UNIVERSIDADE DA BEIRA INTERIOR

Ciências

Determination of amphetamines-type stimulants in urine samples using microextraction by packed sorbent and gas chromatography-mass spectrometry

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Resumo alargado

As anfetaminas e seus derivados pertencem a um grupo de compostos estimulantes ao nível do Sistema Nervoso Central (SNC), na medida em que atuam como substratos para recetores neuronais de monoaminas, como é o caso das catecolaminas (epinefrina, norepinefrina e dopamina) e a serotonina. Esta ligação é estabelecida pela similaridade existente entre as estruturas químicas das anfetaminas e das monoaminas, onde atuam como análogos, inibindo a recatapção de neurotransmissores e exercendo inibição enzimática da monoamina oxidase (MAO), perpetuando assim efeitos de euforia, agressividade, aumento do estado de alerta, supressão de apetite e sono, entre outros. Este facto fez aumentar o interesse por parte da população, mesmo que originárias no ano de 1887, sendo que o consumo e a sua síntese só têm vindo a aumentar, quer em forma de fármacos prescritos para tratamento de narcolepsia e hiperatividade, como em mercados ilegais.

Estima-se que mais de 92 milhões de pessoas, ou seja, um pouco mais de um quarto da população dos 15 aos 64 anos de idade da União Europeia, já tenham experimentado drogas ilícitas ao longo da sua vida. A experiência de consumo de drogas verifica-se com mais frequência no sexo masculino (56,0 milhões) do que no feminino (36,3 milhões). Relativamente ao consumo em particular de estimulantes, segundo o último relatório em matéria de drogas publicado pelo Observatório Europeu da Droga e da Toxicodependência, estima-se que 11,9 milhões de adultos europeus (15-64 anos), ou 3,6 % deste grupo etário, tenham experimentado anfetaminas durante as suas vidas. Os números relativos ao consumo mais recente, no grupo etário em que o consumo da droga é mais elevado, sugerem que 1,2 milhões (1,0 %) de jovens adultos (15-34 anos) consumiram anfetaminas no último ano, com as estimativas nacionais mais recentes relativas à prevalência a variarem de 0,1 % em Portugal a 3,6 % nos Países Baixos. No caso particular do *ecstasy*, este consumo ascende para os 2,6 milhões de indivíduos. Esta tendência de consumo mantem-se estável pelo menos desde os dois últimos anos.

Para além dos seus efeitos nefastos o consumo destas *designer drugs* leva a casos de tolerância e dependência, tornando-se um problema sério para a saúde dos seus consumidores, tanto a níveis físicos como psicológicos. Desta forma, torna-se impreterível o uso de novas técnicas que englobem a eficácia da extração aliada à sua rápida deteção. Este trabalho tem como objetivo o desenvolvimento e validação de um método analítico, visando determinar seis tipos de anfetaminas em amostras de urina, com recurso à microextração com seringa empacotada (MEPS), uma técnica recente que requer menor volume de amostra e solventes, quando comparada a técnicas de extração mais clássicas. Para a deteção e

quantificação dos compostos em estudo foi usado um cromatógrafo de gases acoplado à espetrometria de massa.

Esta técnica foi inicialmente otimizada de modo a maximizar a quantidade de compostos recuperados da matriz, sendo que as condições finais foram: acondicionamento da coluna MEPS (250 µL de água; 250 µL de metanol); número de eluições da amostra (9 ciclos de 100 µL); número de lavagens da coluna (150 µL de água; 150 µL de solução de água: metanol (95:5)); solução de eluição contendo 2% de hidróxido de amónia em acetonitrilo (4 ciclos de 100 µL); assim como a solução de reconstituição da coluna usando hidróxido de amónia em acetonitrilo: metanol (1:1) e 1% de ácido fórmico em isopropanol: água (10:90) (4 ciclos de 100 µL para cada uma das soluções) para se poder prosseguir para uma nova extração.

Este método foi integralmente validado de acordo com as recomendações internacionalmente aceites, baseadas em princípios estipulados para a validação de métodos bioanalíticos pela Food *and Drug Administration* (FDA) e a *Scientific Working Group of Forensic Toxicology* (SGWTOX).

Obteve-se linearidade entre os limites de quantificação (LLOQ) e 1000 ng/mL para todas as anfetaminas estudadas, com coeficientes de determinação superiores a 0,99. O limite mais baixo de quantificação foi de 25 ng/mL para todos os compostos, à exceção da anfetamina e MDMA que apresentaram limites quantificadores de 35 ng/mL e a MDA com 50 ng/mL. As recuperações obtidas variaram entre 19 e 71%. Na avaliação da precisão intra e interdia, o método mostrou-se preciso, exacto e apresentou especificidade.

Por último, mas não menos importante, é de salientar que se trata do primeiro estudo a ser realizado para determinação de anfetaminas em amostras de urina usando a técnica de micro extração com seringa empacotada, com recurso à cromatografia gasosa e espetrometria de massa. Este método visa mostrar a sua potencialidade em futuras abordagens alternativas nos laboratórios de análise toxicológica, uma vez que apresenta um processo de rápida extração (menos de 3 minutos), de fácil execução ainda com a possibilidade de reutilizar os cartuchos (aproximadamente até 100 extrações) trazendo, por sua vez, uma redução de recursos tanto no caso dos solventes usados (escala de microlitros) como de amostra (apenas 200 µL).

Palavras-chave:

Anfetaminas, urina, microextração com seringa empactada (MEPS), cromatografia gasosa acoplada a espetrometria de massa (GC-MS).

Abstract

Microextraction by packed sorbent (MEPS) is a miniaturized technique adapted from the conventional solid-phase extraction (SPE), however allowing the possibility to work with a minor scale of sample volumes (scale of µL instead of mL). MEPS uses the same sorbents as the SPE columns and it can be suitable to use with most of the methods that already exist, with the requirement to readjust the volumes, miniaturizing them. The major advantages of this technique are the reduced volume of organic solvents and sample required, as well as the possibility of reuse the sorbent assuring that carryover effect isn't observed.

With this research project the aim was the development, optimization and validation of an analytical method using MEPS with gas chromatography coupled to mass spectrometry (GC-MS) to determine amphetamine (AMP), methamphetamine (MAMP), *3,4-methylenedioxyamphetamine* (MDA), 3,4-methylenedioxyethylmethamphetamine (MDMA), 3,4-methylenedioxy-N-methyl-α-ethylfenilethylamine (MBDB), and 3,4-methylenedioxy-N-ethylamphetamine (MDE) in urine samples.

In the present work, seven different procedures, adapted from SPE, were tested in order to optimize the extraction, increase the eficiency, and reach low detection and quantification limits using a small volume of sample (200 µL). Regarding the optimization of the MEPS procedure, several parameters were evaluated, namely: type of sorbent, sample dilution, number of strokes, activation of the ion exchange mechanism and composition of both washing and elution solvents.

The method was validated according to the Food and Drug Administration (FDA) and the Scientific Working Group of Forensic Toxicology (SGWTOX) for the validation of bioanalytical methods. The studied parameters included selectivity, calibration model and linearity, limit of detection (LOD) and limit of quantification (LLOQ), precision, accuracy, stability, dilution integrity and recoveries. This method proved to be linear in the range of 25-1000 ng/mL for MAMP, MBDB and MDE, 35-1000 ng/mL for AMP and MDMA, and 50-1000 ng/mL for MDA with coefficients of determination (R^2) greater than 0.99 for all analytes. Intra-and inter-day accuracy and precision were in accordance with the above-mentioned criteria, presenting coefficients of variation typically lower than 15% and mean relative error (RE) within a range of ± 15% of the theoretical concentration. The recoveries of the proposed MEPS procedure ranged from 19 to 71%, allowing LLOQs ≤ 50 ng/mL.

Keywords: amphetamines, urine, microextraction by packed sorbent (MEPS), gas chromatography couppled to mass spectrometry (GC-MS).

Index

Index of figures

Chapter 1| Introduction

Chapter 2| Experimental developments

Index of tables

Chapter 1| Introduction

Chapter 2| Experimental developments

Index of abbreviations

AMP	amphetamine
APCI	atmospheric pressure chemical ionization
BIN	barrel insert and needle
CNS	central nervous system
CC SHLLE	counter current salting-out spectrometry
DAD	diode array detector
DLLME	dispersive liquid-liquid microextraction
DLLME-SFO	dispersive liquid liquid microextraction – solidification of floating organic drop
DUI	drive under influence
EI	electron ionization
FDA	food and drug administration
FIA	flow injection analysis atmospheric
FID	flame ionization detector
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
HPLC-UV	high performance liquid chromatography - ultraviolet detection
HS-HF- LPME	head space hollow fiber liquid phase microextraction
HS-SPME	headspace solid phase microextraction
IS	internal standards
LC	liquid chromatography

LC- ESI MS/MS	liquid chromatography with electrospray ionization coupled with mass spectrometry in tandem
LC- HRMS	liquid chromatography with high resolution mass spectrometry
LLE	liquid liquid extraction
LLME	liquid liquid microextraction
LOD	limit of detection
LLOD	lower limit of detection
LOQ	limit of quantification
LLOQ	lower limit of quantification
MAMP	methamphetamine
MBDB	3,4- methylenedioxy-N-methyl-α-ethylfenilethylamine
MDA	*3,4-methylenedioxyamphetamine*
MDE	3,4-methylenedioxy-N-ethylamphetamine
MDMA	3,4-methylenedioxyethylmethamphetamine
MEPS	microextraction by packed sorbent
MISPE	molecularly imprinted-solid phase extraction
MSD	mass selective detector
RT	retention time
SADLLME	surfactant -assisted dispersive liquid liquid microextraction
SGWTOX	scientific working group of forensic toxicology
SOLA SCX	solid phase cation exchange extraction
SPE	solid phase extraction
SPME	solid phase microextraction
PCI	positive chemical ionisation
U	urine
ULOQ	upper limit of quantification

Objective and justification of the theme

Designer drugs of abuse exist in a dynamic market where new drugs are constantly appearing. They represent a serious health problem to our society as well as a challenge to forensic toxicologists, health professionals and law enforcement agencies. Scientists and authorities need to be aware and constantly updated on the newest drug trends as well as in-depth investigations on chemical analytical and toxicological proprieties of these drugs due to the emerging problem of abuse (2). In this sense, it becomes crucial to have proper techniques that allow their identification and quantification in consumers. Several techniques have been reviewed for the purpose and will be further presented and discussed.

In the present days, it is essential to develop new methods that will decrease the costs associated and the time spent in the analysis. In addition, there is a mandatory requirement for reliable results coupled with a rapid execution. The method should be reliable enough to exclude false positive results that would incriminate innocent patients, and be sensitive enough to detect certain drug consumption in a short period of time after their intake.

In this work, six types of amphetamines were determined in urine samples, a commonly used specimen, with a new extraction technique that requires lower sample and solvent volumes when compared with the classic techniques used in forensic laboratories.

The main goals were:

- Development and optimization of an analytical method in order to detect and quantify six amphetamine-types in urine samples using a gas chromatographer coupled to a mass spectrometer (GC-MS);
- Development and optimization of a microextraction procedure (MEPS);
- Validation of the analytical method accordingly to the guidelines from Food and Drugs Administration (FDA) and Scientific Working Group for Forensic Toxicology (SWGTOX);
- Application of the method developed and validated to authentic samples.

Taking into account that part of this dissertation has been submitted for publication, the present work is divided into two chapters.

Chapter I corresponds to a review of the literature where the main subjects of the dissertation theme will be presented and discussed, namely: the compounds under study; the approaches to detect and quantify them in biological matrices; and ultimately an approach on the statistical incidence in European countries from the last year.

Chapter II describes the entire experimental part of this dissertation and corresponds to the submitted article entitled “Determination of amphetamines-type stimulants in urine samples using microextraction by packed sorbent and gas chromatography-mass spectrometry”.

This article describes a sensitive method for the determination of the above-mentioned amphetamines in urine samples using MEPS and GC-MS.

Finally a conclusion will be presented with all the important points and highlights from this dissertation.

Chapter 1| Introduction

1. Review of the literature

1.1. Drugs of abuse and synthetic drugs

Drug abuse has long been an overall issue. In a global context, Europe is an important market for drugs, supplied both from domestic production and trafficking from other world regions. Recent changes happening in the illicit drug market, being linked to globalisation and new technology, suggest the innovation on the production of new drugs and trafficking methods, establishing new trafficking routes and online markets (2).

Synthetic drugs, also known as designer drugs, present proprieties and effects very similar to an illicit or prohibited drug but the difference resides in a slightly altered chemical structure from the current drug resulting in a similar structure and analogous effects to the illicit drug. These type of compounds require more attention, especially when the aim is to circumvent legislative controls (1). The most common amphetamine-type designer drugs are the ones that go through an introduction of N-alkyl substituent into the molecule of the original drug which activity is already known (1).

Drug precursors are chemicals required to synthesize illicit drugs. Many of these drugs have legitimate use as they are not prohibited, but they need to be monitored and their trade controlled under EU regulations in order to maintain a list of controlled substances (1).

The availability of drug precursors has a huge impact in the synthetic drug market, as well as the creation of new methods used by illicit laboratories. The most common changes include the use of non-scheduled chemicals so synthetic drugs can be produced by their precursors, like the recently detected N-t-BOC-MDMA (N-tert- butyloxycarbonyl-MDMA) (2).

Synthetic amphetamines were synthesized in 1887 by Lazer Edeleanu. Researchers have examined a wide range of catecholamine-like derivates with the intent to raise blood pressure as well as to relieve nasal and bronchial congestion from hay fever and colds. Amphetamine was commercially introduced in 1932 as benzedrine, a free base that was administrated in inhaler form, with an indication for clinical treatment of narcolepsy (4). By the year of 1936 pharmacies began to sell benzedrine without prescription but only in 1946 the pharmaceutical industry promoted more than thirty uses for amphetamines (eg. treatment for schizophrenia, opiate addiction, seasickness and infantile cerebral palsy) (3,5).

Amphetamines were then re-synthesized by Gordon Alles in 1997 bringing the first report of stimulant effects of amphetamines (3). Nowadays amphetamine and methamphetamine are still prescribed for narcolepsy, attention deficit disorder and weight control (6), although with dosage special attention because it can lead to serious dependence and irreversible damage on brain neurons, more specifically to serotonin and dopamine nerve terminals in central nervous system (CNS) (7,8).

1.2. Amphetamines and derivates

Amphetamines are psychoactive compounds obtained by chemical synthesis and present similar structure to some drugs, such as ephedrine, catecholamines (eg. epinephrine, norepinephrine, dopamine) and the neurotransmitter serotonin (see Figure 1). Their active principle is not found in nature, therefore they are known as synthetic drugs.

These drugs are known to be weak bases with pka values ranging from 8.8 to 10.4 and presenting a low molecular weight as they diffuse easily across cell membranes and lipidic layers (9). In this sense, they are effortlessly absorbed through the intestine, airway, muscle and placenta (10). Amphetamines present an elimination half-life varying from 6 to 12 hours, being eliminated by hepatic and renal clearance with a significant portion of the drug remaining unaltered (11).

Since 1980s, a new group of synthetic amphetamines, identified as methylenedioxy analogs, have gained popularity as recreational drugs presenting similar behavioural effects to those described for amphetamines and hallucinogens (11,12).

Amphetamines are indirect monoamine agonists promoting the release of norepinephrine, dopamine and serotonin from the pre synaptic terminals in the CNS and at peripheral level where there is an interaction with the membrane transporters involved in neurotransmitters reuptake and the vesicular storage systems (9). Shortly, amphetamines can be transported into the nerve terminals by passive entrance or even through a reuptake transporter, acting as inhibitors of the reuptake of monoamines. Once they are inside the neuron they reverse the direction of the membrane transporter leaving the norepinephrine, dopamine and serotonin efflux to the synaptic fissure (9). The pre synaptic reuptake of the cathecolamines (dopamine, epinephrine and norepinephrine) and serotonin is blocked causing a hyper stimulation in the selected postsynaptic neuron receptors.

Amphetamines are recognized by membrane transporters and accumulate within the synaptic vesicles, dissipating the proton gradient, and leading to an increase of the catecholamines in cytosol. With the increasing of the reverse transport caused by amphetamines an interaction between these subtracts and the receptors will cause an N-terminal phosphorilation of

dopamine transporters which will promote an efflux through the transporter. The now hyper stimulated neurons will stimulate other non-catecholamine and other pathways that will eventually provoke mood changes, motor movement and appetite, leading to euphoria, increase of alertness, alter the sense of self-esteem, increase aggression and intensify emotions, all mediated by this central dopaminergic alterations (10,13).

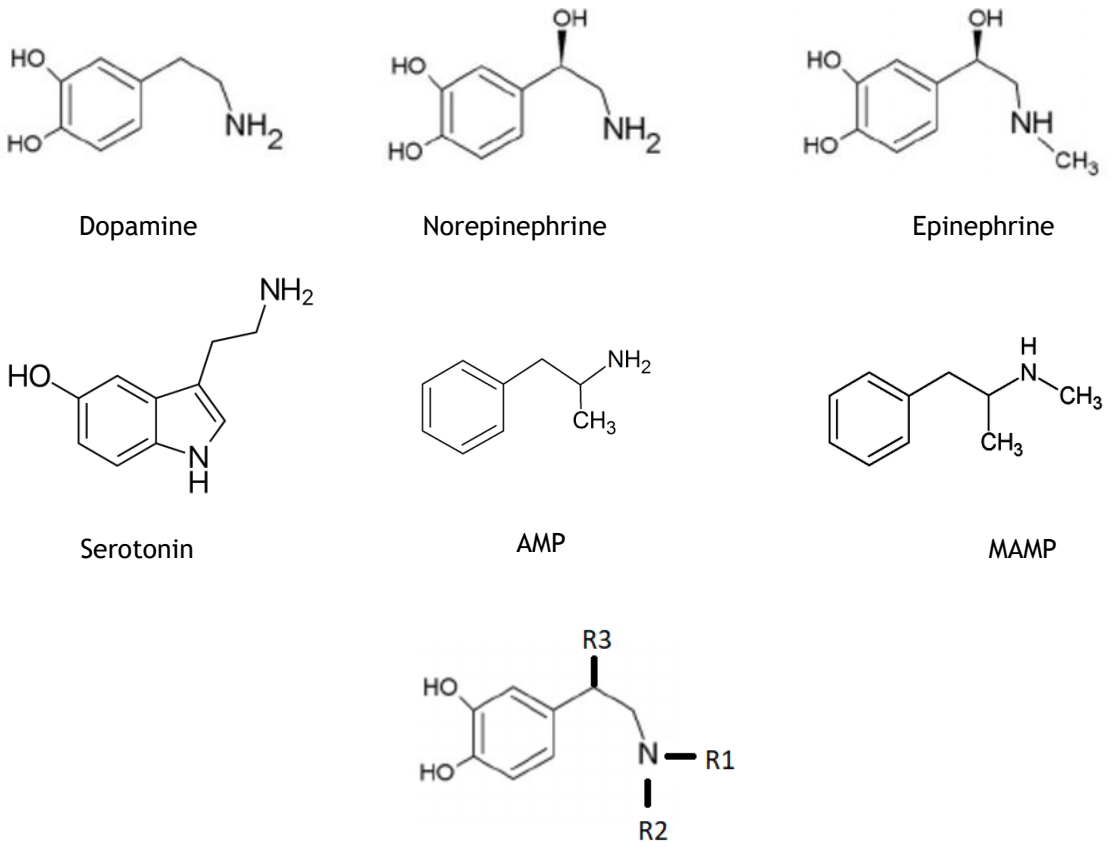

Figure 1-Chemical structures of the monoamines (dopamine, norepinephrine, epinephrine and serotonin) as well as the drugs in study: amphetamine, metamphetamine and amphetamine analogs (Adapted from Turfitt G.E. (1947) (1), Pereira J. *et al.* (2014) (2)).

Table 1- Names and chemical structures of the studied drugs with molecular features in Figure 1 (Adapted from Turfitt G.E. (1947) (1)).

Drug name (chemical formula)	Structure		
	R1	R2	R3
MDA ($C_{10}H_{13}NO_3$)	H	H	CH_3
MDMA ($C_{11}H_{15}NO_2$)	CH_3	H	CH_3
MBDB ($C_{12}H_{17}NO_2$)	CH_3	H	CH_2CH_3
MDE ($C_{12}H_{17}NO_2$)	CH_2CH_3	H	CH_3

Up to the year of 2016 there were identified 14 metabolic precursors of amphetamine and metamphetamine on the market (2,9).

Amphetamines with a side-chain substitution can be psychomotor stimulants or anorectics where the derivatives of these drugs with substitutions in the terminal amine present psychomotor stimulant effects in low doses and hallucinogenic activity at higher doses. Amphetamines with aromatic ring substitutions are weak stimulants, but in higher doses they can present hallucinogenic activity. When methylenodioxi substitutions occur on the phenyl ring, such as MDMA or MDA, the effects are both hallucinogenic and stimulant at relatively low doses (9).

In the present work the compounds that will be studied are: amphetamine (AMP), methamphetamine (MAMP), *3,4-methylenedioxyamphetamine* (MDA), 3,4-methylenedioxyethylmethamphetamine (MDMA), 3,4-methylenedioxy-N-methil-α-ethylfenilethylamine (MBDB) and 3,4-methylenedioxy-N-etilamphetamine (MDE). Their pharmacokinetics will be explained in the following topics.

1.2.1. Amphetamine (AMP)

AMP is more frequently used orally and the peak plasma concentration is attained four hours after ingestion. Due to their low protein binding and high bioavailability the diffusion from

the plasma to the extra vascular compartment is easy to occur (9). Studies have showed that amphetamine-dependent individuals have larger volume of distribution and plasma elimination half-life when compared to drug-naive individuals, which is probably due to an increased affinity of the tissues for the drug as a result of the development of tolerance to the drug (14).

AMP is metabolized by N-deamination and posterior oxidation (where 4% is excreted) after which is conjugated with glycine and excreted as the corresponding hippuric acids (16-27%). It can also be metabolized by hydroxylation in position 4 of the aromatic ring forming 4-hydroxyamphetamine and then conjugated with sulphate or glucuronic acid (2-4% excreted) (15). The plasma half-life of amphetamines will depend on the urine pH since renal excretion is the main elimination pathway. Amphetamines are weak bases which mean that their excretion will increase with urine acidification and will decrease with urine alkylization. With ingestion of bicarbonate (increase pH) the effect of the drug will be prolonged. The fraction of the AMP excreted without suffering biotransformation varies from 3 to 55.5% (16).

1.2.2. Methamphetamine (MAMP)

MAMP is usually consumed by oral ingestion, snorting, intravenous injection, vapour inhalation or by smoking. The terminal plasma half-life is ten hours for all types of administration and the effects can persist after eight hours of the uptake. Smoking and intravenous injection of MAMP results in rapid action and a maximum concentration (C_{max}) is attained after 1-2.5 hours, where if it is taken via orally the C_{max} occurs after three hours (17).

The process of distribution of MAMP is similar to the AMP, and has been shown to accumulate in saliva, nails and hair of drugs abusers (18). MAMP suffers N-demethylation into amphetamine and hydroxylation of the aromatic ring producing 4-hydroxymethamphetamine. ß-oxidation followed by N-demethylation produces norephedrine. All three are psychoactive and their metabolic pathway continues with hydroxylation, deamination or by non-active metabolites conjugation (15). MAMP can be metabolized into methylenodioxyamphetamine derivatives, such as MDMA (9). After twenty-four hours of administration about 75% of the MAMP is excreted in urine, where 50% is in its unchanged form, and its metabolites: 4-hydroxymethamphetamine (15%) and amphetamine (10%) (18). It has an elimination life of twenty-five hours and it can accumulate in urine when repeating doses.

1.2.3. 3,4-methylenedioxyamphetamine (MDA)

There are no pharmacokinetic information available for MDA in humans. It is mostly obtained from MDMA synthesis, where occurs N-demethylation resulting in MDA (9).

1.2.4. 3,4- methylenedioxyethylmethamphetamine (MDMA)

MDMA differs from AMP and MAMP in one important aspect, as shown in figure 1, it has a methylenedioxy (-O-CH_2-O-) group attached to positions 3 and 4 of the aromatic ring of the amphetamine molecule (i.e., it is "ring substituted"). Usually ingested in single doses, the initial effects appear after thirty minutes with maximal peak effects at approximately one hour after ingestion (19). MDMA metabolism can have two different pathways: the opening of the methylenedioxy ring follwed by methylation of one of the hydroxyl groups from the resulting catechol and the conjugation with glucoronid or sulfate (15) or the N-dealkylation that will form MDA. The deamination and side-chain oxidation lead to the formation of phenylketones and then occurs oxidation to benzoic acid derivatives that are conjugated with glycine and then excreted as hippuric acids (9).

The excretion of amphetamines which aromatic ring has suffered a methylenodioxy substitution have a more extended metabolic pathway and about 80% of this drug is eliminated after hepatic metabolism where 20% of the dose is excreted unchanged in urine. The amount of drug excreted in urine without occurring biotransformation is lower (9). The majority of the dose is excreted within twenty-four hours after ingestion and has an elimination half-life of six to nine hours (19).

1.2.5. 3,4-methylenedioxy-N-methyl-a-ethylfeniletilamine (MBDB)

MBDB is the α-ethyl homologue of MDMA and they present similar mechanisms of metabolism and excretion, with a high percentage of compounds being excreted in urine without being metabolized. The main metabolic pathways are O-dealkylation and subsequent methylation, sulphation and glucoronidation. Both toxicological and pharmacological actions of MBDB are smaller than the ones observed for MDMA, therefore difficult to estimate (20).

1.2.6. 3,4-methylenedioxy-N-ethylamphetamine (MDE)

MDE can be administered orally and intranasal and presents rapid absorption where the initial effects appear after ten-twenty minutes with duration of two hours. MDE metabolism occurs in the liver and a large amount is eliminated in urine without suffering biotransformation, depending on the urine pH (the acidification of urine will increase the elimination). As for the absorption and distribution of MDE in the body, data on oral bioavailability and plasma protein binding in humans are not available, therefore the mechanism of action is still not clear (21).

1.3. Toxicological effects

Amphetamines are known as a type of psychotropic compounds, widely abused and searched due its stimulants effects and hallucinogenic proprieties. Their potential toxicological effects are reviewed by Albertson T.E. *et al*. (1999) (10), Kraemer T. *et al*. (2002) (15), Berma S. *et al*. (2008) (22) and Carvalho M. *et al*. (2012) (23), who reported findings of laboratorial studies as follow:

Cardiovascular system

- chest pain
- palpitations
- dyspnoea

Central Nervous System

- agitation
- anxiety
- hallucinations
- delirium
- psychosis
- seizure
- cerebrovascular accidents (due to haemorrhage or vasospasm cerebral edema, cerebral vasculitis)

Infections

- risk of endocarditics
- viral hepatitis
- human immunodeficiency virus (HIV)

All associated with intravenous MAMP use.

Respiratory system

- acute non-cardiogenic pulmonary edema
- pulmonary hypertension

The effects will depend on the amphetamine-type administered, as well as the dosage and the route of administration.

Systemic and dermatologic toxicity

- hyperthermia
- convulsions
- acute renal failure
- hepatocellular damage
- disseminated intravascular coagulation

The most common dermatological manifestations in amphetamine abusers are mainly self-inflicted, meaning that they are induced by trauma, intravenous needles or burns.

1.4. Tolerance, dependence and treatment approaches

The term tolerance is characterized as a progressive reduction of a generated effect caused by the same drug taken in equal doses, where the effectiveness is gradually lost. In order to obtain similar effects it would be necessary the consumption of higher doses that can lead to a dependence development (24).

Amphetamines consumption is approved for treatment of attention-deficit, hyperactivity disorder and narcolepsy and methamphetamine as been approved to treat obesity, both being accepted in medical uses but under control due to their abuse potential, and physiological as well as psychological dependence (22). Their consumption has been increasing in Europe (2) and studies have showed that when administrated in higher doses they can cause convulsions, stereotypic movements and psychosis. Fatigue, anxiety and tiredness can also appear once the main stimulant effects vanish (9).

Amphetamines and its abuse generated a serious public health concern in ways that are consumed most commonly by young adults, being the third drug (marijuana the first and cocaine coming second) with an illicit consumption in young European adults (2). These type of drugs show a high abuse potential and can induce dependence, tolerance and withdrawal symptoms, where the repeated consumption can deplete catecholamine supplies that eventually will produce a decrease in pharmacological effects, called acute tolerance (9).

As it happens in severe overdose episodes, an acute intoxication should require an immediate supportive care (airway control, oxygenation and ventilation as well appropriate monitoring). Other approaches should be performed (10):

- Termination of amphetamine induced seizure activity and arrhythmia;
- Correction of hypotension, hypertension, hyperthermia, metabolic and electrolyte abnormalities;
- Control of possible psychiatric agitation;

Not many patients have required pharmacologic intervention but they can be treated for mild agitation with decreased stimuli. In cases of severely hyperactive persons, the treatment involves the administration of antipsychotic drugs (that will antagonize the central behaviour effects of amphetamines) or benzodiazepines (that are used to terminate seizures caused by amphetamines). If sedation fails, several antihypertensive agents might reverse the amphetamine-induced cardiovascular symptoms (10).

Recommendations like acidifying the urine (to increase the elimination of the drug) as well as monitoring the renal function and fluid intake and output should be considered as well. Finally, both social and psychiatric intervention will be needed in order to reduce the chance of long term dependence (25).

1.5. Statistics in Europe and in Portugal

There has been an increase of amphetamines use since their introduction as medicine in the 1930s. The history of amphetamines use result of many interests, spreading it from the 1960s until the present days where its consumption has become a problem, possibly more than any other major illicit drugs (2).

Their global production of amphetamines is mainly concentrated in Europe, being situated mostly in the Netherlands, Poland and Belgium and the supply ranges from small scale laboratories to a limited international trafficking, based on two regions: north and central Europe (2).

Consumption of crystal methamphetamine by smoking has been increasing in many parts of the world but in Europe is almost completely available in its powder form which makes it difficult to distinguish from other amphetamines. There are similarities in their appearance and effects. Withdrawal from amphetamines is not life-threatening and it's possible to have a successful detoxification. It is estimated that 11,9 million European adults (15-64 years) (3,6% of this age group) have tried amphetamines during their lives. The most recent report estimates a prevalence of 0,1% in Portugal and 3,6% in the Netherlands. In the particular case

of *ecstasy*, the consumption increases up to 2,6 million of individuals in Europe. There are many treatment options and systems that have been specialized in responding to the needs of their consumers in Europe, yet there is no data available for treatment entrants for amphetamines users in Portugal (2).

2. Detection of amphetamines in biological matrices

2.1. Biological matrix

Nowadays there are several biological matrices explored in toxicological and forensic fields with the main goal being its application to detect and quantify several compounds of interest. When choosing a biological matrix it should be taken in consideration the toxicokinetic characteristics from the target analytes as well as the purpose of the analysis.

In the present work, urine samples were chosen to determine this group of drugs. The main reason for urine application is related to the fact that is a well-known sample and widely used in toxicology laboratories for drug testing, also used in the DUI (drive under influence) testing. This specimen also presents many advantages: easy to collect, usually available in great amounts, can be screened for drugs more readily (when compared to blood testing), less complex specimen. However is used for short time detection for most drugs and metabolites (48-72 hours after consumption) being able to screen 1000 types of amphetamines with 500 of them confirmed (26).

2.2. Sample preparation technique (MEPS)

Microextraction by packed sorbent (MEPS) is a new technique adapted from the conventional solid-phase extraction (SPE), where in this recent format it is possible to work with a minor scale of sample volumes (µL instead of mL). MEPS uses the same sorbents as the SPE columns, however it is necessary to readjust the volumes, miniaturizing them.

The MEPS technique has been applied to extract a wide range of analytes in different matrices (urine, plasma, blood). To the date studies have been done with local anaesthetics, anti cancer drugs, neurotransmitters dopamine and serotonin, methadone, cocaine and cocaine metabolites, among others (27).

This extraction technique can be coupled online with GC or LC (gas chromatography or liquid chromatography, respectively) without the need for modifications. MEPS can be automated,

where the sample processing, extraction and ejection are performed online using the same syringe. This differs from the SPE, where the packing is integrated in a separate column (27).

In MEPS the sample extraction, concentration and cleanup are performed in a single device that is composed of a syringe and a MEPS barrel insert and needle (BIN). The BIN is equivalent to the cartridge used in SPE, being a small tube that contains about 2 mg of the thermo-packed sorbent. The syringe loads the sample through the BIN and the analytes are retained and then eluted successively (See Figure 2) (28,29).

To obtain a good extraction efficiency it is important to choose the right sorbent: C2-C18 phases are suitable for analytes with lipophilic proprieties; polymeric phases and the mixed mode phases (anion-cation exchange) are appropriated for non-polar and polar analytes (27).

MEPS has been commercialized by SGE Analytical Science and has a few range of sorbents as the C18, C8, C2, C8/SCX, SCX, SAX and silica. Specialized packing materials such as carbon, PBA and CMD are also used in this technique (27).

With the continuous use of the MEPS cartridge (more than 100 times) it is recommended to renew the sorbent before a new extraction, eliminating the possibility of carry-over. In practice, the functional failure of these sorbents is due to blockage, coagulation of the sample and sorbent degradation caused by aggressive solvents. In this sense, it is important to do a previous sample treatment (e.g. centrifugation, pH adjustment, hydrolysis or precipitation) (27).

With a pre-treated sample it is possible to increase its fluidity as well as reduce the number of interferences that could obstruct the sorbent, so the competitive interactions between the target analyte-matrix can be reduced and the interactions between analyte-sorbent can be enhanced, increasing the effectiveness (27).

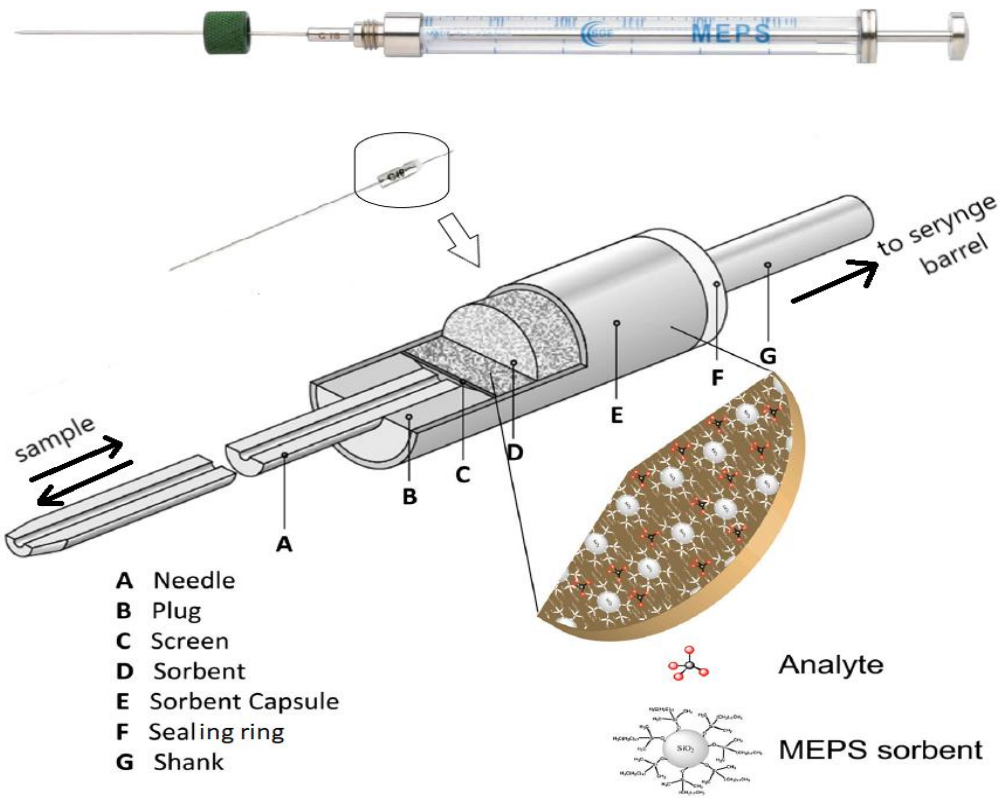

Figure 2 -An overview of the microextraction by packed sorbent (MEPS) syringe and the MEPS BIN (Adapted from Pereira J. *et al*. (2014) (3) and Abdel-Rehim M. *et al*.(2010) (4)).

Before initiating a MEPS procedure, the sample must be diluted in order to reduce its viscosity, prevent coagulations or blockage in the sorbent, and achieve low back-pressure. A general procedure occurs as follows (27):

1st. Conditioning of the column with methanol and water, the methanol will wash the cartridge and the water will prepare the cartridge to absorb the compounds when loaded;

2nd. Sample load, where the same aliquot is aspirated several times for an efficient retention of the compounds. One or more cycles of extraction are used according with the concentration factor and the efficiency of the sorbent solution to retain the analytes;

3rd. Washing step to remove weak interferences. In this step it is important that both solvents pH and percentage are controlled, preventing leakage of the target analytes. Studies reported as optimal washing solution, for basic drugs, methanol or 2-propanol in water (5:10) (30,31) . It's recommended two solutions: the first one should be an organic solvent, such as methanol or acetonitrile with some acid or base so that a strong washing occur; the second solution, a weaker one, should be pure water or a combination of methanol and water;

4th. It is recommended an additional step of drying due to a small bed of sorbent;

5th. Elution, using an appropriated organic solvent like methanol, isopropanol or acetonitrile. The elution solution can be as strong as the washing solution and its purpose is to collect the target analytes.

6th. It is suggested to use of two solutions to reconstitute the column so another cycle of MEPS can be proceeded (28,27).

The MEPS technique presents high sensitivity, precision and accuracy that will enable the quantification of many drugs in different matrices, being very helpful in clinical, forensic toxicology and environmental analysis areas.

Nevertheless, MEPS is not recommended in cases that include large sample volume because only 250 to 500 µL can be loaded each time. This means that all the process would be too long and laborious (28).

The aim in future studies should concern the extraction of many more drugs and its metabolites, so MEPS can be applied in a wide range of different areas such as food and environmental analysis. Using a commercial basis sorbents tailored for specific applications would improve the non-specificity between analytes and sorbent used in MEPS, minimizing the interferents that are co-eluted. Also, would be important to develop new materials with proprieties that are innovative for this technique in order to improve the analytical performance with new methods. Multi-wall carbon nanontubes and graphene are the two recent candidates, according to Pereira *et al*. (2014) (28).

2.3. Seperation and detection techniques

There are several analytical methods, described in the literature, with the goal to determine of amphetamines in biological matrices. In Table 2 is presented a review of the works published along the past years focusing the detection and quantification of the amphetamines under study, using microextraction techniques and the more conventional ones (non- micro scale techniques like SPE and LLE for example) applied to urine specimens.

This research was performed with a data base obtained from US National Library of Medicine National Institutes of Health (Pubmed) with the keywords: amphetamines, extraction, urine, toxicology.

Table 2- Methods for extraction of amphetamines on biological samples.

Sample (volume)	Amphetamines	Extraction tecnique	Instrumental analysis (detector)	LOD (ng/mL)	LOQ (ng/mL)	Recoveries (%)	References
Urine (1 mL)	AMP, MAMP, MDA, MDMA, MDE	HS-SPME	GC, (FID)	30-40	35-40	19.5–47 for AMP; 20–38.1 for MAMP; 5.1–6.6 for MDA; 7–9.6 for MDMA ; 5.4–9.6 for MDE	(32)
Urine (5 mL)	AMP and MAMP	DLLME-SFO	HPLC–UV	2×10^6–8×10^6	2×10^6 – 8×10^6	87.8–113.2	(33)
Urine (5 mL)	AMP and MAMP	SPE	GC-MS	0.17-0.77	0,05-0,23	93.28 for AM and 103.55 for MA	(34)
Urine (0.1 mL)	AMP and MAMP	OCD	HPLC	250	5×10^5	100 for AMP and 102 for MAMP	(35)
Urine (1 mL)	AMP, MAMP , MDA MDMA, MBDB and MDE	LLE	GC-MS	10 for AMP and MDA; 5 for the others	n.s.	62 and 66 for AMP and MDA, respectively; 73 -85 for the other four compounds	(36)
Urine (1 mL)	AMP, MAMP, MDA, MDMA, MDE and MBDB	LLE	LC- MS/MS	0.1	0.08	56–100	(37)

Urine (1 mL)	AMP and MAMP	CCSHLLE joined with DLLME-SFO	HPLC–UV	n.s.	0.5–2	78- 84 and 157-168	(38)
Urine (0.5 mL)	AMP,MAMP and MDMA	SPME	HPLC -FLD	100-1000	1000-2500	n.s.	(39)
Urine (0.5 mL)	AMP, MAMP and MDMA	SPME	HPLC -FLD	6-50 ng/mL	12-100	97-102	(40)
Urine (1 mL)	AMP and MAMP	HF-LPME	GC-MS	10000 for AMP; 20000 for MAMP	20	50-76	(41)
Urine (0.1 mL)	AMP, MAMP and MDMA	SOLA SCX	LC- HRMS	1-5	2.5	51.2-111.2	(42)
Urine (0.1 mL)	AMP	MEPS	LC-MS/MS	3	10	92-106	(43)
Urine (1 mL)	MAMP	SPE	LC–MS-MS	1	5	71	(44)
Urine (10 mL)	AMP, MAMP	SPE	LC- MS/MS	1	1	100.7-102.1 for AMP; 97.3-102.1 for MAMP	(45)
Urine (1 mL)	AMP, MAMP	SPME	GC-MS	30	50	n.s.	(46)
Urine (6 mL)	AMP, MAMP	LLLME	HPLC-UV	0.5	0.5	96.8-99.9 for AMP: 95.1-107.9 for MAMP	(47)

Urine (5 mL)	AMP, MAMP	LPME	HPLC-UV	0.3	0.3	92-105 for AMP; 89-104 for MAMP	(48)
Urine (4 mL)	AMP, MDA	HS-HF-LPME	GC-MS	0.25 for AMP; 1 for MDA	0.25 for AMP; 1 for MDA	100	(49)
Urine (4 mL)	MDMA	DLLME	FASI-CZE	1	1.4	91.1	(50)
Urine (0.5 mL)	AMP, MAMP, MDA, MDMA	SPE	HPLC-DAD	0.1	0.1	60-105 for AMP; 66-102 for MAMP; 75-111 for MDA; 65-93 for MDMA	(51)
Urine (0.5 mL)	AMP, MAMP, MDA, MDMA, MBDB, MDE	LPME	FIA - MS/MS	100 for AMP; 30 for MAMP; 100 for MDA; 8 for MDMA; 2 for MBDB and MDE	100 for AMP; 30 for MAMP; 100 for MDA; 8 for MDMA; 2 for MBDB and MDE	24 for AMP; 44 for MAMP; 20 for MDA; 35 for MDMA; 68 for MBDB; 56 for MDE	(52)
Urine (2 mL)	MAMP, MDMA	MISPE and DLLME	GC (FID)	2 for MAMP; 18 for MDMA	8 for MAMP; 50 for MDMA	86-89 for MAMP; 80-82 for MDMA	(53)
Urine (5 mL)	AMP, MAMP	SADLLME	HPLC-UV	2 for AMP; 3 for MAMP	2 for AMP; 3 for MAMP	91-96 for AMP; 91-95 for MAMP	(54)
Urine (0.1 mL)	AMP	MEPS	HPLC-MS/MS	3	10	92-106	(43)
Urine (0.2 mL)	AMP, MAMP, MDA, MDMA, MBDB and MDE	MEPS	GC-MS	35 for AMP; 50 for MDA and 25 for others	35 for AMP; 50 for MDA and 25 for others	19-71	This article

Abbreviations: AMP, amphetamine; MAMP, methamphetamine; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine;MBDB, N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine; MDE, 3,4-methylenedioxyethamphetamine; HS-SPME, headspace solid phase microextraction; SPE, solid phase extraction; LLE, liquid liquid extraction; LPME, liquid phase microextraction; LLLME, liquid liquid liquid microextraction; HS-HF- LPME, head space hollow fiber liquid phase microextraction; DLLME-SFO, dispersive liquid liquid microextraction – solidification of floating organic drop; MISPE, molecularly imprinted-solid phase extraction; CC SHLLE, counter current salting-out spectrometry; SADLLME, surfactant -assisted dispersive liquid liquid microextraction; GC-MS, gas chromatography coupled to mass spectrometry; FID, flame ionization detector; HPLC-UV, high performance liquid chromatography with ultraviolet detection; HPLC-MS/MS, high performance liquid chromatograpfy coupled with mass spectrometry in tandem; LC- MS/MS, liquid chromatography coupled with mass spectrometry in tandem; FASI-CZE, field amplified sample injection-capillary zone electrophoresis; OCD, on column derivatization; FIA, flow injection analysis atmospheric;; MSD, mass selective detector; SOLA SCX, solid phase cation exchange extraction; LC- HRMS, liquid chromatography with high resolution mass spectrometry; n.s., not specified; LOD, limit of detection; LOQ, limit of quantification.

In the absence of the LOQ value, the lowest point of the calibration curve was considerate.

2.4. Gas Chromatography coupled to Mass Spectrometry (GC-MS): the purpose in analytical and forensic toxicology

The most predominant challenges in forensic toxicology reside in proofing an abuse of illegal drug or a murder case by poisoning. Further, a forensic toxicologist has to detect drugs that eventually may or may not reduce the penal responsibility of a criminal, or which may reduce the condition to drive a vehicle. In doping control, the use or abuse of drugs used for stimulation of the building up of muscles, increase the endurance during competitions and reduce the pain caused by them, must be monitored. The basis of an efficient and reliable analytical and toxicological trial resides in an efficient toxicological analysis. Because a lot of the drugs that arrive at the laboratories are usually unknown, they have to be identified before the determination of clinical or legal consequences. For this reason, a toxicological analysis requires an elevated degree of exactness and GC-MS procedures satisfy most of these requirements (55).

Several procedures for systematic toxicological analysis of relevant drugs have been reviewed using GC-MS in clinical toxicology. Several papers have already described the detection of acute or chronic intoxication and therefore the detection of drugs of abuse such as barbiturates and other sedative-hypnotics, anticonvulsants, benzodiazepines, antidepressants, phenothiazine and butyrophenone neuroleptics, hallucinogens, opioid (narcotic) and other potent analgesics, non-opioid analgesics, antihistamines, cocaine, among others. To obtain results confirmation, the chromatographic techniques are usually applied and GC-MS is most commonly used for the detection of several categories of drugs, in "general unknown analysis" (55).

Initially GC-MS was used for identification of metabolites and in 1980 Tanaka *et al*. developed a GC-MS method to identify 155 compounds in urine samples, proving its potential (56).

Specific and sensitive detection and precise quantification of xenobiotics in biosamples (e.g., blood, urine, saliva, sweat, hair) are the greatest challenges in clinical and forensic toxicology, doping control, and biomonitoring (57). Thin-layer chromatography, gas chromatography with common detectors, among others are used in these fields. Gas chromatography coupled with mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) coupled with a diode-array detector (DAD) are still the most commonly used techniques today. At the present time, GC-MS is considered sensitive, specific, and universal analytic method for compounds that are volatile in GC, providing great separation. The electron ionization (EI) full-scan mode is the method of choice for the systematic analysis procedures, allowing identification of unknown compounds by comparison

of their unequivocal full mass spectrum with large collections of reference mass spectra from more than 6,400 toxicologically relevant compounds (1,57).

GC involves a separation column made from a length gas, fused silica, or metal tubing. The mobile phase flows through the separation column toward a detector. GC presents a mobile phase as an inert gas, such as helium, nitrogen or hydrogen, known as a carrier gas and its function resides in carrying a mixture of analytes. Prior to analysis with GC, one has to consider that compounds: may need to be chemically derivatized to make them volatile enough, less polar or even more stable. This chromatographic system operates with high temperatures (1).

Mass spectrometry (MS) was introduced in organic and analytical chemistry as a powerful spectroscopic method when there is the need to determine the accurate molecular mass and thus the calculation of the chemical composition of the compound. When directly coupled to chromatographic methods, especially with GC, the analysis of complex organic compounds has become an improvement due their high sensitivity and specificity (55).

MS consists of a vacuum system, an ion source, a mass analyzer, an ion detector and a data recording system. Briefly, is based on ionization and fragmentation of molecules in the gas phase. Molecules fragment in their specific way, therefore the ion fragmentation pattern can be used to obtain information about their structure.

GC-MS is widely used in forensic laboratories. The separation of the compounds occurs with chromatography and thus they can be identified by a mass spectrometer, where ions are produced in the ion source and then accelerated to the mass analyser. Then they are separated by their mass-to-charge ratio (m/z), the ion signal is recorded and then amplified, generating the mass spectrum (1).

In forensic analysis, drugs that need to be analysed in suspect abusers can be identified when comparing the obtained mass spectrum with a library data base of mass spectra. Some differences to the data can occur due differences on instrumentation and ionization conditions but, theoretically, the molecular ions should present the same m/z ratio in each spectrum and the base pick should present the same m/z in both spectrum that are being compared (1).

The applicability of GC-MS in analytical toxicology will depend on the requested analysis and the type of drugs that we are working with. Usually the compounds under analysis are unknown, therefore the first analytical step should be the identification of the compounds with a screening test and a second confirmatory test where positive results must be confirmed with high level of confidence (55).
With no doubt GC-MS is the method of choice when confirming the presence of toxicants that present volatility in the GC (55).

Chapter 2| Experimental developments

1. Introduction

Amphetamines are psychoactive compounds obtained by chemical synthesis and present similar structure to some drugs, such as ephedrine, catecholamines (eg. adrenaline, noradrenaline, dopamine) and the neurotransmitter serotonin (8,22). Since they are basic compounds and reveal liposolubility, it makes easy for them to cross over the blood-brain barrier as well as the placenta (23).

The increasing diffusion of these drugs on the European illegal market has raised great concern, mainly due to social and public health problems that are associated with its consumption. In Portugal the consumption of these drugs is much lower than central and northern European countries but there are still 0.1% of the Portuguese population, mostly young male adults (15-34 years old) that consume *ecstasy* and AMP (2). Hence there is a necessity for reliable and valid analytical screening tests to detect amphetamines and related ''designer drugs'' in biological samples.

Urine can be considered as a great biological specimen to determine this group of drugs, a well-known and used matrix in the toxicology field, work place drug testing and DUI , as it is present in great amounts, it is easy to collect, and can be screened for drugs more readily (when compared to blood testing) (26).

Several procedures have been developed for the separation and pre concentration of amphetamines from biological samples, namely liquid–liquid extraction (LLE) (36,37,58,59), solid-phase extraction (SPE) (34,40,44,45,51,60,61), solid-phase microextraction (SPME) (39,46,62,63), liquid-phase microextraction (LPME) (41,48,52,64) and dispersive liquid-liquid microextraction (DLLME) (33,53), among others. Although LLE and SPE are the most commonly used extraction techniques, SPE is time-consuming and expensive, while LLE method requires high volumes of potentially toxic organic solvents.

Microextraction by packed sorbent (MEPS) is a pre-concentration and clean-up technique adapted from the conventional SPE, where in this recent format it is possible to work with a minor scale of sample volumes (µL instead of mL). The MEPS uses the same sorbents as the SPE columns, can be suitable for most biological specimens, is environment-friendly and very straightforward (28,65). It is fast, simple and requires very small amounts of sample to obtain similar/comparable results to more conventional techniques, having the advantage of being interfaced to liquid chromatography coupled to mass spectrometry (LC-MS) and gas chromatography coupled to mass spectrometry (GC-MS) systems.

This technique has been used to extract a wide range of analytes in different matrices (urine, plasma, blood), namely local anaesthetics, anti-cancer drugs, neurotransmitters dopamine and serotonin, methadone, cocaine and cocaine metabolites, among others (27,65), presenting high sensitivity, precision and accuracy that will be very helpful in clinical, forensic toxicology and environmental analysis.

The aim of the present work was to determine amphetamine, methamphetamine, *3,4-methylenedioxyamphetamine* (MDA), 3,4-methylenedioxyethylmethamphetamine (MDMA), 3,4-methylenedioxy-N-methyl-α-ethylfenilethylamine (MBDB), and 3,4-methylenedioxy-N-ethylamphetamine (MDE) in urine samples using MEPS coupled to GC-MS.

2. Materials and methods

2.1. Reagents and standards

The analytical standards of amphetamine (AMP), methamphetamine (MAMP), *3,4-methylenedioxyamphetamine* (MDA), 3,4-methylenedioxyethylmethamphetamine (MDMA), 3,4-methylenedioxy-N-methyl-α-ethylfenilethylamine (MBDB), and 3,4-methylenedioxy-N-ethylamphetamine (MDE) and the internal standads of AMP-d6, MAMP-d9, MDA-d5, MDMA-d5 and MDE-d5 were purchased from LGC Promochen (Barcelona, Spain). Methanol (Merck Co, Darmstadt, Germany), hydrochloric acid, acetic acid, acetonitrile, ethyl acetate, formic acid, hexane, isopropanol and sodium phosphate were all of analytical grade. Deionized (DI) water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA) and ammonium hydroxide was purchased from T.J. Baker (Deventer, Holland).

A MEPS serynge (250 µL) and M1(4 mg; 80% C8 and 20% SCX) and C18 cartridges SGE Analytical Science, Australia, were used. Stock solutions of each analyte were prepared at 10 µg/mL in methanol. Working solutions were prepared by proper dilution of stock solutions with methanol to obtain concentrations of 1 and 0.5 µg/mL.

ISs were prepared at 0.1 µg /mL in methanol. All these solutions were stored refrigerated at 4°C.

2.2. Biological specimens

Drug-free urine samples used in all experiments were provided by laboratory staff. Authentic urine samples used for analysis arrived to the Fármaco-Toxicologia laboratory in UBImedical, provided by the Centro Hospitalar Cova da Beira, Covilhã, Portugal. These samples were stored refrigerated at -21 ºC until analysis.

2.3. Gas chromatographic and mass spectrometric conditions

For this method it was used a gas chromatograph HP 7890A coupled with a mass spectrometer model 7890B from Agilent Technologies. The column used was constituted of 5% de phenylmethylsiloxane (30m x 0,25 mm; 0,25 um i.d.) from Agilent Technologies. The data was acquired in *selected ion monitoring* (SIM) mode using MassHunter WorkStation Rev. B.02.01 from Agilent Technologies. The temperature of the oven started at 90°C for the initial 2 minutes, increasing 20°C per minute up to 300°C where it was maintained for 3 minutes. The total run takes 15.5 minutes. A constant flow of the carrier gas (helium) of 0.8 mL per minute was used, with splitless mode injection, where 2 µL of the extract was injected. 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 ºC and 280 °C, respectively. The ions were chosen based on the selectivity and abundance in order to maximize the signal-to-noise in matrix extracts. Under these conditions, the peaks from each compound are baseline separated with the following retention times: AMP and AMP-d6 (6.55 min); MAMP and MAMP-d9 (7.15 min); MDA and MDA-d5 (8.76 min); MDMA and MDMA-d5 (9.22 min); MBDB (9.70 min); MDE and MDE-d5 (9.74 min). Table 1 resumes the detection conditions for each target amphetamine.

2.4. Sample preparation

Firstly urine samples were centrifuged at 3500 rpm for 15 minutes. Before extraction, 200 µL of urine sample was diluted with 100 µL of ammonium acetate and spiked with 50 µL of ISs. After conditioning the MEPS C18 cartridge sequentially with one cycle of 250 µL of methanol and 250 µL of water, the sample was loaded with nine cycles of 100 µL. The washing step included 150 µL of water and 150 µL of a solution of water: methanol (95:5). Regarding the elution, a solution containing 2% ammonium hydroxide in acetonitrile was used (four cycles of 100 µL). A Final step was added to reconstitute the sorbent using two solutions: ammonium hydroxide in acetonitrile: methanol (1:1) and 1% formic acid in isopropanol: water (10:90) (four cycles of 100 µL, each). After extraction, 100 µL of HCl (0.1M) was added and the solution was then evaporated until dryness under a steam of nitrogen. The dry extracts were derivatized by adding 65 µL of MSTFA 5% TMCS on a microwave at 800 W for 2 minutes and 2 µL were injected into the GC-MS system.

Table 3 -Retention time and selected ions for the identification of analytes. Amphetamine (AMP), methamphetamine (MAMP), *3,4-methylenedioxyamphetamine* (MDA), 3,4-methylenedioxyethylmethamphetamine (MDMA), 3,4-methylenedioxy-N-methyl-α-ethylfenilethylamine (MBDB) and 3,4- methylenedioxy-N-ethylamphetamine (MDE).

Analyte	Retention time (min)	Quantifying ion (m/z)	Qualifying ions (m/z)	Dwell time (µs)
AMP	6.55	116	91 192	50
AMP-d6*	6.55	120	-	50
MAMP	7.15	130	91 206	50
MAMP-d9*	7.15	215	-	50
MDA	8.76	116	100 236	50
MDA-d5*	8.76	120	-	50
MDMA	9.22	130	105 250	50
MDMA-d5*	9.22	255	-	50
MBDB	9.70	144	135 264	50
MDE	9.74	144	135 264	50
MDE-d5*	9.74	269	-	50

**Internal Standards*

2.5. Validation procedure

The method was validated according to the Food and Drug Administration (FDA) and the Scientific Working Group of Forensic Toxicology (SGWTOX) for the validation of bioanalytical methods (66,67). The following parameters were considered: selectivity, calibration curves and limits, intra and inter-day precision and accuracy, recoveries, dilution integrity and stability.

3. Results and discussion

3.1. Optimization of the extraction procedure

3.1.1. Extraction procedure selection

The first step of the extraction procedure was the selection of a technique based on the available literature for the determination of amphetamines in biological specimens. A total of seven different techniques were evaluated using C18 and M1 in order to observe the one resulting in better extraction efficiencies. The seven techniques evaluated (n=3) are resumed in Table 4.

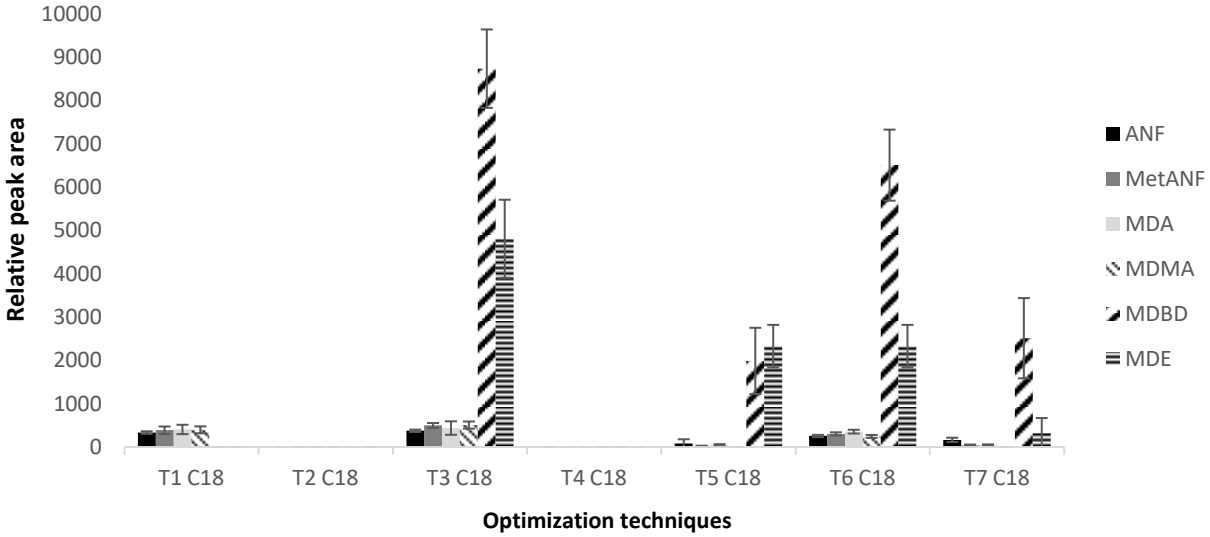

Figure 3-Evaluation of the extraction techniques.

Overall, better results were observed when using the C18 sorbent. Figure 3 shows the relative peak areas obtained for the target amphetamines when the different techniques were tested using C18 sorbent. It is possible to affirm that the third and sixth extraction techniques (T3 and T6) resulted in greater recoveries for the amphetamines under study. These techniques used ammonium hydroxide: acetonitrile (2:98) as the elution solution, but differed in the washing solution. Nevertheless, washing with water followed by water: methanol (95:5) (T3) gives greater recoveries when compared with the acetic acid used in T6. The statistical evaluation when comparing T3 and T6 revealed a significant difference between both techniques, $F(1,4)= 17.23$, $p<0.05$ for AMP; $F(1,4)=24.89$, $p<0.05$ for MAMP; $F(1,4)=27.34$, $p<0.05$ for MDMA; $F(1,4)=8.26$, $p<0.05$ for MBDB; $F(1,4)=17.07$, $p<0.05$ for MDE. Only MDA had no significant difference when comparing these two procedures $F(1,4)=6.64$, $p<0.05$, hence T3 was the chosen one.

Table 4- Different reagents used in the techniques of microextraction by packed sorbent (MEPS).

Technique	Sample (volume)	Dilution of the sample	Packaging	Load	Washing	Elution	Column reconstitution	References
T1	Urine (200 µL)	100 µL Sodium phosphate (0,1M) pH6	250 µL Water; 250 µL methanol	6 x 100 µL	50 µL Acetic acid (0,1M); 50 µL water	Dichloromethane:isopropanol: amonium hydroxide (78:20:2) (6 x 100 µL)	Ammonium hydroxide on acetonitrile: methanol (1:1); 1% formic acid in isopropanol: water (10:90) (4 x 100 µL)	(68)
T2	Urine (200 µL)	100 µL 10% Formic acid	250 µL Water; 250 µL methanol	6 x 100 µL	50 µL 10% Formic acid; 50 µL water	Ammonium hydroxide on methanol (2:98) (6 x 100 µL)	Ammonium hydroxide on acetonitrile: methanol (1:1); 1% formic acid in isopropanol: water (10:90) (4 x 100 µL)	(69)
T3	Urine (200 µL)	100 µL Ammonium acetate (0,1M)	250 µL Water; 250 µL methanol	6 x 100 µL	50 µL Water; 50 µL water:methanol (95:5)	Ammonium hydroxide in acetonitrile (2:98)(6 x 100 µL)	Ammonium hydroxide on acetonitrile: methanol (1:1); 1% formic acid in isopropanol: water (10:90) (4 x 100 µL)	(70)
T4	Urine (200 µL)	100 µL 1% Formic acid	250 µL Water; 250 µL methanol	6 x 100 µL	50 µL Acetic acid (0,1M)	Ammonium hydroxide on methanol (2:98) (6 x 100 µL)	Ammonium hydroxide on acetonitrile: methanol (1:1); 1% formic acid in isopropanol: water (10:90) (4 x 100 µL)	(71)
T5	Urine (200 µL)	100 µL Ammonium acetate (0,1M)	250 µL Water; 250 µL methanol	6 x 100 µL	50 µL Water; 50 µL water:methanol (95:5)	Ammonium hydroxide on methanol (2:98) (6 x 100 µL)	Ammonium hydroxide on acetonitrile: methanol (1:1); 1% formic acid in isopropanol: water (10:90) (4 x 100 µL)	(69)
T6	Urine (200 µL)	100 µL Ammonium acetate (0,1M)	250 µL Water; 250 µL methanol	6 x 100 µL	50 µL Acetic acid (0,1M); 50 µL water	Ammonium hydroxide in acetonitrile (2:98)(6 x 100 µL)	Ammonium hydroxide on acetonitrile: methanol (1:1); 1% formic acid in isopropanol: water (10:90)(4 x 100 µL)	(70)
T7	Urine (200 µL)	100 µL Ammonium acetate (0,1M)	250 µL Water; 250 µL methanol	6 x 100 µL	50 µL Acetic acid (0,1M); 50 µL water	Ammonium hydroxide on methanol (2:98)(6 x 100 µL)	Ammonium hydroxide on acetonitrile: methanol (1:1); 1% formic acid in isopropanol: water (10:90) (4 x 100 µL)	(69)

3.1.2 Optimization of extraction procedure with DOE

In order to optimize the chosen technique and to test the different parameters that could affect the extraction efficiency, a design of experiments (DOE) was considered. This statistical tool allowed a multivariate analysis of 4 factors (number of strokes, washing solvent volume, elution solvent volume, and ammonium hydroxide percentage) in random combinations in order to identify which critical conditions would influence the most. Nevertheless, this evaluation was not conclusive, once all parameters gave a significant influence. In a normal analysis, only two or three factors should reveal as significant. In the best case scenario none of the factors would be significant, that would allow setting different values for each parameter without damaging the final result. In this sense, an optimization process continued in a univariate way.

3.1.3 Optimization of extraction procedure with univariate analysis

Through a univariate analysis it is possible to obtain better results from each parameter independently, hence one single parameter was studied isolated and the results compared after each study.

3.1.3.1. Optimization of number of strokes

In the first univariate study, the number of strokes effect was evaluated (n=3), varying from 3, 6, 9 and 15 (x100 µL). The number of strokes compromises the final recovery of the compounds and for more complex matrices it is preferable to use a greater number of strokes. Nevertheless, there were no significant differences, with exception for AMP and MAMP, when using 6 and 9 (x100 µL) number of strokes [$F(1,4)=61.62$, $p<0.05$ for AMP; $F(1,4)=35.17$, $p<0.05$ for MAMP; $F(1,4)=1.41$, $p<0.05$ for MDA; $F(1,4)=-3.63$, $p<0.05$ for MDMA; $F(1,4)= 0.39$, $p<0.05$ for MBDB; $F(1,4)=0.85$, $p<0.05$ for MDE]. Greater peak areas were obtained for 9 strokes. Figure 4 shows the results obtained in this evaluation and it is possible to observe a raise in the recoveries as the number of strokes increased, however no significant differences were observed when the number of strokes was greater than 9. The comparison between 9 and 15 strokes resulted in [$F(1,4)= 4.32$, $p<0.05$ for AMP; $F(1,4)= 0.93$, $p<0.05$ for MAMP; $F(1,4)= 0.23$, $p<0.05$ for MDA; $F(1,4)= 0.06$, $p<0.05$ for MDMA; $F(1,4)= -3.31$, $p<0.05$ for MBDB; $F(1,4)= -0.07$, $p<0.05$ for MDE] , hence the MEPS procedure optimization continued with 9 strokes.

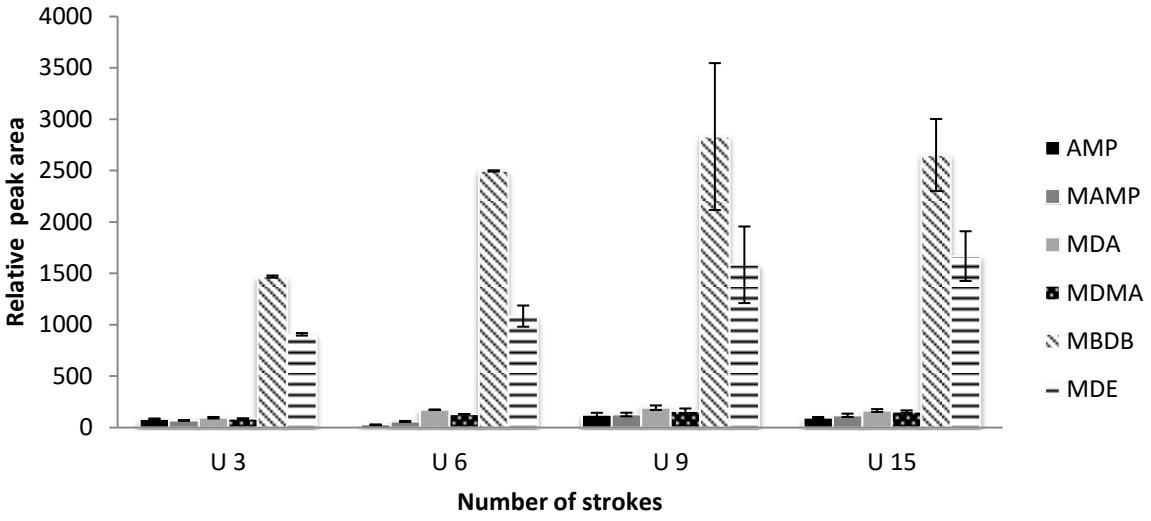

Figure 4-Evaluation of the number of strokes.

3.1.3.2. Optimization of washing volume

The optimization proceeded by varying the volume of washing solution between 50 µL, 100 µL and 150 µL. Figure 5 allows the assumption that for urine samples the cartridge should be washed with 150 µL due to the fact that greater recoveries and cleaner chromatograms were obtained. Although, comparison between 100 and 150 µL revealed no significant differences [$F(1,4)= 0.01$, $p<0.05$ for MAMP; $F(1,4)= 0.03$, $p<0.05$ for MDA; $F(1,4)= -2.04$, $p<0.05$ for MDMA; $F(1,4)= 0.15$, $p<0.05$ for MBDB; $F(1,4)= 0.46$, $p<0.05$ for MDE], with exception for AMP [$F(1,4)=520.89$, $p<0.05$]., in Figure 5 one can observe that in a general way, greater relative peak areas were obtained when 150 µL were applied.

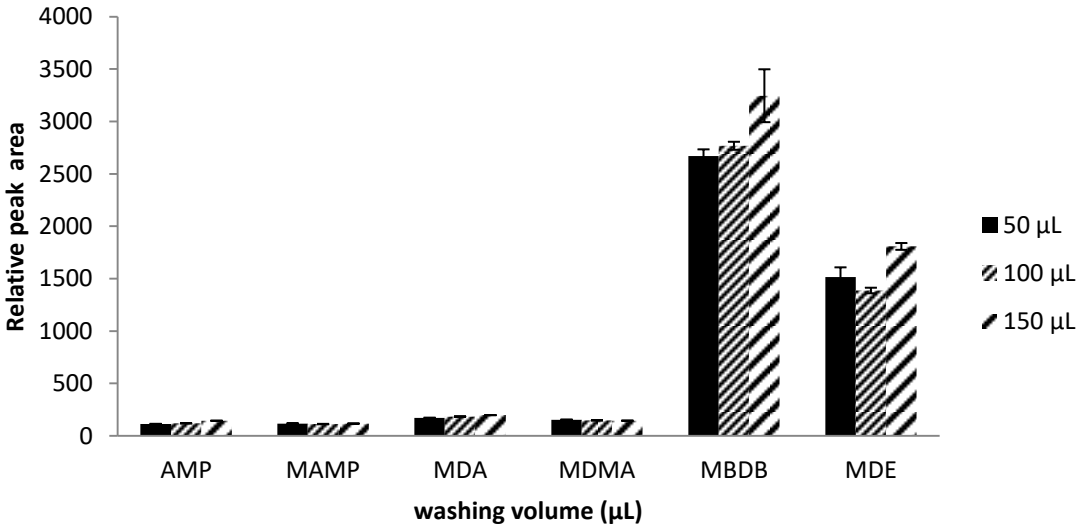

Figure 5- Evaluation of the washing solvent volume.

3.1.3.3. Optimization of percentage of ammonium hydroxide

As the next optimization step, it was evaluated the adequate percentage of ammonium hydroxide for the elution solution applied in the MEPS procedure. The solution with 2% ammonium hydroxide showed better results with greater peak areas. Although no significant differences were shown when using 1.5% and 2% of ammonium hydroxide for most amphetamines [$F(1,4)= 0.04$, $p<0.05$ for MAMP; $F(1,4)= 0.00$, $p<0.05$ for MDA; $F(1,4)= -1.81$, $p<0.05$ for MDMA; $F(1,4)= -3.70$, $p<0.05$ for MBDB; $F(1,4)= 0.08$, $p<0.05$ for MDE], 2% of ammonium hydroxide gave a significant better extraction efficiency for AMP [$F(1,4)= 98.79$, $p<0.05$). Figure 6 is the graphical representation of the obtained results.

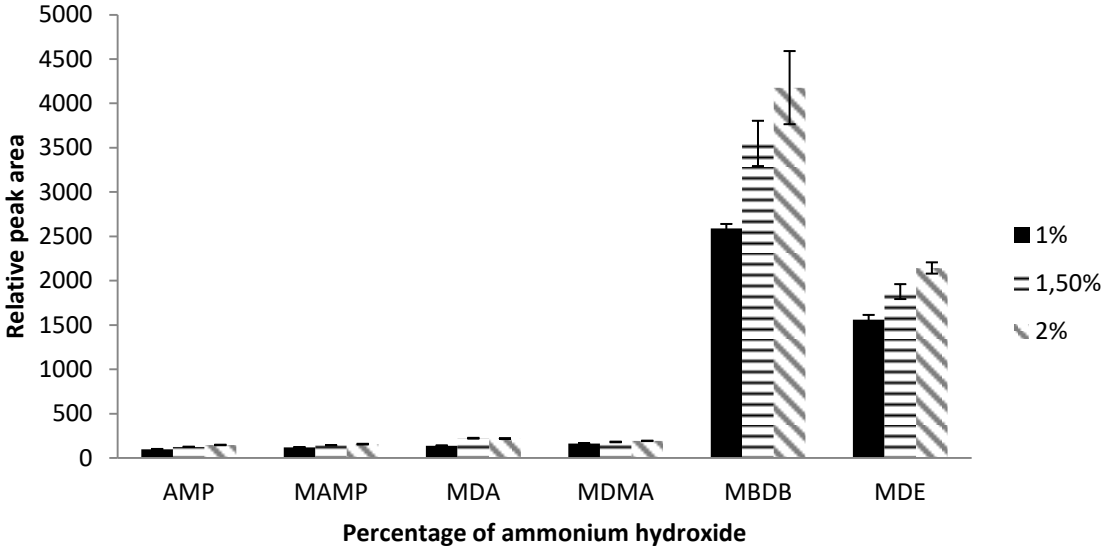

Figure 6-Evaluation of the percentage of ammonium hydroxide.

3.1.3.4. Optimization of number of elutions

Finally, the number of elutions was studied in order to choose which one would result in greater amounts of amphetamines collected. Varying from 4 to 8 cycles of 100 µL , 4 cycles of 100 µL seemed the most appropriate number of elution cycles (see Figure 7). The differences between eluting 6 or 8 times are almost inexistent. Therefore, the results obtained from 2 and 4 elutions were statistically compared. All of the differences were significant [$F(1,4)= 9.60$, $p<0.05$ for AMP; $F(1,4)= 38.32$, $p<0.05$ for MAMP; $F(1,4)= 67.54$, $p<0.05$ for MDA; $F(1,4)= 4901.19$, $p<0.05$ for MDMA), except for MBDB and MDE [$F(1,4)= -1.63$, $p<0.05$ for MBDB; $F(1,4)= -1.52$, $p<0.05$ for MDE]. Also, analysing 4 and 6 elutions, all results presented differences with significance [$F(1,4)= 23.53$, $p<0.05$ for MAMP; $F(1,4)= 26.77$, $p<0.05$ for MDA;

F(1,4)= 82.70, p<0.05 for MDMA; F(1,4)= 59.85, p<0.05 for MDE] with exception for AMP and MBDB [F(1,4)= 0.66, p<0.05 for AMP; F(1,4)= -1.96, p<0.05 for MBDB]. Overall, 4 cycles of elution seemed suitable.

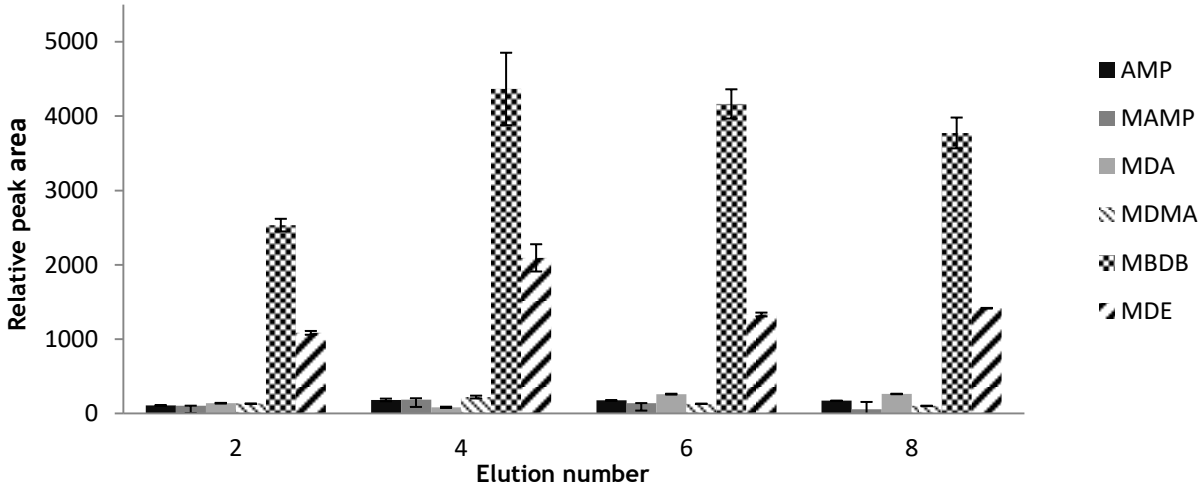

Figure 7- Evaluation of the number of elutions.

Table 5-Variables and final results for the analysis of the extraction procedure, including number of strokes, washing solution volume, percentage of ammonium hydroxide and elution number.

Matrix	Analysis	Variables	Results
Urine	number of strokes	3, 6, 9 and 15 (x 100 µL)	9 (x 100 µL)
	wash volume	50, 100 and 150 µL	150 µL
	% ammonium hydroxide	1%, 1.5% and 2%	2%
	number of elutions	2, 4, 6 and 8 (x 100 µL)	4 (x 100 µL)

3.2. Method validation parameters

The method was validated according to the guiding principles of the Food and Drug Administration (FDA) and the Scientific Working Group of Forensic Toxicology (SGWTOX) for the validation of bioanalytical methods (66,72).

The five day validation protocol, as well the studied parameters, included:

3.2.1. Selectivity

The capacity that the method has to differentiate and quantify the analyte in the presence of other components of the biological matrix is known as the selectivity of the analytical method. In this study blank urine obtained from ten healthy volunteers were checked for endogenous interferences and assured in the lower limit of quantification (LLOQ) (66).

In addition, the specificity was also studied by the analysis of urine samples where it was verified the interferences that other abuse drugs and medication could have in the retention time of the analytes of interest. Moreover, 30 different compounds that could be present in authentic samples and therefore interfere with the analysis, were also injected. No signals were detected at the corresponding retention times and transitions of the studied compounds. These compounds included benzodiazepines, antidepressant, antipsychotic and anticonvulsant drugs, cocaine and metabolites, cannabinoids, opiates, caffeine and nicotine and metabolites.

The criteria considered for a positive identification with associated confidence included an absolute retention time within 2% or ± 0.1 min of the retention time of the same compound in the control sample and also the presence of three ions per analyte. The maximum allowable tolerances for the relative ionic intensities between the transitions (as a percentage of the base peak) were: if the relative ion intensity in the control sample was higher than 50%, an absolute tolerance of ± 10% was accepted; if this value was between 25 and 50%, a relative tolerance of ±20% was permitted; if it was between 5 and 25%, an absolute tolerance of ± 5% was accepted and lastly, for relative ion intensities of 5% or less, a relative tolerance of ± 50% was used (73). As these previously described criteria, the method would be considered selective if no analyte could be identified in the blank samples.

Figure 8 shows the chromatograms obtained for a blank urine sample and one spiked at the LLOQ for each amphetamine. It is possible to observe no interferences at the retention time and selected quantifying ion.

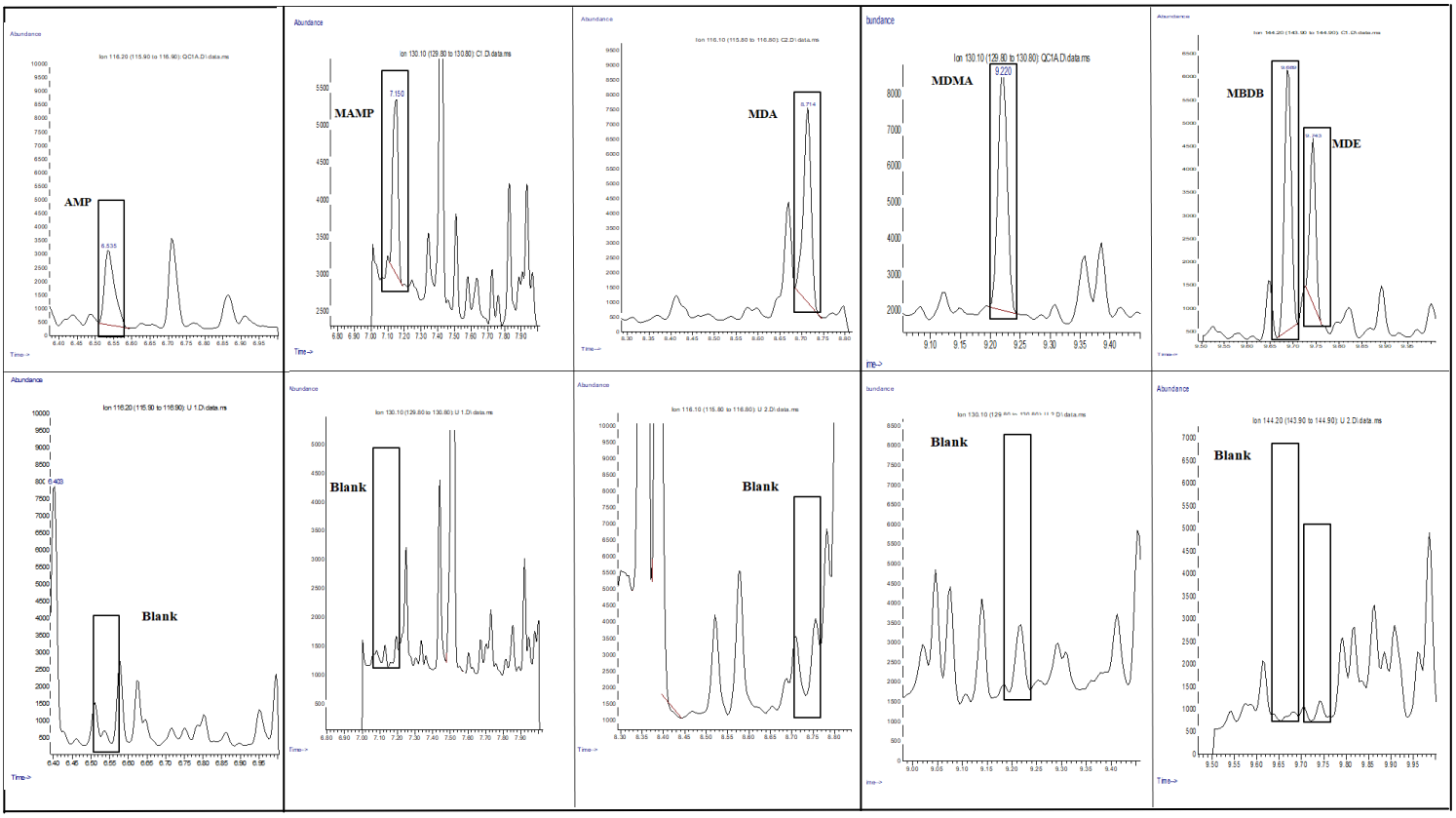

Figure 8-GC-MS chromatograms of selected ions obtained after extraction of blank urine samples and the LLOQ for all compounds.

3.2.2. Calibration and curves and limits

For the study of the linearity were chosen seven calibrators in a range of 25-1000 ng/mL and the analysis was repeated during five days. The linearity was obtained in every single day with $R^2 \geq 0.99$ using a weighting of 1/x in every curve.

There were analysed six different weightings (1/x; $1/\sqrt{x}$; $1/x^2$; 1/y; $1/\sqrt{y}$; $1/y^2$) and the best was chosen, i.e. the one that revealed the lower relative errors (RE). The weighting showing lower RE and $R^2 \geq 0.99$ was the selected to proceed the analysis.

The calibration curves were obtained by plotting the peak area ratio between each amphetamine and the IS against the amphetamine concentration.
The LLOQ was defined by the lowest concentration that could be measured with the adequate precision and accuracy i.e. with a coefficient of variation (CV) of equal or less than 20% and the relative error (RE) within ± 20% of the supposed concentration (66). Table 6 resumes the obtained linearity data.

Table 6-Linearity data (n=5).

Analyte	Weight	Linear range (ng/mL)	Linearity		R^{2*}	LLOQ (ng/mL)
			Slope*m	Intercept*b		
AMP	1/x	35 – 1000	0.0102 ± 0.0017	0.0972 ± 0.0826	0.9952 ± 0.0021	35
MAMP	1/x	25 – 1000	0.1862 ± 0.0721	-0.3248 ± 2.9133	0.9954 ± 0.0031	25
MDA	1/x	50 – 1000	0.0136 ± 0.0023	0.1639 ± 0.2556	0.9961 ± 0.0020	50
MDMA	1/x	35 – 1000	0.1951 ± 0.0518	-1.3423 ± 2.8992	0.9947 ± 0.0028	35
MBDB	1/x	25 – 1000	0.3569 ± 0.0997	-6.6969 ± 4.3824	0.9956 ± 0.0025	25
MDE	1/x	25 – 1000	0.2248 ± 0.1232	-3.2278 ± 4.3232	0.9957 ± 0.0028	25

*Mean values ± standard deviation.

Comparing the LLOQs obtained in the present work with the available literature, it is possible assume the advantages of this new method. Bugamelli F. *et al*. (2006) (40) applied a SPME procedure using 500 µL of urine reaching for limits of detection ranging from 6-50 ng/mL and limits of quantification of 12-100 ng/mL. Franco de Oliveira *et al*. (2016) (41) studied the detection of AMP and MAMP using LPME coupled to GC-MS and obtained LOQ of 20 ng/mL and LOD of 10000-20000 ng/mL using 1 mL of urine sample. The present procedure reaches lower LLOQs using only 200 µL of sample.

Earlier in 2014 a study (43) was made using MEPS technique but coupled to HPLC detecting amphetamine with only 100 µL of urine samples and obtaining LOD of 3 ng/mL and LOQ of 10 ng/mL, presenting recoveries ranging from 92-106%. The present work can be applied to several amphetamines, which may be considered a great alternative.

3.2.3. Intra- and Inter-day precision and accuracy

The precision of a method describes the proximity of individual measurements from the sample analysis where the procedure is repeated in samples with known quantities of the analyte. For that matter were used at least five determinations for each concentration (66).
The evaluation of the Inter-day precision and accuracy was made within a 5-day period at minimum of 6 concentration levels. The CVs obtained were lower than 15% for all analytes at tested concentration levels with a accuracy within a ± 15 % interval.

To evaluate the intra-day precision were analysed, in the same day, six replicates of blank urine samples spiked with the six compounds at three concentration levels (the lowest, 35 ng/ml; the intermediate, 250 ng/mL; and the highest, 1000 ng/mL). Results show a coefficient variation ≤ 14% with RE ≤ ± 13% for the tested concentrations, except MBDB which presented an RE ≤ ± 20% for the LLOQ.

To determine the intermediate precision and accuracy, during 5 days there were analysed 3 different concentrations (n=3) in a range of 25-1000 ng/mL (nominated quality controls (QC) with concentrations of 35, 300 and 900 ng/mL) and the CVs obtained were ≤ 10% with a RE ≤ ± 8%.

It is correct to say that this method presents precision due the consistency of results obtained when repeated measurements were made, as well accuracy because the final obtained concentrations were approximate to the theoretical ones, presenting low percentage of error. Tables 7, 8 and 9 resumes the data obtained.

Table 7-Inter-day precision and accuracy (n=5 days).

Analyte	Spiked	Measured	CV (%)	RE (%)
AMP	**35**	30.66 ± 4.35	14.2	7.37
	50	49.89 ± 3.46	6.9	-0.22
	100	101.06 ± 11.45	11.3	1.06
	250	238.31 ± 11.08	4.7	-4.68
	500	489.52 ± 35.12	7.2	-2.10
	750	738.84 ± 22.94	3.1	-1.49
	1000	1036.69 ± 46.43	4.5	3.67
MAMP	**25**	25.51 ± 3.22	12.6	2.06
	50	50.05 ± 4.23	8.5	0.09
	100	96.92 ± 12.83	13.2	-3.08
	250	247.68 ± 31.17	12.6	-0.93
	500	501.52 ± 42.74	8.5	0.30
	750	753.56 ± 32.93	4.4	0.47
	1000	997.17 ± 72.42	7.3	-0.18
MDA	**50**	49.80 ± 3.23	6.5	-0.41
	100	102.13 ± 8.56	8.4	1.78
	250	237.55 ± 25.41	10.7	-4.98
	500	492.50 ± 22.27	4.5	-1.50
	750	753.40 ± 36.96	4.9	0.45
	1000	1020.11 ± 37.15	3.6	2.01

	35	34.95 ± 2.11	6.03	-0.15
	50	51.75 ± 3.46	6.7	3.51
	100	98.44 ± 9.41	9.6	-1.56
MDMA	**250**	225.96 ± 11.39	5.0	-9.62
	500	499.66 ± 43.39	8.7	-0.07
	750	759.01 ± 33.26	4.4	1.20
	1000	1015.24 ± 39.15	3.9	1.52
	25	26.02 ± 3.02	11.6	4.09
	50	49.00 ± 3.79	7.7	-2.00
	100	92.98 ± 7.31	7.9	-7.02
MBDB	**250**	244.96 ± 23.61	9.6	-2.02
	500	517.53 ± 27.26	5.3	3.51
	750	754.91 ± 42.99	5.7	0.65
	1000	992.19 ± 34.07	3.4	-0.78
	25	24.17 ± 2.66	11.0	-3.32
	50	51.76 ± 4.35	8.4	3.51
	100	107.92 ± 3.58	3.3	7.92
MDE	**250**	245.90 ± 26.24	10.7	-1.64
	500	505.36 ± 27.24	5.4	1.07
	750	743.25 ± 40.06	5.4	-0.90
	1000	1004.49 ± 50.01	5.0	0.45

All concentrations in ng/mL; CV – Coefficient of variation; RE – Relative error [(measured concentration-spiked concentration/spiked concentration)] x 100; *Mean values ± standard deviation.

Table 8-Intra-day precision and accuracy (n=6).

Analyte	Spiked	Measured	CV (%)	RE (%)
	35	36.45 ± 0.48	1.33	4.15
AMP	250	236.78 ± 17.84	7.53	-5.29
	1000	1044.82 ± 84.43	8.08	4.48
	25	27.93 ± 0.61	2.20	11.74
MAMP	250	225.83 ± 11.42	5.06	-9.67
	1000	1104.42 ± 38.53	3.49	10.44
	50	51.69 ± 3.99	7.72	3.37
MDA	250	233.25 ± 17.08	4.64	-6.70
	1000	1007.89 ± 46.76	4.64	0.79

MDMA	35	34.69 ± 1.35	3.88	-0.90
	250	219.32 ± 2.03	0.92	-12.27
	1000	935.53 ± 96.89	10.36	-6.45
MBDB	25	29.95 ± 0.09	0.31	19.78
	250	230.96 ± 8.87	3.84	-7.62
	1000	978.22 ± 84.05	8.59	-4.50
MDE	25	27.71 ± 0.58	2.10	10.84
	250	243.55 ± 33.38	13.70	-2.58
	1000	1021.31 ± 94.61	9.26	2.13

All concentrations in ng/mL; CV – Coefficient of variation; RE – Relative error [(measured concentration-spiked concentration/spiked concentration)] x 100; *Mean values ± standard deviation.

Table 9-Intermediate precision and accuracy (n= 5 days).

Analyte	Spiked	Measured	CV (%)	RE (%)
AMP	**75**	74.81 ± 6.02	8.04	-0.26
	300	303.96 ± 19.47	6.41	1.32
	900	855.58 ± 54.55	6.38	-4.94
MAMP	**35**	34.78 ± 1.66	1.66	1.84
	75	77.24 ± 5.83	7.54	-2.87
	300	293.51 ± 26.67	9.09	-3.12
	900	913.33 ± 68.18	7.46	-1.05
MDA	**75**	78.06 ± 7.20	9.22	4.08
	300	292.76 ± 16.48	5.63	-2.41
	900	906.91 ± 52.74	5.82	0.77
MDMA	**75**	70.83 ± 1.62	4.60	-5.56
	300	295.76 ± 25.41	8.59	-1.41
	900	883.63 ± 54.45	6.16	-1.82
MBDB	**35**	33.62 ± 1.94	5.77	-3.96
	75	74.39 ± 1.96	2.63	-0.81
	300	296.99 ± 25.67	8.64	-1.00
	900	931.83 ± 65.55	7.03	3.54
MDE	**35**	34.69 ± 3.17	9.13	-0.90
	75	76.92 ± 2.99	3.89	2.55
	300	277.79 ± 23.89	8.60	-7.40

900	844.80 ± 75.85	8.98	-6.13

All concentrations in ng/mL; CV – Coefficient of variation; RE – Relative error [(measured concentration-spiked concentration/spiked concentration)] x 100; *Mean values ± standard deviation.

3.2.4. Recoveries

The results from the samples extracted (set 1, where the compounds were spiked and extracted by the MEPS procedure) were compared with samples without extraction of the amphetamines (set 2, where blank samples were extracted and the extracts were spiked after it, representing the 100% of recovery). The ISs mixture was added to both sets of samples after the extraction.

To obtain the recoveries results, the peak areas from set 2 were compared to the peak areas obtained in set 1. For that three different concentrations were tested, the LLOQ, the upper limit of quantification (ULOQ) and an intermediate concentration of 500 ng/mL. The results obtained are shown on table 10.

Comparing with the literature available of microextraction techniques to determine amphetamines in urine samples, the present work seems to give adequate recoveries. Chung *et al.* (2008) (34) obtained recoveries for AMP and MAMP of 93.28-103.55% using a SPE, an extraction technique that is known for resulting in great extraction efficiencies, technique but used 5 mL of urine. Also, Ahmadi-Jouibari *et al.* (2014)(33) obtained recoveries for AMP and MAMP ranging from 87.8–113.2% using DLLME technique but also required 5 mL of urine. Franco de Oliveira *et al.* (2016)(41) reached 50-76% of recovery for AMP and MAMP using 1 mL of urine and LPME and Raikos *et al.* (2003) (32), showed recoveries of 19.5–47% for AMP; 20–38.1% for MAMP; 5.1–6.6% for MDA; 7–9.6% for MDMA ; 5.4–9.6% for MDE using SPME and a volume sample of 1 mL. Analysing the previous examples it is safe to say that MEPS technique presents its advantages not only due the low volume of sample used but as well the percentages of recoveries presented for all the six compounds.

Table 10-Recoveries (%) of the target analytes under the optimized extraction conditions (n=3).

Analyte	**Recoveries(%)**		
	LLOQ	**500**	**ULOQ**
AMP	49.21 ± 4.55	42.13 ± 3.68	31.53 ± 1.69
MAMP	38.80 ± 1.21	18.95 ± 2.50	30.23 ± 1.48
MDA	44.29 ± 6.19	30.16 ± 3.54	47.71 ± 0.48
MDMA	51.75 ± 4.70	45.05 ± 5.21	39.86 ± 0.66
MBDB	37.52 ± 4.12	49.56 ± 4.19	33.97 ± 29.42
MDE	71.20 ± 0.16	52.39 ± 3.03	63.40 ± 11.87

*Mean values ± standard deviation.

3.2.5. Dilution integrity

The study of the integrity after dilution of the sample reveals itself as useful in cases where the real concentration value is greater than the ULOQ (in this case 1000 ng/mL). It was evaluated if the sample could still be stable, precise and viable when a dilution was performed. There were chosen two dilution factors, 1:5 and 1:2 of an initial sample spiked at 2000 ng/mL (the dilution was prepared with blank urine).

The results, resumed in table 11, presented CVs ≤ 12% with RE ≤ ± 13% leading to the conclusion that even with a diluted sample, the compounds still can be detected and their concentration determined with confidence.

Table 11-Evaluation of dilution integrity (n=3).

Analyte	Dilution factor	Measured	CV (%)	RE (%)
AMP	**1:5**	2087.00 ± 27.39	1.31	4.35
	1:2	1987.66 ± 149.59	7.53	-0.62
MAMP	**1:5**	1850.43 ± 145.70	7.87	-7.48
	1:2	2003.36 ± 81.55	4.07	0.17
MDA	**1:5**	1883.14 ± 41.32	2.19	-5.84
	1:2	1939.81 ± 171.30	8.83	-3.01
MDMA	**1:5**	2085.16 ± 43.32	2.03	4.26
	1:2	1986.85 ± 51.89	2.61	-0.66
MBDB	**1:5**	1835.52 ± 124.51	6.78	-8.22
	1:2	1886.09 ± 143.97	7.63	-5.70
MDE	**1:5**	2258.03 ± 46.72	2.07	12.90
	1:2	1968.82 ± 224.72	11.41	-1.56

All concentrations in ng/mL; CV – Coefficient of variation; RE – Relative error [(measured concentration-spiked concentration/spiked concentration)] x 100; *Mean values ± standard deviation.

3.2.6. Stability

In this phase of validation of the analytical method three types of stability were studied: the stability of processed samples, the stability after freeze/thaw cycles and at room temperature.

3.2.6.1. Processed samples, freeze/thaw and room temperature stability

To evaluate the processed samples stability, samples with three different concentrations (n=3) were injected and then maintained in the autosampler during 24 hours. After this period the same samples were re-injected and the results were compared with the ones obtained from freshly prepared ones. Their concentrations were determined on the basis of the original calibration curve. Analysing the results it is clear that the compounds are stable if maintained for 24 hours in the autosampler, presenting values of CV≤ 11% with RE ≤ ± 11%.

To study freeze/thaw stability, spiked samples were stored at -20°C for 24 hours. Then the frozen samples were thawed unassisted at room temperature (completing 1 cycle) being then refrozen for 12-24 hours under the same conditions. This first cycle was repeated twice more and after the third and final cycle of freeze/thaw the samples were analysed. The results showed CV ≤ 12% with RE ≤ ± 15%.

For the room temperature stability, the spiked urine samples were left at room temperature during 24 hours and only then the extraction procedure occurred. Results showed a CV ≤ 14% with RE ≤ ± 12%. All data is shown on Table 12.

Table 12-Evaluation of the stability of processed samples, freeze/thaw and room temperature (n=3).

	Sampler stability 24h (n=3)				Freeze/thaw stability (n=3)				Room temperature stability (n=3)			
Analyte	**Spiked**	**Measured**	**CV (%)**	**RE (%)**	**Spiked**	**Measured**	**CV (%)**	**RE (%)**	**Spiked**	**Measured**	**CV (%)**	**RE (%)**
AMP	**50**	50.59 ± 3.37	6.65	1.18	**75**	69.60 ± 8.00	11.49	-7.21	**75**	66.01 ± 1.38	2.08	-11.99
	300	311.43 ± 19.60	6.29	3.81	**300**	306.09 ± 18.54	6.06	2.03	**300**	283.97 ± 3.49	1.23	-5.34
	900	814.32 ± 24.56	3.02	-9.52	**900**	802.69 ± 13.46	1.68	-10.81	**900**	923.32 ± 22.67	2.46	2.59
MAMP	**35**	35.45 ± 0.87	2.46	1.28	**35**	34.07 ± 2.35	6.91	-2.66	**35**	33.42 ± 3.92	11.74	-4.51
	300	298.34 ± 17.29	5.79	-0.55	**300**	261.98 ± 2.13	0.81	-12.67	**300**	310.67 ± 27.61	8.89	3.56
	900	842.36 ± 30.20	3.59	-6.40	**900**	931.02 ± 25.88	2.78	3.47	**900**	890.68 ± 120.07	13.48	-1.04
MDA	**50**	54.41 ± 0.14	0.26	8.82	**75**	66.35 ± 1.49	2.24	-11.53	**75**	72.49 ± 2.62	0.04	-3.35
	300	288.37 ± 10.65	3.69	-3.88	**300**	271.88 ± 2.10	0.77	-9.37	**300**	299.55 ± 9.37	0.03	-0.15
	900	841.55 ± 39.58	4.70	-6.49	**900**	825.16 ± 27.63	3.35	-8.32	**900**	1004.85 ± 23.06	0.02	11.65
MDMA	**50**	49.87 ± 2.06	4.14	-0.26	**75**	75.90 ± 5.59	7.37	1.21	**75**	77.86 ± 4.50	5.78	3.81
	300	298.71 ± 34.14	11.43	-0.43	**300**	304.16 ± 6.97	2.29	1.39	**300**	298.41 ± 21.16	7.09	-0.53
	900	939.32 ± 21.70	2.31	4.37	**900**	974.35 ± 31.16	3.20	8.26	**900**	935.05 ± 82.82	8.86	3.90
MBDB	**35**	33.10 ± 0.58	1.74	-5.43	**35**	35.21 ± 1.71	4.85	0.59	**35**	32.38 ± 1.98	6.11	-7.49
	300	270.03 ± 4.57	1.69	-9.99	**300**	257.44 ± 0.29	0.11	-14.19	**300**	319.61 ± 12.70	3.97	6.54
	900	985.57 ± 35.30	3.58	9.51	**900**	954.72 ± 89.09	9.33	6.08	**900**	912.58 ± 120.3	13.16	1.40
MDE	**35**	33.40 ± 2.13	6.37	-4.58	**35**	36.58 ± 1.49	4.07	4.52	**35**	35.13 ± 3.90	11.10	0.37
	300	281.22 ± 26.02	9.25	-6.26	**300**	270.63 ± 11.48	4.24	-9.79	**300**	289.39 ± 22.16	7.66	-3.54
	900	799.88 ± 24.99	3.12	-11.12	**900**	865.61 ± 48.82	5.64	-3.82	**900**	953.80 ± 59.64	6.25	5.98

All concentrations in ng/mL; CV – Coefficient of variation; RE – Relative error [(measured concentration-spiked concentration/spiked concentration)] x 100; *Mean values ± standard deviation.

3.2.7. Method applicability to authentic samples

After validation of this analytical method, in order to demonstrate the applicability of the method, it was successfully applied to routine analysis of hospital samples with suspicion of drug of abuse intoxication. The samples were homogenized for 20 minutes and were analysed in triplicate according to the described method. As an example, Figure 9 shows a chromatogram obtained from the analysis of a urine sample positive for MDMA (*ecstasy*).

The MDMA concentration found in this sample was 110 ng/mL. As it is possible to observe, the chromatogram shows a neat peak at the retention time of 9.2 min with the three selected ions (105, 130 and 250 m/z) that correspond to MDMA.

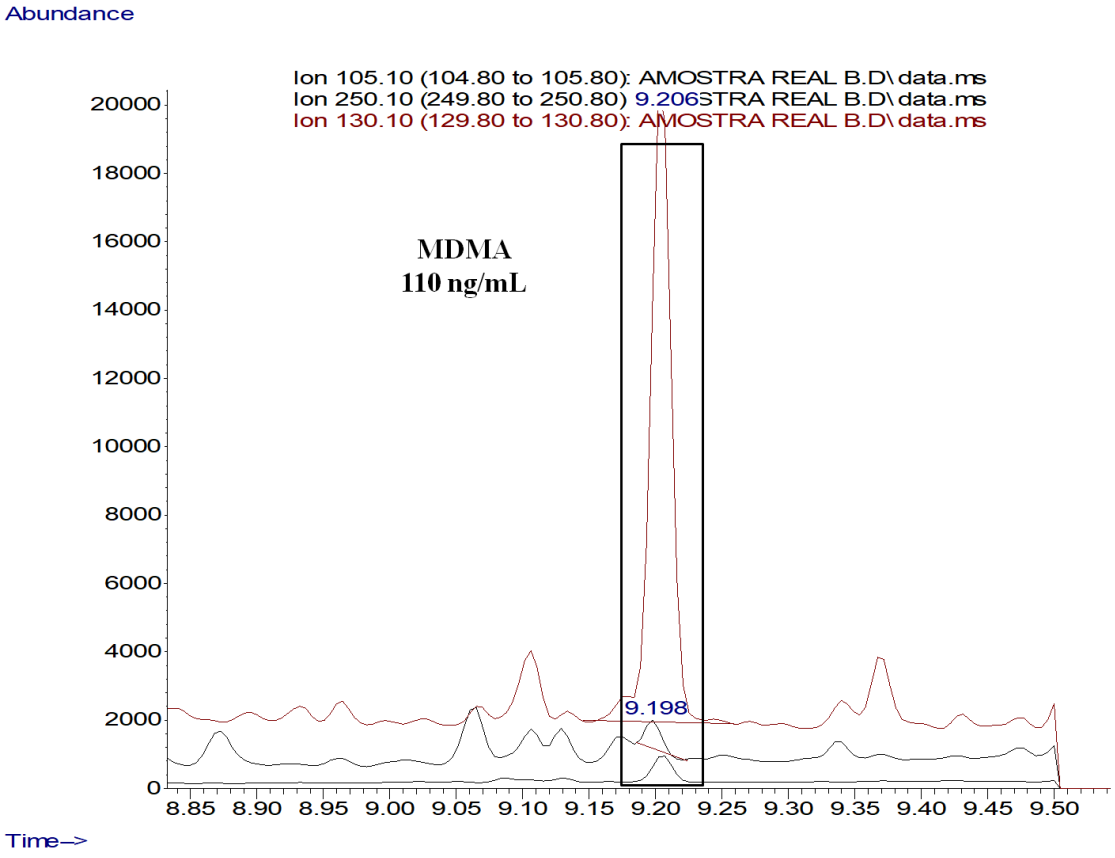

Figure 9-Chromatogram of an authentic sample positive for MDMA (110 ng/mL).

4. Conclusions

- The final developed and optimized MEPS procedure was successfully validated according to international guidelines.
- Linearity was established from LLOQ up to 1000 ng/mL for all the target amphetamines, obtaining coefficients of determination (R^2) greater than 0.99.
- The lower limit of quantification (LLOQ) was 25 ng/mL for all the compounds, except for AMP and MDMA that presented a limit of 35 ng/mL and MDA with 50 ng/mL.
- The recoveries obtained ranged from 19 to 71%. Both intra-and inter-day showed as precised and accurate, presenting sensitivity, specificity, being considerated adequate according to guidelines.
- This is the first study where amphetamines are extracted and detected in urine samples by microextraction by packed sorbent using a gas chromatographer coupled to mass spectrometry.
- The present method has a rapid extraction process (less than 3 minutes) and it is easy to operate with the possibility to reutilize the cartridge (approximately 100 extractions) bringing, consequently, minimization of resources like solvents, compounds and sample volumes (only 200 µL), being cost-effective.

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Conclusions

The present study represents a final and optimized analytical method for the determination and quantification of six different types of amphetamines in urine samples using a microextraction technique.

This method was successfully validated according to international guidelines from Food and Drug Administration (FDA) and Scientific Working Group for Forensic Toxicology (SWGTOX) where linearity was obtained from the lower limit of quantification (LLOQ) to 1000 ng/mL for all the compounds, obtaining coefficients of determination (R^2) higher than 0.99.

The obtained recoveries ranged from 19 to 71% and the LLOQ was 25 ng/mL for all the compounds except for AMP and MDMA that presented a limit of 35 ng/mL and MDA with a limit of 50 ng/mL.

This current MEPS procedure can be considered quite advantageous, requiring a low volume of sample (200 µL) and allowing the determination of all target analytes with a rapid extraction process (less than three minutes).

The proposed method is easy to operate with the possibility to reutilize the cartridge (approximately 100 extractions) bringing, consequently, minimization of resources like solvents, hence considered cost-effective.

The present method was successfully applied to the analysis of authentic samples.

Attachments

The present dissertation was disseminated in different congresses in the area of toxicology, as well as was submitted for publication.

Presentations in congress:

Poster

DETERMINAÇÃO DE ANFETAMINAS EM AMOSTRAS DE URINA COM RECURSO À MICROEXTRAÇÃO EM SERINGA EMPACOTADA

S. Malaca, T. Rosado, M. Barroso, E. Gallardo.

Sixteenth National Congress of Legal Medicine and Forensic Sciences (Coimbra, November 2017)

Oral communication

DETERMINATION OF AMPHETAMINES-TYPE STIMULANTS IN URINE SAMPLES USING MICROEXTRACTION BY PACKED SORBENT AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

S. Malaca, T. Rosado, J. Restolho, J. Rodilla, L. Silva, C. Margalho, M. Barroso, E. Gallardo.

Third Regional Meeting of The International Association of Forensic Toxicologists (TIAFT) (Turkey, October 2018)

Submitted articles:

DETERMINATION OF AMPHETAMINES-TYPE STIMULANTS IN URINE SAMPLES USING MICROEXTRACTION BY PACKED SORBENT AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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