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Determination of amphetamines in hair samples using microextraction by packaged sorbent and gas chromatography coupled to mass spectrometry

VERSÃO APÓS DEFESA

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Dedicatória

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Resumo Alargado

Na literatura científica encontram-se descritos diferentes protocolos de extração e de *clean-up* para a determinação de estimulantes relacionados às anfetaminas (ATS) em cabelo. A microextração em seringa empacotada (MEPS) é uma versão miniaturizada da extração em fase sólida (SPE) que tem sido aplicada para determinar diversas drogas em cabelo (como por exemplo, opiáceos, cocaína, cetamina, etc.). No entanto, no que diz respeito aos ATS, a MEPS só foi utilizada no cabelo para a determinação de anfetamina (AMP) e metanfetamina (MAMP). O objetivo deste trabalho foi o desenvolvimento e validação de um método para determinar anfetamina (AMP), metanfetamina (MAMP), 3,4-metilenodioxianfetamina (MDA), 3,4-metilenodioximetanfetamina (MDMA), 1-(1,3-benzodioxol-5-il)propan-2-il(etil)amina (MDE) e *N*-metil-1-(1,3-benzodioxol-5-il)-2-aminobutano (MBDB) no cabelo usando a MEPS. Após lavagem e corte, 50mg de cabelo foram incubados com 500 µL de NaOH 1M. Com o objetivo de promover a extração dos compostos foram estudadas diferentes condições: (1) 80 ºC durante 1 hora, (2) 60 ºC durante a noite e (3) 45 ºC durante a noite. Após a incubação, os extratos foram neutralizados com 50 µL de HCl 10M e posteriormente centrifugados. A melhor condição da etapa de hidrólise foi 45 ºC durante a noite.

Adicionalmente após a hidrólise foi estudada a necessidade ou não de uma precipitação/diluição do extrato obtido. Assim, foram estudadas as seguintes condições: adição de 500 µL de H2O; filtração da sobrenadante seguida de adição de H2O; e precipitação com acetonitrilo congelado seguido de centrifugação. A melhor condição foi a adição de 500 µL de H2O.

O sobrenadante foi submetido a um processo de MEPS com o objetivo de ter um extrato mais limpo. Nesta etapa foram estudados diferentes parâmetros a fim de maximizar a eficiência desta etapa. Os parâmetros estudados foram: número de aspirações da amostra (variando entre 5 a 15 aspirações de 100 µL); número de aspirações da etapa de lavagem (variando de 1 a 3 aspirações com 50 µL de H_2O e 50 µL de H_2O:metanol 95:5); e número de aspirações na etapa de eluição (variando de 2 a 6 eluições com 100 µL de 2% NH4OH em acetonitrilo). Este procedimento foi otimizado com recurso aos *softwares* MiniTab e SPSS, mediante a realização de um Desenho Experimental (DOE), Superfície Resposta (SR) e uma análise não paramétrica. Através desta análise estatística, foi possível concluir que os parâmetros otimizados para o procedimento de MEPS foram: 18 aspirações na etapa de passagem da amostra; zero aspirações para a etapa de lavagem e 7 aspirações com 100 µL de 2% NH_4OH em

acetonitrilo para a etapa de eluição. Uma análise não paramétrica foi posteriormente realizada para a lavagem, a fim de confirmar que zero lavagens eram, de facto, a melhor opção para o método. Para isso, foram realizados ensaios com zero lavagens, lavagens com H_2O e, por fim, lavagens com H_2O e H_2O:metanol 95:5. Foi possível confirmar que, efetivamente, houve perda de compostos ao utilizar algum tipo de lavagem pelo que no método final não foi incluída esta etapa.

As recuperações de 8-14% para a AMP, 14-20% para a MAMP, 10-15% para a MDA, 18-28% para a MDMA, 25-43% para a MDE e 34-52% para a MBDB. O método foi validado conforme *guidelines* da *ANSI/ASB* permitindo ainda alcançar os cutoff propostos pela *Society of Hair Testing* (0,2 ng/mg). Obteve-se linearidade no intervalo de concentrações entre 0,2 - 5,0 ng/mg com coeficientes de determinação superiores a 0,99. A precisão e a exatidão do método desenvolvido cumpriram as diretrizes internacionais para validação de métodos (coeficientes de variação ≤ 20% e BIAS±20%).

Este é o primeiro método analítico que utiliza a MEPS acoplada à cromatografia gasosa –espetrometria de massa (CG-MS) para determinar a determinação de anfetaminas e derivados em amostras de cabelo, provando ser uma excelente alternativa aos procedimentos de *clean-up* clássicos.

Palavras-chave

Anfetaminas;Cabelo;Microextração em seringa empacotada;Cromatografia de gases espetrometria de massa.

Abstract

Different extraction and clean-up protocols have been used for Amphetamine-type psychostimulants (ATS) determination in hair. Microextraction by packed sorbent (MEPS) is a miniaturized version of solid-phase extraction (SPE) which has been applied to determine a number of drugs in hair (e.g., opiates, cocaine, ketamine, etc.). However, concerning Amphetamine-type psychostimulants, MEPS has only been used in hair for the determination of amphetamine (AMP) and methamphetamine (MAMP). The aim of this work was the development and validation of a method to determine AMP, MAMP, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 1-(1,3-benzodioxol-5-yl)propan-2-yl(ethyl)amine (MDE) and *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-aminobutane (MBDB) in hair using MEPS for sample clean-up. Hair (50 mg) was incubated with NaOH 1M at 45 ºC overnight, after which HCl 10M was added for neutralization and the solution was centrifuged. The MEPS procedure for extract clean-up was optimized using the Design of Experiments (DoE) approach, and the final steps were: conditioning (1 x 250 µL of methanol and 1 x 250 µL of deionized water); loading (18 x 100 µL); and elution (7 x 100 µL of 2% NH_4OH in acetonitrile). To the eluted extract, 30 µL of MBTFA was added, and extracts were evaporated to dryness, following microwave-assisted derivatization with 50 µL of MBTFA. A gas chromatography coupled to mass spectrometry (GC-MS) system was used for determination. The MEPS procedure resulted in recoveries of 8-14% for AMP, 14-20% for MAMP, 10-15% for MDA, 18-28% for MDMA, 25-43% for MDE and 34-52% for MBDB, in the linear range of 0.2 - 5.0 ng/mg. Precision and accuracy of the developed method were in accordance with the statements of international guidelines for method validation. This is the first analytical method using MEPS coupled to GC-MS to determine the selected amphetamines in hair samples, proving to be a great alternative to the classic procedures, being rapid, eco-friendly and less expensive.

Keywords

Amphetamines;hair analysis;microextraction by packed sorbent;Gas chromatography-mass spectrometry.

Índex

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List of abbreviations

2C-B	4-bromo-2,5-dimethoxyphenylethylamine
4-CB	4-carbethoxyhexafluorobutyryl chloride
5-HT	Serotonin
ACTH	Adrenocorticotropic
ADH	Antidiuretic
ADHD	Attention-Deficit/Hyperactivity Disorder
AMP	Amphetamine
ATS	Amphetamine-type psychostimulants
BDB	1-(3,3-benzodioxol-5-yl)-2-aminobute
BF_3	Boron trifluoride
BIN	Barrel inserted needle
BSA	Bis(trimethylsilyl)–acetamide
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
CNS	Central Nervous System
COMT	Cathecol-*O*-methyltransferase
CV	Coefficient of variation
DA	Dopamine
DAT	Dopamine transporters
DHEA	3,4-dihydroxyethylamphetamine
DLLME	Dispersive liquid-liquid microextraction
DOE	Design of experiment
ECD	Electron capture detector
FID	Flame ionization detector
GC	Gas chromatography
GC/MS	Gas Chromatography coupled with mass spectrometry
GHB	Gamma-hydroxybutyrate
HFBA	Heptafluorobutyric anhydride
HFBI	Heptafluorobutyrylimidazole
HF-LPME	Hollow Fiber Liquid-phase Microextraction
HMDS	Hexamethyldisilzane
HMEA	4-hydroxy 3-methoxyethylamphetamine
LC-ESI-MS/MS	Liquid Chromatography - Electrospray Ionization - Tandem Mass Spectrometry
LC-HRMS	Liquid chromatography-high resolution mass spectrometry

LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LLOQ	Lower limit of quantification
MAMP	Methamphetamine
MAO	Monoamine oxidase
MBDB	*N*-methyl-1-(1,3-benzodioxol-5-yl)-2-aminobutane
MBTFA	*N*-methyl-bis(trifluoroacetamide)
MDA	3,4-methylenedioxyamphetamine
MDE	1-(1,3-benzodioxol-5-yl)propan-2-yl(ethyl)amine
MDMA	3,4-methylenedioxymethamphetamine
MDP2NP	3,4-methylenedioxyphenyl-2-nitropropene
MDP2P	3,4-methylenedioxyphenykpropan-2-one
MEPS	Microextraction by packed sorbent
MIPs	Molecular imprinted polymers
MMDPPA	Alphamethyl-3,4-methylenedioxyphenylpropionamide
MS	Mass spectrometry
MSTFA	*N*-methyl-trimethylsilyltrifluoroacetamide
MTBSTFA	*N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide
NE	Norepinephrine
NET	Norepinephrine transporters
NPS	New psychoactive substances
P2P	Phenyl-2- propanone
PFBCI	Pentafluorobenzoyl chloride
PFBHA	Pentafluorobenzyl-hydroxylamine hydrochloride
PFPA	Pentafluoropropionic anhydride
PFPI	Pentafluoropropanylimidazole
PFPOH	Pentafluoropropanol
PMA	*p*-methoxyamphetamine
pOH-AMP	*p*-hydroxyamphetamine
*p*OH-MAMP	*p*-hydroxymethamphetamine
PS-DVB	Polystyrene–divinylbenzene copolymer
PTSD	Post-traumatic stress disorder
RAM	Restricted accesses materials
RE	Relative error
SCX	Strong cation exchange silicas
SERT	Serotonin transporters

SIADH	Syndrome of inadequate diuretic hormone
SPE	Solid phase extraction
SPME	Solid phase microextraction
SR	Surface response
SULT	Sulfotransferases
SUPRAS	Supramolecular solvents
TBH	Tetrabutylammonium hydroxide
TCCA	Trichloroisocyanuric acid
TCD	Thermal conductivity detector
TFAA	Trifluoroacetic anhydride
TFAI	Trifluoroacetylimidazole
TMCS	Trimethylchlorosilane
TMS-DEA	Trimethylsilyldiethylamine
TMSI	Trimethylsilylimidazole
UDP	Uridine diphosphate
UGT	Glucuronyltransferases
UHLC–MS/MS	Ultra high performance liquid chromatography–tandem mass spectrometry
VMAT-2	Vesicular monoamine transporter 2

Chapter 1 | Review of the literature

1. Introduction

1.1. Drugs of abuse and synthetic drugs

New psychoactive substances (NPS) are a diverse group of substances commonly known as designer or synthetic drugs [1]. Generally, these substances are analogues of existing controlled drugs or pharmaceutical products, created to mimic the actions and effects of classical drugs, such as cocaine, cannabis, heroin, LSD, and amphetamines, however, these NPS often times are not regulated [1,2]. Also referred to as "legal highs," these NPS pose a significant challenge to researchers, drug services, and drug control policies, due to their number, nature, and everchanging composition [1].

NPS frequently make use of well-established templates, like phenethylamines, piperazines, aminoindanes and cathinones, with some modifications to bypass international or domestic drug legislations, or even to add new psychoactive effects with different potencies [2]. These changes often result in increased harm to users, and if the substances are subsequently prohibited through new drug control regulations, an array of chemical and structural alterations are sought to circumvent this control, which highlights the significant problem of chemical diversity in the NPS domain [2]. The constant evolution of NPS has prompted some countries to introduce a more generic method to control psychoactive substances, including those that consider biological effects over chemical structures [2].

1.2. Amphetamines and derivatives

Amphetamine-type psychostimulants (ATS) are a group of natural or synthetic substances related to amphetamines, being synthetic chemically derived from β-phenethylamine, and producing stimulating effects on the central nervous system (CNS) [3,4]. Amphetamines are classified as psychotropic agents, being widely used and abused for their stimulant, euphoric, anorectic, empathogenic, and hallucinogenic properties [5,6].

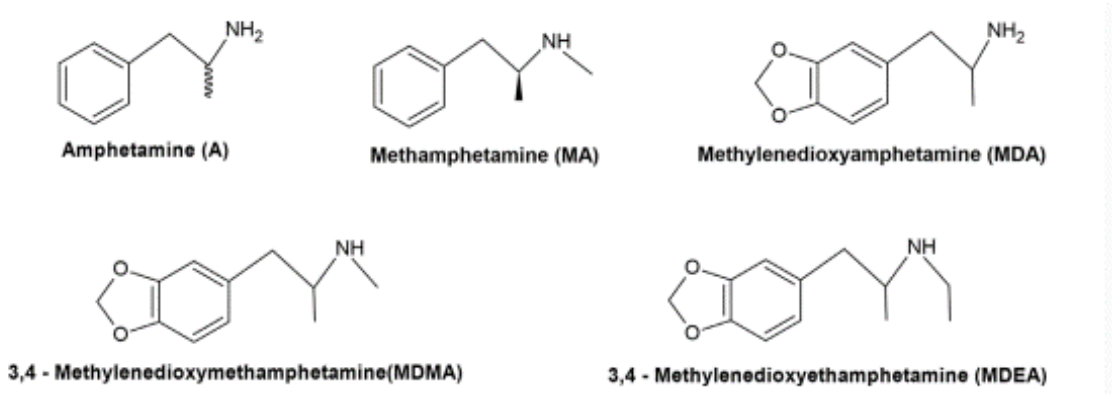

Figure 1- Structure of some amphetamine-type psychostimulants (Adapted from Novais, Arantes, Almeida et al. [4])

Although most amphetamines are considered synthetic drugs, among which amphetamine (AMP), methamphetamine (MAMP), and 3,4-methylenedioxymethamphetamine (MDMA) are the most well-known, the use of naturally occurring amphetamines by humans dates back to thousands of years [5,6]. *Catha edulis* (Khat) is an evergreen plant present in Africa and in the Arabian Peninsula that contains an array of active compounds in its fresh leaves, capable of increasing energy levels, alertness, self-esteem, and elation [5,6]. These effects are attributed to the presence of amphetamine-like compounds in Khat [5,6]. The most potent of these compounds is cathinone, an amphetamine with a keto functional group in the aliphatic chain, but other weaker amphetamines are also present in Khat, such as norpseudoephedrine (cathine) and norephedrine [5,6].

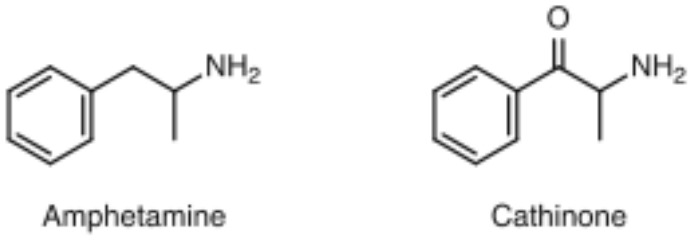

Figure 2- Chemical structure of Amphetamine and Cathinone (Adapted from Simmler and Liechti. [7])

Another plant containing natural amphetamines is *Ephedra sinica*, which is one of the oldest medicinal herbs known to mankind [5,6]. The aerial parts of different *Ephedra* species contain at least 6 different and optically active amphetamines [5,6]. Ephedrine and pseudoephedrine are the main psychoactive constituents of the plant, with ephedrine being an amphetamine with an aliphatic hydroxyl substitution and an extra nitrogen-methyl group [5,6]. It is popularly used as an appetite suppressant and performance booster [5,6].

Amphetamines were first synthetized in 1880 by the chemist Lazăr Edeleanu, but it was only in 1927, that it would be resynthetized and studied by a scientist named Gordon Alles, while in search of a substitute for ephedrine [3]. Clinical use of AMPs began in 1935 when a pharmaceutical company named Smith, Kline and French Co. introduced it to the market with the name Benzedrine® as a way to treat narcolepsy, post-encephalitic parkinsonism, and depression[3].

As Benzedrine® was freely available as a decongestant inhaler, and people soon realized that they could take advantage of AMP to experience psychostimulant effects, making it an extremely popular drug[3]. In 1939, four years after the commercialization of Benzedrine®, a prescription started being a requirement for the purchase of both Benzedrine® and Dexedrine®, the latter being a more potent isomer of AMP known as dextro-amphetamine (d-AMP) [3].

World War II soldiers would later use AMP and MAMP to endure long fighting journeys, which resulted in addiction to these substances [3]. Recognizing the problem and dependence caused by the misuse of AMPs, the Unites States (U.S.) Drug Enforcement Administration (DEA) and the United Nations nominated AMP as a strictly controlled substance. Since then, and except for some prescribed drugs, most AMPs have become illegal substances [3].

1.2.1. Amphetamine (AMP)

Amphetamines are one of the most widely used stimulant drugs in the European market [8]. The Leuckart process has been the most predominant method to synthetize this drug due to its relative simplicity [8]. In as little as 3 steps, this synthesis is able to produce amphetamine from a precursor known as benzylmethylketone (BMK) [8]. However, this precursor has been controlled since 2004 by the EU drug precursor chemical legislation, leading producers to use inventive ways to circumvent the legal restrictions involved with importing and producing precursors [8]. Several uncontrolled precursors, such as BMK-bisulphite salt, alpha-phenylacetoacetonitrile (APAAN), BMK-methyl glycidate, and BMK glycidic acid, have been found over the years as ways to mask the production of AMP, even though they can all be easily converted into BMK [8]. Most of these precursors have later been added to the existing EU precursor legislation, but novel precursors are still being created and emerging in the market (Figure 3) [8].

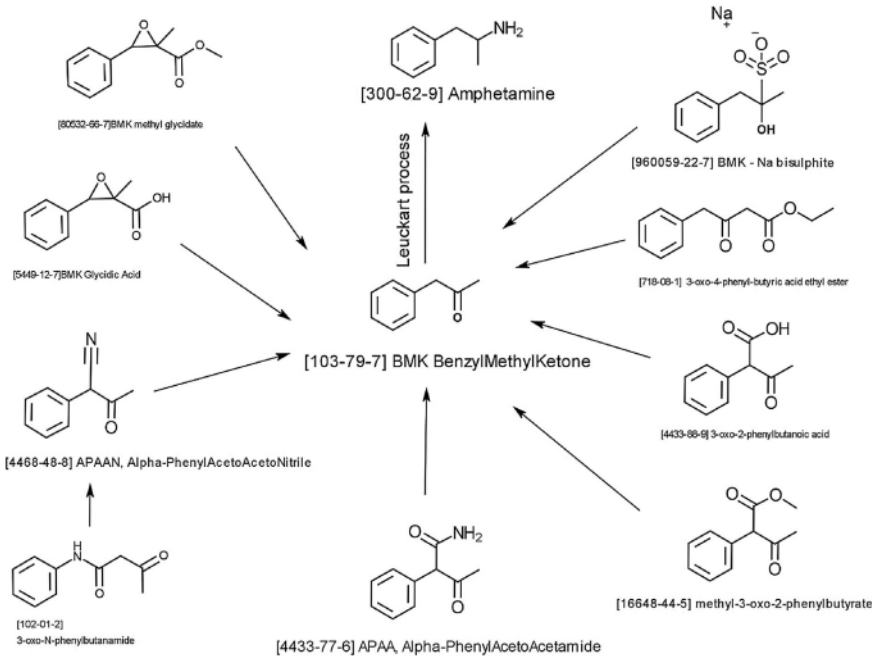

Figure 3 - Precursors of BMK which in turn is a precursor of AMP (Adapted from Emke et al. [8]*)*

1.2.2. Methamphetamine (MAMP)

Methamphetamine (MAMP) is an illicit recreational drug with a strong stimulant property on the CNS [9]. Belonging to the ATS, MAMP was first synthetized in 1893, being spread following World War II, being widely abused and presenting a significant neuropsychotoxicity [3,10]. Named by the IUPAC as (2S)-*N*-methyl-1-phenylpropan-2-amine, MAMP is a monoamine possessing one chiral center, most often encountered on the streets as a racemate in the form of the (S)-(+) or D-stereoisomer form and (R)-(−) or L-isomer [3,9]. The (R)-(−) or L-isomer are a legal decongestant readily available in several over-the-counter products, while the (S)-(+)-enantiomer is the more active stimulant and the illegal street drug [9]. There are several brand names for medical use around the world with desoxyn and methampex in the U.S., methedrine in the UK and Pervitin/Temmler in Germany [3].

With a pKa of 9.87 and a molecular weight of 149.24 g/mol, the (S)-(+)-methamphetamine is usually found in the form of a hydrochloride salt that looks like white crystalline powder, known in the streets as speed, meth or chalk [3,9]. Recrystallization of this hydrochloride results in colourless crystals, hence the street name crystal, crystal meth or ice [3,9]. Synthetic routes to produce MAMP utilize a palladium-catalysed reduction, which uses a chlorine derivative originating from the reaction between (−)-ephedrine and SOCL2, PCl5, POCl3, or PCl3 [9]. Other compounds such as (+)-pseudoephedrine or phenyl-2- propanone (P2P) have also been

used as starting materials, and it is also possible to obtain MAMP with a straight acid and red phosphorus reduction of (–)-ephedrine [9]. Other reduction methods are also known to have been used, including Nagai, Moscow, Rosenmund, Hypo, and Birch reductions (Figure 4) [9].

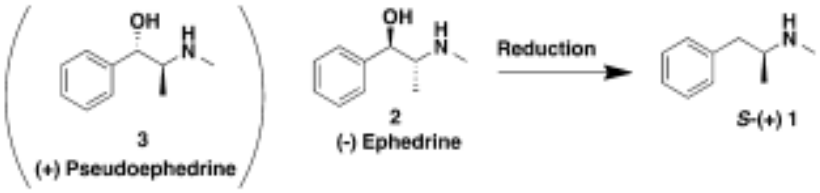

Figure 4- Synthesis of MAMP by reduction of ephedrine or pseudoephedrine (Adapted from Abbruscato and Trippier [9])

Since (+)-pseudoephedrine is a common ingredient in over-the-counter decongestants, it is commonly used as a replacement for (–)-ephedrine, which utilizes a modified Birch reduction, known as "shake and bake" to obtain MAMP [9]. Restrictions on the amount of products containing (+)-pseudoephedrine which an individual can buy were imposed to combat and reduce the production and use of MAMP [9]. Due to these restrictions, the use of P2P became a common way to produce MAMP [9]. There are two main methodologies to accomplish this goal: the reductive amination of P2P with methylamine, obtaining the (E)-*N*-methyl-1-phenylpropan-2-imine intermediate, which in combination with aluminium foil and anhydrous ammonia as a source of hydrogen produces MAMP (Figure 5) [9]; And the Leuckart method, which takes P2P to form an intermediate amide, which in turn can transform into MAMP (Figure 6) [9]. The synthesis routes utilizing P2P as a base material are only able to provide the racemic form of MAMP, while the use of (–)-ephedrine and (+)-pseudoephedrine is able to achieve an enantiomerically pure (S)-(+)-methamphetamine, which is much more potent and provides a greater stimulant effect[9].

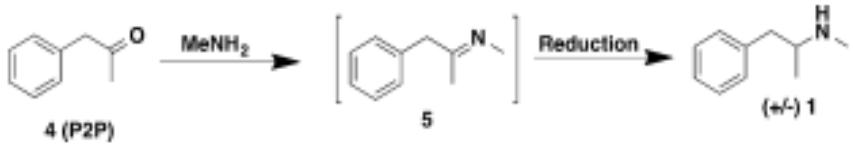

Figure 5- Synthesis of racemic MAMP from P2P(Adapted from Abbruscato and Trippier [9])

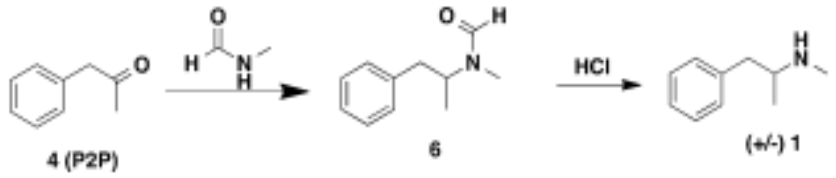

Figure 6- Synthesis of racemic MAMP by the Leuckart Method (Adapted from Abbruscato and Trippier [9])

The overall chemical structure of MAMP is very similar to that of dopamine, and since it is highly liposoluble, it can easily cross the blood-brain barrier and enter the brain [10]. Due to these characteristics, MAMP increases the central and peripheral release of monoamine neurotransmitters, which leads to the consumer experiencing feelings of euphoria, enhanced mental acuity, positive moods, systemic sympathomimetic effects, and social/sexual disinhibition [10]. Since dopamine release is essential in the human reward system, it is only natural that an increase in its levels may be largely accountable for the abuse and over-consumption of MAMP [10].

1.2.3. 3,4-methylenedioxyamphetamine (MDA)

MDA (3,4-methylenedioxyamphetamine), also known as R,S-1-(3',4'-methylenedioxyphenyl)-2-propanamine or "Love Pills" is a ring-substituted phenethylamine derivative, that was the first methylenedioxyamphetamine synthesised in 1910 by two German scientists named Mannich and Jacobsohn [11,12]. MDA was later patented as an antitussive, an ataractic and an appetite suppressant [11]. MDA can be manufactured, illicitly, using commercially available precursors and reagents, these include safrole, isosafrole and piperonal, with 3,4-methylenedioxyphenykpropan-2-one (MDP2P) as a common synthetic intermediate [13]. When synthetising MDA using safrole and isosafrole as starting material, there are two main methods that are followed to obtain the desired drug: the Leuckart-Wallach synthetic route and a bromination/amination [13]. It is also possible to use 3,4-methylenedioxyphenylacetic acid to obtain MDA through the already mentioned Leuckart-Wallach route and through a simple reductive amination [13]. Another known method is by utilizing 3,4-methylenedioxyphenyl-2-nitropropene (MDP2NP) to synthetize the MDA by using piperonal as a precursor [13]. All of these methods produce a great number of impurities which in turn help link to seizures, track routes of distribution, and identify criminal networks [13]. Since safrole, isosafrole and piperonal are restricted in many jurisdictions, new precursors and synthetic routes are emerging, being helional one of those substances [13]. Globally used as a perfume, soap and detergent fragrance due to its watermelon-like smell, helional is readily available and can be purchased in bulk,

making it the perfect clandestine precursor for drug production[13]. A now popular synthetic method called “two dogs” is being used to produce MDA, utilizing helional as a precursor[13]. Figure 7 illustrates this synthesis[13].

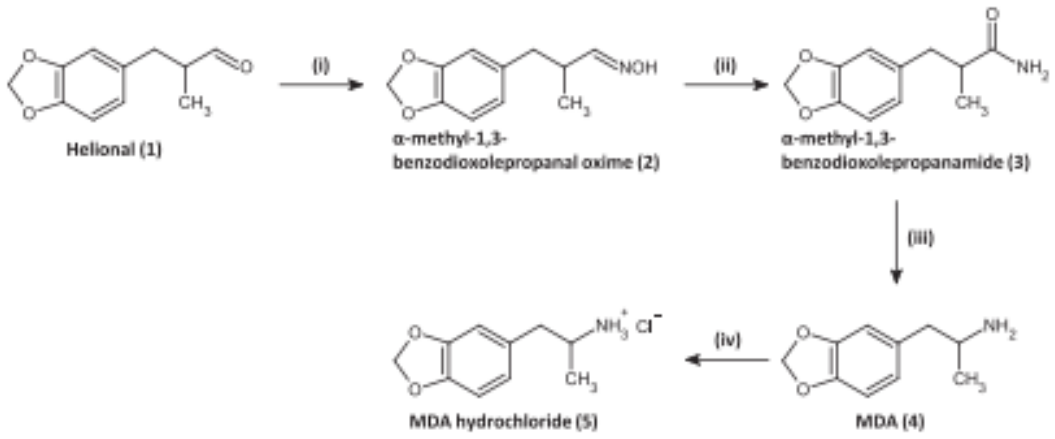

Figure 7- Synthesis of MDA from helional via the "towdogs" method. (i) Hydroxylamine (NH2OH), Sodium carbonate (NaCO3); (ii) Nickel Acetate (Ni(OAc)2); (iii) Trichloroisocyanuric acid (TCCA) or Sodium hypochlorite (NaOC)l; (iv) Hydrocloric HCl. (Adapted from Mercieca et al. [13])

Some other methods to synthetize MDA can be found in literature, such as the use of alpha-methyl-3,4-methylenedioxyphenylpropionamide (MMDPPA) [14]. This unusual precursor suffers an Hofmann Degradation (Hofmann Rearrangement) reaction using a sodium hypochlorite solution (bleach), and at the end of the five step synthesis it is able to produce MDA (Figure 8) [14].

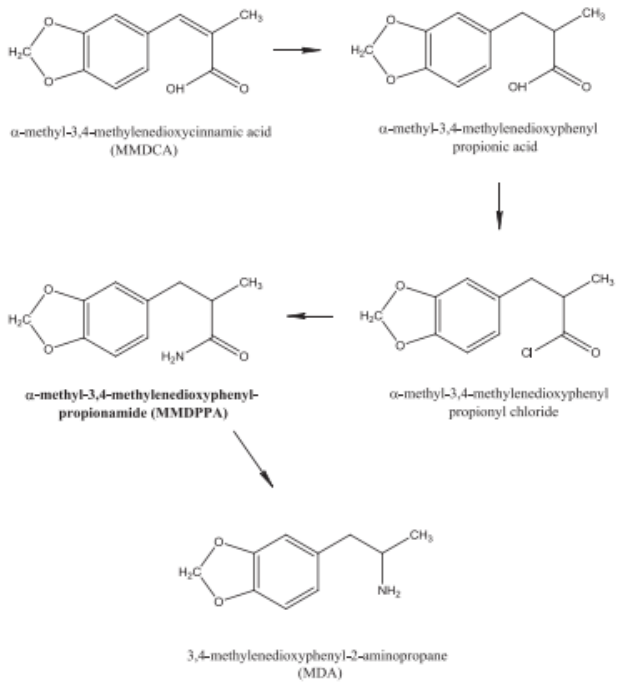

Figure 8- Sythesis of MDA from MMDPPA though an Hofmann Degradation (Adapted from Cason et al. [14])

MDA gained the street name of the “love” drug due to its effects in empathy and emotion enhancement, without the hallucinogenic properties, being also recommended as a drug of use in psychotherapy [11,12]. Although it has been known that in higher doses MDA produces hallucinations, these effects are usually diminished to visual distortions rather than well-formed hallucinations[11].

1.2.4. Methylenedioxyethylmethamphetamine (MDMA)

MDMA is an amphetamine derivative that has been widely abused for decades [7]. Discovered in 1912 by a chemist named Anton Köllisch, later in 1914 it was patented as an appetite suppressant [15]. Also known as ecstasy or Molly, this psychoactive substance is extremely similar to endogenous catecholamines, making it a powerful agonist of serotonin, dopamine and noradrenalin, increasing their concentration in synaptic clefts and leading to synaptic and hallucinogenic stimulant effects on the Central Nervous System (CNS)[15] . This substance is a 3,4-methylenedioxy derivative of AMP but it presents distinct pharmacological profiles and effects from the AMP [7]. While the main psychostimulant action of AMP is related to euphoria, MDMA is a prototypical empathogenic or entactogenic drug that induces reduced psychostimulant effects, enhancing the feelings of love, empathy, sociability, happiness, self-esteem and closeness to others [6,10]. Ecstasy had its therapeutical effects redirected thanks to this entactogenic ability, but it was quickly banned due to its adverse effects and potential for abuse [15]. Some cardio stimulant effects are also common and those include an increase in blood pressure, heart rate, hyperthermia and even bruxism from increased muscle tension [7,11].

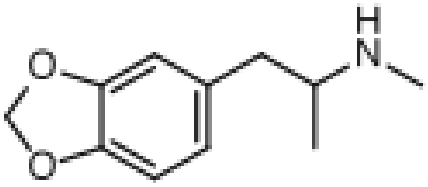

Figure 9- Chemical structure of MDMA.

Since MDMA has a moderate potential to cause dependency and addiction, it is considered harmless to most of its users, having a mortality rate of is 0-2% in admissions [15]. Obviously, since this drug is illegally manufactured, there is no pharmaceutical control, and thus it is impossible to know its integral composition, concentration, and degree of purity that a user might be consuming [15]. Other drugs of

abuse may work in combination with MDMA, allowing for an increased cumulative effect of the drug [15]. The lethal dose from this drug is variable, which relates the toxicity to frequency of use, individual vulnerability and external conditions [15]. The most reported cases involving deaths related to MDMA toxicity are very rare and usually relate to hyperthermia and hyponatremia [15].

1.2.5. 1-(1,3-benzodioxol-5-yl)propan-2-yl(ethyl)amine (MDE)

MDE, also known as MDEA, "EVE", and even "intellect", is a ring-substituted AMP chemically and pharmacologically related to MDMA [16,17]. Its proper chemical name according to the IUPAC is 1-(1,3-benzodioxol-5-yl)propan-2-yl(ethyl)amine and when consumed, this compound can provoke effects similar to MDMA, leading to psychomotor stimulation, alterations in one's perception, positive and embracing emotional state as well as other sympathomimetic physical effects [17]. The name "ecstasy" is no longer only associated to MDMA, but also to the whole group of ATS, such as MDA, MDE and *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-aminobutane (MBDB) due to their chemical and pharmacological similarities [17]. After the federal government ban on MDMA in the late 1980s, MDE made its first appearance as a substitute followed closely by MBDB in the 1990s [17]. Of course this MDE zenith was short lived and the US categorized it as a controlled drug in 1987 due to the emerging law prohibiting controlled substances analogues [17]. Just like other phenylethylamines, MDE can be easily synthetized in clandestine laboratories with minimal equipment and reagents [17]. Over 20 pathways for the synthesis of MDMA or MDE are known, some of which have already been mentioned above [17]. The simplest synthesis known uses MDA and transforms it into MDE through a simple *N*-alkylation [17]. Since the pathways for the synthesis of MDE and MDMA are similar, the same popular precursors can be used (such as safrole and MDP2) [17]. An "EVE" pill can contain pure MDE, but mixtures of MDA, MDMA, MDE and MBDB are becoming increasingly common, even in combination with other drugs of abuse, such as cocaine, opiates, LSD, benzodiazepines, phencyclidine, ketamine, gamma-hydroxybutyrate (GHB), e, 4-bromo-2,5-dimethoxyphenylethylamine (2C-B), paramethoxyamphetamine (PMA), and other types of drugs [17]. With this in mind, the assumed consumption of ecstasy or MDE cannot be associated with a particular substance, hence the importance of analysing and confirming what users are really consuming, in order to understand which compounds have been consumed [17]. MDE can be injected due to its solubility on water and alcohol, but it is usually taken orally

with a rare nasal or rectal routes of administration [17]. Since it presents a high boiling point, it is somewhat impossible to turn it into vapor and inhale this drug [17].

1.2.6. *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-aminobutane (MBDB)

N-methyl-1-(1,3-benzodioxol-5-yl)-2-aminobutane, also known as MBDB, "Methyl-J" or "EDEN" is an homologue of MDMA, presenting the entactogenic properties of the latter, endorsing a pleasant state of introspection, enhanced communication and a pronounced sense of empathy [11,18,19]. MBDB can be synthetized using as a precursor piperonal, 1-nitropropane, 1-bromopropane and 1-(3,3-benzodioxol-5-yl)-2-aminobute (BDB), with the latter possessing an extra carbon when compared to other MDMA precursors, preventing the accidental synthesis of MDMA when attempting to produce MBDB and vice-versa [18]. As it was previously mentioned, MBDB is almost always sold as "ecstasy" and it is usually ingested in this form [18].

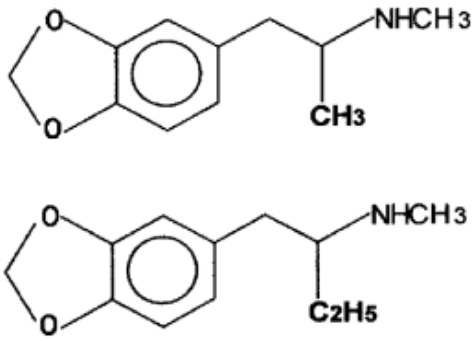

Figure 10- Chemical structure of MDMA (above) and MBDB (bellow) (Adapted from van Aerts et al. [18])

1.3. Mechanisms of action, pharmacokinetics, and toxicology

The metabolism of MDA, MDMA, MDE and MBDB is well studied in humans and described schematically in (Figure 11) [20,21]. There are two main overlapping metabolic pathways: *O*-demethylation to dihydroxy derivatives, called catechols, with a consequent methylation of one hydroxy group, catalyzed by the cathecol-*O*-methyltransferase (COMT) and/or glucuronidation via uridine diphosphate (UDP) glucoronyltransferases or sulfation by sulfotransferases [20,21], followed by a successive degradation of the side chain, originating *N*-dealkyl and diamino oxo metabolites through a *N*-dalkylation of MDMA, MDE and MBDB and a deamination of MDMA, MDA, MDE and MBDB, respectively [15,18]. Neurotoxicity from MDMA can

also occur mainly due to the formation of superoxide or other radicals rather than just the hydroxyl free radical [20]. Propylamines like MDA, MDMA and MDE are subsequently metabolized to glycine conjugates called hippuric acid derivatives which correspond to 3,4-disubstituted benzoic acids [20].

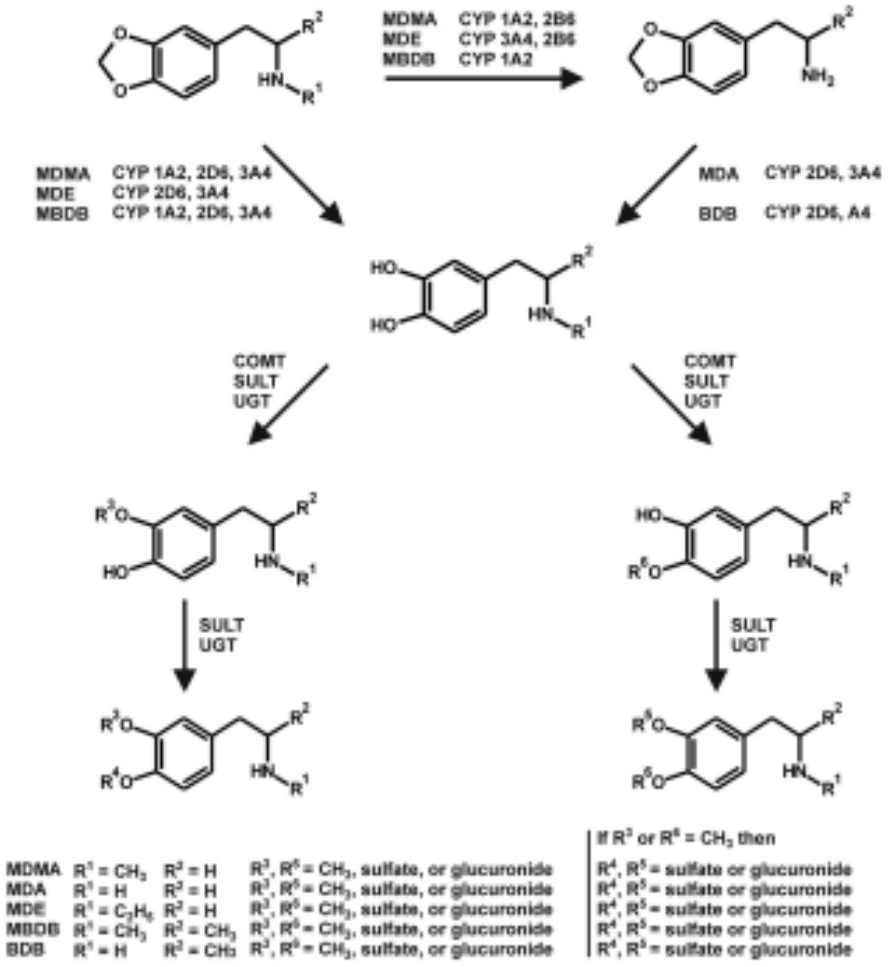

Figure 11- Metabolic pathways of MDA, MDMA, MDE and MBDB (Adapted from [21])

Demethylation and consequent formation of catechol derivatives is mainly catalysed by CYP3A4 and by the polymorphic CYP2D6, however, other CYP isoenzymes are involved in these processes [20–22]. Studies showed that *N*-demethylation of MDMA and MBDB had also the involvement of the CYP1A2 to a minor extent [20–22]. In contrast, *N*-dealkylation of MDMA and MBDB was catalysed by CYP1A2 while MDE was catalysed by CYP3A4 [20–22]. CYP2B6 was later described and confirmed as the main enzyme catalysing the *N*-dealkylation by Meyer et al. [23] on is study of the enantioselective metabolism of MDMA [21]. For the R,S-MDMA, R-MDMA and S-MDMA, *N*-demethylation was mainly catalysed by the CYP2B6, CYP1A2, and CYP2B6 respectively [21,23]. When it comes to the demethylation, the highest contributing

isoenzyme was CYP2D6 [21,23]. Figure 12 resumes these isoenzyme-dependent major metabolic pathways.

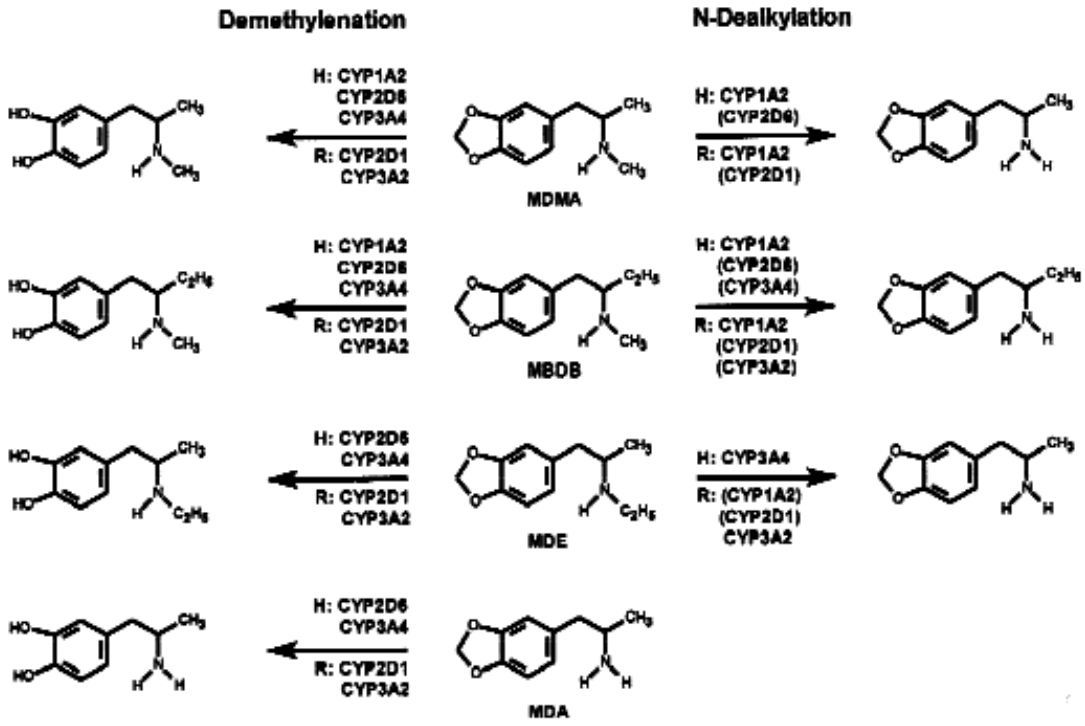

Figure 12- Isoenzyme-dependent major metabolic pathways of racemic MDA, MDMA, MDE and MBDB. (Adapted from Kraemer and Maurer [20])

In order to uptake and store neurotransmitters like monoamines into nerves, a transporter protein is needed in order to cross the nerve synaptic/plasmalemmal membrane, and allow the monoamines to enter the cell [24]. Subsequently, a monoamine transporter protein called vesicular monoamine transporter 2 (VMAT-2) is necessary to uptake and store the neurotransmitters in their respective storage vesicles[24]. In the CNS the transporters that are targeted are the norepinephrine transporters (NET), dopamine transporters (DAT) and serotonin transporters (SERT), but in the periphery the main target for stimulants is the NET, which is found at the adrenergic neuroeffector junction (Figure 13) [24]. Amphetamines act as a substrate for the transporters, competing with the monoamines for the reuptake transport and also directly displacing monoamines from their respective storage vesicles through the VMAT-2 [24].

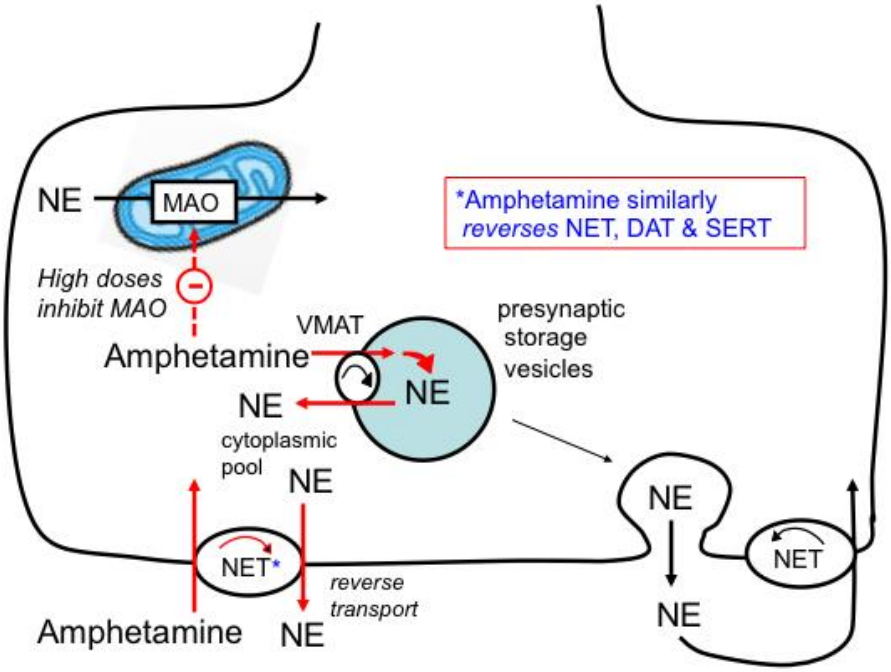

Figure 13- Amphetamine synaptic mechanisms (Adapted from tmedweb.tulane.edu [25])

Though this mechanism, AMP causes the release and accumulation of NE unrelated to any nerve activity [24].

Affinity studies of the AMP, MAMP, MDMA and MDE for each monoamine transporter can be seen in Table 1 [24]. Additionally Table 2 shows the potency of stimulants at inhibition of uptake of monoamines by their transporters [24]. Lastly, Table 3 shows the potency of stimulants at causing a release of monoamines by their transporters [24].

Table 1- Affinity of stimulants at monoamine transporters NET, DAT and SERT in ligand-binding sites (Adapted from Docherty and Alsufyani [24])

Agent	NET	DAT	SERT	DAT/NET	SERT/NET
Amphetamine	1.0	5.68	>25	*5.7*	*>25*
Methamphetamine	1.39	3.96	240	*2.85*	*173*
	4.28	1.85	26.7	*0.43*	*6.2*
	2.84	**2.91**	**133**	***1.64***	***(89.6)***
MDMA	30.9	22	14.7	*0.71*	*0.48*
	30.5	6.5	13.3	*0.21*	0.44
	30.7	**14.2**	**14.0**	**0.46**	**0.46**
MDEA	6.17	1.35	4.32	*0.22*	*0.70*

Table 2- Potency of stimulants at inhibition of uptake of monoamine neurotransmitter by NET, DAT and SERT (Adapted from Docherty and Alsufyani [24])

Agent	NET	DAT	SERT	DAT/NET	SERT/NET
Amphetamine	0.039	0.034	3.8	0.9	97
	0.07	0.64	38	9.1	543
	0.094	1.30	>10	14	>106
	0.07	1.3	45	19	265
	0.067	0.093	3.4	1.4	50.8
	0.068	**0.67**	**20.0**	**8.9**	**212**
Methamphetamine	0.048	0.11	2.1	2.3	44
	0.11	0.46	32	4.2	291
	0.16	0.65	27	4.1	169
	0.026	0.026	4.1	1.0	244
	0.064	1.05	>10	16.4	>156
	0.14	1.1	18	7.9	129
	0.091	**0.566**	**15.5**	**6.0**	**172**
MDMA	0.46	1.57	0.24	3.4	0.52
	1.2	8.3	2.4	6.9	2.0
	0.024	0.2	0.11	8.3	4.6
	0.45	17.0	1.36	38	3.0
	2.1	12.6	7.6	6.0	3.6
	0.85	**7.93**	**2.34**	**12.5**	**2.74**
MDEA	1.02	9.3	1.27	9.1	1.25

Table 3- Potency of stimulants at causing release of monoamine neurotransmitter by NET, DAT and SERT (Adapted from Docherty and Alsufyani [24]

Agent	NET	DAT	SERT	DAT/NET	SERT/NET
Amphetamine	0.0071	0.025	1.8	3.5	255
	0.006	0.006	0.7	1.0	117
	0.038	**0.016**	**1.25**	**2.25**	**186**
Methamphetamine	0.012	0.024	0.74	2.0	62
	0.152	0.43	27.5	2.8	181
	0.082	**0.23**	**14.3**	**2.4**	**122**
MDMA	0.077	0.376	0.057	4.9	0.75
MDEA		(>100)	(2.88)		/
Mephedrone	0.058	0.051	>10	0.9	>172
Cathinone	0.012	0.018	2.4	1.5	200
	0.014	0.025	9.3	1.8	664
	0.013	**0.022**	**5.8**	**1.65**	**432**
Cathine	0.015	0.068	>10	4.5	>667
	0.013	0.015	1.77	1.2	136
	0.014	**0.042**	**>5.9**	**2.85**	**>102**
Methylphenidate	*	*	*		
Cocaine	*	*	*		
Modafinil		*			
Bupropion	*	*			

AMP, MAMP, and methylphenidate show selectivity for inhibition of NET and DAT, being AMP and MAMP nearly inactive at the SERT [24]. MDMA and MDE seem to be potent inhibitors of the SERT, mainly as competitive substrates [24]. MDMA maintains a similar potency for the 3 transporters, while AMP and MAMP cause a release though the NET and DAT transporters [24]. MDMA also has high potency as a substrate for the SERT (inhibiting it) and at releasing it [24]. This all goes in accordance with MDMA effects due to the involvement of SERT and the stimulation of the 5-HT2A receptor

linked to it[24]. For MDMA analogues, the potency for releasing dopamine (DA) is MDMA, followed by MDA and then MDE but when it comes to releasing serotonin (5-HT) the potency is MDA, MDMA and then MDE [24]. With this in mind, MDMA is the most potent of the 3 at NET and DAT, while MDA is the most potent at SERT [24].

AMP, MAMP and MDMA also inhibit the monoamine oxidase (MAO), leading to an elevation on monoamine levels available for release since MAO has the function of breaking down monoamines [24]. MAMP also interferes with the VMAT-2, increasing the cytoplasmatic levels of monoamines which leads to auto-oxidation damages[24]. MDA, MDE and MDMA will also inhibit the α2-adrenoceptors which reduce neural release of norepinephrine (NE) [24].

An intranasal delivery of MAMP can present a bioavailability of up to 79% while inhalation and oral consumption of the drug has a maximum of around 67% [9]. Peak plasma levels by intranasal delivery are achieved after only 4H and drug distribution occurs with high accumulation in the liver and lungs, 23% and 22% respectively, and with an intermediate accumulation of 10% in the brain [9]. The half-life of MAMP is between 8 and 13 hours, with the effects lasting for the same period [9]. A highly acidic urine is usually related with an higher MAMP level, and around 43% of MAMP is excreted in its unaltered form in urine [9]. A smaller amount is excreted in the form of AMP (4 to 7%)[9]. Peak methamphetamine concentrations are seen in 2.6-3.6 hours after oral administration, with a mean elimination half-life of 10.1 hours (range 6.4-15 hours) [26].

In humans, MAMP is metabolized primarily by CYP2D6, which results in an aromatic hydroxylation and an *N*-demethylation [9]. From this metabolization, two major compounds are produced, being those para-hydroxymethamphetamine (pOH-MAMP) and AMP [9]. After this reaction, AMP suffers yet another metabolic change, which will produce para-hydroxyamphetamine (pOH-AMP), phenylacetone, *N*-hydroxyamphetamine, and norephedrine (Figure 14) [9] .

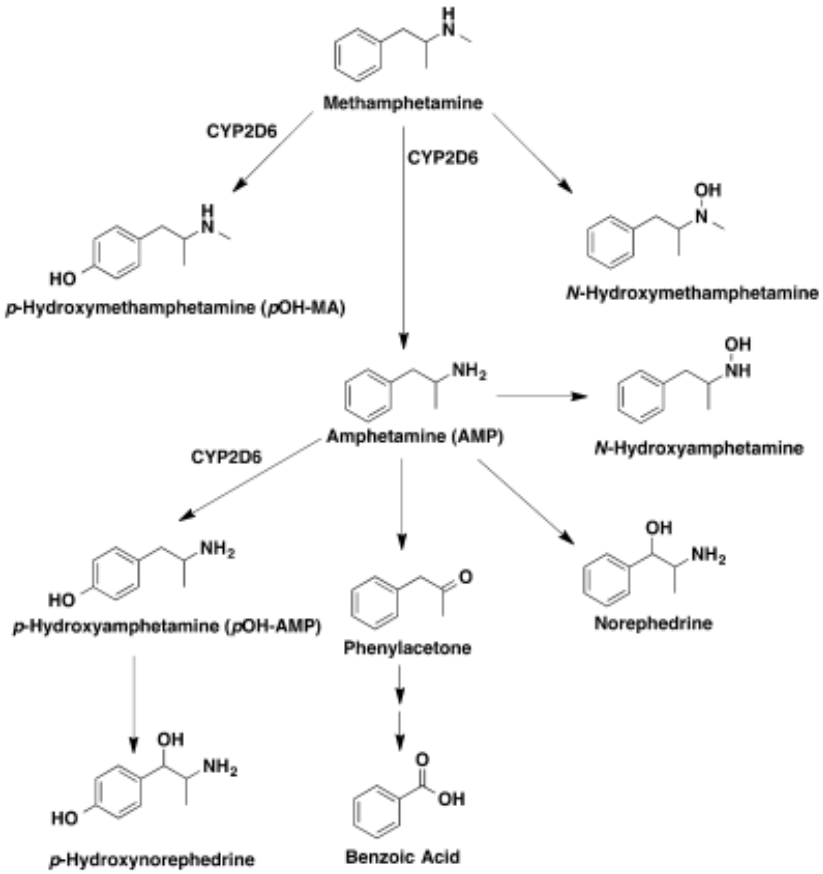

Figure 14- Metabolic pathway of methamphetamine in humans (Adapted from Thomas J. Abbruscato and Paul C. Trippier [9])

MDA produces a sympathomimetic effect, mediated by the release NE, leading the consumer to experience tachycardia, elevated blood pressure, mydriasis, tremor, palpitations and diaphoresis [11]. Increased salivation, bruxism (grinding of the teeth) and trismus (tight jaw muscles) are also common effects of MDA, being the last two most likely related to the release of 5-HT [11]. After effects from MDA (MDMA and MDE) are usually drowsiness, muscle aches and general fatigue, 24-48h lasting depression, low concentration, paranoia, anxiety and irritability [11]. Since MDA (MDMA and MDE) produce such subjective effects, it is labelled a entactogen drug. This term insinuates de connotation of producing a "touching within" effect, in reflection to the drugs ability to enhance the positive self-assessment and esteem while promoting an inward reflection to the consumer [11]. MDA is also an active metabolite of MDMA, presenting a much higher agonistic potency for the 5-HT2A receptor that the latest [6,10]. MDA gained the street name of the "love" drug due to its effects in empathy and emotion enhancement, without the hallucinogenic properties, being also recommended as a drug of use in psychotherapy [11,12]. Although it has been known that in higher doses MDA produces hallucinations, these effects are usually diminished to visual distortions rather than well-formed hallucinations[11].

While the main psychostimulant action of AMP is related to euphoria, MDMA is a prototypical empathogenic or entactogenic drug that induces reduced psychostimulant effects, enhancing the feelings of love, empathy, sociability, happiness, self-esteem and closeness to others [6,10].

Just like AMP, MDMA behaves as an indirect monoamine agonist, acting on monoamine reuptake transporters, causing an increase of neurotransmitters in the synaptic cleft [6,15]. In this way, 5-HT, DA, epinephrine and NE are the neurotransmitters that accumulate due to this action, in their respective transporters [7]. Unlike AMP, which predominantly acts on the human DAT and NET transporters, MDMA preferentially exerts its action on the SERT and NET transporters, promoting the release of, mainly, 5-HT which is associated with the induction of acetylcholine release in several neuronal regions [6,15]. By blocking the SERT transporters, 5-HT is prevented from returning to the interior of the presynaptic neuron, which in turn results in an increased release in the nerve terminals and the adrenal spinal cord [15]. MDMA is also able to increase the levels of extracellular monoamines though the inhibition of MAO, being the MAO enzymes responsible for the degradation of 5-HT and other catecholamines [15]. Due to this different course of action, and since different neurotransmitters are differently involved in the control of behaviour, distinct psychotropic effects are to be expected when the drugs are consumed separately [7]. Some mild hallucinogenic effects have also been reported with the consumption of MDMA and are attributed to the low-potency and partial agonist effect that the substance has over the 5-HT2A receptor [7]. A direct interaction between the serotonergic and dopaminergic systems also exist, in which activation of 5-Ht receptor in GABAergic neurons lead to an increase of DA production [15]. MDMA will also increase the blood levels of some hormones like cortisol, dehydroepiandrosterone, antidiuretic (ADH), adrenocorticotropic (ACTH) and prolactin [15].

Even though MDMA as a low binding affinity for adrenergic receptors, it increases the NE levels via NE release and NET uptake inhibition, originating an indirect NE-mediated effect at the adrenergic receptors which in turn contribute to the action of MDMA [7]. Heart rate increase is related with the β-Adrenoceptors while hyperthermia and vasoconstriction are implicated by both α1- and β-adrenoceptors [7].

Serotonergic toxicity can be induced by MDMA consumption and it's a result of the increase in the levels of 5-HT, which in some extreme cases can lead to serotonin syndrome [7]. This syndrome usually presents with symptoms including neuromuscular hyperactivity, clonus, hyperthermia, sweating, confusion, and agitation

[7]. MDMA consumption can also lead to a syndrome of inadequate diuretic hormone (SIADH) which results in hyponatremia [7].

Noradrenergic toxicity derived from stimulants often presents with symptoms of hyperthermia, hypertension, tachycardia, and agitation [7]. AMP is usually the associated drug when it comes to cardiovascular sympathomimetic toxicity but MDMA can also be the cause though induction of NE release and increase in plasma levels [7]. Like it was mentioned before, NE-mediated hyperthermia and vasoconstriction are implicated by both α1- and β-adrenoceptors [7]. The mechanisms behind this symptoms are the activation of the α1-adrenoceptors, which increase vasoconstriction and lead to a decrease in heat dissipation with an activation of the β3-adrenoceptors, that leads to heat generation though mitochondrial uncoupling and consequent hyperthermia [7].

Metabolism plays an important role in the neurotoxicological aspects of a drug and the main metabolomic steps for MDE are the same as those of MDMA (Figure 15) [16]. *O*-demethylation by cytochrome P450 (CYP) occurs giving origin to 3,4-dihydroxyethylamphetamine (DHEA) [16]. Next, DHEA can suffer a methylation by the COMT to 4-hydroxy 3-methoxyethylamphetamine (HMEA) or undergo conjugation, either catalysed by sulfotransferases (SULT) or glucuronyltransferases (UGT). HMEA will later be conjugated as well by the SULT and UGT [16].

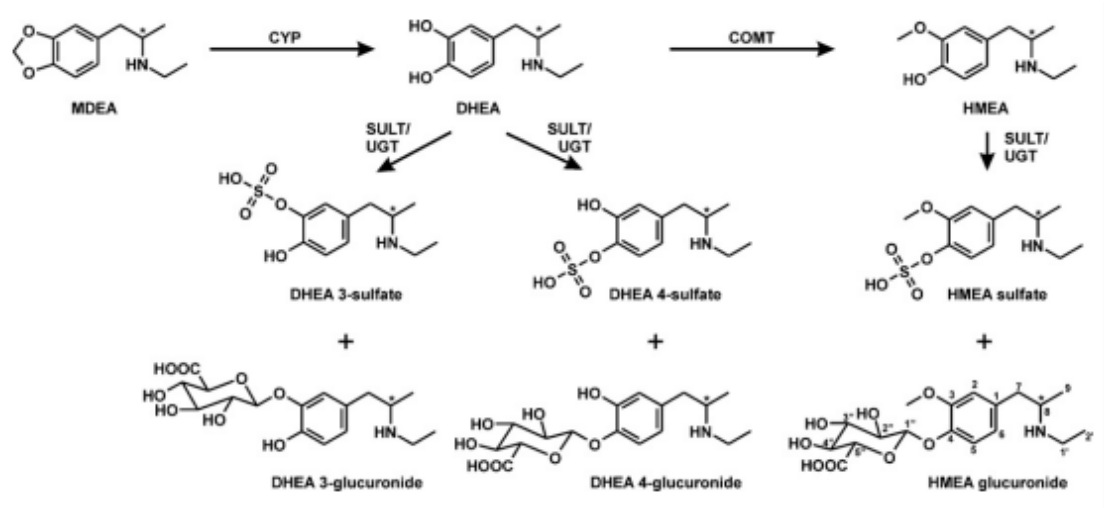

Figure 15- Main metabolic steps of MDE (Adapted from A.E. Schwaninger et al. [16])

1.4. Therapeutic Potential

As previously indicated, amphetamines are a group of central nervous system stimulant drugs that have both therapeutic and recreational uses. Their therapeutic importance lies in their ability to affect certain neurotransmitters in the brain, primarily dopamine and norepinephrine. The administration of illicit drugs in therapy is an intricate and controversial issue. While most drugs of abuse present harmful effects, alongside being illegal, some of their compounds have shown potential therapeutic benefits when administered under regulated conditions[27].

Some amphetamine-type stimulants, such as lisdexamphetamine dimesylate, mixed amphetamine salts, MDMA, and other amphetamine derivatives, including dextroamphetamine and phentermine have shown promising results in treating several conditions in adults as well as in children.

Briefly are described several significant therapeutic applications and their relevance:

(1) Treatment of Attention-Deficit/Hyperactivity Disorder (ADHD): Amphetamines, such as methylphenidate and dextroamphetamine, are commonly prescribed to individuals with ADHD. These drugs help improve attention, focus, and impulse control in people with this neurodevelopmental disorder. The importance here is in enhancing the quality of life for those with ADHD, enabling them to function better in academic, occupational, and social settings. Lisdexamphetamine dimesylate has shown promising results in treating ADHD in both children and adults, as well as in addressing drug dependency and withdrawal, making it a versatile treatment option [28–37]. Mixed amphetamine salts have also demonstrated efficacy in alleviating ADHD symptoms in adults, and further research is needed to explore their potential use in treating bipolar disorder and cocaine use disorder[38–42]. MDMA-assisted psychotherapy has emerged as a novel approach in the treatment of post-traumatic stress disorder (PTSD) and has exhibited significant and sustained reductions in PTSD symptoms [43–48]. Furthermore, it has shown promise in promoting post-traumatic growth and managing anxiety related to life-threatening illnesses [43–48]. Lastly, dextroamphetamine and phentermine have demonstrated efficacy in treating conditions such as cocaine and opioid dependence, ADHD, and obesity [49–53]. These substances require careful consideration and monitoring by medical professionals due to their potential risks and benefits.

(2) Narcolepsy Management: Amphetamines can help manage the symptoms of narcolepsy, a sleep disorder characterized by excessive daytime sleepiness, sudden episodes of muscle weakness (cataplexy), and sleep paralysis. By increasing wakefulness and alertness, amphetamines can improve the patient's ability to function during the day [28–37].

(3) Weight Loss and Obesity Management: Amphetamines were historically used as appetite suppressants due to their ability to reduce hunger and increase metabolism. While their use for weight loss has declined due to safety concerns and the development of alternative medications, they still have a role in managing obesity in some cases [49–53].

(4) Treatment of Depression and Fatigue: In certain cases, amphetamines have been prescribed off-label to treat depression and fatigue. They can boost mood and energy levels, which can be beneficial for individuals with severe depression or fatigue when other treatments have been ineffective [43–48].

(5) Enhancing Cognitive Performance: Amphetamines have been used to enhance cognitive performance in some situations, such as improving alertness and concentration in military personnel or shift workers. However, their use for this purpose is often controversial due to potential side effects and risks [43–48].

It is important to note that the use of amphetamines as therapeutic drugs should always be under the supervision of a qualified healthcare professional, as they can have potential side effects and carry a risk of misuse and addiction. Additionally, the long-term safety and effectiveness of these drugs are still a subject of ongoing research and debate, particularly in the context of their use in treating ADHD and other conditions.

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1.5. Statistics in Europe

The European drug report of 2022 [54] shows that 83.4 million adults, ages between 15-64, are estimated to have consumed an illicit drug in their lifetime with stimulants being the second most reported category. Last year, around 2 million adults have consumed AMP and 2.6 million MDMA [54]. MAMP is found in powdered form and its consumed orally, nasally, or even injected [54]. Between 2010 and 2020, MDMA and MAMP have shown the largest increase (129% for MDMA and 107% for MAMP) in the

number of seizures [54]. The pandemic caused a slight decrease in numbers in 2020, but recent years the production of AMP and MAMP has seemed to remain stable or even increased [54]. In Portugal, the European web survey on drugs of 2021, showed that most amphetamine users are young adults who still attend secondary or higher education and still reside with their parents in big cities (45% live in Lisbon and *Vale do Tejo*). Regarding the ways of consumption, 68% of amphetamines are used in a powered/crystal form while 45% are used in the form or tablets/pills [55]. These statistics are in accordance with the forms of consumption being that 63% are consumed though nasal snorting, 55% are consumed orally, and the rest are consumed in other ways like smoking and injection [55]. The motives for the consumption of amphetamines vary, but still most of the users use it to get high or "for fun" (77%), to socialize (37%) or even to enhance performance and reduce stress (both 20%) [55]. Consumption is episodic, with 57% consuming 1 to 5 days in a period of 12 months, being the average consumption on a typical day, 1 tablet or 0,7g of powered/crystal amphetamines [55].

2. Detection of amphetamines in Hair Samples

Forensic toxicology aims to use chemistry, toxicology and pharmacology knowledge to investigate compounds of interest in collected samples, aiding legal investigations like drug-facilitated crimes, doping and even deaths [56]. Traditional biological matrices like whole blood, plasma, serum, and urine are used, but recent research has focused on finding alternative matrices for drug detection [56,57]. One of these matrices and the focus of this work is hair, providing a larger window of detection and less invasiveness for the patient. Moreover, it has already been used for the detection of several drugs, medication and biomarkers, as part of criminal investigations (drug-facilitated crimes, deaths, child protection) and as a way to monitor drug misuse in rehabilitation programs and the workplace [56,58].

2.1. Hair matrix

Hair is a complex tissue composed of keratin (65-95%), lipids (1-9%), minerals (<1%) and water (15-35%) [57,59]. It covers most of the human body, and helps with the regulation of body temperature and protects the skin from injuries [58]. Hair follicles are rooted 3-4 mm deep, covering a high percentage of body surface, with around 5 million hair follicles present in an adult. [57–59]. The variable amount and distribution

of hair pigments, primarily melanin, is associated with hair colour, which is determined by four types of melanin, being eumelanin, pheomelanin, oxyeumelanin, and oxypheomelanin [58,59]. Hair has a thick coating of cuticles, which protect and attach the hair shaft to the follicle [57,59]. The cell membrane complex and medulla, which includes medullar cells are also important structures in hair [57,59].

Hair analysis primarily involves head hair – from the scalp but different types of hair can be used as a substitute for analysis when scalp hair is not present or in conditions to be analysed [57,59]. Studies have shown differences in drug concentration in the different types of hairs, such as armpit hair (axillary), pubic hair, arm hair, and beard hair [57,59]. A lower drug concentration for opioids, methadone, cocaine, MAMP and cannabinoids has been described when comparing hair coming from the scalp with hair from other locations [57,59]. Considering all this, to perform a proper interpretation of results from hair testing it is imperative to consider the biological differences that might be present in hair sampled from different locations in the body [57,59].

Scalp hair is easy to collect and grows at the fastest growth rate of 0.2-1.12mm/day, which [57–60]. Sampling is performed by collecting hair from the vertex posterior area, due to the fact that it is the most uniform area [59]. Head hair is also exposed to several external contaminants, like sebaceous secretions, sweat, powders, fumes, solutions, dusts, and even cosmetic treatments, which can alter the toxicological results [59]. The Society of Hair Testing (SoHT) recommends that hair is collected from the posterior vertex region of the head [58,60].

Normally, human hair grows in three developmental stages: anagen, catagen and telogen [57–59]. The anagen stage, which can last up to 6 years and is promoted by the papilla, being estimated that 85% of the hair found in the human scalp is currently on this phase [57,58]. Hair growth occurs in the cell zone, where protein synthesis is realized [59]. The catagen or transitional phase, lasts up to 2-3 weeks [57,58]. During this phase, the bulb migrates upwards in the skin through the epidermal surface, shrinking as it is pushed out of the hair follicle and giving the roots an elongated appearance while the metabolic activity diminishes and slows down [57,59]. As the catagen phase finishes and telogen begins, where hair growth halts, causing a club-shaped appearance as the follicle atrophies and hair starts to fall off [57,59]. Around 10-15% of all hairs are estimated to be in the telogen phase, with this duration increasing with age and changing with hair type[58].

Three routes have been proposed to identify drugs and other compounds incorporation into hair, but the exact mechanism remains partially unknown, and the extent to which each of the described routes contribute to this incorporation is still unclear [57–59].

The passive diffusion model describes the drug incorporation into hair from blood to hair (via passive diffusion) [57–59]. This process occurs during keratogenesis, where cells grow in the hair root, and substances diffuse into the hair shaft in a tightly bound form[57]. Drug concentration in hair will depend on the drug concentration in the blood and if the hair growth is constant[57]. In hair, parent drugs are more common and are generally found in higher concentrations than their metabolites[58].

Sweat, sebum and secretions of apocrine glands can be vehicles for the excretion of drugs which may influence drug incorporation (Figure 16) [57–59].

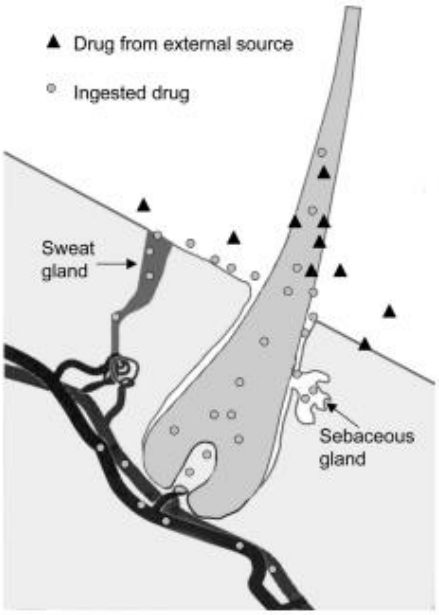


Figure 16- Incorporation routs of compounds into the hair follicle (Adapted from Kintz et al. [58])

External environmental contamination can also occur due to the high surface-to-volume ratio that hair presents and exposure to elements[59]. Chemicals in aerosols, smoke or even direct contact with powders or drugs can lead to contamination and incorporation[58,59].

Intradermal transfer of lipid soluble drugs happen when the main drug and metabolites get accumulated into the skins layers and subsequently pass into the hair though the same mechanism[57].

Physicochemical characteristics of a substance directly affect its ability to be incorporated into hair [58,59]. Incorporation and binding of drugs is known to be

affected by factors like drug pKa, structure, size, lipophilicity, protein binding capacity and melanin affinity [58]. Highly hydrophobic and small molecules, with basic ionizable groups in their constitution, tend to be the best at binding into the hair structure [59]. Keratin plays a prominent role in this binding, with basic drugs, like amphetamines and cocaine, are incorporated into hair to a much greater extent than other acidic/phenolic substances [58,59].

Hair offers a great advantage over other matrices due to its stability, allowing for detailed history of an individual's exposure to drugs. This high stability is always dependent on the morphology of the hair and its physicochemical properties in conjunction with any damage or harsh conditions that the hair might be exposed to through natural elements (rain, wind, sun) or cosmetic treatments [57–59].

2.2. Hair Analysis

Hair should be collected by a competent professional, who should wear gloves and use clean tools to avoid external contamination. Sample should be of about the thickness of a pencil and collected from the posterior vertex region, as close as possible to the scalp (Figure 17) [57–60]. When scalp hair is unavailable, alternative hair sampling regions like the beard and pubic hair can be used as long as this fact is taken into account when interpreting results [57–60]. Samples should be stored at room temperature in a dry and dark environment, inside an envelope, aluminium foil, or a plastic bag [57–60].

Figure 17- Vertex region of the scalp, where hair samples should be colected (Adapted from Kintz et al. [58])

Due to the high exposure of hair to external contaminants it is essential a decontamination procedure (washing) is performed [57–60]. This washing step helps reduce some contaminants while improving the extraction recovery from the hair sample though the removal of external residues like sweat, dust, sebum, and hair products [57–60]; The SoHT recommends that every decontamination procedure involve an organic and aqueous washing step, with the most commonly used organic solvents being acetone, dichloromethane, methanol, ethanol, hexane, pentane and

diethyl ether [57–60]. Non-protic solvents like dichloromethane and acetonitrile do not cause swelling of the hair and are believed to decontaminate a sample without the loss of compounds of interest [59]. On the other hand, protic solvents like methanol and water can cause the hair to swell which may lead to a removal and reduction of the incorporated compounds from the hair matrix [59].

Extraction and clean-up methods must be developed and optimized to solubilize the analytes of interest while minimizing its loss through hydrolysis [57,59]. After washing, hair is cut it into small pieces (1-3mm) or by griding it with special equipment (ball mill) [57–60]. The SoHT recommends the use of 10-50mg of hair, accurately weight for each analysis [58,60].

Compounds can be solubilized by utilizing a variety of methods that must abide by the characteristics of the targeted drugs and the chosen analytical technique [57,59]. This solubilization, or hydrolysis, leads to the disintegration of the hair matrix in order to liberate the incorporated substances for further extraction, clean-up or direct analysis [58]. Extraction will depend on the surface area, incubation time, solvents, analytes and all extraction conditions in general, and several methods can be applied:

Methanolic incubation at 45ºC for several hours, with an optional ultrasonic bath; allowing the extraction of almost every drug that is neutral, hydrophilic, and moderately lipophilic [57,59].

Aqueous incubation with acids (0.01-0.5M of HCl) or with buffer solutions (1M phosphate buffer with a pH around 6.4-7.6), overnight and with temperatures rounding the 60ºC has been shown to be an efficient way to extract basic drugs, although some drug conversions have been reported [57,59].

Alkaline digestion with aqueous NaOH (1M) performed at several temperatures and times (1h 80ºC or 45-60ºC overnight) is advantageous for basic substances that are stable under alkaline conditions, like nicotine and amphetamines [57,59]. With this digestion the protein contents of the hair are damaged so it's essential to control the parameters of temperature, time and concentration for the incubation[57].

Enzymatic solutions: which usually contain pronase, arylsulfatase, proteinase K or glucuronidase and digest hair by acting on the main hair protein (keratin) without altering or destroying the drug and metabolites of interest [57]. Of course, the true recovery of drugs from the hair matrix cannot be easily determined and as so, investigations comparing the different extraction procedures can only give some insight

about the extraction kinetics and what method seems to fit best for that particular analysis [58].

After extraction, sample clean-up procedures like liquid-liquid extraction (LLE), dispersive liquid-liquid microextraction (DLLME), solid phase extraction (SPE), solid phase microextraction (SPME) and microextraction by packed sorbent (MEPS) are applied to the obtained solutions in order to isolate our target drugs and reduce the interferents present in the hair matrix.

2.3. Sample preparation technique (MEPS)

Microextraction by packed sorbent, also known as MEPS, is a miniaturized version of conventional solid phase extraction (SPE), allowing for purification and pre-concentration of various analytes from different biological matrices, while maintaining a minimal sample and solvent volume [61,62]. This technique is able to combine sample extraction, pre-concentration and clean-up in one single device that can be divided into two parts: the MEPS syringe (with a volume of 100µl to 250µl) and the packed sorbent bed, also known as barrel inserted needle (BIN) [61,62]. MEPS is similar to SPE in almost every aspect and, since they work both with the same sorbent principals, being those normal phase, reversed phase, mixed mode and ion exchange interactions, pre-existing SPE methods can easily be adapted to MEPS through a simple scale down of solvent and sample volume [4]. Unlike conventional SPE columns, in MEPS, the sample flows in both directions (up and down) and the sorbents are directly integrated into the liquid handling syringe [61,62]. Due to this bidirectional motion of the sample, washing and elution steps on a MEPS protocol are of extreme importance to reduce loss of analytes and efficiently isolate from matrix interferents [61,63]. Around 1-4mg of sorbent is either inserted directly into the syringe barrel as a plug or between the barrel and the needle (BIN) (Figure 18), working as a cartridge and allowing for low void volume sample manipulation [61,62]. As the sample passes through the sorbent, analytes of interest get trapped, allowing for the removal of interferents and separation of the analytes from the rest of the sample [61,62].

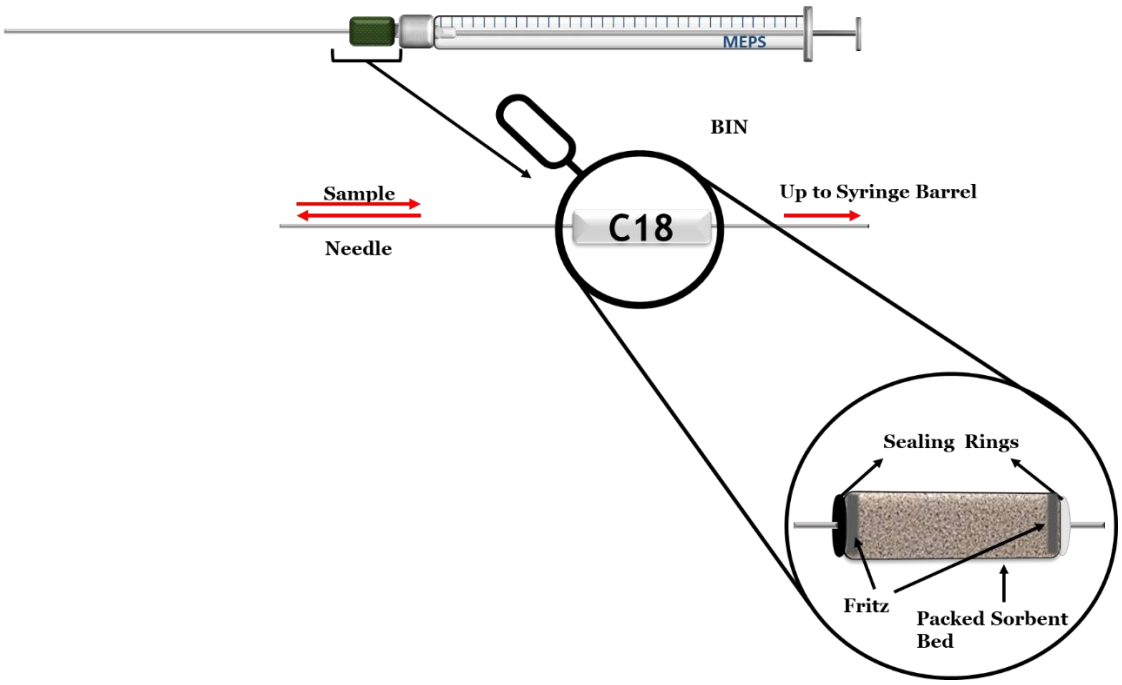


Figure 18- Representation of a MEPS device: The syringe and the packed sorbent (BIN).

Sorbents integrated in the MEPS cartridge can be made from different materials, depending on the analyte being studied. These materials range from silica based sorbents (C_2, C_8, C_{18}), strong cation exchange silicas (SCX), restricted accesses materials (RAM), mix mode sorbents (C_8/SCX), HILIC, carbon, polystyrene–divinylbenzene copolymer (PS-DVB) and even molecular imprinted polymers (MIPs) for a more specific approach [61–63]. The low solvent and sample volumes (mL to µL, from SPE to MEPS) combined with the reusability (up to 100 times) and the small amounts of sorbent involved in this procedure, makes MEPS a very efficient and environmental-friendly sample-preparation technique [61]. The low elution volumes required to fully elute the sorbent (10-50 µL) also means that MEPS is perfect for on-line use with both GC and LC and for immunoassays or even other colorimetric techniques in 96 well-plates [61,62]. In summary, the advantages of MEPS are the low sample and solvent volumes, its simplicity and ease of use, the reduced total analysis time, the reusability which in combination with the low volumes, make MEPS a relatively inexpensive technique and also the fact that it can all be fully automated and directly implemented on-line into GC and LC equipments without the need of user interaction [61,62]. The MEPS protocol consists of 5 essential steps (Figure 19): sorbent conditioning; sample loading; sorbent washing; elution; and sorbent regeneration for re-use [61,62].

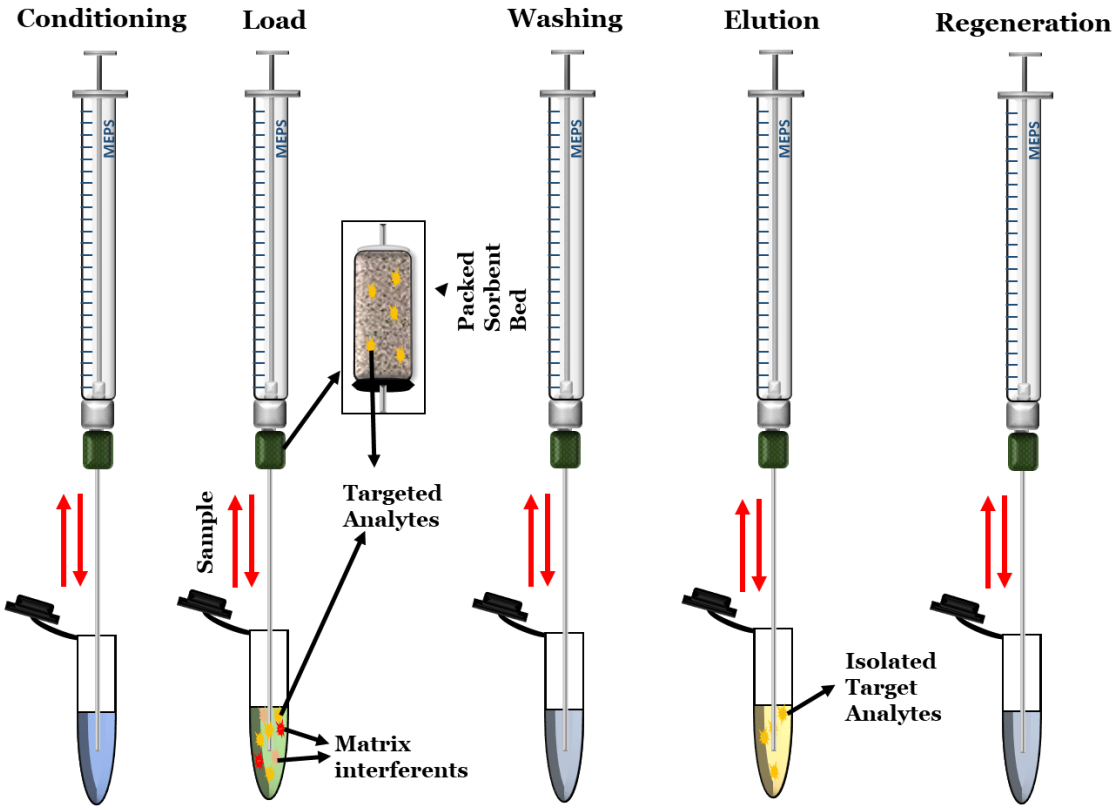


Figure 19- Representation of a MEPS protocol.

Conditioning is the 1st step in MEPS, activating and preparing the sorbent for the sample. A strong organic solvent, and then water that may or may not be slightly acidic or basic (2% HCOOH or 2% NH4OH are recommended) are used for preparing the sorbent and facilitating analyte retention. After each aspiration, the solvent in question is discarded [61,62]. Thereafter comes the 2nd step, the loading of the sample, which can be directly loaded into the MEPS device or after being properly diluted, precipitated, centrifuged or any other pre-treatment technique that might be required [61]. The 3rd step is washing, which aims to remove as many matrix interferents from the sorbent as possible, allowing for a pure elution with only the target analytes thereafter [61,62]. For this step it is usually recommended that a small volume of water with 5-10% organic solvent is used to elute the unwanted compounds while keeping the targeted analytes immobilized in the sorbent [61,62]. The 4th step in the MEPS protocol is elution, which consists of passing an organic solvent, like acetonitrile or methanol, through the sorbent bed to displace and elute our target analytes [62]. This solvent can be pure or mixed with acid/base solutions (0.1-3% NH_4OH for bases or HCOOH for acids), depending on the analyte in question. This solvent also needs to be volatile, for GC injections, and miscible with sample solvent or and LC mobile phase, for LC analysis [62]. Regeneration of the sorbent bed is the 5th and final step in a MEPS protocol, allowing for the re-use of the sorbent with a different sample while attempting

to minimize the carry-over effects that may present due to vestigial concentrations of analytes in the sorbent [62]. This step usually involves 2 washing solutions: A strong and a weak washing. The strong solution should be a mixture of an organic solvent (methanol or acetonitrile) with isopropanol (10-20%) and a 0.2% formic acid/ammonium hydroxide depending on the nature of the analytes while the weak solution can be pure water or 5% methanol [62]. It is also recommended that in between washing and the elution, a drying cycle is added to dry the sorbent bed, although it is not strictly necessary [62]. Every single step and condition of a MEPS protocol should be optimized in relation to the matrix and analytes being studied. Choice of solvents, solvent volumes, and number of strokes are important parameters that need to be studied in order to optimize sample clean-up and analyte pre-concentration [61,62].

2.4. Gas Chromatography coupled to Mass Spectrometry (GC-MS)

In the following lines, a brief description of the discovery of gas chromatography and its mechanism of separation and detection is provided.

Developed by Archer Martin, a Nobel Prize laureate, along with his colleagues Richard Synge and Anthony James in 1952, modern gas chromatography (GC) has evolved into a widely employed and essential analytical tool in the field of chemistry [64,65]. The earliest known use of gas chromatography dates back to around 1512 when Hieronymus Brunschwig detailed a method for purifying alcohol using a metal column filled with cotton or sponge, pre-saturated with olive oil [65]. This represented a gas-liquid form of gas chromatography, with the cotton or sponge serving as a solid stationary phase and the olive oil acting as a liquid stationary phase, while the alcoholic fumes acted as the mobile phase. Subsequently, Tobias Lowitz introduced the science community to the adsorption properties of substances in charcoal [65]. In the 18th century, when reliable purification techniques were scarce, Lowitz delved into the absorptive capabilities of charcoal for the purification of medicines, drinking water, vodka, honey, saltpeter, and various volatile substances. This research had such a significant practical impact that by the 19th century, the use of these absorbents had become widespread for purifying solutions and gaseous substances [65].

Paul Schuftan is widely credited as the first scientist to apply gas-solid chromatography in the form we recognize today [65]. In the early 1930s, Schuftan proposed and implemented a method known as "adsorption analysis," which enabled the separation

and subsequent analysis of a gas mixture comprising several known gases, including hydrogen, nitrogen, oxygen, carbon monoxide, methane, ethylene, ethane, propane, propylene, isobutane, and n-butane. He employed the principle of low-temperature activated-carbon adsorption, permitting gases with lower boiling points and molecular weights to be displaced by others with higher densities and boiling temperatures, resulting in distinct fractions. Some overlap was inevitable in practice due to the imperfect apparatus used; nevertheless, this methodology can be seen as the starting point for applying chromatography principles to gas analysis [65].

The concept of retention time as we understand it today was initially determined by a scientist named Nikolay Shilov, who developed the first formula allowing for the calculation of the then-referred-to "time of protective effect" of gas masks [65]. The formula was $\theta = kL - \tau$, where θ represents the time of protective effect, L is the length of the layer, k is the coefficient of the protective effect, and τ is a correction factor accounting for the loss of protective effects over time. Building on this work, Mikhail Dubinin, a researcher specializing in the kinetics and dynamics of sorption, applied frontal analysis to analyze gases, laying the groundwork for calculating adsorbers in the fractional separation of gas/vapor mixtures, with assistance from Yavich and Khrenova. Expanding on this research, Dubinin described the principle of frontal-type gas chromatography, stating that the front of each component would move at a constant speed along the sorbent layer, subsequently presenting the concept of the external chromatogram. In 1941, Hesse, widely regarded as the first person to use a carrier gas, reported his initial experiments in GC, employing a rudimentary device with silica gel as the stationary phase and carbon dioxide as the mobile phase [65].

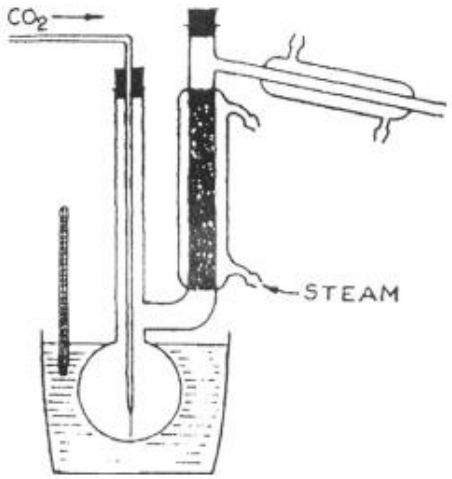


Figure 20- Hesse´s instalation for the first gas-chromatic separation. Steam was used as a way to heat the column (Adapted from Kolomnikov et al. [65])

In order to develop a quick and accurate method for determining acetylene and ethylene, a German-Austrian chemist named Erika Cremer, along with her postgraduates Fritz Prior and Müller, turned to the chromatographic method. However, her completed work was only published 30 years after its conclusion due to a bombing incident at the print shop where the journal was supposed to be printed. Despite the challenges of post-war recovery, a lack of funding, and the need to salvage components from the ashes, Cremer and Prior successfully created a system that encompassed all the essential features of gas chromatography. This system involved a pre-purified carrier gas passing through a column at various rates, controlled temperature settings, and the use of a detector based on differences in heat conductivity to determine the elution times and quantities of components (Figure 21) [65].

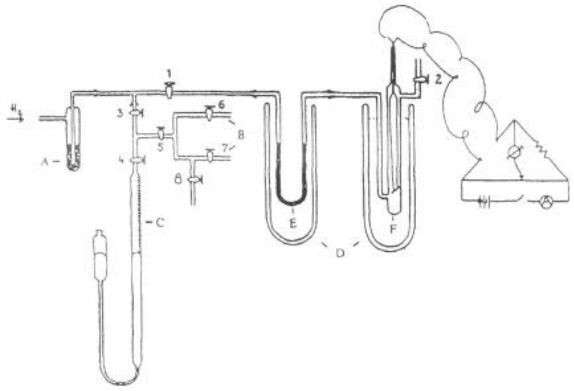


Figure 21- Cremer and Prior chromatographic device. The carrier gas was purified though an adsorbent (A) and the column was made of a 20cm thick silica gel or activated coal with a diameter of 1cm (E) (Adapted from I.G. Kolomnikov et al. [65])

In 1948-49, Glueckauf conducted the first gas-chromatographic isotope separation, which involved separating 22Ne and 20Ne isotopes through adsorption on charcoal at -196°C [65]. From his experiments, he derived several equations capable of determining the purity of the extracted substances and constructing the adsorption isotherm based on the adsorption curves. This was achieved by determining the concentration of the extracted substances and using constants like column length [65].

Nusin Motelevich Turkeltaub, a Russian chemical scientist, began developing a chromatographic method for detecting microconcentrations of hydrocarbons in the air. His analyzer was one of the earliest examples in the world to utilize gas chromatography principles. He used a stationary phase of water-moistened activated charcoal with air as the carrier gas (Figure 22) [65].

While 1952 is commonly recognized as the birth year of modern GC, thanks to the renowned work of Martin, earlier research had already described the use of gas chromatography at various levels and with different types of equipment[65].

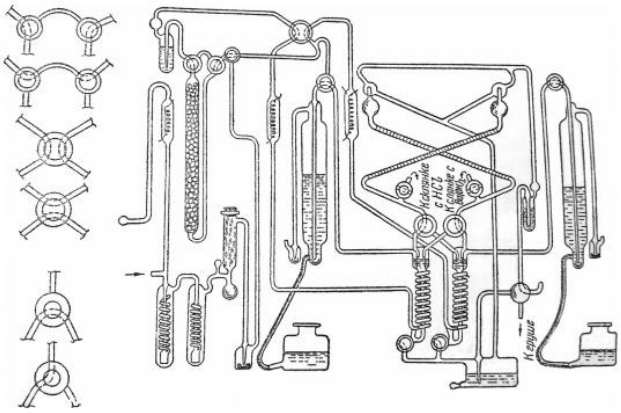


Figure 22- Diagram of a chromatograph by Turkeltaub (Adapted from Kolomnikov et al. [65])

Nowadays, GC is the best choice for analyzing volatile and semi-volatile small molecules from various matrices, with applications in the fields of food, the environment, and toxicology [66]. A modern GC system comprises a mobile phase gas, an inlet for sample injection into the column, a column where separation occurs, an oven for temperature control according to the desired method, a detector to register and analyze the column effluent, and software to record detections and control the GC itself [67].

The carrier gas facilitates the movement of sample constituents through the column, typically using nitrogen or helium, depending on the laboratory and the type of analysis being performed. In some cases, air can be used, particularly with portable or on-site chromatographs, although it's less common. As most GC columns are sensitive to moisture and oxygen when operating at high temperatures, it's crucial to pay special attention to gas purity and the tubing used for transportation to extend column longevity and ensure result reproducibility [67].

Figure 23- Display of a modern chromatograph

Several detectors can be coupled with GC to enhance the analysis of the substances being studied and separated [67]. Flame ionization detector (FID), thermal

conductivity detector (TCD), and electron capture detector (ECD) are among the most commonly used detectors in conjunction with GC. However, over the years, affordable mass spectrometers have gained prominence and revolutionized GC analysis [67]. Gas Chromatography coupled with mass spectrometry (GC/MS) was a natural combination, as both methods have complementary features, utilizing small sample amounts in the gaseous phase [68].

Mass spectrometry (MS) is typically considered an instrumental technique for the separation of electrically charged species in a gas. It detects and sorts ions based on their mass-to-charge ratios (*m/z*), either in space or in time [69]. These ions can correspond to fragmented species formed during the ionization process or to their original molecules. This technique is highly selective because it allows for the direct identification of molecules solely based on their *m/z* or fragmentation patterns [69]. Several instrument and sample-related parameters, such as ion transmission efficiency or ionization interferences, can affect MS, making it crucial to study each factor to maximize MS capabilities. Sample preparation is one of the most critical factors to investigate, as it can mitigate sample-related issues and enable a clean, low-interference analysis [69].

2.5. Derivatization

Though the use of derivatizing agents, GC can also be applied to some non-volatile compounds, and better the detection of some substances [66]. In GC, derivatization is defined as the procedural technique that primarily modifies the functionality of an analyte in order to enable a better separation [70]. In GC analysis, the volatility of a substance is a required characteristic, and with derivatization, highly polar materials can be transformed into sufficiently volatile products, allowing for an elution without the risk of thermal decomposition or molecular re-arrangements [70]. The derivatization process can, not only, increase the volatility but also decrease it. Compounds with -SH, -NH, -COOH, -OH, or any other functional groups with active hydrogens need to be carefully studied due to the inherent intermolecular hydrogen bonds these groups tend to form [70]. These bonds affect the volatility and thermal stability of the substance while also interacting with the column packing [70]. Derivatization is also capable of reducing analyte adsorption in the GC, improving detector response, peak separations and peak symmetry [70]. Usually, derivatization is aimed with the goal of improving 3 main aspects of GC: The suitability of the compounds being analysed, making sure that they present the necessary characteristic for a successful GC analysis [70]; The efficiency in the production of good peaks, with

good resolution and symmetry for an easy identification and posterior analysis [70]; And the detectability, allowing for the production of a better signal from the interaction between the analyte and the GC detector of choice [70]. There are three main reaction types when it comes to derivatization [70]. These are alkylation, silylation and acylation [70].

Alkylation is primarily used as a first step in further derivatizations or to protect active hydrogen groups in a molecule [70]. The general process that occurs in alkylation is the esterification (Figure 24), where the active hydrogen group in the molecule is replaced by an aliphatic or aliphatic-aromatic group [70]. Some common derivatization agents used in this reaction are dialkylacetals (dimethylformamide, DMF), diazoalkales (Diazomethane, N2CH2), pentafluorobenzyl bromide (C7H2F5Br), pentafluorobenzyl-hydroxylamine hydrochloride (PFBHA), benzylbromide, tetrabutylammonium hydroxide (TBH), and boron trifluoride (BF3) [70].

$$RCOOH + PhCH_2X \rightarrow RCOOCH_2Ph + HX$$

Figure 24- General reaction for the esterification process. X: Halogen or Alkyl group; H: Alkyl group. (Adapted from Orata [70])

Silylation volatizes the sample, making it the most prevalent derivatization method and suitable to prepare non-volatile compounds for GC analysis [70]. This derivatization consists in the introduction of a silyl group into a molecule, usually replacing an active hydrogen in the process [70]. This substitution reduces hydrogen bonding and polarity, allowing compounds regarded as non-volatile or unstable to be successfully analysed by GC [70]. Silylation occurs though a nucleophilic SN2 attack and as so, the better the leaving group, the better the reaction (Figure 25) [70]. Some reagents used in silylation are bis(trimethylsilyl)–acetamide (BSA), bis(trimethylsilyl)trifluoroacetamide (BSTFA), *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA), hexamethyldisilzane (HMDS), trimethylchlorosilane (TMCS), trimethylsilylimidazole (TMSI), trimethylsilyldiethylamine (TMS-DEA), and *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide (MTBSTFA), being BSA and BSTFA the most popular and used reagents [70].

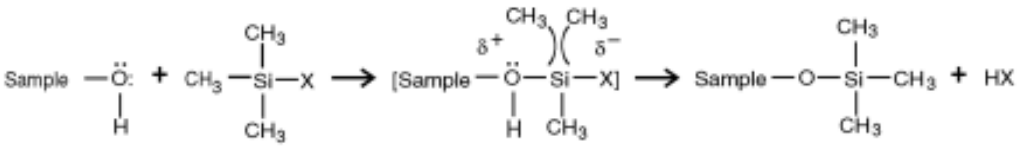


Figure 25- General reaction for the formation of trialkylsilyl derivatives for trimethylchlorosilane. X: Cl. (Adapted from Orata [70])

Last but not least, in acylation, an acyl group is added to the compound, with active hydrogen containing molecules turning into esters, thioesters and amides [70]. Acylation is popular for the creation of volatile derivatives, while also improving the stability of compounds that are thermally labile through the insertion of protection groups [70]. It can turn extremely polar compounds into GC ready molecules, allowing for their analysis, and it can also be a useful tool in combination with silylation or as an alternative [70]. Some of the common reagents used in this process are Fluorinated anhydrides like trifluoroacetoic anhydride (TFAA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA), fluoracylimidazoles which include trifluoroacetylimidazole (TFAI), pentafluoropropanylimidazole (PFPI) and heptafluorobutyrylimidazole (HFBI), pentafluorobenzoyl chloride (PFBCI), pentafluoropropanol (PFPOH), 4-carbethoxyhexafluorobutyryl chloride (4-CB) and *N*-methyl-bis(trifluoroacetamide) (MBTFA) [70].

MBTFA is the derivatization reagent used in this work and it rapidly reacts with primary and secondary amines, while also reaction slowly with thiol and hydroxyl groups (Figure 26) [70]. It is often used for amine drugs like stimulants, amino acids, and alcohols and it is recommended for the analysis of sugars [70]. The conditions necessary for the reaction are mild an relatively inert, with the formation of non-acidic by-products, keeping column damage to a minimum [70].

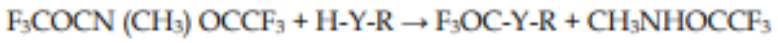


Figure 26- Derivatization reaction of amines, hydroxyl groups and thiols using MBTFA.Y: O, S, NH, NR, R; R: Alk, Ar. (Adapted from Forata [70])

Chapter 2| Aims

The first goal of this work was to develop a method to determine amphetamine-like stimulants in hair samples. In this sense, the entire process of optimizing the extraction and sample clean-up conditions for AMP, MAMP, MDA, MDMA, MDE, and MDE in hair samples using MEPS as the sample clean-up method is described, followed by their subsequent quantification by GC-MS.

Chapter 3| Experimental Procedure

1. Materials and methods

1.1. Reagents and standards

The analytical standards of AMP, MAMP, MDA, MDMA, MBDB, and MDE and the internal standards of AMP-d6, MAMP-d9, MDA-d5, MDMA-d5 and MDE-d5 were purchased from Sigma Aldrich (Merck Co, Darmstadt, Germany). All the analytical grade chemicals used were methanol, hydrochloric acid, acetonitrile, ethyl acetate, formic acid (Merck Co, Darmstadt, Germany). Deionized water was acquired from a Milli-Q System (Millipore, Billerica, MA, USA) and ammonium hydroxide from T.J. Baker (Deventer, Holland). A MEPS syringe (250 µL) and M1 (4 mg; 80% C_8 and 20% SCX) cartridge from SGE Analytical Science, (ILC, Portugal), were used. Each analyte stock solution was prepared in methanol at 10 ng/mL. Working solutions were created by effective stock dilution.

1.2. Biological specimens

Blank hair samples used during the experimental procedure were obtained from personnel working in the laboratory, who are neither consumers nor are exposed to amphetamine type-stimulants in their day-to-day life. Authentic hair samples were obtained from drug users and from proficiency testing schemes. Each hair sample was given willingly after reading and accepting the informed consent (Ethical Committee project: CE- UBI-Pj-2021-046:ID1005).

The hair specimens were collected from the posterior vertex region of the scalp, as close as possible to the root without leaving any signs of the collection. Clean and uncontaminated scissors were used to cut the hair. Each sample was saved in a paper envelope and correctly identified.

1.3. Gas chromatographic and mass spectrometric conditions

In this work it was used a gas chromatograph HP 7890A coupled with a mass spectrometer model 7890B from Agilent Technologies. A capillary column (30m x 0,25 mm; 0,25 µm i.d.) with 5% phenylmethylsiloxane (HP-5 MS) was supplied by J & W Scientific (Folsom, CA, USA). The data was acquired in selected ion monitoring (SIM)

mode using MassHunter WorkStation Rev. B.02.01 from Agilent Technologies. The chromatographic conditions were as follows: the oven temperature was held at 90°C for 2 minutes, then increased 20°C per minute up to 300°C; maintained at 300°C for 3 minutes. The total run took 15.5 minutes. Helium was used as carrier gas with a constant flow of 0.8 mL per minute. Only 2 µL of the derivatized extract was injected in a splitless mode. The mass spectrometer was operated with a filament of 70 µA in the positive electron ionization mode. Inlet and ion source temperatures were set at 220 °C and 280 °C, respectively. The respective ions were chosen based on abundance and signal to noise ratio with the matrix. Table 4 presents the retention times and ions used for each amphetamine.

Table 4- Retention times and MS conditions for each ATS.

	Retention Time (min)	*Quantifying ion (m/z)*	*Qualifying ions (m/z)*	*Dwell Time*
AMP	6.45	140	91/118	
AMP-d6	6.43	144	_	
MAMP	7.20	154	110/118	
MAMP-d9	7.17	161	_	
MDA	8.60	135	162/275	
MDA-d5	8.58	280	_	50
MDMA	9.28	154	162/135	
MDMA-d5	9.26	158	_	
MDE	9.52	168	162/140	
MDE-d5	9.50	173	_	
MBDB	9.63	168	135/176	

1.4. Sample preparation

Firstly, the hair samples were washed with methanol, ultra-pure water (H2O Milli-Q) and dichloromethane for 15 minutes each, in a tube roller, while respectively discarding each solvent before adding the next. The samples were then segmented into small pieces, after air drying, using clean scissors and a piece of paper in the shape of a cone. For the extraction, an alkaline hydrolysis was performed as follows: 50 mg of the previously cut and washed hair were placed in a glass tube along with 500 µL of NaOH (1M), the tubes were then left to hydrolyse overnight at 45°C. After this step, the tubes were cooled to room temperature before adding 50 µL of HCl (10M) to neutralize the

pH of the mixture. Following this procedure, the samples were transferred to conical tubes and proceed to be centrifuged at 4500 rpm for 15 min. The supernatant was recovered and 500 µL of H2O Milli-Q was added to allow for a better MEPS clean-up procedure. After the sample clean-up is performed, 10 µL of an internal standard mixture solution (AMP-d6, MAMP-d9, MDA-d5, MDMA-d5 and MDE-d5) at 1 µg/mL was added followed by 30 µL of *N*-Methyl-bis(trifluoroacetamide) (MBTFA). The extracts were then evaporated to dryness under nitrogen stream, after which 50 µL of MBTFA was added and microwave-assisted derivatization was performed at 800W for 2 min. A 2 µL aliquot of the sample was injected into the GC-MS.

1.5. MEPS Optimization

For the optimization of the MEPS protocol, two different approaches were used, namely Design of Experiments (DOE) and Surface Response (SR). Three variables were studied resorting to the software MiniTab®: load cycles; wash cycles; and elution cycles. After this statistical analysis, a non-parametric test of a Friedman's Two-Way Analysis of Variance Related Samples was performed, in SPSS, for the number of washes.

1.6. Validation

This method was fully validated according to the ANSI/ASB guidelines [71]. The studied parameters in this validation were selectivity, limit of detection (LOD), lower limit of quantification (LLOQ), linearity, inter-day, intra-day and intermediate precision and accuracy, and recoveries. The whole validation process was carried out for 5 days.

2. Results and discussion

2.1. Optimization of the extraction and sample clean-up procedure

2.1.1. Extraction procedure selection

The extraction procedure allows the solubilization of the analytes from the tough hair matrix into a chosen solution for better workability and consequent analysis. Earlier in the work, some hair hydrolysis for amphetamine type-stimulants were described and from those, an alkaline hydrolysis/digestion was chosen as the extraction procedure

[59]. Since ATS are basic compounds, an extraction through basic hydrolysis using NaOH seemed to be the best fit for the job, not only due to the recommendation in the literature [59] but also because in this case, other types of hydrolysis might lead to loss in analytes due to the fragile nature of the ATS.

For this reason, 3 extractions (each in triplicate) using NaOH (1M) were tested, changing only the time of incubation and temperature. The extractions were the following:

- A) Incubation at 80ºC with 500 µL of NaOH 1M for 1h;
- B) Incubation at 60ºC with 500 µL of NaOH 1M overnight;
- C) Incubation at 45ºC incubation with 500 µL of NaOH 1M overnight.

Results showed that extractions B) and C) were the best hydrolysis for our target compounds, perhaps because the temperature used was not so drastic. While observing the chromatogram obtained when incubation at 80ºC was performed, the peaks for each analyte were not so clear and defined when compared with the others, also there was an interferent on the MDA peak. Despite no significant differences were observed between extractions B) and C), extraction C) showed a slightly cleaner and prominent chromatogram (Figure 27). Due to this fact, and since 45ºC is a more acceptable and secure temperature for the ATS, the extraction chosen was incubation C).

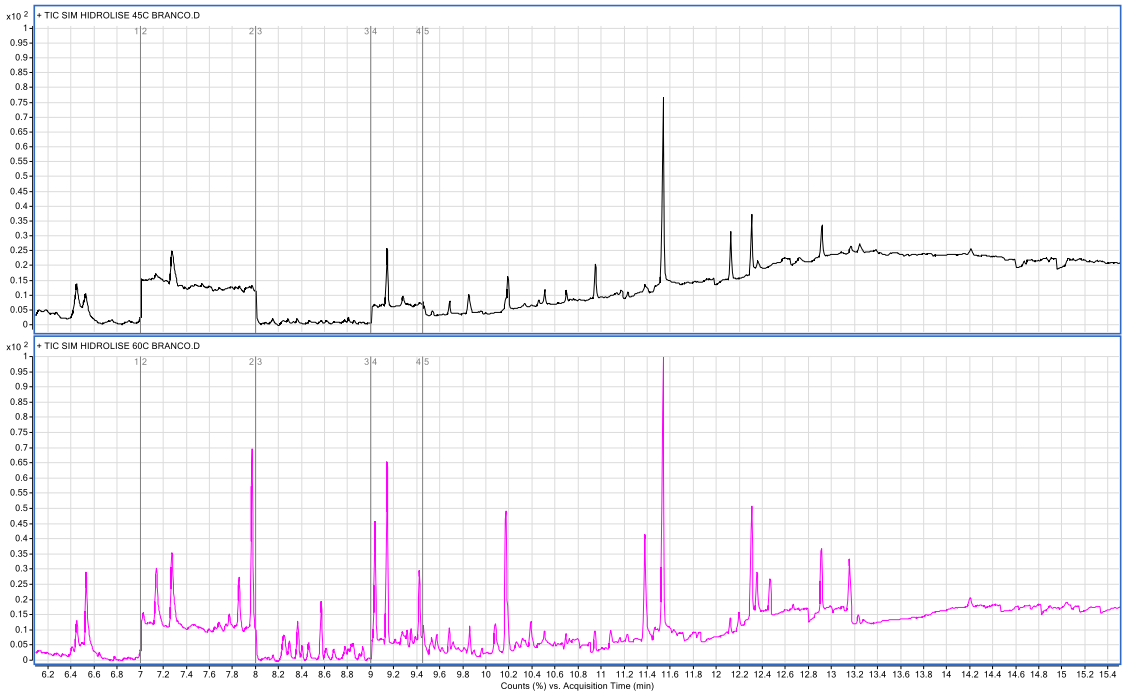


Figure 27- Chromatogram of a blank sample after the 45ºC overnight extraction (above) vs the 60ºC overnight extraction (below)

2.1.2. Optimization of the MEPS procedure using a design of experiments-Factorial Design

As previously mentioned, the MEPS protocol is made up of 5 essential steps: Sorbent conditioning; Sample Loading; Sorbent Washing; Elution; and sorbent regeneration. To perform a correct sample clean-up while maximizing the analytes recovered from the matrix, it is essential to optimize the primary changing steps in each MEPS protocol in order to best fit the sample and the compounds being analysed. The chosen steps to optimize were the load, wash and elution cycles. A first attempt using a design of experiments (DOE) approach with a factorial design was performed and although the results were promising, this approach was not enough to define an optimal number of cycles for each parameter studied. Even still, the work performed, and the results are showed below (Figures 27-29).

↓	C1	C2	C3	C4	C5	C6	C7
	StdOrder	RunOrder	CenterPt	Blocks	Load	Wash	Elution
1	6	1	1	1	15	1	6
2	4	2	1	1	15	3	2
3	2	3	1	1	15	1	2
4	8	4	1	1	15	3	6
5	7	5	1	1	5	3	6
6	10	6	0	1	10	2	4
7	1	7	1	1	5	1	2
8	11	8	0	1	10	2	4
9	3	9	1	1	5	3	2
10	9	10	0	1	10	2	4
11	5	11	1	1	5	1	6

Figure 28- Conditions obtained for the DOE-Factorial design

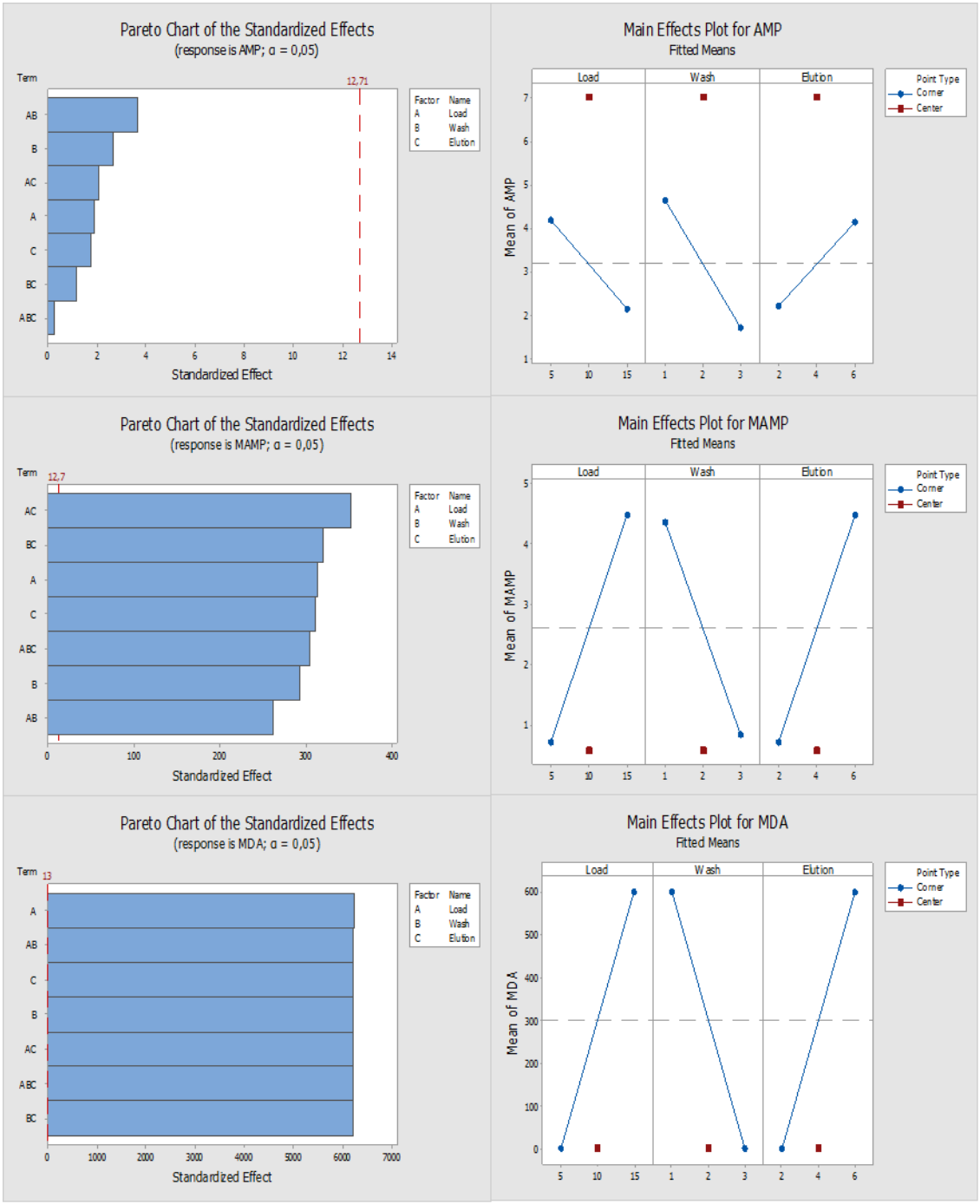


Figure 29- Pareto Chart of Standardized Effects (Left) and Main Effects Plot (Right) of AMP, MAMP and MDA

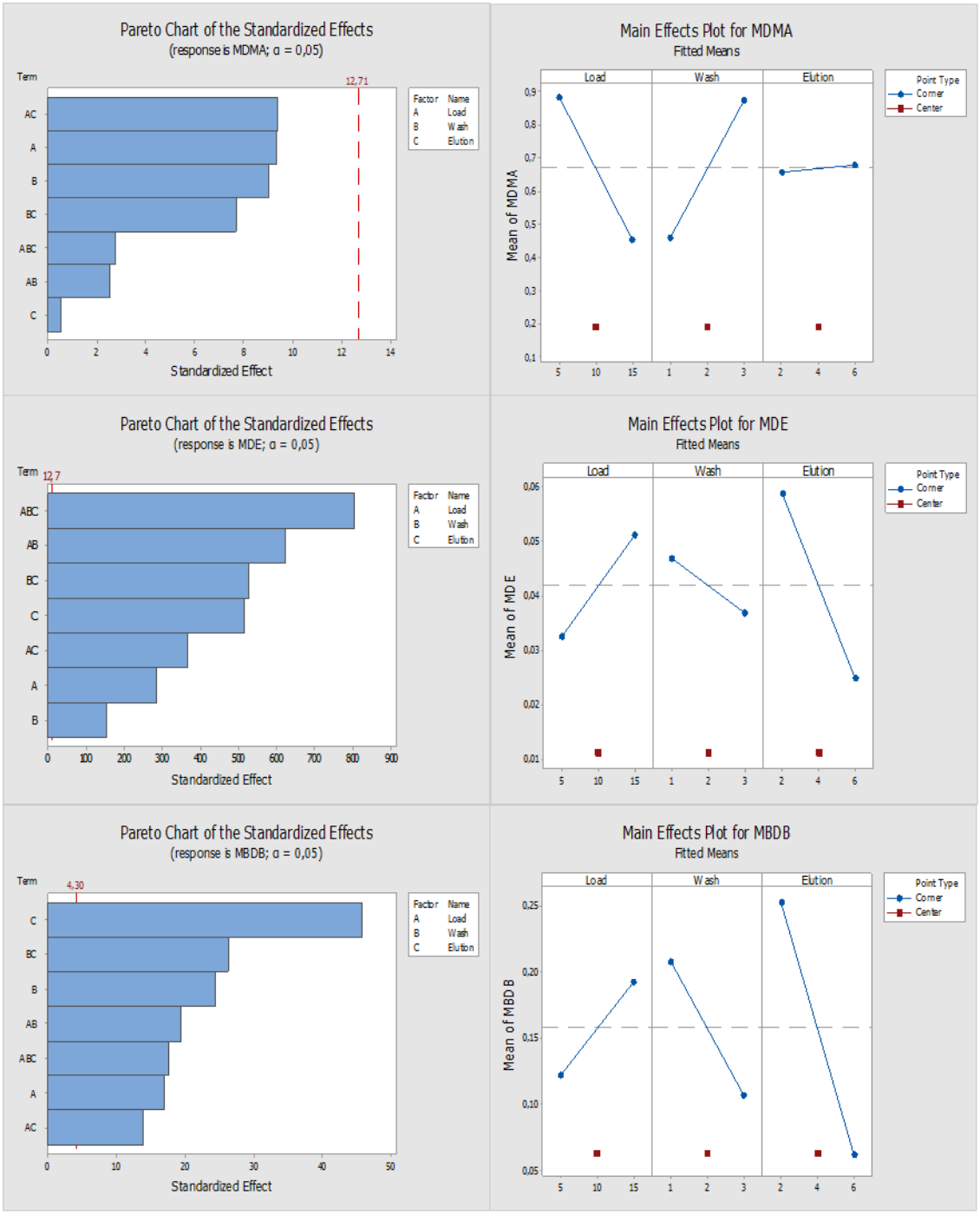


Figure 30- Pareto Chart of Standardized Effects (Left) and Main Effects Plot (Right) of MDMA, MDE and MBDB

The results from this statistical analysis showed inconsistency and lacked statistical significance for AMP and MDMA. The Pareto charts for AMP and MDMA revealed no significant variations, as the studied parameters did not cross the red line. In contrast, the Pareto charts for MAMP, MDA, MDE, and MBDB demonstrated that each parameter had an impact on the recovery of these compounds throughout the MEPS protocol. Since the goal of this optimization was to develop a method suitable for all selected ATS, these findings were disregarded. Instead, a DOE-SR (Design of

Experiments - Screening and Response Surface) was employed to delve deeper into the investigation of each selected parameter.

2.1.3. Optimization of the MEPS procedure with DOE- SR and application of the non-parametric test

Since the DOE-Factorial design test was not conclusive, a separate and more comprehensive test called surface response was employed. This test behaves similarly to the previous, but it allows for a higher range of cycles to be studied, demonstrating which number of cycles presented the most optimal result. Figure 31 shows the studied conditions for the statistical analysis and Figures 32 and 33 show the results obtained.

↓	C1	C2	C3	C4	C5	C6	C7
	StdOrder	RunOrder	PtType	Blocks	LOAD	WASH	ELUTION
1	14	1	-1	1	11,0000	3,50000	10,3863
2	3	2	1	1	2,0000	6,00000	1,0000
3	6	3	1	1	20,0000	1,00000	8,0000
4	18	4	0	1	11,0000	3,50000	4,5000
5	15	5	0	1	11,0000	3,50000	4,5000
6	7	6	1	1	2,0000	6,00000	8,0000
7	4	7	1	1	20,0000	6,00000	1,0000
8	12	8	-1	1	11,0000	7,70448	4,5000
9	13	9	-1	1	11,0000	3,50000	-1,3863
10	9	10	-1	1	-4,1361	3,50000	4,5000
11	1	11	1	1	2,0000	1,00000	1,0000
12	10	12	-1	1	26,1361	3,50000	4,5000
13	2	13	1	1	20,0000	1,00000	1,0000
14	5	14	1	1	2,0000	1,00000	8,0000
15	19	15	0	1	11,0000	3,50000	4,5000
16	11	16	-1	1	11,0000	-0,70448	4,5000
17	20	17	0	1	11,0000	3,50000	4,5000
18	16	18	0	1	11,0000	3,50000	4,5000
19	8	20	1	1	20,0000	6,00000	8,0000

Figure 31- Conditions obtained for the SR

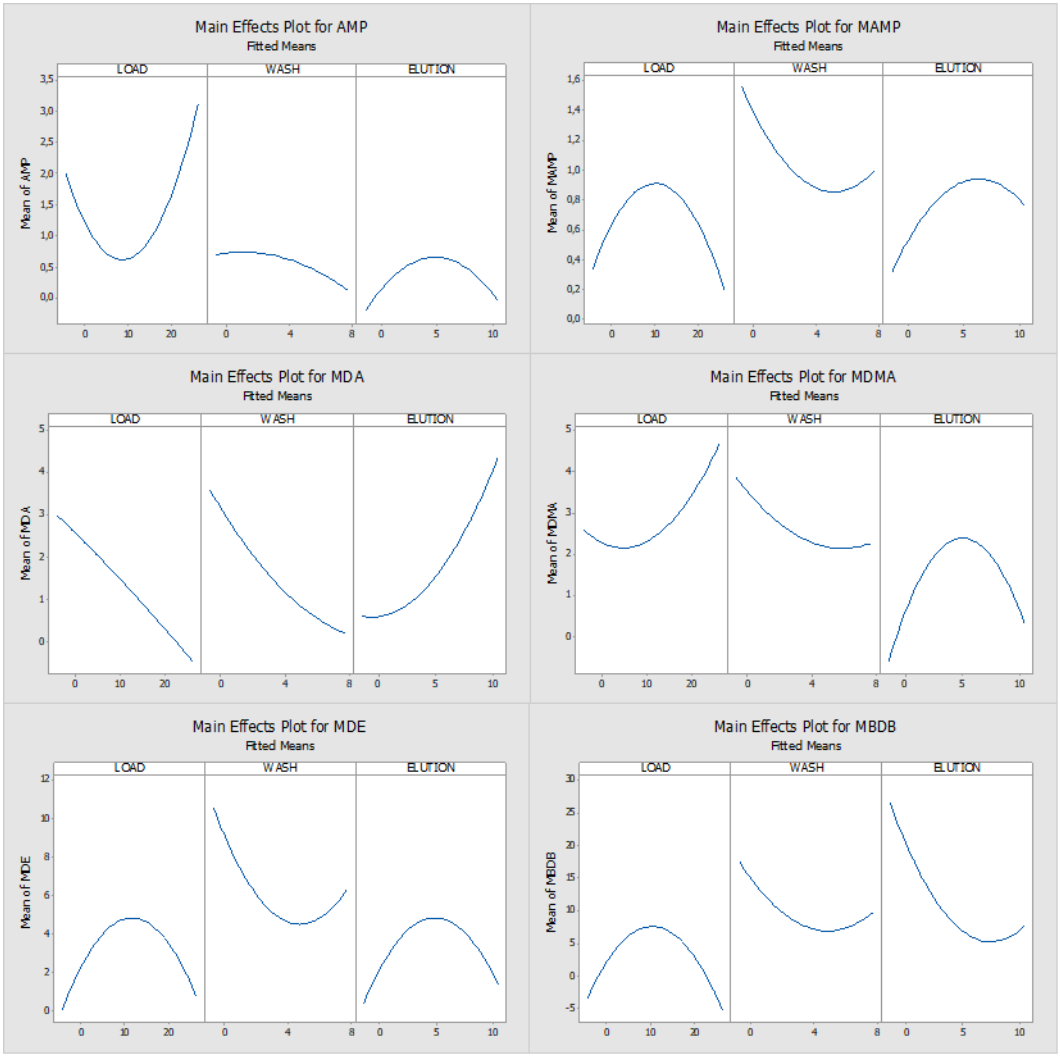


Figure 32- Main effects plots obtain from the SR analysis for AMP, MAMP, MDA, MDMA, MDE and MBDB

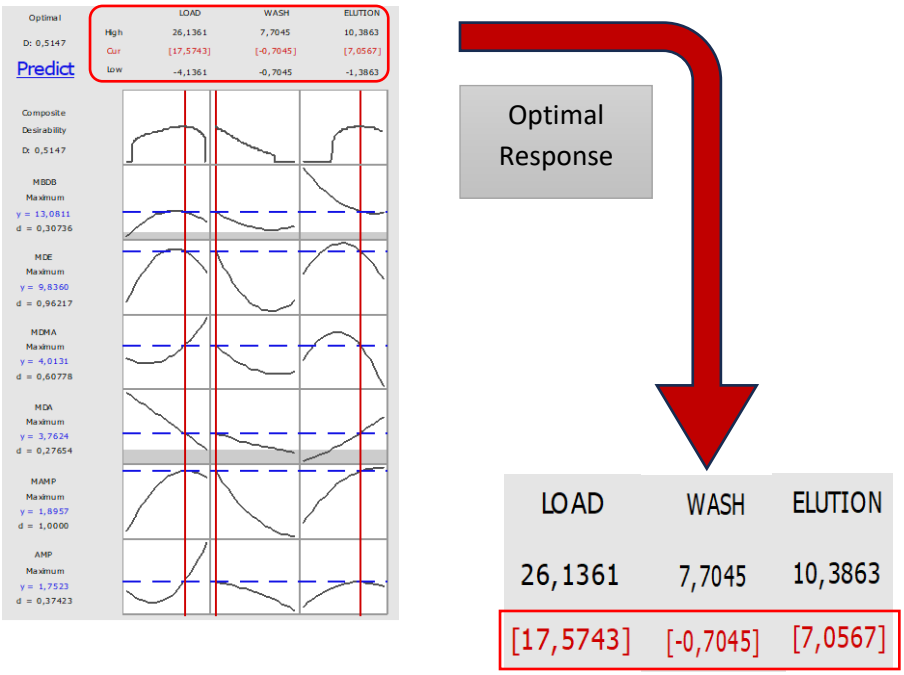


Figure 33- Results of the response optimizer for AMP, MAMP, MDA, MDMA, MDE and MBDB

Due to the critical importance of removing unwanted interferents, a thorough investigation of the sorbent washing step in the MEPS protocol is essential to minimize analyte loss and enhance interferent removal. To achieve this goal, a non-parametric test was specifically applied to assess the effectiveness of different washing approaches. In this study, a triplicate analysis was conducted for three distinct protocols: the first protocol involved no washes, the second protocol utilized only 50 µL of H2O, and the third and final protocol employed 50 µL of H2O followed by 50 µL of a 5% MeOH solution in H2O. Subsequently, the results obtained from this analysis were subjected to a Friedman's Two-Way Analysis of Variance Related Samples in SPSS (Figure 34).

	Hipótese nula	Teste	Sig.[a,b]	Decisão
AMP	As distribuições de Sem_Lavagem, Lavagem_H2O e Lavagem_MeOH são iguais.	Amostras Relacionadas de Análise de Variância de Dois Fatores de Friedman por Postos	,050	Rejeitar a hipótese nula.
MAMP	As distribuições de Sem_Lavagem, Lavagem_H2O e Lavagem_MeOH são iguais.	Amostras Relacionadas de Análise de Variância de Dois Fatores de Friedman por Postos	,097	Reter a hipótese nula.
MDA	As distribuições de Sem_Lavagem, Lavagem_H2O e Lavagem_MeOH são iguais.	Amostras Relacionadas de Análise de Variância de Dois Fatores de Friedman por Postos	,097	Reter a hipótese nula.
MDMA	As distribuições de Sem_Lavagem, Lavagem_H2O e Lavagem_MeOH são iguais.	Amostras Relacionadas de Análise de Variância de Dois Fatores de Friedman por Postos	,097	Reter a hipótese nula.
MDE	As distribuições de Sem_Lavagem, Lavagem_H2O e Lavagem_MeOH são iguais.	Amostras Relacionadas de Análise de Variância de Dois Fatores de Friedman por Postos	,135	Reter a hipótese nula.
MBDB	As distribuições de Sem_Lavagem, Lavagem_H2O e Lavagem_MeOH são iguais.	Amostras Relacionadas de Análise de Variância de Dois Fatores de Friedman por Postos	,607	Reter a hipótese nula.

Figure 34- Results from the non-parametric test applied to the wash step. AMP is negatively affected by the washes.

Based on the results obtained in both statistical analysis, the washing step was excluded from the protocol due to its negative impact on the recovery of the AMP. Furthermore, this step did not exhibit significant differences in the extraction process of the other compounds.

The final MEPS conditions were as follows: 18 strokes of 100 µL for sample loading; no washing; 7 strokes of 100 µL as the elution.

This sample clean-up method was fully and correctly optimized though this complete statistical analysis, being able to achieve the cut-off concentration of 0.2ng/mg for each compound defined by the SoHT [60].

2.2. Method validation parameters

2.2.1. Selectivity

Selectivity represents a methods ability to detect the compounds of interest in the presence of matrix derived interferents. Endogenous substances present in the biological matrix may create similar hight molecular weight ions which, if presenting with retention times close to the compounds of interest, may negatively affect their detection. For this study, the ANSI/ASB guidelines [71] were followed. A blank matrix sample from 10 different sources was analysed without the addition of any internal standard. The samples were provided by laboratory staff who did not consume any of the ATS being studied. Additionally, 3 characteristic ions were chosen for each analyte of interest, where no interferences were observed, to guarantee a correct and reliable analysis.

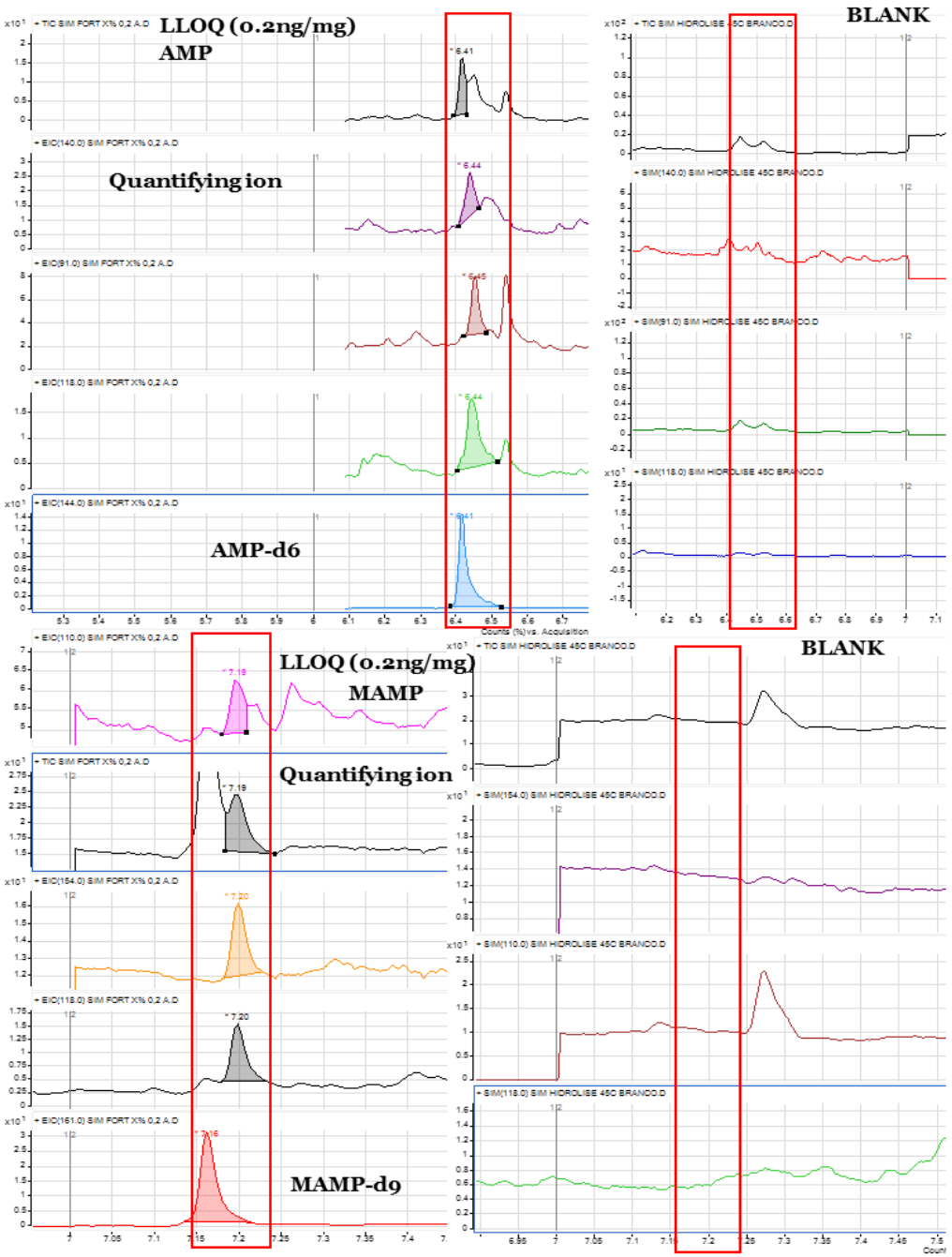
The criteria used for a confident positive identification included several factors: first, the absolute retention time had to be within 2% or ± 0.1 min of the retention time of the same compound in the control sample. Additionally, there needed to be the presence of three transitions per analyte. Furthermore, there were specific tolerances set for the relative ionic intensities between these transitions, based on the relative ion intensity in the control sample:

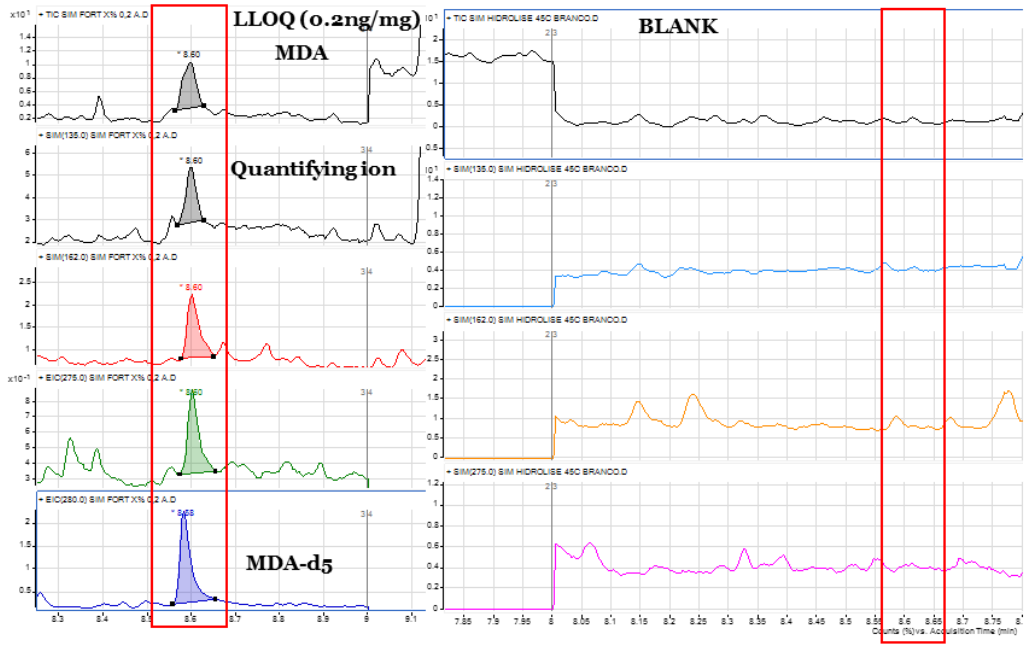
- If the relative ion intensity in the control sample exceeded 50%, an absolute tolerance of ± 10% was accepted.
- If the relative ion intensity in the control sample ranged between 25% and 50%, a relative tolerance of ± 20% was permitted.

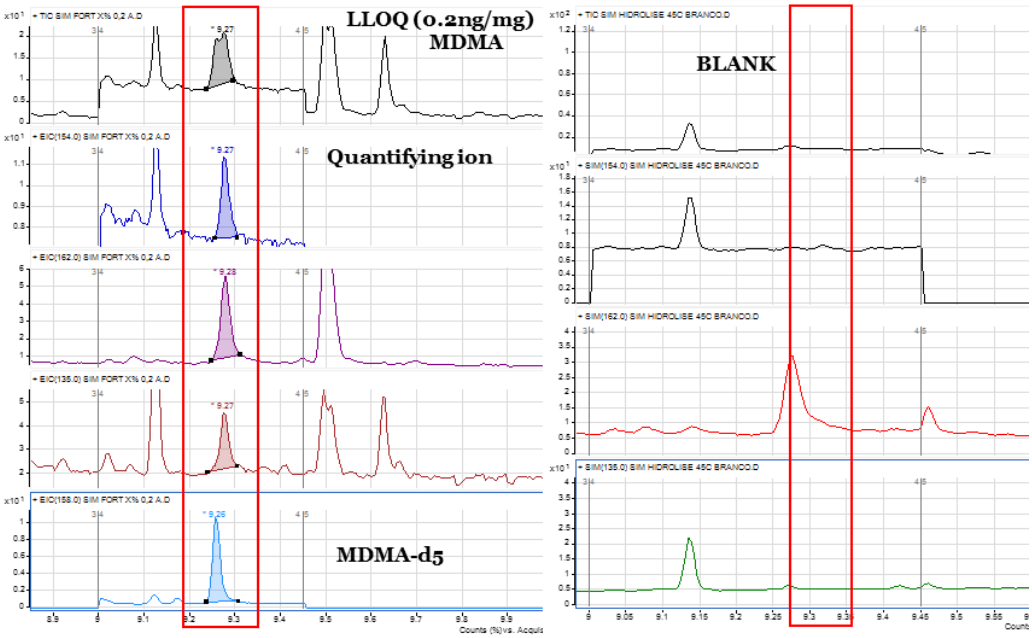
- If the relative ion intensity in the control sample fell between 5% and 25%, an absolute tolerance of ± 5% was accepted.
- Finally, for relative ion intensities of 5% or less, a relative tolerance of ± 50% was applied.

According to these criteria [72], the method would be considered selective if no analyte could be identified in the blank samples.

Figure 35 shows a chromatogram for the chosen ions, of each compound, at the LLOQ concentration (0.2ng/mg) against a blank sample.




LLOQ (0.2ng/mg)
MDA
Quantifying ion
MDA-d5
BLANK


LLOQ (0.2ng/mg)
MDMA
Quantifying ion
MDMA-d5
BLANK

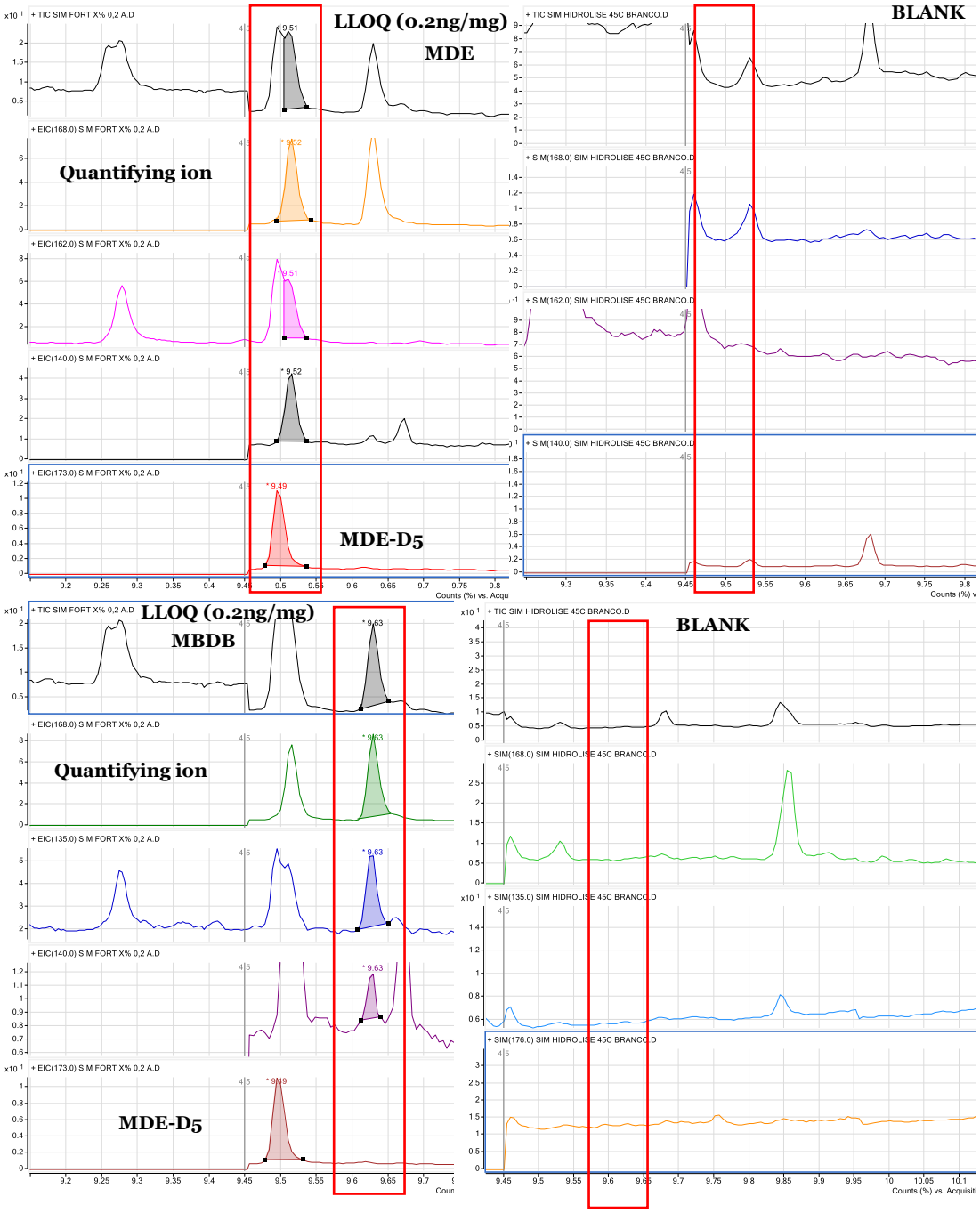


Figure 35- Chromatogram of the LLOQ of 0.2ng/mg (left) vs a blank sample for each compound

2.2.2. Calibration curves and limits

The blank hair samples were spiked before extraction in a concentration ranging from 0.2-5 ng/mg for all studied compounds. To evaluate the linearity of the method, 6 calibrators were chosen and evenly distributed inside this range, with this being repeated 5 times [71]. The relationship between the peak area of each compound and

the respective internal standard (IS) was studied to establish a good and acceptable calibration curve for each compound.

According to specifications by the ANSI/ASB guidelines [71], the maximum acceptable bias for any calibrator was ±20% and the coefficient of determination (R^2) had to be at least 0.99.

To fulfil this criterion, several regressions ($1/x$; $1/\sqrt{x}$; $1/x^2$; $1/\ y$; $1/\sqrt{y}$; $1/y^2$) were studied to find out which presented the best R^2 value. The adopted calibration ranges were wide, and as such weighted least squares regressions had to be used to compensate for heterocedasticity. The optimal regression was $1/x$ and table 5 shows all the calibration data.

Table 5- Linearity (n=5)

Analyte	Weight	Linear Range (ng/mg)	Linearity		R^{2*}	LLOQ (ng/mg)
			Slope*	Intercept*		
AMP	1/x	0.2-5.0	0.3412±0.0825	0.0463±0.0161	0.9937±0.0031	0.2
MAMP			0.4305±0.0687	0.0416±0.0215	0.9954±0.0018	
MDA			4.3669±0.6948	0.3702±0.4687	0.9951±0.0031	
MDMA			1.1700±0.2365	0.1568±0.0371	0.9936±0.0033	
MDE			2.3450±0.2160	0.4165±0.1245	0.9948±0.0040	
MBDB			2.1010±0.1408	0.1382±0.1936	0.9935±0.0022	

*Mean values ± standard deviation

The lower limit of quantification (LLOQ) represents the lowest concentration of analyte that can be measured with an acceptable bias and precision [71]. For this work, a recommended cut-off of 0.2 ng/mg by the SoHT was adopted and we were able to reach a LLOQ of 0.2 ng/mg.

The limit of detection (LOD) was defined as the lowest concentration at which the analytes in the sample can be reliably differentiated from the blank matrix and identified. LOD is usually calculated based on the signal-to-noise ratio of a measurement: LOD = (3.3 x SD) / S

Where: LOD is the Limit of Detection; SD is the standard deviation of the blank (noise); S is the slope of the calibration curve (response). In this work, LOD was not systematically studied due the limit of quantifications (LOQs) are 0.2 ng/mg

(recommended cut-off by the SoHT). At the 0.2 ng/mg the signal-to-noise is higher than 10 times.

Although this method utilizes such a low sample volume and similar quantities of hair when compared with other non-miniaturized extractions and clean-ups, it is still able to achieve the minimum LLOQ imposed by the SoHT. On the other hand, other methods sacrifice the greenness, renewability and rentability of their analysis by utilizing higher sample volumes and disposable clean-up columns, like SPE, to obtain lower LODs and LLOQs [73–75]. In this sense, MEPS is a great option since it is able to reach guidelines limits with minimal sample and solvent volume and allowing for a reutilization of its columns for several extractions (in this work, MEPS BINs lasted at between 40-50 extractions). Table 6 shows the comparison of LOD, LLOQ and recoveries some methods for some of the ATS studied in this work.

Table 6- Comparison of LOD, LLOQ and recoveries of several methods for ATS

Analyte	Sample Volume	LOD (ng/mg)	LLOQ (ng/mg)	Recoveries (%)	Extraction method	Method of detection	Reference
AMP MAMP MDA MDMA MDE	50mg hair 1 mL	0.02	0.05	48%(LLOQ) >72%	cryogenic grinding	LC–MS/MS	[76]
AMP MAMP MDMA	50mg 3mL	0.01	0.2	N.S	HF-LPME	GC-MS	[77]
AMP MAMP MDA MDMA MDE	25mg Hair 400µL	0.0005 0.0006 0.0007 0.0009 0.0007	0.02 0.05 0.04 0.13 0.06	107±5 99±2 101±5 99±4 98±3	SUPRAS	LC-ESI-MS/MS	[78]
AMP MAMP MDA MDMA MDE	10mg Hair 3mL	0.002 0.0024 0.0023 0.002 0.0027	0.01	>88.9 ±7.4	Liquid extraction (Methanol)	LC-HRMS	[79]
AMP MAMP	50mg Hair 3mL	0.002	0.01	62.8±5.6 66.3±1.8	SPE	LC-MS-MS	[75]
AMP MAMP MDA MDMA MDE	25mg Hair 2mL	0.03	0.05	> 88.6	SPE	GC-MS	[74]
AMP MAMP MDA MDMA	20mg Hair 2.5mL	N.S	0.031	<35	SPE	UHLC–MS/MS	[73]
AMP MAMP MDA MDMA MDE MBDB	50mg Hair 1.05mL	0.2	0.2	8.8±0.9 - 51.6±4.8	MEPS	GC-MS	This work

Hollow Fiber Liquid-phase Microextraction (HF-LPME); LC-ESI-MS/MS: Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry; LC-HRMS: Liquid chromatography-high resolution mass spectrometry; MEPS: Microextraction by packed sorbent; NS: Non Specified; SUPRAS:

Supramolecular solvents); SPE: Solid Phase Extraction; UHLC–MS/MS: ultra performance liquid chromatography–tandem mass spectrometry.

2.2.3. Intra-day, Inter-day and Intermediate Precision and Accuracy

Precision and accuracy are required in every analytical method to ensure that the values obtained from such method are viable and as close to the real values as possible. To evaluate these parameters, a triplicate analysis of a low (0.2 ng/mg), medium (2.5 ng/mg), and high (4.0 ng/mg) concentration was performed in triplicate over 5 different runs [71]. The precision is then measured by the coefficient of variation (%CV) (Equation 1) and the accuracy is measured by the relative error (%RE/BIAS) (Equation 2), while not exceeding ±20% at each concentration in order to be acceptable [71].

$$(1)\%CV = \frac{Standard\ deviation}{mean} \times 100$$

$$(2)\%RE = \frac{Obtained\ Value - Nominal\ Value}{Nominal\ Value} \times 100$$

The intra-day precision and accuracy is analysed all on the same day, with the triplicate (5 days) of the low, medium, and high concentrations obtaining overall %CV and %RE values ranging from 3.99-18.41% and -15.48-12.34%, respectively (Table 7) [71]. These are acceptable values for intra-day precision and accuracy since they present under the defined limit of ±20% at each concentration.

Table 7- Intra-day precision and accuracy (n=5)

Analyte	Spiked	CV (%)	RE/BIAS (%)
AMP	0.2	18.41	-2.05
	2.5	3.99	1.20
	4	4.49	12.34
MAMP	0.2	11.08	-0.71
	2.5	10.21	7.47
	4	8.67	-3.82
MDA	0.2	5.86	-9.72
	2.5	15.58	-1.89
	4	9.08	5.73
MDMA	0.2	11.08	-0.71
	2.5	10.21	7.47

	4	5.30	-15.9
MDE	0.2	11.08	-0.71
	2.5	10.21	7.47
	4	5.90	-15.48
MBDB	0.2	11.08	-0.71
	2.5	10.21	7.47
	4	8.34	-4.31

Concentrations in ng/mg; CV: Coefficient of variation; RE: Relative error

The evaluation of inter-day precision and accuracy was made for all calibrators during a 5-day period. obtained %CVs and %RE were lower than 15% for all analytes at every tested concentration (Table 8).

Table 8- Inter-day precision and accuracy (n=5)

Analyte	Spiked	CV (%)	RE/BIAS (%)
AMP	0.2	12.76	0.83
	0.5	13.85	-0.90
	0.7	6.35	3.44
	1	6.39	-4.43
	2	3.21	1.00
	5	6.54	-3.45
MAMP	0.2	11.93	4.46
	0.5	9.95	-5.63
	0.7	7.16	-1.67
	1	7.54	1.77
	2	5.61	-0.35
	5	2.01	0.49
MDA	0.2	4.97	-1.38
	0.5	11.09	0.41
	0.7	4.15	-2.79
	1	3.75	1.74
	2	6.50	3.40
	5	3.66	-4.94
MDMA	0.2	8.15	-1.10
	0.5	5.01	-3.51
	0.7	12.14	0.89

	1	2.80	1.00
	2	8.63	3.36
	5	1.91	-2.24
MDE	0.2	7.09	-1.06
	0.5	3.91	0.19
	0.7	10.35	-2.88
	1	3.86	1.65
	2	7.65	3.46
	5	1.99	-2.13
MBDB	0.2	12.04	-4.7
	0.5	12.10	2.5
	0.7	8.19	-1.3
	1	7.31	4.9
	2	5.07	0.6
	5	10.64	-8.1

Concentrations in ng/mg; CV: Coefficient of variation; RE: Relative error

For the intermediate precision and accuracy, 3 quality control samples with concentrations of 0.2, 2.5, and 4.0 ng/mL were evaluated with a n=3 for a period of 5 days. Obtained %CVs and %RE were all acceptable and bellow ±20%.

Table 9- Intermediate precision and accuracy (n= 15)

Analyte	Spiked	CV (%)	RE/BIAS (%)
AMP	0.2	15.37	-6.14
	2.5	7.77	-11.49
	4	7.25	-14.31
MAMP	0.2	15.54	13.11
	2.5	5.32	-8.98
	4	5.32	-17.86
MDA	0.2	19.79	-5.56
	2.5	1.40	-4.40
	4	5.43	-10.82
MDMA	0.2	6.67	7.04
	2.5	7.14	-3.27
	4	8.17	-12.43
MDE	0.2	3.47	-1.88
	2.5	1.84	1.42

	4	7.12	8.69
MBDB	0.2	7.65	8.37
	2.5	1.98	-5.62
	4	3.23	8.69

Concentrations in ng/mg; CV: Coefficient of variation; RE: Relative error

Since the method presents %CVs and %RE under the value defined by the *ANSI/ASB* guidelines it can be concluded that the developed and optimized MEPS method is both precise and accurate.

2.2.4. Recoveries

To evaluate the recovery of the developed method for all compounds, a triplicate analysis of 3 concentrations (0.2 ng/mg, 2.0 ng/mg and 5.0 ng/mg) were performed and compared with a blank spiked after the extraction. The relative area obtain from each spiked extraction was divided by the mean of the relative areas obtain from the spiked blank samples. The recoveries were as follows: 8-14% for AMP, 14-20% for MAMP, 10-15% for MDA, 18-28% for MDMA, 25-43% for MDE and 34-52% for MBDB (Table 10).

When compared with other methods, the recoveries obtain by this MEPS protocol are generally low. Going back to Table 6 we can see that recoveries for non-miniaturized and more dispendious methods are usually above the 80-90%, but it is obvious that the extra sample and solvent volume plays an important role in obtaining those recoveries [74–76,78,79]. We cannot forget that these recovery values may also be due to the fact that most methods resort to single use extraction devices, that don not suffer the risk of being worn out and losing the ability to separate the compounds of interest. In turn, this is in line with the lower LODs and LLOQs obtained by those same methods. By having a higher percentage of recovered analyte, it is logical that an easier detection and consequent, lower limit is obtained. It is important to mention that some of these publications calculate relative recovery and not absolute recovery (as calculated in the present study). With this in mind, MEPS is still an amazing technique, that even with low sample volume and recoveries, it is able to reach the minimum cut-off of 0.2ng/mg employed by the SoHT, as it was mentioned before. Not to mention that even some SPE methods, like Fernández et al. [73], present recoveries as low as the ones obtain with this method (<35), without presenting any of the advantages of MEPS. Unfortunately, it is not possible to compare the method developed in this work with others published in

the literature because there is no existing work that utilizes MEPS as a clean-up technique for all the ATS studied in this work.

Table 10- Recoveries obtain with the optimized MEPS protocol for AMP, MAMP, MDA, MDMA, MDE and MBDB

Analyte	Concentration (ng/mg)	Recovery (%) *
AMP	0.2	14.2±1.4
	2.0	11.8±1.5
	5.0	8.8±0.9
MAMP	0.2	16.8±1.9
	2.0	19.7±2.1
	5.0	14.6±1.1
MDA	0.2	13.4±2.7
	2.0	15.2±0.6
	5.0	10.9±1.2
MDMA	0.2	23.9±4.6
	2.0	27.5±4.1
	5.0	18.8±1.3
MDE	0.2	42.8±6.8
	2.0	38.4±5.3
	5.0	25.3±3.1
MBDB	0.2	51.6±4.8
	2.0	42.7±7.9
	5.0	34.1±3.9

*Mean values ± standard deviation

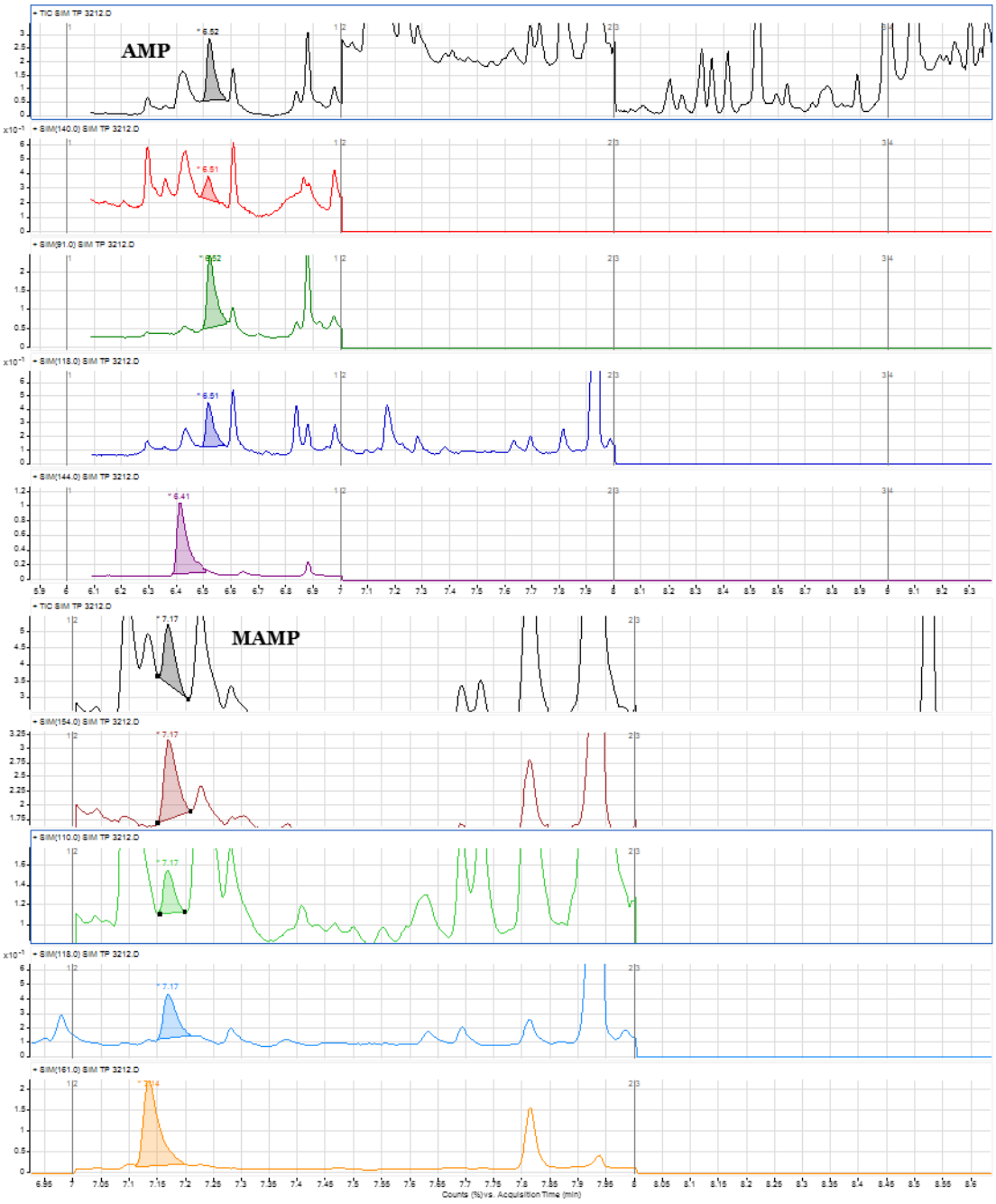
2.3. Method applicability

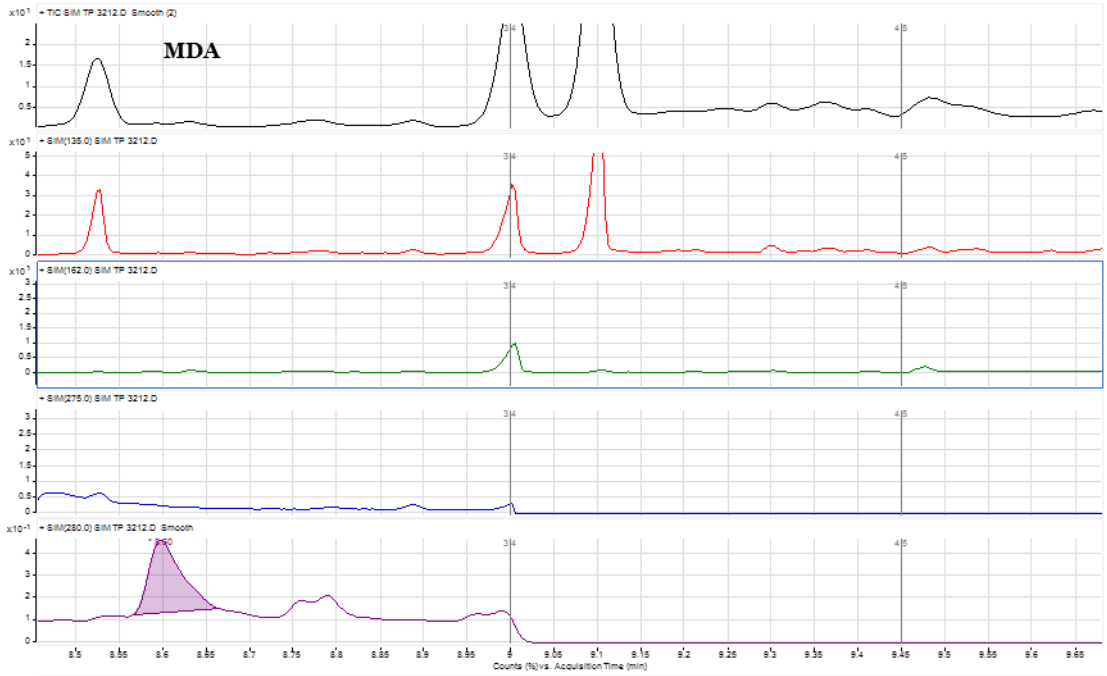
After the validation and optimization of the full method was complete, all that it was left to do was apply it to real samples. The present method was successfully applied to authentic hair samples obtained from ATS consumers.

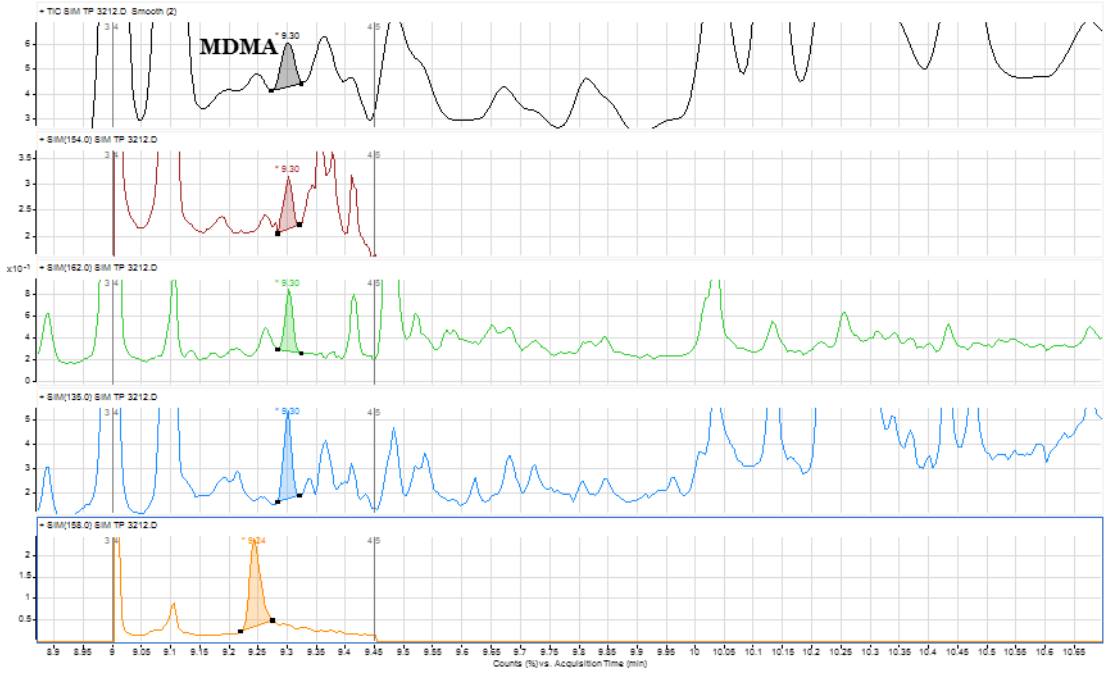
The table 11 describes the concentrations obtained in some of these samples, and figure 36 presents a chromatogram as an example.

Table 11- Concentrations of ATS found in authentic hair samples

Sample number	Concentration (ng/mg)					
	AMP	MAMP	MDA	MDMA	MDE	MBDB
1	0.46	0.44	Negative	Negative	Negative	Negative
2	0.73	1.48	Negative	0.21	<LLOQ	Negative
3	Negative	Negative	0.26	<LLOQ	Negative	<LLOQ
4	<LLOQ	Negative	Negative	Negative	<LLOQ	Negative
5	0.20	Negative	Negative	Negative	<LLOQ	Negative


+ TIC SIM TP 3212.D
AMP
* 6.52
+ SIM(140.0) SIM TP 3212.D
* 6.51
+ SIM(91.0) SIM TP 3212.D
* 6.52
+ SIM(118.0) SIM TP 3212.D
* 6.51
+ SIM(144.0) SIM TP 3212.D
* 6.41
+ TIC SIM TP 3212.D
* 7.17
MAMP
+ SIM(154.0) SIM TP 3212.D
* 7.17
+ SIM(110.0) SIM TP 3212.D
* 7.17
+ SIM(118.0) SIM TP 3212.D
* 7.17
+ SIM(161.0) SIM TP 3212.D
* 7.14
Counts (%) vs. Acquisition Time (min)


MDA
+ TIC SIM TP 3212.D Smooth (2)
+ SIM(135.0) SIM TP 3212.D
+ SIM(162.0) SIM TP 3212.D
+ SIM(275.0) SIM TP 3212.D
+ SIM(280.0) SIM TP 3212.D Smooth
Counts (%) vs. Acquisition Time (min)


MDMA
+ TIC SIM TP 3212.D Smooth (2)
+ SIM(154.0) SIM TP 3212.D
+ SIM(162.0) SIM TP 3212.D
+ SIM(135.0) SIM TP 3212.D
+ SIM(158.0) SIM TP 3212.D
Counts (%) vs. Acquisition Time (min)

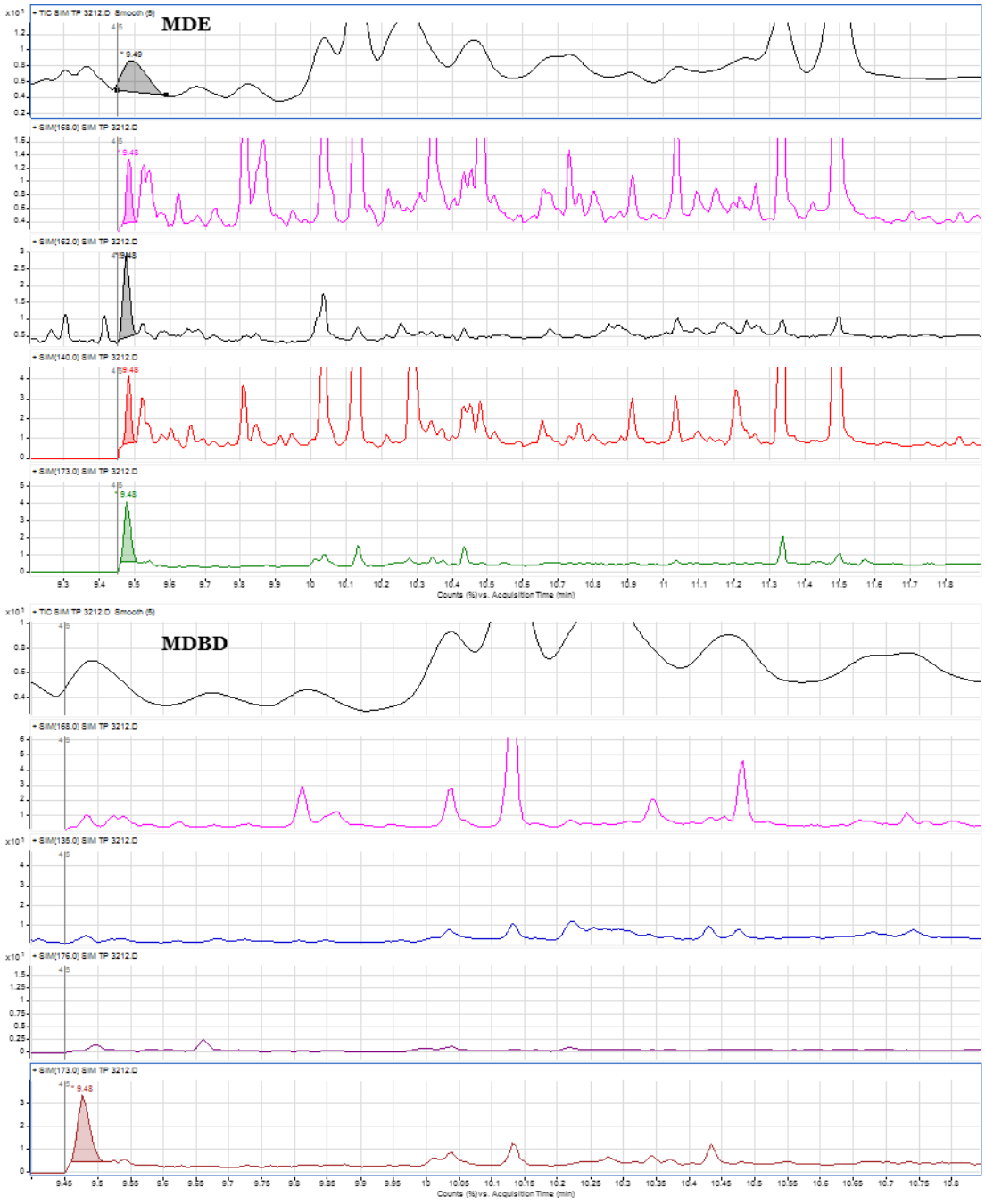


Figure 36- Chromatogram of sample 2

Meng et al. [80] employed a combination of high-speed grinding and Solid-Phase Microextraction (SPME), a miniaturized technique akin to MEPS and SPE, for the detection of amphetamines, specifically AMP, MAMP, MDA, and MDMA, along with other drugs like ketamine and its metabolites in hair, using LC-MS analysis. By utilizing 20 mg of hair and 2 mL of saturated sodium carbonate solution, the authors successfully extracted the target analytes from the hair matrix, employing SPME as a complementary clean-up method [80]. Their methodology was optimized and validated, demonstrating its effectiveness when applied to real samples sourced from

suspected drug addicts. The concentrations of target analytes in the real hair samples ranged from 0.8–3.2 ng/mg for AMP and 1.9–7.9 ng/mg for MAMP.

Interestingly, the method developed by Meng et al. [80] bears some resemblance to the MEPS method established in this study. However, it exhibits certain limitations that MEPS effectively overcomes. Noteworthy advantages of MEPS include reduced solvent volume, more controllable extraction temperatures, and overall simplicity in the extraction process. Additionally, MEPS offers the capability to reach and detect the concentrations achieved in the real samples analysed by Meng et al. [80].

As previously mentioned in section 2.2.4, despite presenting lower recovery rates, this MEPS protocol consistently attains performance levels on par with both miniaturized and non-miniaturized techniques. Importantly, it does so without compromising the method's detection and quantification limits or the precision and accuracy of the analytical procedure. Meng et al. [80] is not the only example able to compare and distinguish the capabilities MEPS. Burgueño et al. [81] and Skender et al. [82] used SPE for the detection of AMP, MAMP, MDA, MDMA and MDE with GC-MS, being this a dispendious and laborious work that can be performed by the here developed MEPS protocol in a much greener, cleaner and economic way. While Burgueño et al. [81] achieved the LLOQ established by the SoHT and tested 2954 real hair samples, Skender et al. [82] used SPE on a smaller group, mentioning only the LOD obtained (0.5–0.30 ng/mg) for the work. With such a high number of samples, the utilization of MEPS in the work of Burgueño et al. [81] would have allowed for reduced economic strain, due to the MEPS BIN reusability and low solvent and sample volumes. Interesting results were obtain by Burgueño et al. [81], having a total of 21.94% positive samples for amphetamines, with AMP showing with the hights rate (16.38%). No sample showed a positive result for MDE, with MAMP also being found in very low quantities (less than 1%) [81]. MDA and MDMA showed in 4.10% and 12.09% of samples, respectively [81]. No quantification appears to have been done, other than do identify positive samples. On the other hand, Skender et al. [82] applied the method to a much lower sample size (36 subjects) and got only 4 positive samples for MAMP, 2 for MDA and 12 for MDMA. Its important to say that these authors did indeed calculate de concentration of each compound in the hair samples, with the lowest registered at 0.30 ng/mg of hair (MAMP) [82]. It is fair to say that this MEPS method would have also been a great alternative to SPE for the realization of this work, if not the best method for the job. With a sample pool of this size, a single MEPS BIN would have been enough to analyse all the authentic hair samples, reducing variability between columns and monetary expenses by cutting out every single use SPE column needed from the work. With the

LLOQs obtain, MEPS would have easily detected and quantified every single hair sample with great accuracy and precision, while also being environmentally friendly and cheaper.

The established applicability of this method, especially in comparison with other techniques, which demonstrate superior sample volumes and recoveries, serves as evidence of the genuine potential that MEPS holds as a sample clean-up procedure.

3. Conclusions

Using MEPS and GC-MS, a new technique for determining ATS in hair samples was devised and refined. The technique was thoroughly adjusted utilizing the experimental design methodology to increase analyte recovery and hence get lower detection and quantification limits. The improved MEPS process proved to be simple, fast, sensitive, and precise. The suggested analytical approach was validated and applied to actual samples with success.

The present analytical technique uses just 50mg of hair, with minimal sample and solvent volumes, utilizing only 500µL of NaOH and 50µL of HCl for the extraction, and 100 µl of sample for the loading step of the clean-up method. It has also been shown to be linear throughout the concentration ranges chosen, allowing for the LLOQs of 0.2 ng/mg designated by the SoHT to be attained. Because of the ease of extraction and reusability, the suggested approach is particularly useful, user and environmentally friendly, and economically attractive for laboratories that work with this alternative matrix.

To the best of our knowledge is the first study to employ MEPS for the determination of amphetamines in hair.

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Attachments

The present dissertation was disseminated in one congress in the area of toxicology, as well as was submitted for other presentations and a publication.

Presentations in congress:

Congresso internacional - Poster

Pires, Bruno; Simão, Ana; Rosado, Tiago; Gallardo, Eugenia; Barroso, Mário." DETERMINATION OF AMPHETAMINE-RELATED DRUGS IN HAIR USING MEPS AS SAMPLE CLEAN-UP".

27th Meeting of the Society of Hair Testing (Lisbon), 7-9 June 2023, Portugal. ISBN: 978-989-654-929-9

This work was awarded "Best Poster" by the SoHT evaluation committee.

Accepted as poster presentations to come:

Congresso Nacional:

Pires, Bruno; Simão, Ana; Rosado, Tiago; Gallardo, Eugenia; Barroso, Mário- "Desenvolvimento e otimização de uma técnica de microextração em seringa empacotada para a determinação de anfetaminas e derivados em cabelo".

21º CONGRESSO NACIONAL DE MEDICINA LEGAL E CIÊNCIAS FORENSE (Lamego), 12 a 14 outubro 2023, Portugal.

Congresso Internacional:

Pires, Bruno; Simão, Ana; Rosado, Tiago; Gallardo, Eugenia; Barroso, Mário. "Evaluation of hair hydrolysis and MEPS columns for the detection of amphetamines derivatives in hair"

X Congresso Iberoamericano de Ciências Farmacêuticas (Coimbra), 26 a 28 outubro 2023, Portugal.

Publications

The sub-chapter "Therapeutic Potential" was accepted for publication as a review article entitled "The therapeutic potential of Amphetamine-like psychostimulants" in Journal Life (2023).