-up. Addition of ascorbic acid to the whole blood sample and extraction with the Captiva EMR and Captiva ND methods, allowed the extraction of the hydroxy tryptamines with satisfactory yield, as well as the other tryptamines. Ascorbic acid addition was not tested on the other techniques. Of the two Captiva methods, EMR provided best recovery and precision compared to Captiva ND, but not faster operation time because an evaporation step had to be added. The Captiva EMR method with ascorbic acid addition provided sensitive and precise extraction for all the 21 tryptamines.

A complete validation of the developed method was not achieved within the time limits of the master period. However, three complete series were analysed, and an evaluation of the method could be made. The evaluation shoved that the tryptamines could be analysed with satisfactory precision, accuracy, and linearity, according to current guidelines [152], with LODs between 0.14 and 0.48 ng/mL.

Further work should include a full validation of the method in line with current guidelines [152], including matrix effects, studies of possible interferences from other medicinal drugs or drugs of abuse and testing of dilution integrity. Furthermore, addition of ascorbic acid to the other sample preparation methods, LLE, SPE and EME, to see if the hydroxy tryptamines could be extracted would also be of great interest.

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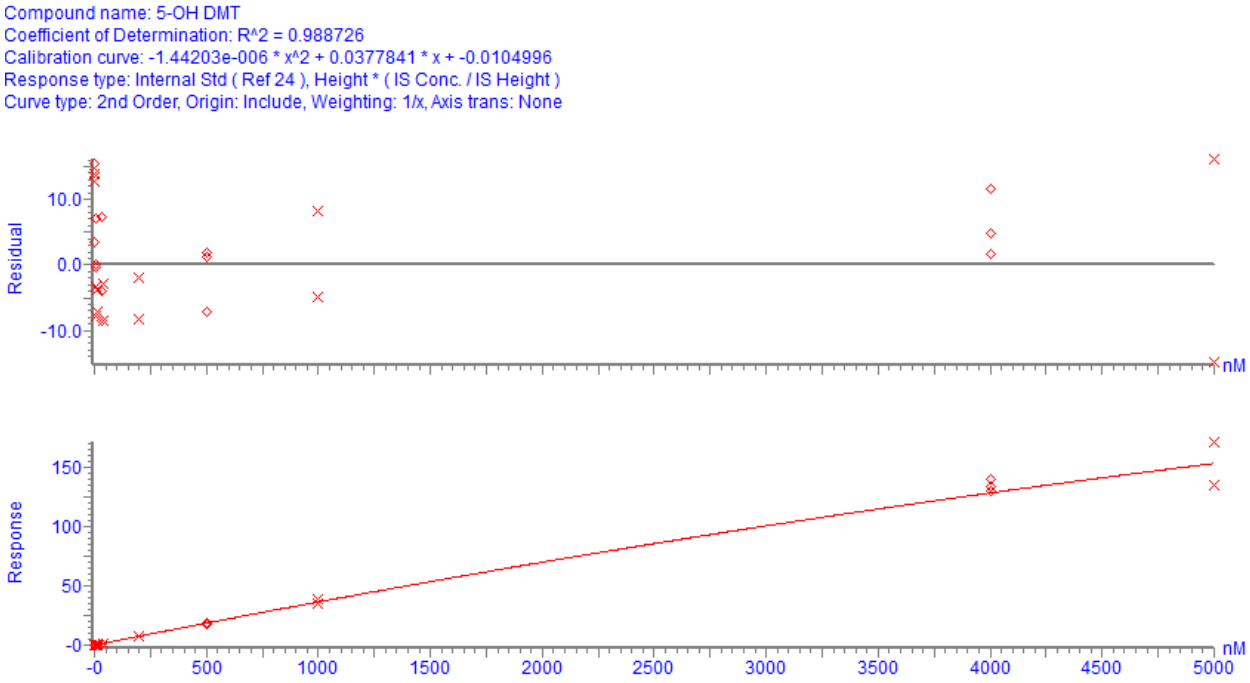
6 Appendix

6.1 Validation data

This chapter contains calibration curves for each analyte from one of the three validation series. The results from each replicate, both from the two replicates of the calibration standards (x) and the three replicates from the quality control samples (o) for each compound, are shown. The results are discussed in Chapter 3.



Compound name: PSILOCIN
Coefficient of Determination: R^2 = 0.998510
Calibration curve: -2.98472e-007 * x^2 + 0.0122094 * x + -0.00629231
Response type: Internal Std (Ref 24), Height * (IS Conc. / IS Height)
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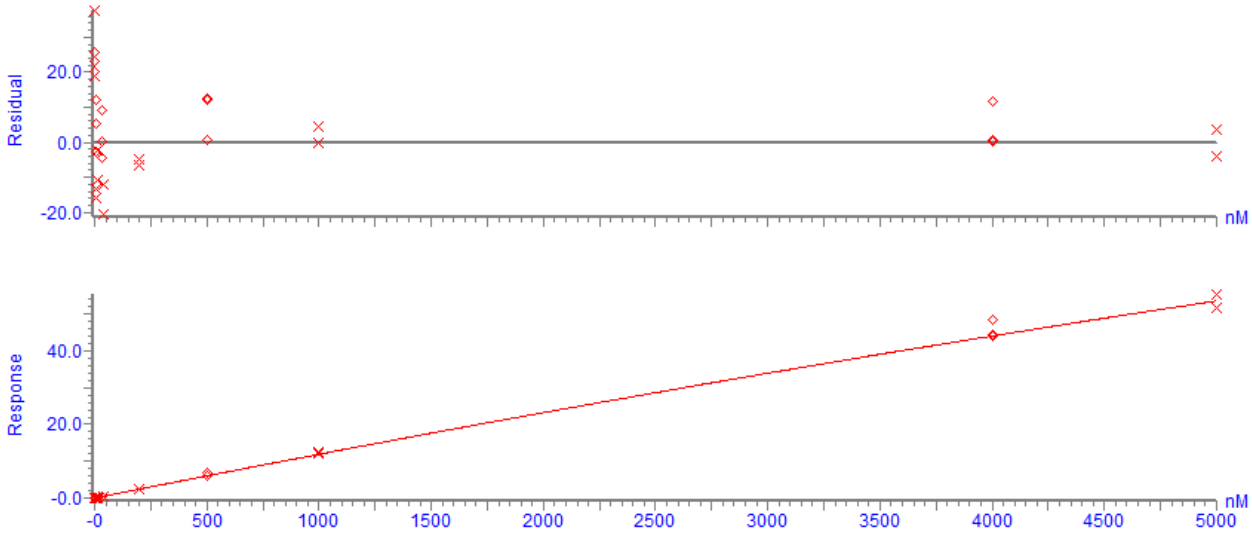

Compound name: 4-OH-MET
Coefficient of Determination: R^2 = 0.994667
Calibration curve: -1.23581e-006 * x^2 + 0.0208954 * x + -0.012538
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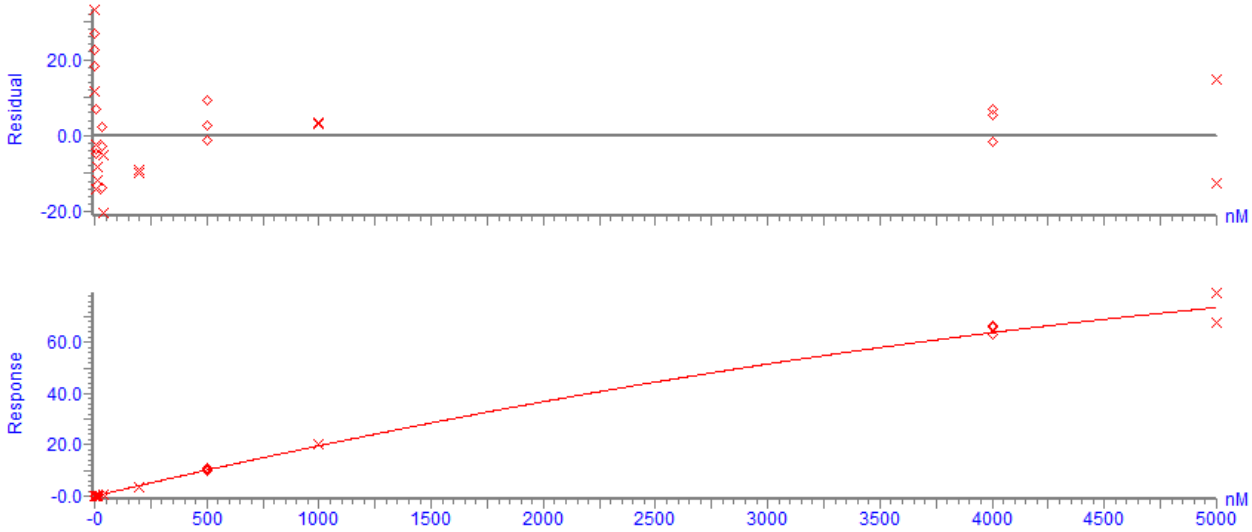

Compound name: DMT
Coefficient of Determination: R^2 = 0.999736
Calibration curve: -1.31922e-008 * x^2 + 0.00125242 * x + -0.000209244
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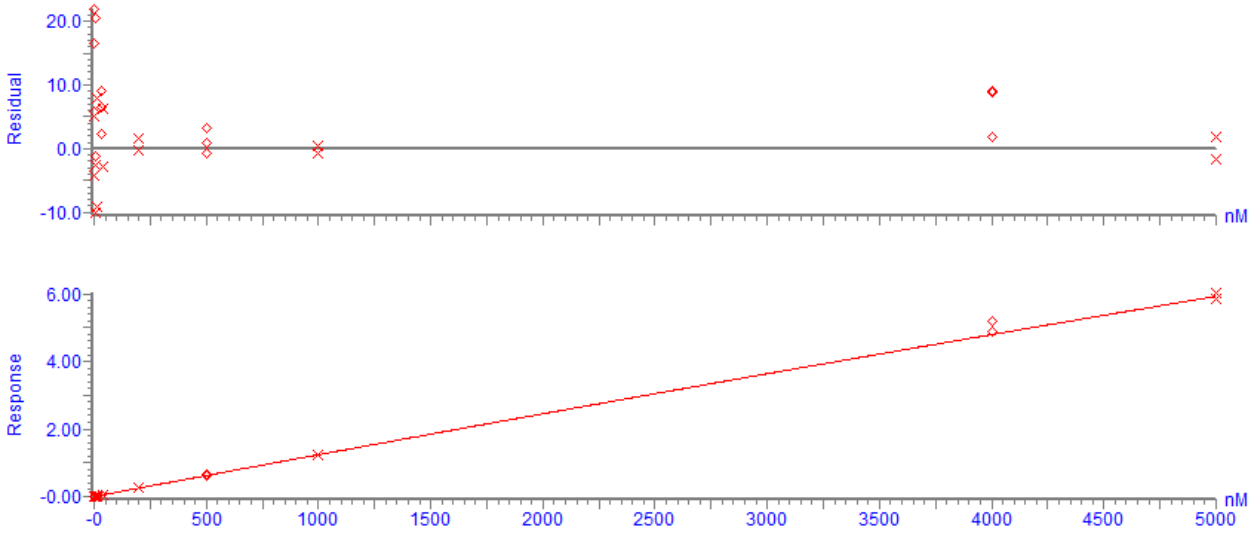

Compound name: 4-OH-DET
Coefficient of Determination: R^2 = 0.996221
Calibration curve: -6.78662e-007 * x^2 + 0.022541 * x + -0.0175776
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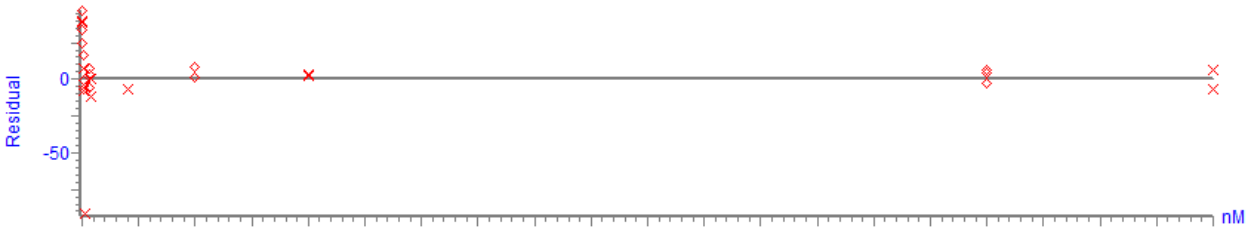

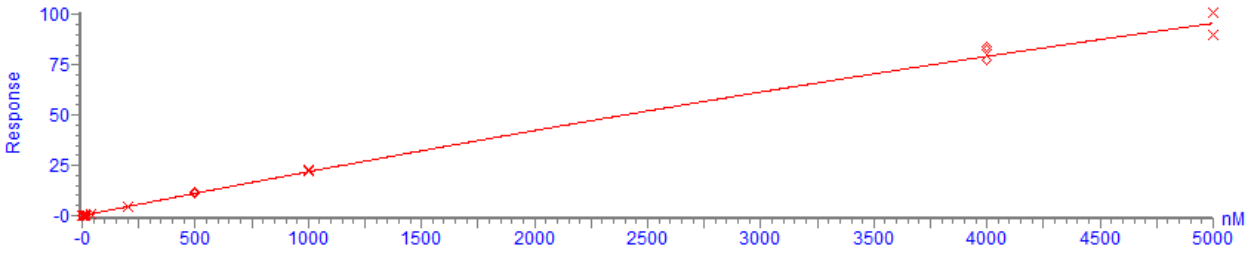

Compound name: 4-OH-MIPT
Coefficient of Determination: R^2 = 0.830845
Calibration curve: -1.06561e-006 * x^2 + 0.00533901 * x + 0.0326579
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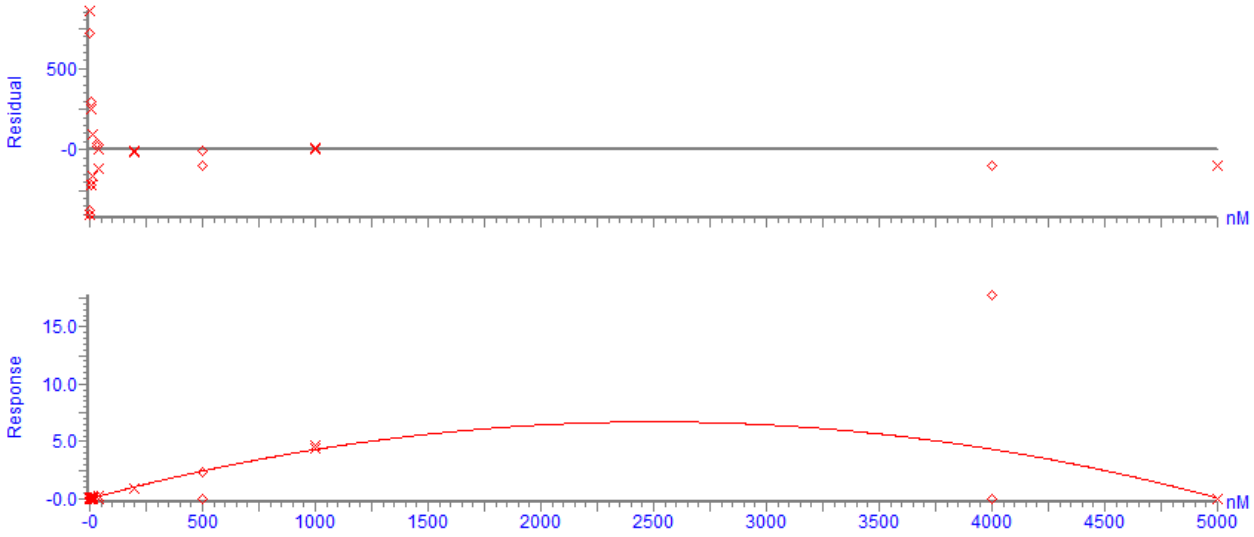

Compound name: 5-MeO-DMT
Coefficient of Determination: R^2 = 0.998844
Calibration curve: 1.64627e-006 * x^2 + 0.078716 * x + 0.0133784
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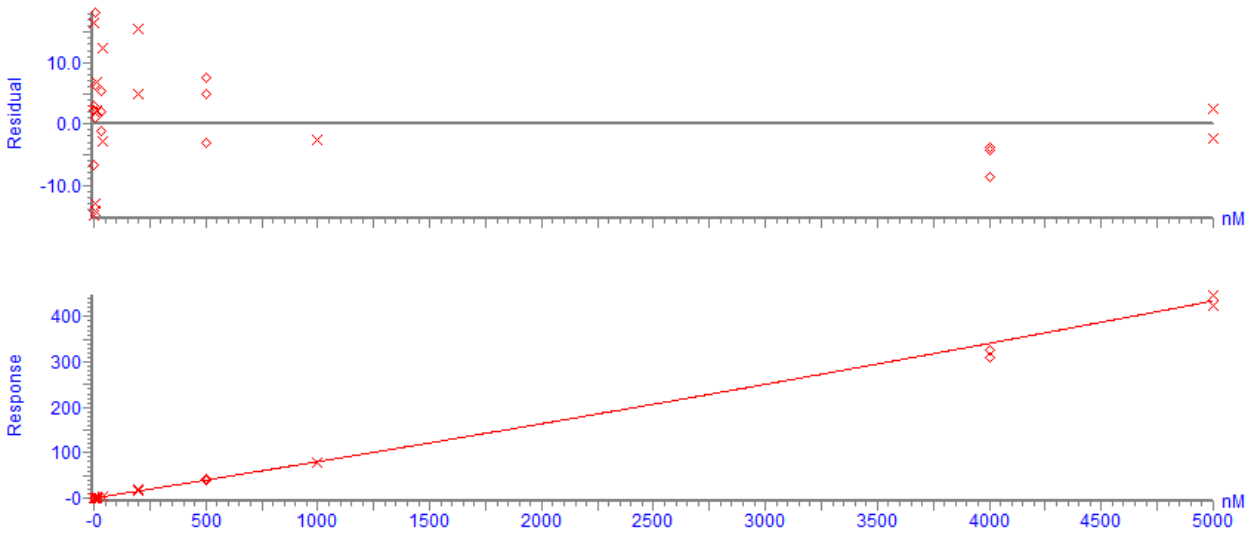

Compound name: MET
Coefficient of Determination: R^2 = 0.999581
Calibration curve: -1.2937e-007 * x^2 + 0.0041098 * x + -0.000260747
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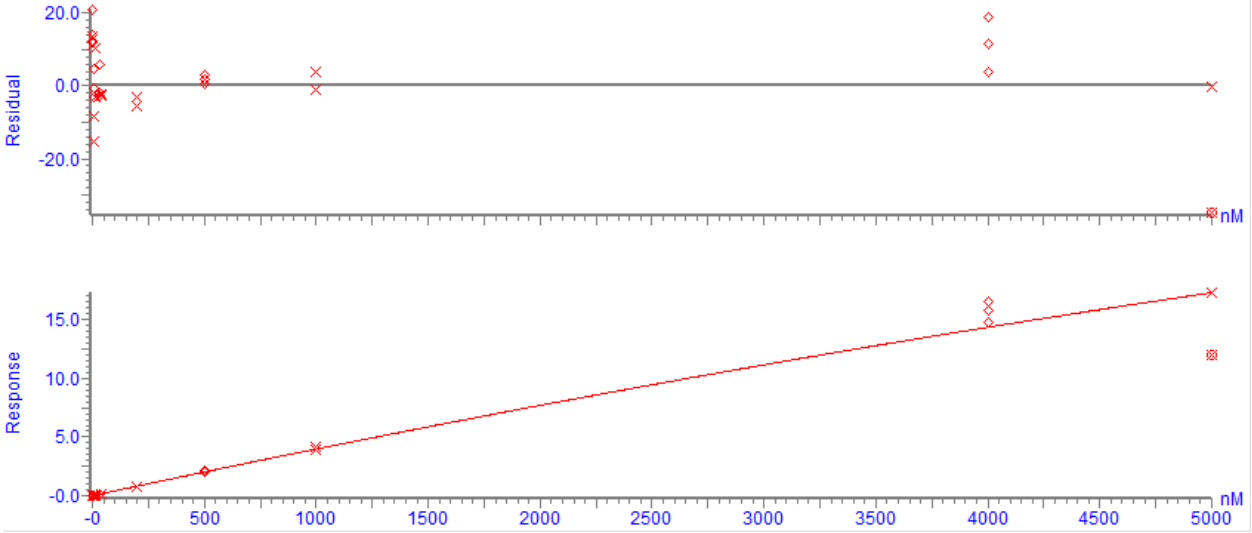

Compound name: DMT-N-OKSYD
Coefficient of Determination: R^2 = 0.999385
Calibration curve: -5.98323e-008 * x^2 + 0.00332307 * x + -0.000840524
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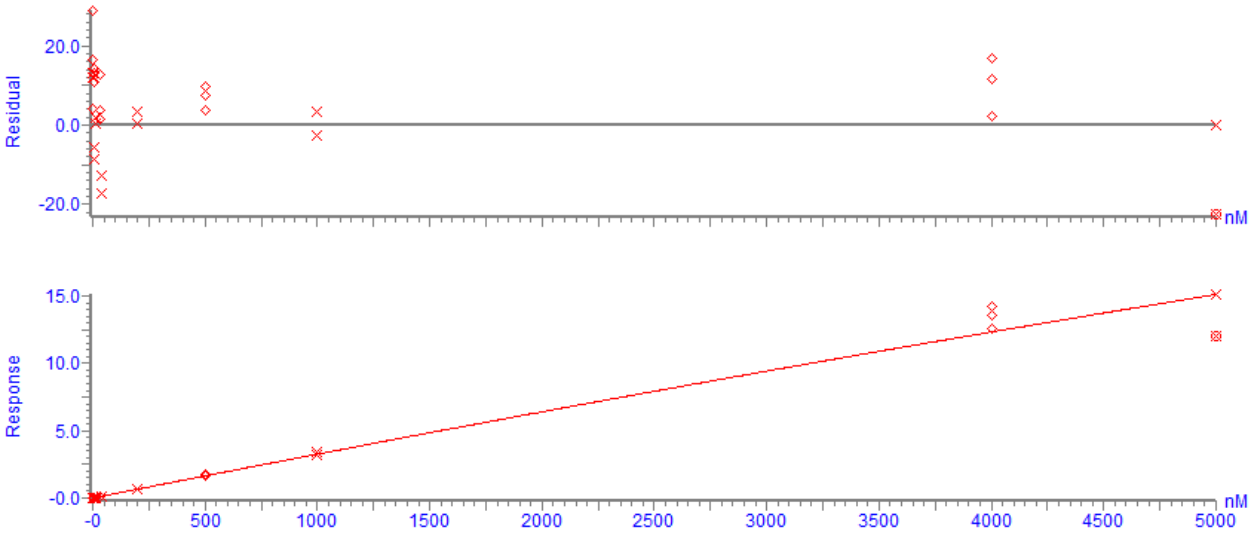

Compound name: 4-AcO-DMT
Coefficient of Determination: R^2 = 0.998608
Calibration curve: 4.13328e-006 * x^2 + 0.0615793 * x + 0.01234
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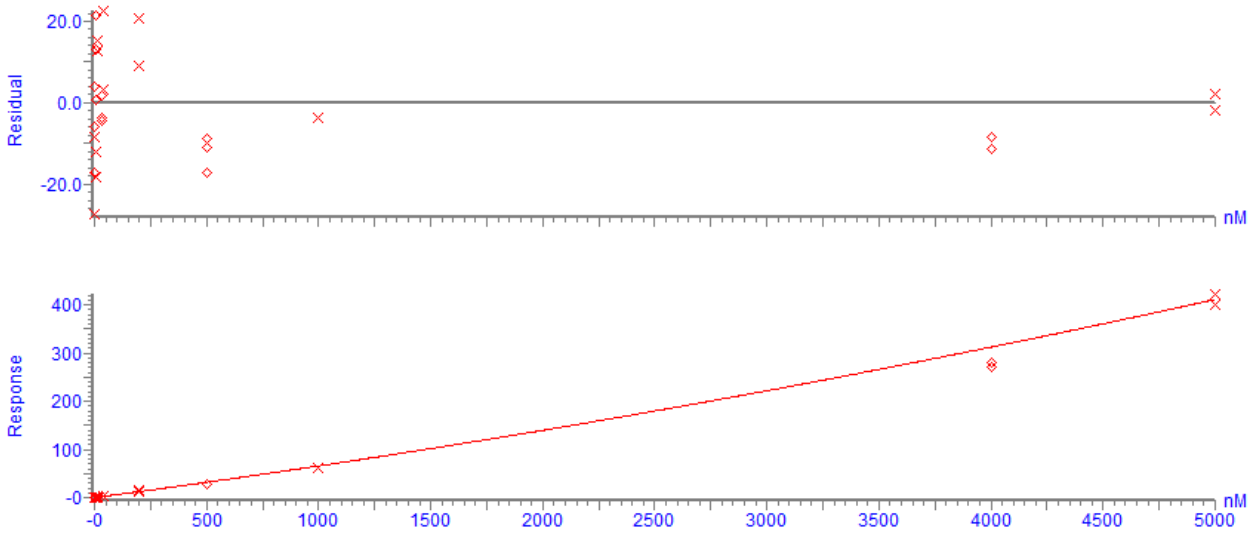

Compound name: 4-OH-EPT
Coefficient of Determination: R^2 = 0.995992
Calibration curve: -2.33976e-007 * x^2 + 0.0293868 * x + -0.0258395
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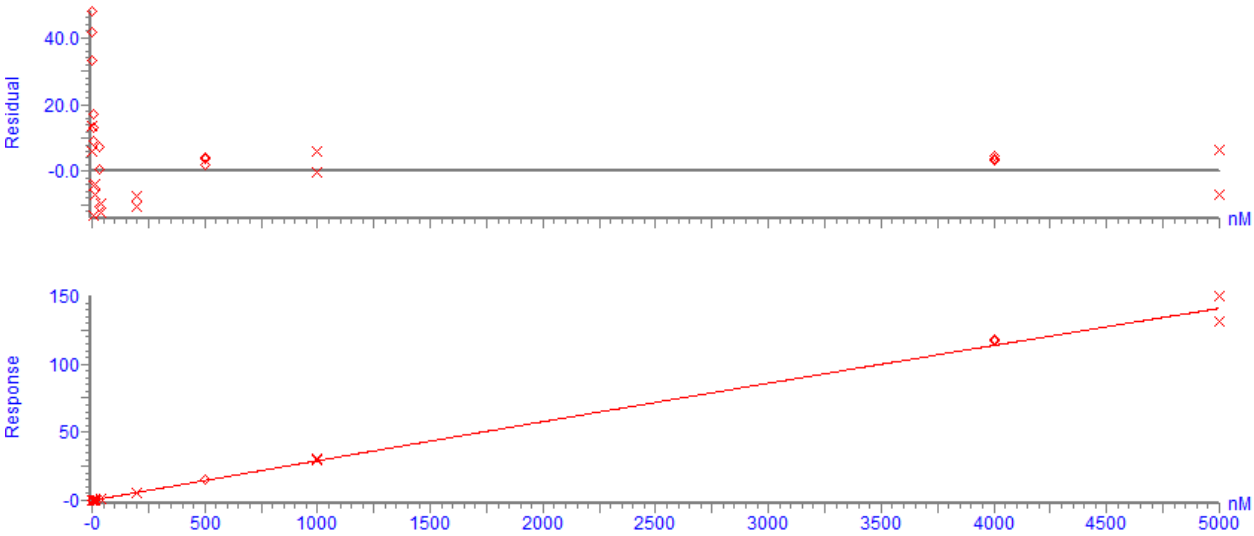

Compound name: 4-AcO-MET
Coefficient of Determination: R^2 = 0.999198
Calibration curve: 2.41114e-007 * x^2 + 0.0245829 * x + -0.0149106
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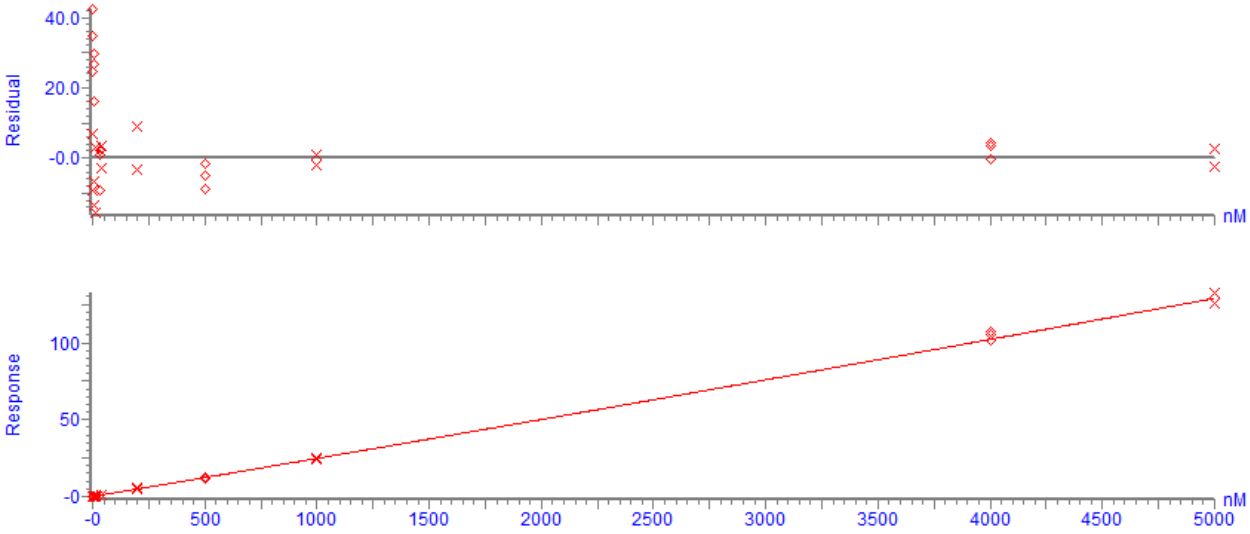

Compound name: MIPT
Coefficient of Determination: R^2 = 0.999701
Calibration curve: -8.13434e-008 * x^2 + 0.00259662 * x + -0.000374164
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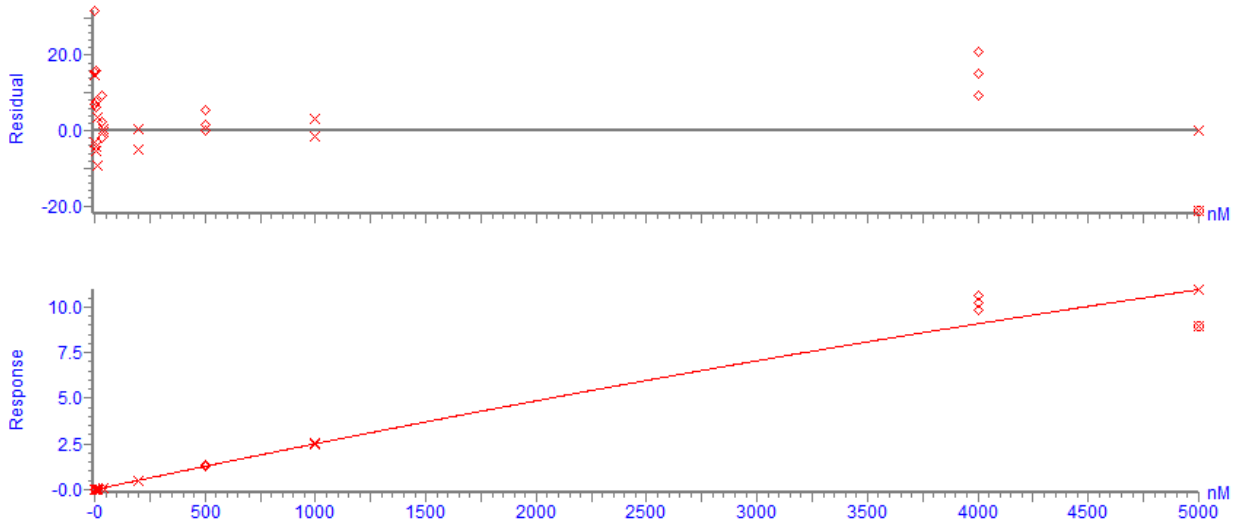

Compound name: 4-MeO-DMT
Coefficient of Determination: R^2 = 0.999697
Calibration curve: -3.71428e-006 * x^2 + 0.151601 * x + 0.108722
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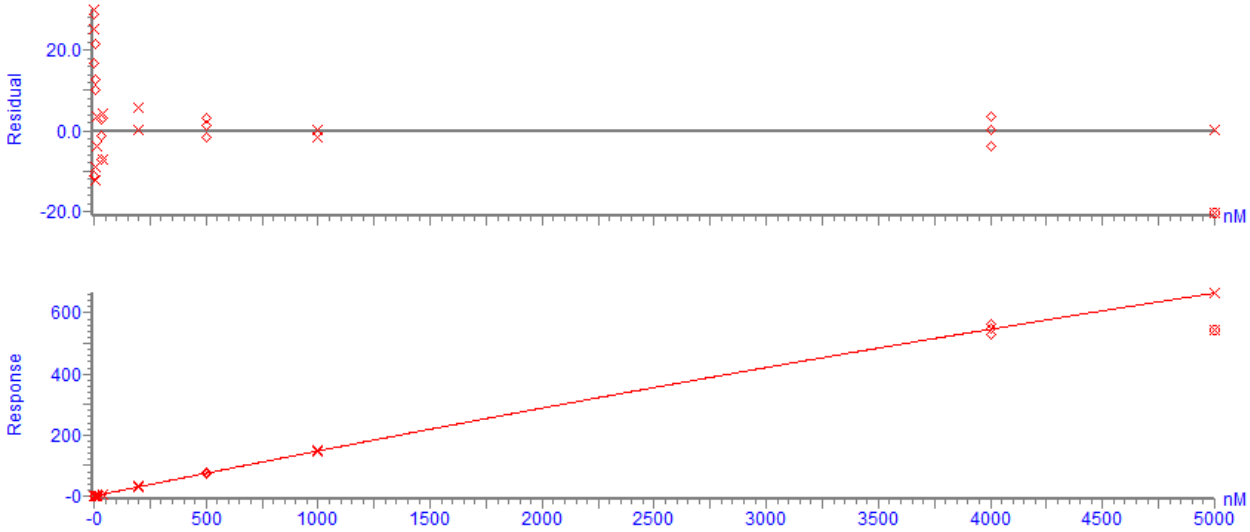

Compound name: 4-AcO-DET
Coefficient of Determination: R^2 = 0.999228
Calibration curve: 2.32672e-006 * x^2 + 0.146609 * x + -0.125208
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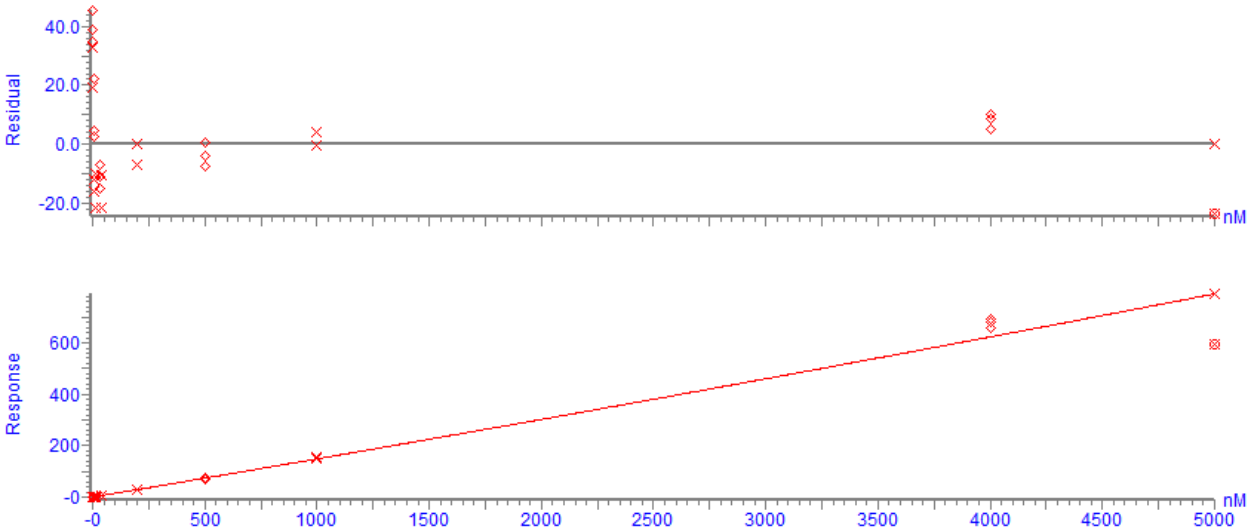

Compound name: 5-MeO-EPT
Coefficient of Determination: R^2 = 0.999310
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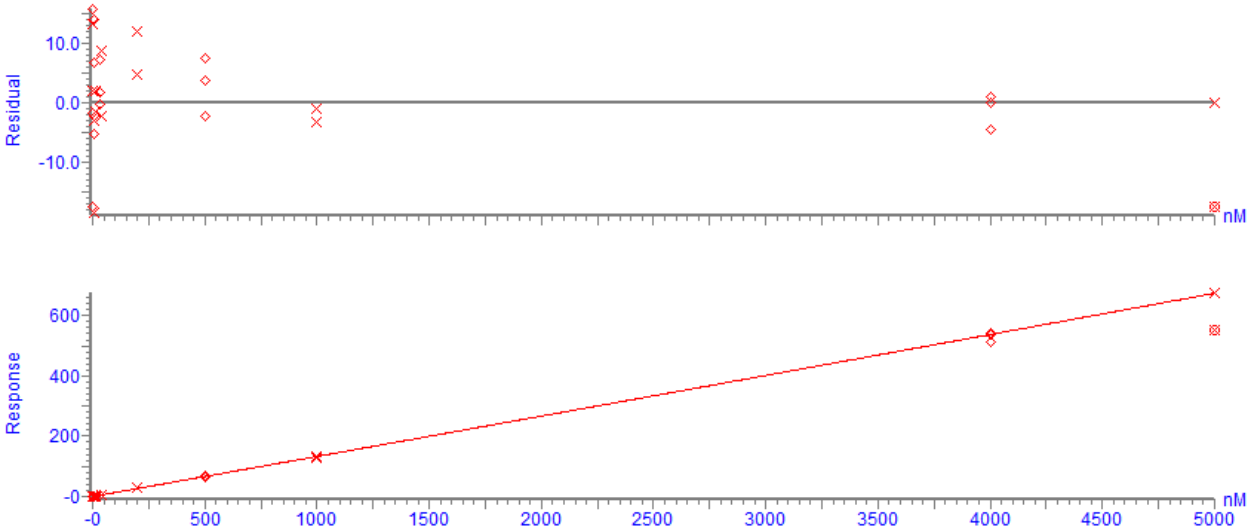

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Coefficient of Determination: R^2 = 0.999829
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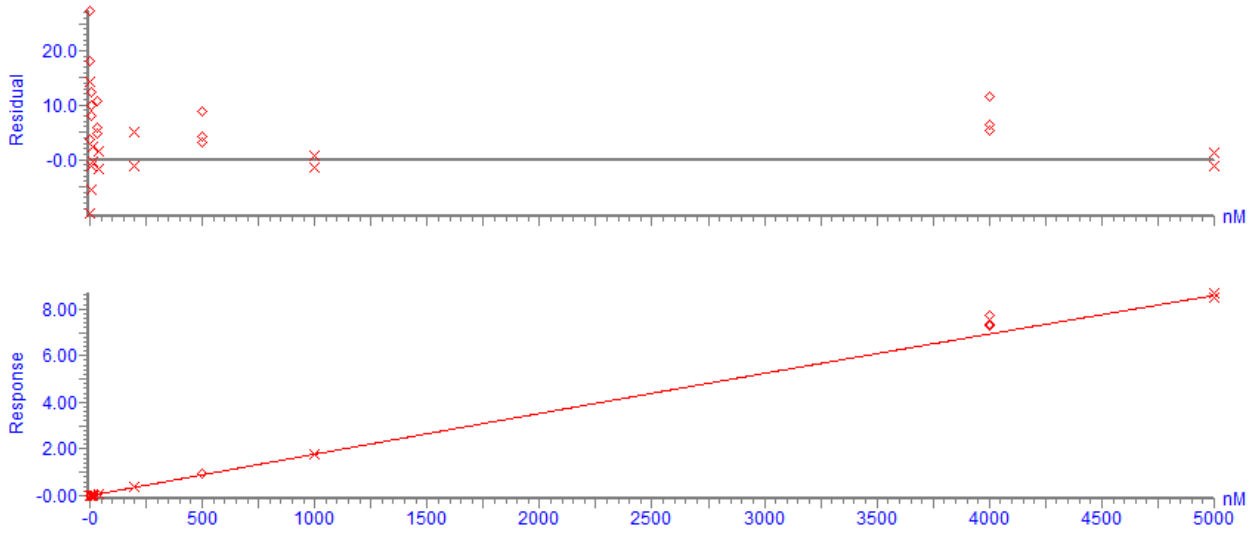

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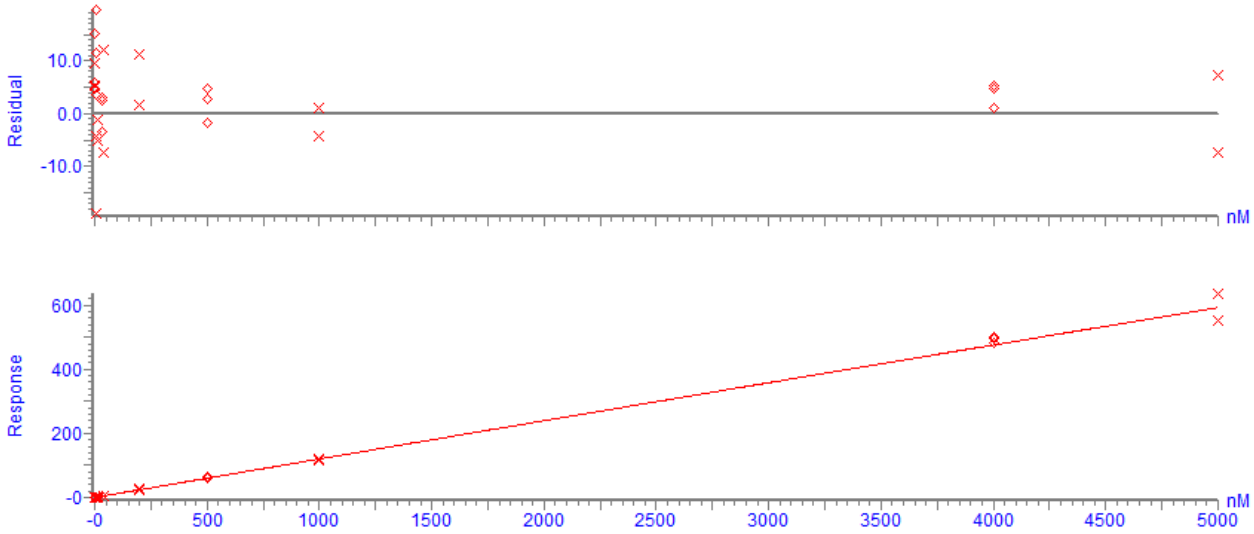
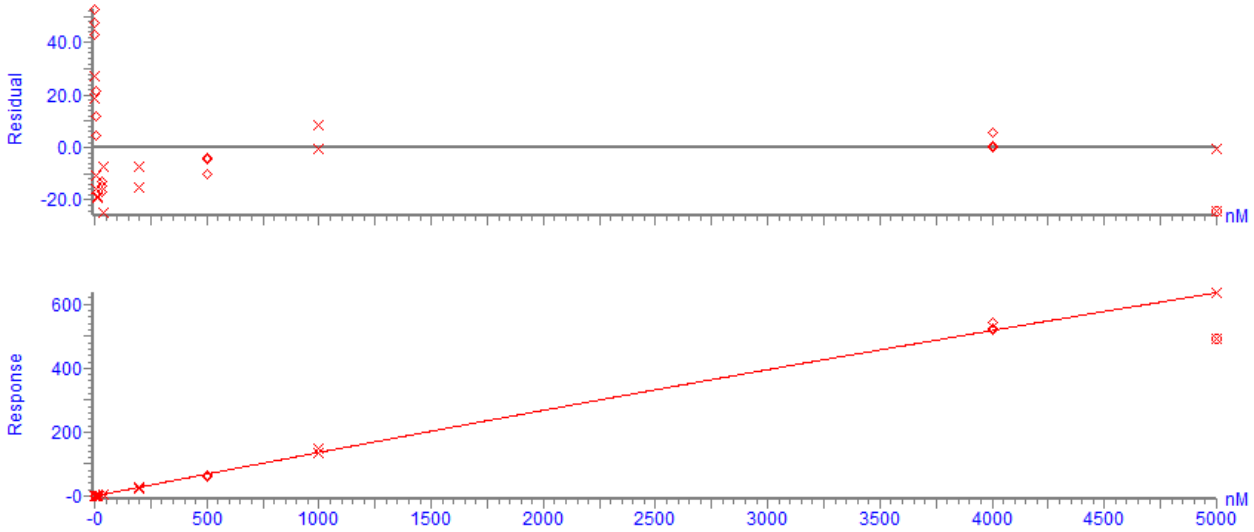
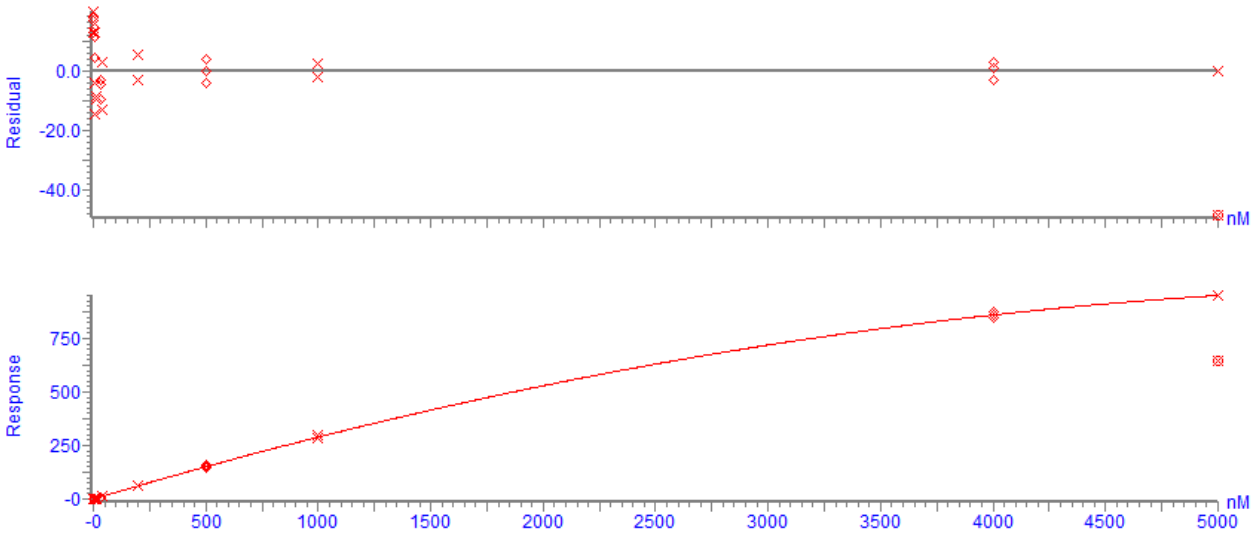
Coefficient of Determination: R^2 = 0.995423
Calibration curve: -4.40092e-007 * x^2 + 0.120712 * x + -0.000662011
Response type: Internal Std (Ref 25), Height * (IS Conc. / IS Height)
Curve type: 2nd Order, Origin: Include, Weighting: 1/x, Axis trans: None



Compound name: 4-ACO-DIPT
Coefficient of Determination: R^2 = 0.997417
Calibration curve: -2.33635e-006 * x^2 + 0.13896 * x + -0.184265
Response type: Internal Std (Ref 25), Height * (IS Conc. / IS Height)
Curve type: 2nd Order, Origin: Include, Weighting: 1/x, Axis trans: None



Compound name: 5-MeO-DPT
Coefficient of Determination: R^2 = 0.999350
Calibration curve: -2.48086e-005 * x^2 + 0.314243 * x + -0.0831875
Response type: Internal Std (Ref 25), Height * (IS Conc. / IS Height)
Curve type: 2nd Order, Origin: Include, Weighting: 1/x, Axis trans: None



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