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The role of miniaturized systems in analytical toxicology: new psychoactive substances

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Resumo alargado

O consumo de substâncias químicas com ação sobre o sistema nervoso central (SNC) é tão antigo como a Humanidade; de facto o consumo destes compostos quer para rituais religiosos, como medicamento ou simplesmente pelo prazer que podiam proporcionar ao consumidor, esteve e está presente em várias sociedades e culturas. No entanto, e apesar de ao longo dos tempos a utilização de substâncias com o poder de modificar o humor ter sido comum, o seu uso tem vindo a generalizar-se, sendo na era da globalização que o consumo excessivo de drogas se intensifica e diversifica. Nos últimos anos têm surgido novas substâncias psicoativas (NSP), inicialmente comercializadas através da Internet como drogas de abuso lícitas. Estes compostos, também denominados "*legal highs*", são concebidos de modo a serem diferentes das drogas ilegais, provocando efeitos similares e criando assim um vazio legal que permite a sua comercialização. Uma vez que não se encontram regulamentados na sua totalidade, estão fora do âmbito dos controlos governamentais, constituindo um desafio para as atuais abordagens de monitorização.

Em Portugal, assim como no resto do mundo, o seu uso abusivo bem como os seus efeitos e consequências na vida do indivíduo e da sociedade, constituem uma das principais situações de risco da população atual. A situação tem vindo a tornar-se cada vez mais alarmante e com grande impacto social, reivindicando maior atenção por parte dos profissionais de saúde. Em termos de saúde pública, as mortes envolvendo *overdoses*, intoxicações, acidentes de viação, homicídios e suicídios relacionados com o consumo de drogas, são a mais grave consequência deste consumo abusivo.

Como tal, e devido à falta de informação científica neste contexto, de serem de fácil aquisição e apresentarem um elevado potencial de uso e abuso, existe uma clara necessidade de dotar os laboratórios de instrumentos que permitam combater esta situação, através do desenvolvimento de métodos para identificar e quantificar estas substâncias psicoativas emergentes.

Assim, os objetivos desta tese foram o desenvolvimento, otimização e validação de técnicas extrativas e cromatográficas para a identificação e quantificação de algumas das novas substâncias psicoativas disponíveis em Portugal, nomeadamente salvinorina A, ketamina (K) e o seu principal metabolito norketamina (NK) e a metoxetamina (MXE) em amostras de interesse toxicológico (plasma e urina). A técnica cromatográfica usada foi a cromatografia gasosa acoplada à espectrometria de massa em tandem (GC-MS/MS). Quanto à preparação da amostra, esta foi efetuada através da microextração em seringa empacotada (MEPS), que não é mais que a miniaturização da técnica de extração em fase sólida (SPE), sendo um procedimento que, em relação às técnicas de extração convencionais, apresenta grandes vantagens, como a redução do volume de solventes orgânicos e amostra, bem como a possibilidade de reutilização da coluna extrativa sem se verificarem efeitos de *carryover*

(arrastamento). Por permitir a redução de solventes orgânicos, MEPS é considerada uma técnica amiga do ambiente. Para uma melhor eficiência do processo extrativo, todos os parâmetros suscetíveis de influenciar o procedimento foram otimizados recorrendo a uma poderosa ferramenta estatística, o *Design of Experiments* (DOE). Esta ferramenta permitiu avaliar simultaneamente a influência dos diversos fatores intervenientes com recurso a um menor número de experiências, o que permite poupar tempo e dinheiro aos laboratórios. Para verificar se o método analítico desenvolvido era adequado à sua finalidade, este foi validado segundo critérios internacionalmente aceites. As metodologias mostraram ser seletivas, lineares dentro das gamas de concentrações estudadas, com coeficientes de determinação superiores a 0.99 para todos os analitos e apresentando limites de deteção de 5 ng/mL para salvinorina A, K e NK, e 1 ng/mL para MXE. Sob as condições otimizadas, os valores de recuperação variaram entre 71 a 80% para salvinorina A, 73 a 101% para K e NK em urina e 63-89% em plasma, 80-110% e 81-88% para MXE em urina e plasma, respectivamente. Por último, para avaliar a aplicabilidade dos métodos desenvolvidos e validados, estes foram aplicados a amostras reais provenientes de consumidores de drogas recreativas. Nenhuma das substâncias psicoativas foi detetada nas amostras analisadas.

A MEPS mostrou ser um procedimento rápido e de fácil utilização para a determinação das substâncias selecionadas em amostras de urina e plasma, permitindo reduzir os custos e tempo de preparação da análise. Além disso, o uso de reduzidos volumes de amostra biológica, torna o método um valioso instrumento para a determinação dos compostos estudados, por exemplo em situações de âmbito clínico e forense.

Palavras-chave:

Novas substâncias psicoativas; Microextração em seringa empacotada; GC-MS/MS; Amostras biológicas.

Abstract

In recent years new psychoactive substances (NPS) have emerged, sold via the Internet as licit drugs of abuse. These compounds, also called "legal highs", are designed as substitutes for illegal drugs, causing similar effects. In Portugal, as in the rest of the world, their abuse as well as their effects and consequences in the life of individuals and society, has become increasingly alarming and with great social impact, claiming more attention from health professionals. As such, due to lack of scientific information in this context, there is a clear need to provide laboratories with tools for combating this situation, by means of methods development to identify and quantify these emerging psychoactive substances. The objectives of this thesis were the development, optimization and validation of extractive and chromatographic techniques for the identification and quantification of new psychoactive drugs available in Portugal, namely salvinorin A, ketamine (K) and its major metabolite norketamine (NK) and methoxetamine (MXE) in samples of toxicological interest (plasma and urine). Gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) was the chosen chromatographic technique. Sample preparation was carried out through microextraction in packed sorbent (MEPS), and for a better efficiency of the extraction process, all parameters susceptible of influencing the procedure were optimized using a powerful statistical tool, the Design of Experiments (DOE). To verify that the analytical method was suitable for its intended purpose, it has been validated according to internationally accepted criteria. The methodologies proved to be selective, linear within the studied concentration ranges, with determination coefficients greater than 0.99 for all analytes and presenting limits of detection of 5 ng/mL for salvinorin A, K, and NK and 1 ng/mL for MXE. Under optimized conditions, recovery values ranged from 71 to 80% for salvinorin A, 73-101% for K and NK in urine and 63-89% in plasma, 80-110% and 81-88% for MXE in urine and plasma, respectively. To evaluate the applicability of the present methods, they were applied to real samples; however, none of the psychoactive substances was detected, with exception of mCPP. MEPS proved to be a fast and easy-to-use procedure for the determination of selected drugs in urine and plasma samples, reducing costs and time of analysis. Furthermore, the use of reduced volumes of biological sample makes the method a valuable tool for the determination of the studied compounds, for example in situations of clinical and forensic context.

Keywords:

New psychoactive substances; Microextraction by packed sorbent; GC-MS/MS; Biological samples.

Thesis Overview

This doctoral thesis is structured into five main chapters:

The *first chapter* consists of a brief review of the literature related to the emergence of a new class of drugs known by new psychoactive substances and to the proposed objectives, in which the review article (Article I) was included.

The *second chapter* covers the purpose of development and the overall objectives of this PhD work.

The third *chapter* discusses the work during the PhD in the form of original research articles, organized as follows:

> Article II – Analysis of Salvinorin A in urine using microextraction in packed syringe and GC–MS/MS
>
> Article III – Determination of ketamine and its major metabolite, norketamine, in urine and plasma samples using microextraction by packed sorbent and gas chromatography-tandem mass spectrometry
>
> Article IV – Analysis of methoxetamine in urine and plasma samples using microextraction by packed syringe and gas chromatography-tandem mass spectrometry

The *fourth chapter* includes a general discussion.

Finally the *fifth chapter* summarizes the conclusions of the doctoral work.

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Abbreviations

4-MeO-PCP	4-methoxyphencyclidine
5-HT	Serotonin
ACMD	Advisory Council on the Misuse of Drugs
ADHD	Attention deficit hyperactivity disorder
BZP	Benzylpiperazine
C_{18}	Octadecyl-silica
C_8	Octyl-silica
CE	Capillary electrophoresis
CE	Collision energy
ChD	Chemical derivatization
CVD	Cardiovascular system
DA	Dopamine
DAD	Diode array detector
DEA	Drug Enforcement Administration
DFSA	Drug-facilitated sexual assaults
DHNK	Dehydronorketamine
DIMS	Drug Information and Monitoring System
DMT	Dimethyltryptamine
DOE	Design of experiments
EMCDDA	European Monitoring Centre for Drugs and Drugs Addiction
FDA	Food and Drug Administration
FL	Fluorescence
GC	Gas chromatography
GC–MS/MS	Gas chromatography-tandem mass spectrometry
HF-LPME	Hollow-fiber liquid-phase microextraction
HLM	Human liver microsomes
I.v.	Intravenous
ICH	International Conference on Harmonization
IRD	Infrared detection
K	Ketamine
K-*d4*	Ketamine deuterated
LC	Liquid chromatography
LLE	Liquid–liquid extraction
LOD	Limits of detection
LOQ	Limits of quantification
LSD	Lysergic acid diethylamide

m/z	Mass-to-charge ratio
M_1	Mixed mode sorbent
MAE	Microwave-assisted extraction
MBZP	1-benzyl-4-methyl- piperazine
mCPP	1-(3-chlorophenyl) piperazine
MDA	3,4-methylene-dioxy-amphetamine
MDMA	3,4-methylenedioxy-methamphetamine, Ecstasy
MeOPP	1-(4-methoxyphenyl)-piperazine
MEPS	Microextraction in packed sorbent
MRM	Multiple reaction monitoring
MXE	Methoxetamine
NA	Noradrenalin
NSC	Nervous system central
NK	Norketamine
NK-*d4*	Norketamine deuterated
NMDA	N-methyl-D-aspartate
NPD	Nitrogen phosphorous detector
NPS	New psychoactive substances
PCP	Phencyclidine
pFBT	3-(p-fluorobenzoyloxy)tropane
pFPP	1-(4-Fluorophenyl)piperazine
PI	Product ion
PMA	p-methoxy-amphetamine
PMMA	p-methoxy-methamphetamine
PP	Protein precipitation
SALLE	Salting-out liquid-liquid extraction
SCX	Strong cationic exchange
SERT	Serotonin transporter
SIL	Silica
SIM	Selected ion monitoring
SOFT	Society of Forensic Toxicologists
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SULT	Sulfotransferase
TCDO	Temporary class drug control
TFMPP	1-(3-trifluoromethylphenyl) piperazine
UNODC	United Nations Office on Drugs and Crime
UV	Ultraviolet
WADA	World anti-doping agency
WHO	World health organization

Chapter I

1. Introduction

1.1. Drugs of abuse

Drugs have always seduced mankind. Since ancient times, each society and culture developed and integrated within it the consumption of toxic or potentially toxic chemicals, without any therapeutic purposes, for example, associated with spiritual rituals, religious cults or merely recreational use. But in the 20th century, due to the confluence of multiple factors (cultural, economic, media, etc.), abusive consumption of drugs generalized and intensified [1,2].

According to the present conception and in accordance with the classical definition of the World Health Organization (WHO), the term drug refers to any substance that once introduced into a living organism is capable of modifying one or more of its functions. In general, the term refers to psychoactive substances that modify behavior, affectivity and awareness, and are prone to consumption with non-therapeutic and/or illicit purposes [1]. Throughout time, drugs eventually assume three dimensions: the merchandise, as the connection point between the legal, economic and fiscal components; the playful and therapeutic dimension, as favoring source of disinhibition of social coexistence and as medical procedure tool; and finally the dimension object and source of crime, an emerging perspective, especially from the mid 20th century [3,4].

In the last decades, in contemporary societies, is notorious an increase in the abuse of new substances [5,6], clandestinely produced and cleverly modified in the illicit drug market. This growing abuse has become a cause for major concern in modern societies, leading to serious safety and public health hazards with a significant impact on road accidents, labor, medical costs and in general throughout the social context [5,7,8]. The most commonly used drugs are amphetamine-like compounds, such as 3,4-methylene-dioxy-amphetamine (MDA), 3,4-methylenedioxy-methamphetamine (MDMA, Ecstasy), p-methoxy-amphetamine (PMA) and p-methoxy-methamphetamine (PMMA) [9].

However, a new class of drugs known by new psychoactive substances (NPS) has recently emerged in the market. These compounds are regarded as new psychotropic drugs which are

not controlled by the 1961 United Nations Single Convention on Narcotic Drugs, or the 1971 Convention on Psychotropic Substances, and that may pose a threat to public health [10,11]. In this perspective, the term “novel” doesn’t essentially refer to new creations, but to substances that have newly become accessible [12]. These new drugs are usually sold via the Internet or in smart shops as "legal" and cheaper alternatives to classic drugs, which are controlled internationally. NPS are known in the market by terms such as "designer drugs", "party pills", "legal highs", "herbal highs" or "research chemicals" [12–15]. NPS are intentionally designed to cause certain effects which are similar to those of regulated drugs through slight changes in their chemical structure in order to bypass legal controls, therefore the frequent name of “legal highs” [16,17]. These drugs are normally produced in chemical laboratories outside Europe and legally imported, either as chemicals or already packaged products [16]. NPS, based on their source, may be classified as natural or synthetic drugs. Natural drugs are those which are extracted from a plant, such as *Salvia divinorum*, Khat and Kratom. Synthetic drugs are those whose production is entirely made in laboratory, and these include the following sub-categories: synthetic cannabinoids (JWH-018, JWH-081, JWH-250, RCS-4, CP-47,497-C8, etc), derivatives/analogs of cocaine [pFBT (3-(p-fluorobenzoyloxy)tropane), dimethocaine], synthetic cathinones (mephedrone, dimethylcathinone, buthylone, methylone, ephedrone, among others), phencyclidine-type substances [4-methoxyphencyclidine (4-MeO-PCP)], tryptamines and derivatives [dimethyltryptamine (DMT), psilocin, psilocybin], phenethylamines and derivatives (2C-E, 2C-I, 4-FA, Bromo-Dragonfly, 2C-B-Fly), piperazines [1-benzylpiperazine (BZP), 1-(3-chlorophenyl) piperazine (mCPP), 1-(3-trifluoromethylphenyl) piperazine (TFMPP), 1-Benzyl-4-methyl- piperazine (MBZP), 1-(4-methoxyphenyl)-piperazine (MeOPP), 1-(4-Fluorophenyl)piperazine (pFPP)], and a group of miscellaneous substances that contains identified NPS which do not fit into any of the aforementioned groups, such as ketamine, methoxetamine and aminoindanes (2-Aminoindane) [18–21]. Regarding the psychoactive effects of NPS, these can be classified into 3 categories: stimulants, hallucinogens and synthetic cannabinoids [17]. The stimulant drugs increase the levels of motor and cognitive activities, leading to a state of euphoria taking as examples synthetic cathinones and piperazines. Hallucinogenic drugs act on the nervous system by changing some activities, without causing any stimulation or depression, such as salvinorin A (*Salvia divinorum*). Synthetic cannabinoids, known as "spice" have a category of their own, since they are the only compounds with action on the cannabinoid receptors [17].

The market of these drugs is distinguished by the speed at which suppliers avoid toxicological controls, creating new alternatives to banned products and promoting them with sophisticated and aggressive marketing strategies (essential oils, bath salts, fertilizers) [13,16]. In addition, these products often lack information on their composition, namely concerning the presence of such substances [22]. Their specific psychotropic effects, which support their use as drugs of abuse, are reported as increased communicability, empathy,

visual and auditory hallucinations, self-awareness and states of euphoria [9]. Due to the effects of NPS, there has been a great demand mainly by young people, which made the consumption of these substances leave the “club scene” and enter the mainstream of night recreation. They are consumed mostly alone, but to enhance the effects, the combined use with others substances, phenomenon known as polydrug use, has also become the most noticed behavior between users, leading the authorities to the need of new developments in control and monitoring programs. Their price and availability as tablets, powders or liquids also guarantee great popularity, thus enhancing all the illicit market associated to these compounds [23].

Scientists and health care professionals agree that these substances are hazardous and pose serious risks to the health, and therefore it was deemed essential to establish sanitary measures of immediate effect against NPS. In Portugal, through the Decree-Law n.º 54/2013, April 17th, it is illegal to produce, import, export, advertise, distribute, sell and possess known new psychoactive substances, as well as others that may appear in the market [20].

The continuous research on the synthesis of new substances, as well as their large scale consumption, results in a growing number of reports on seizures, abuse and intoxications (Figure 1) [5].

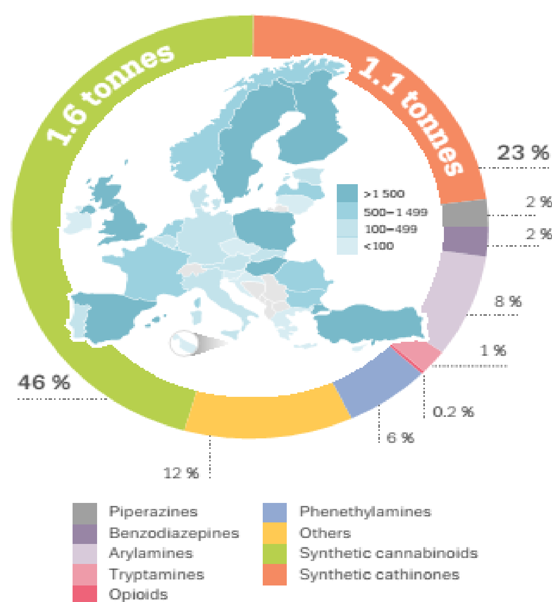

Figure 1. Number of seizures of NPS per country and proportion of seizures by category of substance, 2013 [24].

In recent years we have witnessed a growth of NPS becoming available in Europe, and this fact can be verified by the increase of reports of novel substances to the EU Early Warning System, from just 14 in 2005 to 101 in 2014, being synthetic cannabinoids and synthetic cathinones the largest groups of NPS supervised by the European Monitoring Centre for Drugs and Drugs Addiction (EMCDDA) [24,25]. This was the largest number of substances ever reported in a single year. The increase of the number of officially notified substances in the market takes place in the context of the continued growth of the legal highs phenomenon, which is due not only to an increase in the available substances but also to an improved reporting capability of national early-warning systems (Figure 2) [16].

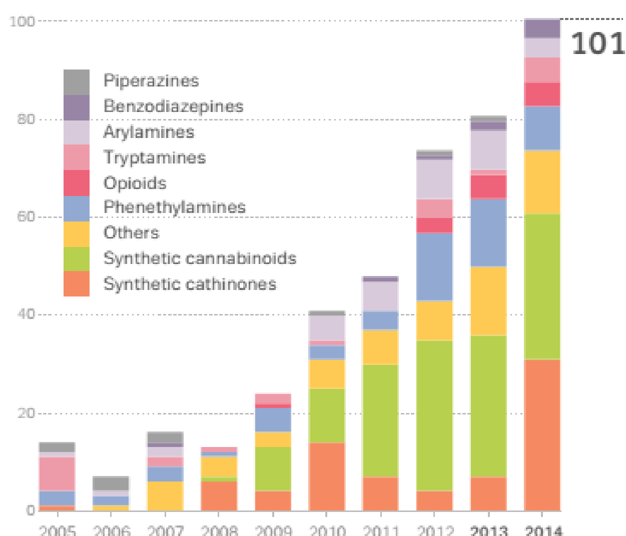

Figure 2. Number and main groups of new psychoactive substances reported to the EU Early Warning System, 2005–14 [24].

To control the prevalence of NPS' use in the general population is often a challenge, leading to a growing need for monitoring these substances, and this is why several countries have opted for the inclusion of NPS in population surveys. The 2014 Flash Eurobarometer, a survey of 13128 young adults aged between 15 and 24 in the EU Member States, provides some useful information about the consumption of these substances. The survey shows that 8% of respondents had consumed "legal highs" at least once, with 3% using them in the last year, which represents an increase relatively to the 5% that related having consumed them at least once in a similar survey performed in 2011 [12,24]. The highest levels of consumption in the last year were reported in Ireland (9%), Spain, France (both 8%), and Slovenia (7%), while in Cyprus and Malta no one mentioned having used NPS in the last year. From the people who mentioned this consumption, 68% said they had received the substance from a friend, 27% said they obtained them through a drug dealer, while 10% purchased them from a specialized shop and 3% bought them on the Internet [12,24]. The report of EMCDDA [26] notes several concerns related to increased consumption of these substances in European Union countries,

relating them even with deadly poisoning cases [27-30]. However, the European Union and its control mechanisms refer the lack of studies that systematically assess the risks associated with the consumption of these compounds [5,8]. Since little is known about the pharmacological, toxicological and safety profiles of these "legal highs", the potential implications of these substances on health are also unpredictable [2,5], so further investigations directed to a better understanding of toxicity associated with the use of these drugs are necessary [5].

From an analytical point of view, the difficulty in analyzing these new drugs by established methods is due to the versatility of modifications in molecules and the speed at which they appear in the market (practically each month), and whose rapid onset is faster than the development of suitable methods for their identification. Contrary to what happens with classic drugs that are measured in frequent drug screening assays, there are no specific methods to efficiently screen for most of these compounds in laboratory routine [9,31]. For this reason, to follow the evolution of the illicit drug market it is mandatory to continuously adapt existing analytical methods, or develop new ones, for the qualitative and quantitative determination of these compounds, which is of great importance in both drug monitoring and risk prevention. The challenge has been met through the use of techniques such as high performance liquid chromatography-diode array detector (HPLC–DAD), gas chromatography-mass spectrometry (GC–MS), liquid chromatography-mass spectrometry (LC–MS) or even using tandem mass-spectrometry (MS/MS) [32]. Each technique offers a benefit, and the analytical toxicologist must take into account the different analytical individualities and drawbacks associated to them [32]. Nevertheless, the absence of reference materials complicates identification and measurement and, even if they are obtainable, acquiring such compounds can be cost-prohibitive, and respective deuterated analogues for quantification by mass-spectrometry may not be available [32].

Since the objective of this thesis was the detection and identification of NPS, a brief review of the available literature regarding some of these particular drugs was carried out, as well as the published methods for their detection, with special focus on drugs whose consumption is higher or more problematic; in addition, conventional and/or alternative biological specimens currently used in their analysis were also addressed.

Table 1. Chemical and physical data of the compounds.

	Compounds			
	Salvinorin A	**K**	**NK**	**MXE**
CAS(*)	83729-01-5	6740-88-1	35211-10-0	1239943-76-0
Molecular mass (g/mol)	432.46	237.73	223.70	247.33
Formula	$C_{23}H_{28}O_8$	$C_{13}H_{16}ClNO$	$C_{12}H_{14}ClNO$	$C_{15}H_{21}NO_2$
Melting point (°C)	238-240	92-93	-	227-233
Boiling point (°C)	-	-	-	-
pKa(**)	-	7.5	7.48	-

(*) CAS: *Chemical Abstracts Service;* (**) to 37 ºC.

Table 2. Methods for the determination and quantification of ketamine, methoxetamine and metabolites in biological specimens.

Blood/Plasma/Urine/Oral fluid matrix						
Compounds	**Matrix**	**Matrix volume (mL)**	**Matrix preparation**	**Detection mode**	**LOD ; LOQ (ng/mL)**	**Ref.**
K, NK, DHNK	Urine	2	HF-LPME (polypropylene fiber)	GC-MS	0.25 ; 0.5 (K) 0.1 ; 0.5 (NK,DHNK)	[33]
K	Urine	1	SPME (PDMS 100 µm)	GC-MS (EI)	100 ; 100	[34]
K, NK	Urine	1	SPE (DAU columns)	GC-MS	10 ; 25 (K) 30 ; 30 (NK)	[35]
K, NK	Urine	1	LLE (hexane)	GC-NPD	5 ; 5 (K) 5.6 ; 10 (NK)	[36]
K, NK	Urine	4	SPE (Bond Elut Certify I)	UHPLC–MS/MS (ESI)	0.03 ; (K) 0.05 ; (NK)	[37]
K	Oral fluid	0.09	µ-SPE (OMIX C18 tips)	LC–MS/MS	0.5 ; 1.5	[38]
K	Plasma, Oral fluid	0.15	Protein precipitation	LC–MS/MS	0.2 ; 0.5 (plasma) 0.2 ; 0.6 (oral fluid)	[39]

K	Blood	1	SPE (OASIS® MCX)	GC-MS	4.73 ; 14.32	[23]
K, NK, DHNK	Urine	-	PVDF syringe filter	LC-MS (ESI,APCI)	0.95 ; 5 (K) 0.48 ; 5 (NK) 0.33 ; 5 (DHNK)	[40]
K, NK	Urine	2	SPE (SPEC DAU)	GC-MS (EI)	15 ; 15 (K) 5 ; 20 (NK)	[41]
K, NK	Urine	0.5	SPE (OASIS® MCX)	LC-MS (ESI)	0.125 ; 0.5 (K) 0.5 ; 2 (NK)	[42]
K	Urine	3	HF-LPME (polypropylene fiber)	GC-FID	8 ; 30	[43]
K	Blood, Urine	0.3 (blood) 4 (urine)	SPE (Oasis MCX)	UPLC-MS/MS (ESI)	- ; -	[44]
K, NK	Urine, Plasma	0.25	MEPS (M_1)	GC-MS/MS (EI)	5 ; 10	[45]
K	Plasma	1	SPE (Chromabond Drug)	LC-MS/MS (ESI)	5 ; - 2.5 ; -, K) 1 ; -	[46]
K	Urine	0.2	SALLE	LC-HR-QTOFMS (ESI)	6 ; 17 (K)	[13]
K	Urine	1	SPE (Focus ™ column)	GC-MS	- ; -	[47]

K	Blood	0.5	SPE (Evolute™ CX)	LC-MS/MS (ESI)	- ; -	[48]
MXE	Urine	0.05	-	LC-MS/MS	0.5 ; 0.5	[49]
MXE	Urine	-	SPE LLE	LC-MS/MS	-	[50]
MXE	Urine	-	SPE LLE Protein Precipitation	GC-MS LC-HR-MS	-	[51]
MXE	Plasma	0.1	Turbulent flow on-line extraction	LC-MS/MS	1 ; 2	[52]

Hair					
Compounds	**Sample amount (mg)**	**Sample preparation**	**Detection mode**	**LOD ; LOQ (pg/mg)**	**Ref.**
K, NK, DHNK	10	MAE	LC-MS/MS (ESI)	0.5 ; 2	[53]
K, NK	2	Micropulverized extraction	(LC-MS) (EI)	20 ; 50	[54]
K, NK	25	SPE (Bond Elut™ Certify)	GC-MS (EI)	50 ; 80	[55]
Other matrices					

Compounds	Sample, amount	Sample preparation	Detection mode	LOD ; LOQ	Ref.
K	Vitreous humour, 0.2 mL	LLE (Ethyl Acetate)	CE-DAD	2 ; 5 ng/mL	[56]

APCI- atmospheric pressure chemical ionization; DAD- diode array detector; DHNK- dehydronorketamine; EI- electron ionization; ESI- electrospray ionization; FID- flame ionization detector; GC- gas chromatography; HF-LPME- hollow fiber liquid phase microextraction; HR-QTOF- high-resolution quadrupole-time-of-flight; K- ketamine; LC- liquid chromatography; LLE- liquid-liquid extraction; MAE- microwave-assisted extraction; MEPS- microextraction by packed sorbent; MS- mass spectrometry; MS/MS- tandem mass spectrometry; NK- norketamine; NPD- nitrogen-phosphorus detector; PDMS- polydimethylsiloxane; PVDF- poly(vinylidene fluoride); SALLE- salting- out liquid-liquid extraction; SPE- solid-phase extraction; SPME- solid-phase microextraction; UHPLC- Ultra high performance liquid chromatography.

1.1.1. Ketamine

Ketamine (K), one of the most widely used club drugs, is a fast-acting agent with sedative, amnesic, and analgesic properties, that has been widely used in clinical practice for the induction of anesthesia [35,57–59]. It was first synthesized in 1962 by Calvin Stevens at the Parke Davis Laboratory as an alternative to its analogue, phencyclidine (PCP, "angel dust") [54,60–62], since it has a shorter duration of action and fewer side effects [54]. It was first marketed in 1970 under the trade name Ketalar™ [58,60,63] following FDA approval for diagnostic and surgical procedures in adults, obstetric patients, children and also used in veterinary surgery [60,62,64,65]. Due to the rapid onset of effects and short duration of action, it is a preferred agent for short-term surgical procedures, in both animals and humans [40,66,67]. Ketamine, chemically known as 2-(2-chloro-phenyl)-2 methyl aminocyclohexanone contains one chiral center at the second carbon of the cyclohexanone radical, resulting in a racemic mixture, i.e., a 50:50 mixture of its enantiomers S(+)-ketamine [(S)-K] and R(–)-ketamine [(R)-K] (Figure 3) [63,68–71].

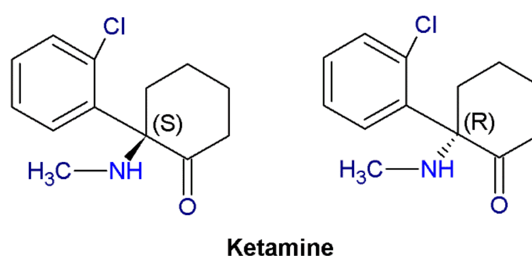

Figure 3. Chemical structure of ketamine's enantiomers.

In most countries it is usually administered as racemate, but the more active (S)-K is becoming increasingly present in commercially available preparations (e.g. in Germany for humans and Switzerland for cats) [68,69]. Moreover, these two enantiomers have significantly different pharmacodynamic activities, as the therapeutic potency of (S)-K is 2–4-times greater than that of the (R)-enantiomer. (S)-K is also a more potent analgesic agent than (R)-K and exhibits a greater clearance and faster anesthetic recovery compared to the racemate, whereas the post-hypnotic stimulatory properties and agitated behavior are more associated with (R)-K [63,69–71]. Ketamine is structurally related to PCP and has three modifications from the PCP main structure (Figure 4).

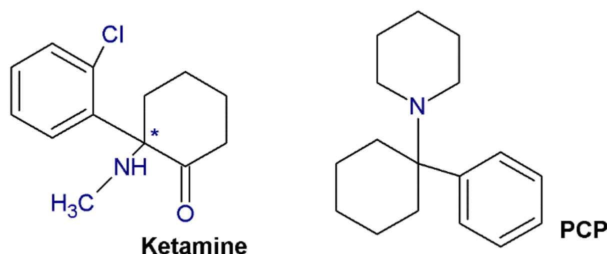

Figure 4. Chemical structures of K and PCP.

The first alteration involves the replacement of the piperidine ring by a methylamine, which gives the same potency as PCP but an increased tendency to induce nausea. The second modification involves addition of two chlorine atoms to the phenyl ring, which decreases the potency but increases the analgesic activity. Finally, the third substitution involves the addition of a carbonyl group to the cyclohexyl ring, which increases the elimination and decreases the duration of action of the anesthesia [71].

As medical use, K was synthesized to induce and maintain general anesthesia in combination with sedative drugs [54,72], both in human and veterinary medicine, especially in developing countries, because it is easy to use and has a wide margin of safety when compared to other anesthetic agents [68,72]. The ease of parenteral administration gives K an advantage when anesthetic gases are impossible to use due to limited equipment and a lack of appropriately trained specialists [68]. Another advantage of the use of K is related to the fact that less monitoring equipment is needed for K as opposed to other anesthetics [73]. But the main advantage of K is that it produces analgesia, amnesia, immobility and sedation, which are not accompanied by actual loss of consciousness [54,71–73], and at the same time it maintains cardiopulmonary function and protective airway reflexes without any slowing of heart breath rates [72–74]. Today K plays a medical role in pain management, being commonly used in pediatrics when conducting painful procedures such as those performed at emergency departments [72,73]. K may have also some antidepressant effects, so the possibility that many chronic users take ketamine as self-medication cannot be ruled out [54,72]. Off-label uses of ketamine include refractory neuropathic pain, and nociceptive pain [73]. Ketamine causes mild stimulation of the cardiovascular (CVD) system without suppression of the respiration and gag reflex; thus, it has a good safety record. Furthermore, K has been used as a therapeutic tool in a range of conditions, including assisted psychotherapy for people with heroin and alcohol addiction, and refractory depression [71,75]. In veterinary medicine, K is the most widely used anesthetic agent in all animal species. Its popularity in equine medicine is reflected in a common street name: “the horse tranquillizer” [72]. In humans, low doses of K can be particularly effective for neuropathic pain and it has also been used in intensive care management of cases of prolonged epileptic seizures [72]. Because of its short half-life (180 min) and its good safety profile (relative protection of airway reflexes and hemodynamic

stability; spontaneous ventilation) it remains a desirable anesthetic drug when compared to other anesthetic agents [64,72].

The above mentioned K effects are well-known for years, however anesthesiologists are also familiar with its side-effects, like the increase of saliva and bronchial secretions, the possible increase of intracranial pressure and the dysphoric effect, that may produce vivid and sometimes unpleasant dreams, due to its probable interaction with the opioid s-receptors [74]. Furthermore, it has been reported that low-dose K can also recreate a number of physiological abnormalities characteristic of schizophrenia [71].

Patients who have undergone surgeries also often report a variety of unusual symptoms when recovering from ketamine anesthesia. These “emergence phenomena” include prolonged hallucinations, delirium and confusion [62,67] and a dissociative state that involves the sensory loss, a trancelike and cataleptic state called the “K-hole” [37,76], that is characterized by a physical immobilization and a sense of a “near-death” and “out-of-body” experience [62,65,71,73].

Precisely those effects that limited the clinical use of K made the drug appealing to drug users, becoming a popular recreational drug in a variety of social settings [54,77], and reports on K abuse appeared soon after its introduction into clinical use [37,61,76]. Indeed, it was initially abused by medical personnel due to its hallucinogenic properties, and, then, gradually became more popular as a drug of abuse among the young users population to increase sensory stimulation and social intimacy [33,35]. It is mostly diverted from pharmaceutical supplies to hospitals, veterinary clinics or from the pharmaceutical distribution network, and it is often distributed at the party scene (nightclubs, dance and rave parties), being one of those recreational drugs known as “club drugs”. Teenagers are the major abusers [65,78–80]. This recreational use of K has widened in Europe in the early 1990s, but quickly spread to other parts of the world, increasing public concerns about the potential hazards of this drug — especially for those using it heavily — including physical harms and addiction [37,40,60,61,76,81]. K is cheap, easily available on the black market under street names like “Special K”, “jet”, “super-acid”, “green”, “K”, “Kit-Kat” and “cat Valium” [72,73,77,78], and it is easily shared because of the small size of the pills [35]. In addition, K is an odorless, tasteless and colorless drug and due to these pharmacological properties it can be added to drinks, without being perceived by the victim, promoting stupor and sedation, which together with amnesia and difficulty in fighting off an assailant, has led to its recent implication in drug-facilitated sexual assaults (DFSA) [33,54,58,73]. DFSA incidents are often reported later than 24h after the alleged assault, when very little drug will remain in the victim’s body fluids [37].

Ketamine is obtained in powder or liquid forms and has many routes of administration. The most popular, when it is used recreationally, is the intranasal route, i.e. snorting (nasal insufflation) the powder, or a solution from a vaporizer. It can be also administered by addition to smoking material or sublingual application [71–74]. Other forms of administration include

intramuscular, subcutaneous or occasionally intravenous injection of a liquid formulation [71,72]. Ketamine is rarely taken orally, as by this route it is rapidly metabolized to norketamine, producing a more sedative and less psychedelic experience [72]. Consumers have reported simultaneous intake of K with other chemical drugs to enhance their effect, including gamma-hydroxybutyric acid (GHB), LSD, marijuana, alcohol, ecstasy, heroin, and various benzodiazepines, which is sold as "Trail Mix". The combination of cocaine and K, known in the party circuit as “CK” or “Calvin Klein”, was also reported [35,40,81,82]. For nonmedical use, a typical dose is 50 and 100 mg respectively for intranasal and oral administration [73].

Norketamine (NK), a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, might also exhibit enantioselective pharmacological activity. For example, (S)-NK has an 8-fold higher affinity than (R)-NK [71], exhibits depressant effects similar to those of K [41], and has shown an anesthetic potency equal to 20–35% of that of K, probably contributing to the long-lasting anesthetic effects [59,66,67].

1.1.1.1. Metabolism

Regarding pharmacokinetics, K is rapidly absorbed when administered through the intramuscular (T_{max} 5-15 min), or nasal (T_{max} 20 min) route with a bioavailability of 93% [63,71]. However, its bioavailability following an oral dose is very low, because of an extensive first-pass metabolism, only 17% of an oral dose is absorbed which also may well explain why K is typically not ingested [71]. Due to its high liposolubility and low plasma proteins binding (about 10–30%), facilitating the rapid transfer across the blood-brain barrier, K is distributed to highly perfused tissues, including the brain, to achieve levels 4-5 times higher than those in plasma [63]. After the entry in the body, ketamine undergoes an extensive liver first-pass metabolism [54,59]. It is metabolized to at least two compounds, with the most important pathway involving N-demethylation to its main active metabolite NK (Figure 12) [63,66,67,78]. On the other hand, NK undergoes dehydrogenation to produce dehydronorketamine (DHNK) [41,67], which is then conjugated with glucuronic acid before excretion in bile and urine [33,54,58,60]. Apart from NK and DHNK, K is also metabolized in the liver to other metabolites, namely alkylhydroxy-ketamine, arylhydroxy-ketamine, alkylhydroxy-norketamine and arylhydroxy-norketamine [62,76]; these compounds were detected in urine for the following times after application: isomers of alkylhydroxy-norketamine for 3–4 days, isomers of the arylhydroxy metabolites for 1–2 days, isomers of alkylhydroxy-ketamine for 24–30 h, and isomers of arylhydroxy-ketamine for 12–24 h [76]. Ketamine is not completely metabolized in humans and other organisms. About 90% of a dose is excreted in urine in 72 h; approximately 2% is excreted as unchanged drug, 2% as NK, 16% as DHNK and 80% as conjugates of hydroxylated metabolites [33,58]. Ketamine and its primary metabolite, norketamine, are metabolized through the hepatic microsomal cytochrome P450 enzymes [66,67,78], mainly CYP3A4, but CYP2B6 and CYP2C9 isoforms are also involved [73]. A study performed by Hijazi *et al.*[83] in human

lymphoblast-expressed CYPs, reported that the general activities of CYP2B6 were higher than those of CYP3A4 and CYP2C9. When these results were extrapolated using the average relative content of these CYP isoforms in humans, CYP3A4 was found to be the main enzyme responsible for K N-demethylation in human liver microsomes (HLM) [76,83]. Ketamine has an inhibiting action on some cytochromes belonging to P450 complex, and this could partly explain the observed tachyphylaxis during the repeated use of the compound [63]. This metabolism does not simply involve the liver, particularly in animals: the kidneys, the intestine, and the lungs are also sites of significant metabolism [63]. The basic K metabolic pathway is shown in Figure 5.

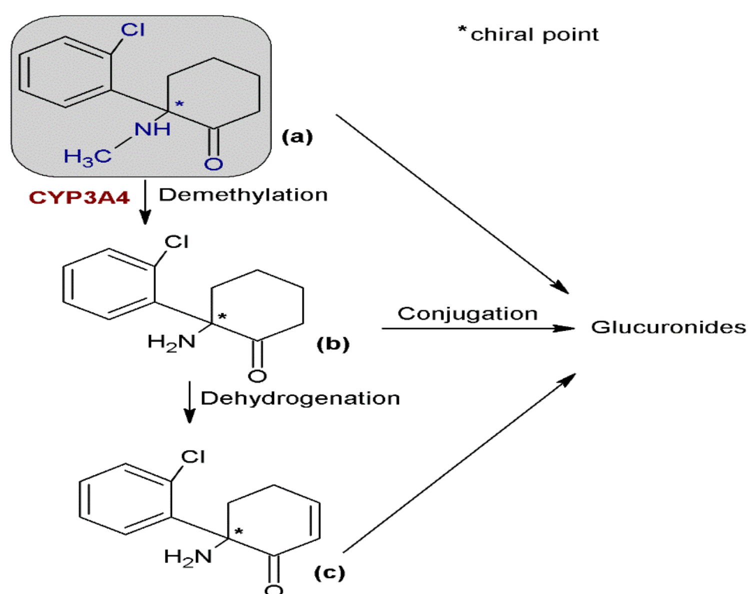

Figure 5. Proposed metabolic pathways for K. (a) ketamine, (b) norketamine and (c) dehydronorketamine.

1.1.1.2. DHNK as a controversial metabolite

As stated above, the ciclohexanone ring undergoes oxidative metabolism to form DHNK, although some authors suggest that DHNK is an artifact of the analytical process, resulting from the GC or GC–MS temperature programming process [80,84,85] or non-enzymatic dehydrogenation of K metabolites [86].

White *et al.* [87] agreed that dehydronorketamine was most likely an artifact but felt that it was due to the instability of the hydroxylated metabolites at high temperatures. This view was further supported by Adams *et al.* [88], who previously theorized that DHNK is a methodological artifact resulting from thermal dehydration of NK and this transformation may be due to the high temperature reached during GC analysis [88]. However, a study performed by Moore *et al.* [81], detecting DHNK under relatively benign conditions (the LC was maintained at 35°C, eliminating the possibility of thermal dehydration), adds evidence to the establishment of DHNK as a true metabolite of ketamine [81]. Cheng *et al.* [57] also found that

DHNK concentrations were generally higher than those of K and NK, being the DHNK concentration range the widest in real urine specimens. Also, internal standards K-d4 and NK-d4 analyzed individually by GC–MS showed no DHNK analogue. Thus, these authors state that DHNK could not be an artifact but a biometabolite of K [57]. Furthermore, Bolze *et al*. [89] also verified that DHNK was not a product due to transformation but is a metabolite recovered in human plasma in significant concentrations [89]. Because dehydronorketamine is not commercially available, only a few articles [35,40,66] studied its quantification in biological samples, contradicting again the idea that DHNK is an artifact.

1.1.1.3. Mechanism of action

Ketamine is a dissociative anesthetic agent that exercises its pharmacologic effects by means of interactions with glutamate, opioid, nicotinic, and non-NMDA receptors, among others [63,69,70,82]. The nature of the subanesthetic K experience has led to the use of the term “dissociative” in a more psychological sense, referring to a feeling of dissociation of the mind from the body [90,91].

Pharmacologically, the primary mode of action of K is on glutamate, the major excitatory neurotransmitter in the brain, by binding to the so-called PCP-binding site of the NMDA-receptor complex (one of the three ionotropic glutamate receptors) [72,92], located within the ion channel, thereby blocking the transmembrane ion flux [93]. This makes K able to block noncompetitively the glutamate NMDA receptor [33,40,71]; consequently, it inhibits the excitability of pain neurons to induce its dissociative anesthetic activity [37,62,71,92]. NMDAR’s role in the pharmacology of K and related compounds was first reported in the early 1980s by Lodge and colleagues [92]. NMDA-receptors (NMDAR) are ligands and voltage calcium-gated channel receptors, containing several competitive (glutamate, aspartic acid, and glycine) and non-competitive binding sites (primary amine, Zn^{2+}, PCP) [91,92]. Activation of the receptor results in opening of the ion channel and depolarization of the neuron. The NMDA-receptor is involved in sensory input at the spinal, thalamic, limbic and cortical levels. Ketamine would be expected to block or interfere with sensory input to higher centers of the CNS with the emotional response to these stimuli, and with the process of learning and memory [72,93]. The antagonism of NMDA receptor is responsible for the specific K properties (amnesic and psychosensory effects, analgesia, and neuroprotection) [63,69,74]. Several studies indicated that K acts as a weak agonist at σ and μ opioid receptors, involved in the pharmacological effects of opiates [71,72,94], and that its analgesic effect may largely be attributed to the activation of these central and spinal receptors [95]. It also inhibits nitric oxide synthase, hence further contributing to analgesia [40]. Furthermore, K acts as a noradrenergic and serotonergic uptake inhibitor, both neurotransmitters being involved in descending antinociceptive pathways [40,71,73]. Some effects of K may be due to its actions on catecholamine systems, notably it may directly or indirectly enhance dopamine activity in the

brain [72,73,96]. Experiments realized by Hancock *et al.* [97] on the effects of K on uptake and efflux of DA demonstrated that it improves dopamine efflux not by blocking dopamine uptake, autoreceptors or NMDA receptors, but by recruitment of the dopamine storage pool to releasable sites. The dopaminergic effects may be of relevance for the euphorigenic, addictive and psychotomimetic properties of K [91]. Other neuropharmacological actions are an agonistic effect at the σ-receptor. Because K binds to both σ(1) and σ(2) receptors with high affinity, this may suggest that σ receptor-mediated neuronal remodeling may contribute to the antidepressant effects of K [71,92].

The two optimal isomers S(+)- and R(-)-K interact with different affinities at the NMDA-receptor [72]. The S-enantiomer is shown to have higher binding affinity for the PCP-site of the NMDA-receptor, being the more potent one, with an approximately 3-4 fold anesthetic potency compared to that of R-ketamine. For this reason the psychotomimetic properties of K are mostly caused by the S-ketamine, even though subanesthetic doses of R-enantiomer can provoke a state of relaxation [91]. The principal metabolite, NK, is also pharmacologically active. Its binding affinity to the NMDA-receptor and anesthetic properties are just about one third of the parent compound contributing significantly to the analgesic effect of K [98].

Notwithstanding years of research, therapeutic treatment using NMDAR antagonists remains incomplete. This may be due to the fact that NMDAR antagonists display contradictory activity: neuroprotection and neurotoxicity, sedation and stimulation, anti-addictive or reinforcing addictive activity [92,99]. Despite of these limitations, the number of potential usages of NMDAR antagonists, including the treatment of neuropathic pain, depression, attention deficit hyperactivity disorder (ADHD) and neurodegeneration, continues to increase [92,100]. Moreover, the use of NMDAR antagonists in neuropharmacological studies is important in the investigation of the neural mechanisms in perception and psychosis [92].

Ketamine also has less prominent actions at other receptor sites. It blocks muscarinic acetylcholine receptors of the CNS and may potentiate the effects of gamma-aminobutyric acid (GABA) synaptic inhibition [72,92].

1.1.1.4. Effects

Ketamine has been found to cause a variety of both negative and positive effects [65]. The hallmark of psychodysleptic effects (also called psychedelic effects) is disturbances in mood, in visual and auditory perceptions, which can include color changes, out-of-body experiences, distortion of time and space and a sense of invulnerability and sociability increase [63,73]. At subanesthetic doses, K possesses sedative and potent analgesic properties [59,67], inducing mild dissociative effects and post-hypnotic emergence reactions, such as prolonged hallucinations [72]. It also produces narcotic effects similar to those of PCP and visual effects like LSD [57,61,65,81]. However, it is less toxic than PCP, demonstrating a lower and shorter effect than the former, and with less marked “psychic effects” [58,61,63]. When K is consumed

at large doses, it generates a more severe dissociation, usually stated to as a “K-hole”, where the user experiences a kind of vivid dreams and delirium as well as out-of-body experience, that has been described as similar to a near-death experience [72,73]. Whoever tries K reports a feeling of seeing the world in a different way, a complete dissociation of body with loss of the notions of time and space, delayed or reduced sensations, entry into other realities, euphoria and ecstasy [63,73]. A survey of drug abusers demonstrated that K has the highest degree of out-of-body experiences compared to the other drugs such as cannabis, alcohol, LSD and amphetamines [73]; nonetheless, only less than 10% of users had out-of-body experiences at “almost always” or “always” [73,101]. The administration in high doses for recreational purposes can cause respiratory toxicity [71].

Concerning the negative effects, these include dizziness, anxiety, amnesia, delirium, negative sociability, paranoia [63,73] convulsions, hypertension and cystitis [65]. Deficiency of speech has also been observed [73,102]. More severe effects reported are impaired memory, impaired motor functions and increased muscle tone [73]. Consumers with a history of K abuse or misuse reported negative physical effects that can include dry mouth, blank stare, slurred speech, respiratory problems like bronchodilation and increased bronchial secretions, nausea, vomiting and blurred vision [73,103]. Cerebral blood flow and intracranial pressure are also increased. Frequent use can also induce neurosis, aggression and mental disorders [73].

According to users, the most appealing aspects of K use are ‘melting into the surroundings’, ‘visual hallucinations’, ‘out-of-body experiences’ and ‘giddiness’ [72,104]. Many users - astronauts of the psyche or “psychonauts” - find the experience spiritually significant, whereas others find it frightening [72].

Abstinence symptoms included shudders, palpitations, restlessness, lacrimation, sweating and nightmares [73]. A substantial decline in the use of K in chronic consumers can produce some repair in cognitive functions [73].

Ketamine is known to be more addictive, both physically and psychologically, than most of psychedelics substances [73,76]. The frequency of its abuse is increasing and fatal poisoning cases with K and its metabolites have been reported [66,67,105].

1.1.1.5. Doses and Concentration-Effect Relationship

Depending on the concentration, form, and method of administration, doses of K for recreational use range from 50 to 300 ng/mL [106]. With a concentration of 70 ng/mL it changes memorization, while 200 ng/mL provokes a lateral nystagmus [63]. On the other hand, 100–250 mg by inhalation, 50–70 mg by intramuscular injection or 200–525 mg of K by oral administration induce hallucination [58]. Paranoid and anxiety feelings appear around 500 ng/mL [63]. The K analgesic effect continues for steady-state plasma concentrations higher than 100–160 ng/mL [63].

1.1.1.6. Legal status

The availability of K associated with the absence of international restrictions led to a dramatic increase in the spread of this drug worldwide [72]. Concerning the legal status, currently there is a variation around the world and therefore K is not under international controls [72,91]. This could be explained by its availability for both human and veterinary use [68,91], especially in countries where no affordable alternative anesthetics are available. Moreover, in veterinary medicine the inaccessibility would be a problem, because there is no substitute for the drug [68,91]. Some countries have already placed this medicine under control [40], but must guarantee prompt access to K for surgery and anesthesia for human and veterinary care [68].

In the United States, since the 1970s, K has been marketed as an injectable anesthetic for humans and animals use, but in August 1999 it became a Schedule III controlled substance under the Federal Controlled Substances Act. Substances on this list have an accepted medical use as a treatment within the USA and people convicted of possession of K are subject to a detention for a period of up to five years [72,73].

In the United Kingdom it became labeled a Class C drug under the Misuse of Drugs Act, since January 2006 [60], suggesting that its injuries are less severe than some other drugs such as cannabis (class B) or ecstasy (class A) [72].

Since drugs such as K may be used for scientific purposes, the British Home Office determines who is legally able to possess and work with it. Possessing K without authorization may be punished by a jail term of 2 years and up to 14 years imprisonment for supplying [60].

In Canada, K is classified as a Schedule I narcotic since 2005 under the Controlled Drugs and Substances Act, belonging to the same category as morphine, codeine and cocaine. In China, K has been categorized as a Class One psychotropic drug, as well as its salts or preparations [78]. Finally, in Portugal, since April 2013, K is under legislation of the Decree-Law n. º 54/2013 of 17th April that prohibits the production, importation, exportation, advertisement, distribution, sale, possession, or availability of new psychoactive substances [107].

1.1.1.7. Analytical techniques

The request for sensitive and specific analytical procedures for the identification of club drugs in several biological samples derives from the field of forensics and drug enforcement [82]. Techniques for club drug analysis provide the means to determine and to quantify drug exposure in cases of misuse or addiction and to evaluate the toxicological effects of the drugs in post mortem scenarios [82].

In recent years, the recreational abuse of K as a “rave”, party, and nightclub drug has dramatically increased worldwide, leading to an augmented public concern about its potential

risks [57]. This abuse tendency has created a requirement for clinical laboratories to develop fast and effective screening and confirmation methods for the analysis of K and metabolites in biological matrices [57,67,105]. Notwithstanding the fact that K and NK are the target analytes in toxicological analysis with suspected involvement of this drug, according to the Society of Forensic Toxicologists (SOFT) and United Nations Office on Drugs and Crime (UNODC), DHNK has also been indicated as a biomarker of consumption of K [33].

Since the 1970s, some analytical methods have been described for the determination of K and its main metabolites, alone or in combination with other abused drugs [40] (Table 2). The traditionally techniques of choice, in different matrices, are GC-MS [23,33,108] and LC-MS [13,40,54]. GC-MS/MS [45] and LC-MS/MS [48,50,53,109] methods are also utilized. The analytes were also detected by gas chromatography coupled to flame ionization detector (GC-FID) [43] and nitrogen phosphorous detection (GC-NPD) [36,110]. Other methods for K determination include ultra-high performance liquid chromatography coupled to MS/MS (UHPLC-MS/MS) [37,44,111], capillary electrophoresis (CE) [112], CE–MS [113] and with diode array detector (CE–DAD) [56].

Some human samples, including urine, blood, saliva, hair and sweat, have been used for the determination of the residues of abused drugs. Among them, urine continues to be a widely used specimen to monitor psychoactive substances in some situations, such as forensic cases [33,60,77], since this sample includes non-invasive method of collection, wide drug detection window compared to blood, and a large sample volume that can be collected for analysis [33,60]. Moreover this sample contains usually high concentrations of drugs and metabolites, compared to other biological matrices [43,55]. However, as a disadvantage, urine samples can be easily adulterated and/or tampered [55,77]. Saliva seems less liable to adulteration, compared to urine sample [77], but the quantity of drug and metabolites are lower in saliva than in urine [77]. Blood is also an important specimen, as it offers information of toxic substances acting in the body at the time of collection [48]. A common drawback of these biological samples is the short-window of drug abuse record, so the use of alternative samples to monitor illicit drug use is required. Hair specimen differs from other biological samples because of a long surveillance window (from months to years, depending on the length), enabling retrospective investigation of chronic and past consumption [53,55,77]. Another advantage over other biological samples is that it can be easily collected, transported and stored, and it is not easily adulterated [53,55]. Additionally, in post-mortem circumstances, in which other biological specimens are inaccessible, hair analysis may be advantageous [53].

Many sample preparation techniques have been developed to isolate and concentrate K and metabolites. Amongst the available techniques, the most common are LLE in urine [50,65], hair

[55], plasma [65] and vitreous humor [56]; and SPE in urine [44,50,62,114], blood [23,44], plasma [115] and oral fluid [38].

Other extraction techniques include salting-out liquid-liquid extraction (SALLE) [13] in urine and microwave-assisted extraction (MAE) in hair [53]. Over the last decade, miniaturized techniques, such as hollow-fiber liquid-phase microextraction (HF-LPME) [33,43,108], solid-phase microextraction (SPME) [34,110] and microextraction by packed sorbent (MEPS) [45], have also been employed for K isolation from biological specimens.

1.1.2. Methoxetamine

Methoxetamine, commonly referred to as “MXE” or “3-MeO-2-Oxo-PCE [116,117] is a dissociative anesthetic derived from K, with greater intensity of effects and a longer half-life than K [118,119]. The substance is said to be developed by an UK-based research chemist with the intention to produce a safer alternative to K, particularly in respect to urinary tract problems that can arise from recurrent use of K [118].

This substance is a dissociative drug belonging to the arylcyclohexylamine family [117,120] that shares some structural similarities to existing illegal drugs of abuse, namely K and PCP [121-124]. Methoxetamine (MXE) differs from K by two modifications (Figure 6). The first involves the removal of the 2-chloro group on the phenyl ring and replacement by a 3-methoxy moiety and the second involves the replacement of the N-methyl group on the amine by an N-ethyl group. The first modification gives MXE less analgesic and anesthetic properties than K, while the second change leads to more potency and duration of action. It has also been suggested that, due to the N-ethyl group, chronic use of MXE has a lower risk of being associated with the urinary tract pathology usually observed with K abuse [51,117,120-122,125]. Concerning the modifications from PCP (Figure 6), the first implicates the removal of the piperidine ring and its replacement by an ethyl amino group which gives more potency than PCP, but increases the tendency to induce nausea. In turn, the 3-methoxy substitution on the phenyl ring increases the μ-opioid receptor affinity, while at the same time removes or at least reduces the mood-altering effects [71,121,122].

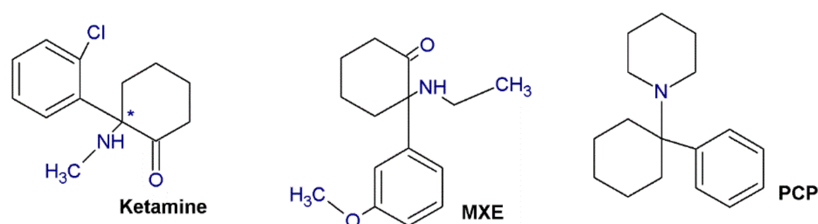

Figure 6. Chemical structures of K, MXE and PCP.

MXE is one of the new chemicals that are increasingly available on the Internet. In 2010, MXE was first found in recreational products marketed by “head shops” as “legal highs” [71,116,118]. Recently, MXE has emerged as a new potential drug of abuse and it has been sold via the Internet to consumers as “legal K” [52,71,123]. Despite being marketed as a research chemical and labeled “not for human consumption” to circumvent the regulations regarding recreational drugs, this substance is abused recreationally for its dissociative and psychedelic effects [52,71,120]. MXE is typically sold as a free base and hydrochloride salt, in a white powder with different brand names. The common street names are “Special M”, “Mexxy”, “MXE”, “Kmax”, “M-ket, “Minx”, “Jipper”, “Roflcoptr” and “MA” [71,117,123]. According to the EMCDDA report, the following “legal highs” product names have been associated with methoxetamine: “Kwasqik”, “Hypnotic”, “Panoramix”, “Magic”, “Lotus”, “Special K” and “X” [124]. Information provided from seizures and collected samples have noted the presence of MXE in powders, tablets, powder-filled capsules, liquids and plant material. [124]

1.1.3.1. Administration

The most common ways of administration for MXE include nasal insufflation [“sniffing” or “snorting” (with a common dose of 20 up to 100 mg being reported by users)] and oral consumption [where it is swallowed either as a powder wrapped in a cigarette paper (so-called “bombing”) or dissolved in a solution with doses of 40–60 mg]. There are also reports on intravenous or intramuscular injection, where dosages can range from 15 to 30 mg, and also rectal administration [51,71,117,121,123]. The threshold doses for MXE are approximately 10 or 15 mg, and some users suggest the increase in the dosage gradually without exceeding 50 mg on the first occasion when administered orally [138,187]. Tolerance builds over time, and heavy users can consume more than 100 mg in a single dose [121]. The desired effects of MXE and dosages are influenced generally by the routes of intake; injection requires the lowest doses with a duration of 2-3h, followed by rectal, sublingual and oral intake with 3-5h, and intranasal administration from 2.5 to 4h [71,117,121]. The effects of MXE are described as beginning after 10 to 20 min [121,123], but the perceived effect could be delayed of some 30–90 min after insufflation. This might be dangerous as it frequently causes the user to ingest another dose of the substance, thinking that the first dose was insufficient [71].

1.1.3.2. Metabolism

Since MXE is a recent drug of abuse, little is known in detail about its metabolism. In 2013, Meyer *et al*. [51] identified both phase I and II metabolites of MXE in rat and human urine. The authors postulated that MXE undergoes a complex phase I metabolism including N-deethylation to N-desethylmethoxetamine or normethoxetamine, probably the most important metabolite of MXE in humans [51], oxidative metabolism to dehydronormethoxetamine, hydroxylation to 3-

hydroxymethoxetamine, O-demethylation to O-desmethylmethoxetamine and reactions via sulphation or glucuronidation producing phase II metabolites as O-desmethylmethoxetamine glucuronide, O-desmethylnormethoxetamine glucuronide and hydroxynormethoxetamine glucuronide [51,117,121]. Kinetic studies with human hepatic CYP isoenzymes showed that N-deethylation is catalyzed by the enzymes CYP2B6 and CYP3A4, O-demethylation by CYP2B6 and CYP2C19, and hydroxylation by CYP2B6 [51]. Figure 7 shows a proposed metabolic pathway for MXE.

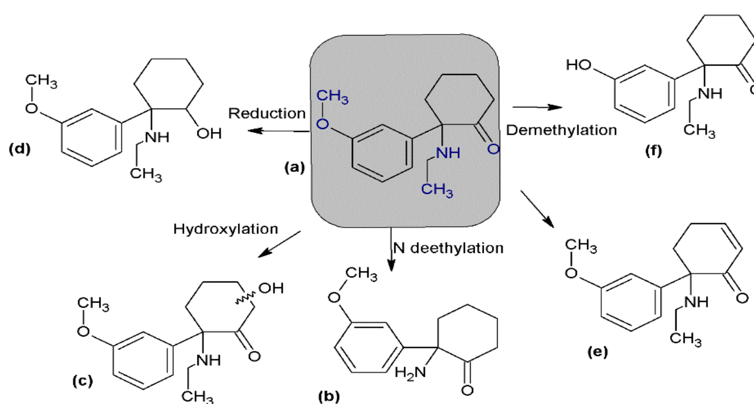

Figure 7. Proposed metabolic pathways for MXE. (a) MXE, (b) normethoxetamine, (c) hydroxymethoxetamine, (d) dihydromethoxetamine, (e) dehydromethoxetamine and (f) O-desmethylmethoxetamine.

1.1.3.3. Mechanism of action

Although there are few studies demonstrating the mechanism of action of MXE, and since it is structurally similar to K, it has been supposed that it acts on the NMDA receptor as an “open channel” blocker [121], and also on the inhibition of dopamine reuptake. Moreover, MXE acts as agonist at 5-HT2 receptors, μ and κ -opioid receptors, δ receptors, and muscarinic cholinergic receptors [51,52,117,120,121].

1.1.3.4. Effects

The clinical effects of MXE are poorly known, and most studies describe symptoms obtained from Web forum discussions, in which consumers describe their experience [52]. As reported by users, effects are similar to those of K [52,71,121], but with longer delay in onset (90 min) and longer duration (5–7 h) when administered orally [71]. Amongst the intended effects, the most common include euphoria, increased empathy and social interaction, feelings of peacefulness and calmness, hallucinogenic symptoms of sleep while awake, the so-called “closed-eye hallucination”, pleasant intensification of sensory experiences (especially to music), mild to strong sense of dissociation from the physical body (described as “M-Hole”), brief antidepressant effects and spiritual or even near-death experiences [51,71,117,125]. However, the consumption of MXE also may be associated to several adverse reactions, such as dizziness,

anxiety, vomiting, sweating, confusion, time distortion, aphasia, synesthesia, tachycardia, hypertension, agitation, stupor, lack of coordination, mydriasis and nystagmus [51,71,118,121,126]. Moreover, slurred speech has been regularly reported [117,123]. Low mood and/or depressive thoughts and potential suicidal attempts are also symptoms associated to the ingestion of methoxetamine [71]. The United Kingdom reported on 21st October 2010 the first overdose by MXE [92]. Fatal intoxication with MXE can also be found in scientific publications; for example, Sweden [127] and Poland [121] reported one death each in 2012, and the EMCDDA report about MXE [124] relates that the United Kingdom reported a total of 15 deaths between 2011 and January 2013; Austria and Finland reported one death each in August 2012, and France reported one death in February 2013.

1.1.3.5. Legal status

MXE is a prevalent psychoactive substance in many countries, but at present, it is still not subjected to restrictive regulations; for example, it isn't controlled in Canada and it is not scheduled under the U.S. Controlled Substances Act [116,121,128] as well. In the United Kingdom, on the 5th April 2012, MXE was subjected to a temporary class drug control (TCDO), prohibiting its importation and sale for 12 months [51,92,122], but authorizing its possession and use [121]. However, on the 26th February 2013, resulting from the recommendation of the Advisory Council on the Misuse of Drugs (ACMD), MXE became a Class B drug under the Misuse of Drugs Act [92,122]. In Switzerland and Russia in 2011, and Japan in 2012, MXE was scheduled as a controlled substance [6]. In Portugal, since April 2013, MXE is under legislation of the Decree-Law n. º 54/2013 of 17th April that prohibits the production, importation, exportation, advertisement, distribution, sale, possession, or availability of new psychoactive substances [107].

1.1.3.6. Toxicological analysis

The last decade has witnessed an alarming increase on the number of novel psychoactive compounds introduced in the drug market as alternatives to controlled substances of abuse, and MXE is one of them. This phenomenon was accompanied by an urgent need to develop fast and effective analytical methods for qualitative and quantitative measurements of new drugs in biological samples. GC–MS [51,128,129] and LC–MS [51] or LC–MS/MS [116,117,119,196,197] have been applied to the analysis of MXE in plasma [52], blood [128–131], urine [49–51] [130,131] and vitreous humor [130,131]. In order to isolate the compound of interest from samples, eliminating the interferences, an adequate sample preparation is required. The sample preparation techniques used to isolate MXE from biological specimens are SPE [50,51], LLE [50,51], protein precipitation [51], turbulent flow on-line extraction [52], and more recently a microextraction by packed sorbent (MEPS) was developed [132]. The miniaturized techniques seem to be a good choice because they provide some advantages when compared to

the more traditional LLE and SPE. One of its greatest achievements is the reduction of sample amounts and organic solvent consumption, and also the possibility of being reused several times; indeed, more than 100 extractions have been reported using plasma or urine samples. By comparison, conventional SPE cartridges are recommended for single use only [133,134].

1.2. Sample preparation

In circumstances where drugs of abuse are investigated, like in clinical and forensic toxicology and workplace drug testing, it is important to select the best specimen to be sampled before optimizing the sample preparation procedures. The detection of abused drugs is generally performed on urine, plasma or blood samples taken from living subjects and forensic cases [135–137]. Urine is the biological sample most widely employed in toxicological analyses, and one of its main advantages is the fact that it can be easily collected from living individuals, mainly when compared with blood or plasma, and its sampling is much less invasive to the examinee [135,138]. In addition, the large volumes that can be obtained for analysis play an essential role for forensic applications because conserving part of the specimen for an independent re-test is vital for approval of the testing process [135,139]. Other advantages include its window of detection, from a few days to weeks depending on the technique, and the higher concentrations commonly encountered in urine when compared to those detected in other specimens, making the identification of drugs easier [135,138]. Furthermore, urine is also of particular significance, since it allows obtaining information on the elimination kinetics of the involved substances. [137]. However, the main drawback associated to urine sampling is the challenging collection under observation, which can be very time consuming and infringes on the examinee’s privacy [135].

In the case of blood and plasma, these allow the correlation between the concentrations and the clinical condition or observed symptoms, and are useful to assess recent and short-term exposure to drugs [140,141].

Other specimens, such as, oral fluid, hair, sweat or meconium are of particular interest in toxicological analysis, providing an alternative to traditionally used samples. In fact, those specimens have the advantage of non-invasive sampling, and are easy to perform [137,140].

With respect to these alternative or unconventional samples, hair is the most widely used and useful specimen to assess exposure to drugs, having the most overwhelming advantage over all the other biological samples, the possibility of assessing drug exposure from weeks to months or even years after consumption, depending on the length of the hair shaft [134]. Based on its large window of detection and because of its sustainable nature, hair analysis enables a retrospective investigation of chronic and recent use of drugs by an individual. Another of the

great hair advantages is the ability to collect another identical sample of the individual in case of suspicion of switched samples or leaks in the chain of custody [142,143].

However, hair samples have also some important drawbacks, being the most relevant the possibility of reporting false-positive results for a drug in circumstances where it was not actively used by the individual, but was present in the surrounding environment [134]. When scalp hair is not available to collect, for instance if hair is too short, axillary and/or pubic hair have been suggested as alternative sources in the detection of drugs [134].

Concerning oral fluid, sample collection is achieved in a fully non-invasive way, moreover, this collection can be made under near surveillance, and consequently the collected sample is less prone to adulteration or substitution. In addition, the concentrations of drugs in oral fluid depend on their non-protein-bound fraction and this is important in the evaluation of the impairment degree [138]. Two main apparent limitations are the smaller amount of matrix collected when compared to urine, and the level of drugs are in general low [139]. Meconium also has a relevant importance as a biological matrix in cases of toxicological analysis, as it allows assessing prenatal exposure to drugs of abuse. The formation of meconium starts between the 12th and 16th weeks of gestation, so the detection of drugs in this matrix corresponds to the last two trimesters of pregnancy [144].

Upon selecting the appropriate specimen, the isolation of toxicologically relevant compounds from the biological matrix is essential for their successful detection, identification and quantification.

Due to the complexity of the samples from which the compounds are analyzed, a sample preparation stage is desirable prior to any biological analysis. Sample preparation, also known as sample pretreatment, sample clean-up or sample extraction, is a very important process for bioanalytical method development, since biological matrices as blood, plasma, or urine are quite complex due to the presence of proteins, small organic molecules like lipids and lipoproteins, salts and other substances with similar chemistry to the analytes [145]. In simple terms, sample treatment is a process which aims at selective isolation of the analytes of interest from the matrix, elimination of matrix components in the processed sample, which are able of prejudicing the performance of analysis and, if required, concentration of the analyte of interest [145,146]. Conventionally, the most used techniques are LLE, protein precipitation (PP) or SPE. However, these sample preparation techniques, generally consisting of several steps, involve time- (approximately 80% of the whole analysis time) and organic solvent-consuming procedures, and are unsuitable in the field of emergency medicine, which demands rapid and sensitive detection. For this purpose, more efficient and less time- and solvent-

consuming techniques compared with those above-mentioned have been developed, in particular in which the isolation process has been miniaturized. These kinds of techniques take into account the need to switch to green analytical methodologies aimed at minimizing the use of toxic organic chemicals and produce less laboratory waste [111,134,146,147]. The major example of the miniaturized techniques is microextraction in packed sorbent (MEPS). This modern isolation technique is based on the miniaturization of a traditional SPE and exhibits various advantages like the decrease of the sample volume and the organic solvent consumption. Moreover, the MEPS sorbent may be used about 30–100 times for human urine [133,148]. By comparison, the conventional SPE cartridges are recommended for single use only. Additionally, the MEPS may be connected off-line or on-line with a LC or GC system [134].

1.3 Design of experiments (DOE)

Developing an analytical method is one of the more time-consuming tasks in a laboratory and where many human and material resources are usually spent. It can be done using the trial and error approach or by the analysis of one factor-at-a-time (univariate analysis). In the latter approach, optimization involves changing one factor while the others remain constant. This approach is very time-consuming and laborious, especially if there are many variables to evaluate, and can lead to inappropriate conclusions since the interactions between factors are not taken into account [149]. DOE is a powerful statistical tool that allows planning the whole process, evaluating in a multivariate way the various factors involved, decreasing the effects of uncontrolled factors and the number of experiments, simultaneously obtaining the best results and a rational use of resources, saving time and money to the laboratories. This statistical tool has already been used successfully in other areas, but its application in the field of forensic toxicology is very scarce [149].

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3. Article I

Role of microextraction sampling procedures in forensic toxicology

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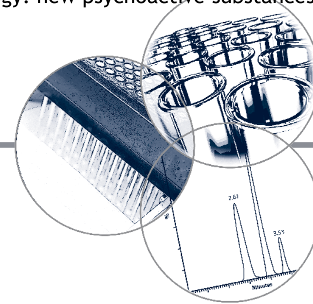

Review

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Role of microextraction sampling procedures in forensic toxicology

The last two decades have provided analysts with more sensitive technology, enabling scientists from all analytical fields to see what they were not able to see just a few years ago. This increased sensitivity has allowed drug detection at very low concentrations and testing in unconventional samples (e.g., hair, oral fluid and sweat), where despite having low analyte concentrations has also led to a reduction in sample size. Along with this reduction, and as a result of the use of excessive amounts of potentially toxic organic solvents (with the subsequent environmental pollution and costs associated with their proper disposal), there has been a growing tendency to use miniaturized sampling techniques. Those sampling procedures allow reducing organic solvent consumption to a minimum and at the same time provide a rapid, simple and cost-effective approach. In addition, it is possible to get at least some degree of automation when using these techniques, which will enhance sample throughput. Those miniaturized sample preparation techniques may be roughly categorized in solid-phase and liquid-phase microextraction, depending on the nature of the analyte. This paper reviews recently published literature on the use of microextraction sampling procedures, with a special focus on the field of forensic toxicology.

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Where **forensic toxicology** is concerned, the last two decades have provided scientists with more sensitive technology, as a dramatic increase in the capabilities of MS were observed. For these reasons, it is not surprising that MS-based techniques have become routine tools in most laboratories, enabling the detection and quantitation of small amounts of analytes in complex matrices, including biological specimens. These advances in analytical instrumentation have surely allowed the determination of several compounds (including new drug metabolites) in new and alternative specimens (e.g., hair, oral fluid and sweat), in which drug concentrations are often low.

More sensitive instrumentation allows a reduction in sample volume, and this assumes particular relevance in forensic toxicology, since sample availability is often limited and several exams need to be performed on the same sample.

However, despite the development of highly sensitive and specific analytical instrumentation, sample pretreatment is usually needed for the extraction and/or concentration of the compounds of interest from complex matrices [1]. In fact, sample preparation is the most time consuming step in bioanalysis and is also prone to errors from analyte losses.

Sample preparation aims at eliminating eventual matrix-borne interferences, which are capable of impairing the performance of the assay. Ideally, sample preparation should be simple, rapid, easily automated and cost effective, providing simultaneously adequate extraction efficiency.

Classic sample preparation techniques, for example, liquid–liquid extraction (LLE) or SPE, have been widely used for drug analysis in biological specimens. However, these methods have various drawbacks, including complicated and time-consuming operations. In addition, they usually require the use of certain amounts of organic solvents (at least a few milliliters, in the case of SPE using minicartridges). Those solvents need to be discarded, which has obvious implications to the environment and in the costs of analysis due to additional operational costs for waste treatment [2,3].

Therefore, there is a growing trend to use 'greener' extraction procedures, namely using fully automatic and/or miniaturized techniques, which provide new operational paradigms. These **microextraction sampling procedures** usually allow performing rapid measurements on small sample volumes, automation, high-throughput and online coupling with analytical instruments. In addition, they take into account the necessity to resort to green analytical methodologies that aim to minimize the use of toxic organic chemicals and produce less laboratory waste.

This review will deal with the most used miniaturized sample preparation techniques. The main analytical and chromatographic problems

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Key Terms

Forensic toxicology: The application of toxicology to situations with legal implications, usually involving the court-of-law. Those situations may include driving under the influence of drugs and/or alcohol, intoxications (suicides, homicides), drug trafficking and so on.

Microextraction sampling procedures: A miniaturized sample preparation approach, which uses little or no organic solvents, being more environmentally friendly. These procedures are often rapid and simple, and usually allow some degree of automation and online coupling to analytical instruments, providing greater sample throughput. However, their absolute extraction efficiency is low.

Solid-phase microextraction: A sampling procedure that extracts and concentrates the analytes of interest into a solid material (usually of polymeric nature). Several variations of this technique exist, and the most renowned is fiber solid-phase microextraction, which was developed in the late 90s.

Liquid-phase microextraction: A sampling procedure that extracts and concentrates the analytes of interest into a liquid extractant (of organic or aqueous nature). Several variations of this technique exist, and one of the most used is single-drop microextraction, where the analytes are extracted and concentrated into a drop of an organic solvent.

that these methodologies present, as well as their advantages and disadvantages over traditional sample preparation techniques, will be discussed with special focus on forensic toxicological analysis.

The studies were selected through the references list of known published papers and using the public MEDLINE database, PubMed, with the following search strings: "Microextraction techniques"; "Analytical microextraction methods and biological fluids"; "Toxicology and microextraction techniques"; "In-tube **solid-phase microextraction**"; "Microextraction and plasma/urine samples"; "**Liquid-phase microextraction**"; "Single-drop microextraction"; "Microextraction in packed sorbent"; "Stir-bar sorptive extraction"; "Dispersive liquid–liquid microextraction"; and, "Polymer monolithic microextraction". Due to the high number of published material, only articles from 2007 onwards were included in the tables.

Solid-phase microextraction

Solid-phase microextraction (SPME) techniques can be basically divided into static batch equilibrium microextraction and dynamic flow through equilibrium microextraction methods. There is no doubt that the most widely used SPME technique is the conventional SPME or fiber SPME, but other approaches can be efficiently used, namely in-tube SPME (or capillary microextraction), solid-phase dynamic extraction (SPDE), microextraction in a packed syringe (MEPS), thin films microextraction (TFME), pipette tips microextraction (in-tip SPME) and stir-bar-sorptive extraction (SBSE). All of those approaches have been used for forensic, clinical and pharmaceutical analysis [3,4].

■ Fiber SPME

SPME, which is an absorption/desorption technique, was developed in the early 1990s at the Waterloo University (Ontario, Canada) by Pawliszyn and collaborators, and concentrates the extraction and enrichment of the analytes into a single solventless step [5]. It is a quick and easy-to-perform extraction technique, which does not require complicated equipment and that can be used to isolate and concentrate volatile and nonvolatile compounds in liquid, gaseous or solid samples, allowing the production of linear and reproducible results within a wide concentration range. It consists of a fused-silica capillary fiber of approximately 1 cm long, coated by a stationary phase, which can be liquid (usually a polymer) or solid (adsorbent substance). The fiber is connected to a stainless steel needle, which allows it to move freely, as well as offers protection throughout the extraction and desorption processes. This technique is based on principles of thermodynamic and mass transference and, in opposition to other extraction methods, the whole of the extracted analyte is introduced in the chromatographic system [6,7].

The extraction procedure is quite simple: first, the septum of the sample vial is pierced and, with the SPME holder inside the vial, the fiber is exposed to the sample (direct immersion) or to the headspace (HS) above it (Figure 1A). This latter approach is advantageous, since fiber damage by aggressive or irreversibly adsorbed matrix components is minimized, which allows more than 100 extractions using the same fiber. In particular, newly developed metal fibers can extend this extraction size to 500 samples [1].

The analytes are then adsorbed/absorbed in the fiber's coating and, after the equilibrium is reached (usually between 2 and 30 min), the device is withdrawn from the vial. Finally, the needle is introduced into the injection port of the chromatographic system, where the analytes are either transferred into the injector for GC, where they are thermally desorbed, or dissolved in the mobile phase for LC [8].

It should be stressed that SPME is an equilibrium process, rather than an exhaustive one, and quantitative analysis can be performed at virtually any extraction time (although SPME has a maximum sensitivity at the partition equilibrium), provided that adequate sensitivity is obtained [9]. It is not the purpose of this review to deal in-depth with SPME kinetics; however, data related to this issue can be found elsewhere [7–14].

Obvious appealing points of SPME include the fact that no organic solvents are required to accomplish the analysis and all the extracted material can be directly analyzed [15]. In addition, the fact that the extracting device is portable allows field sampling, provided that the analytes are stable in the fiber coating during transportation. Potential disadvantages include competition between drug and endogenous compounds for the fiber, particularly when the extraction mechanism is adsorption rather than partitioning [16].

Furthermore, absolute recoveries are, in general, low, since the extraction is not exhaustive; however, this is counteracted by the fact that all the extracted analyte is introduced in the chromatographic system. Nevertheless, analyte

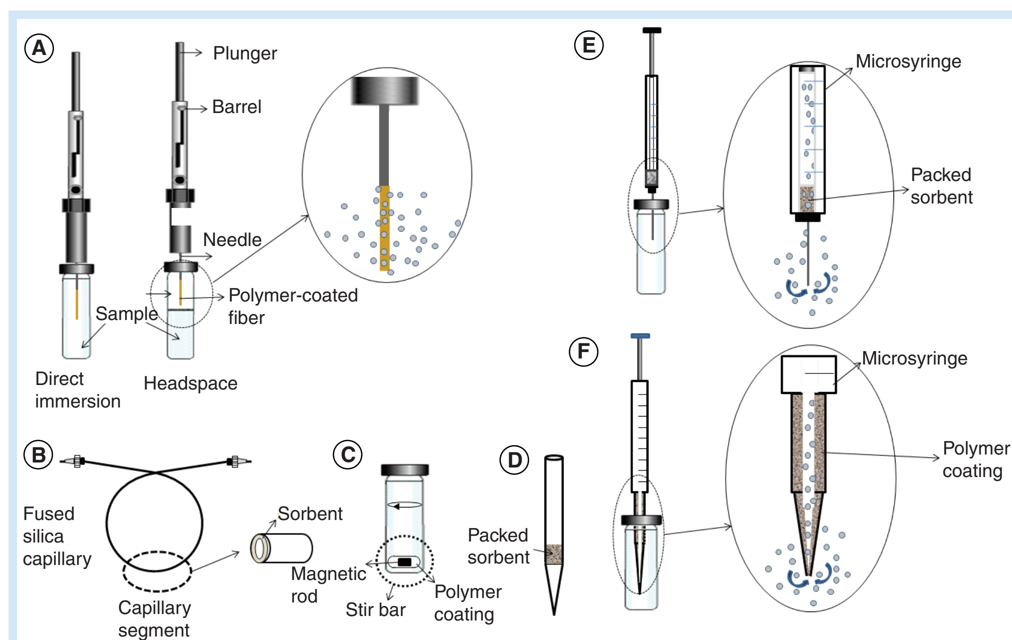

Figure 1. Solid-phase microextraction procedures. (A) Fiber solid-phase microextraction (SPME); **(B)** in-tube SPME; **(C)** stir-bar-sorptive extraction; **(D)** in-tip SPME; **(E)** microextraction in a packed syringe; and **(F)** solid-phase dynamic extraction.

recoveries should be previously optimized to enhance the method's sensitivity. Parameters that may affect analyte recovery include fiber coating, extraction and desorption temperatures, extraction time, pH and ionic strength of the sample [1,8,9,11].

One additional feature that deserves consideration when dealing with fiber SPME is the fact that some of the analyzed compounds cannot be directly analyzed without chemical derivatization (e.g., GC analysis of polar substances, such as 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid). Derivatization of the analytes can be performed in several ways [9]:

- Directly in the sample matrix, where the derivatizing reagent is first added to the vial containing the sample followed by extraction of the derivatives;
- On the GC injector at high temperatures;
- On the SPME fiber coating.

Concerning the latter, it is possible to either perform analyte derivatization following extraction or to make this procedure simultaneous with analyte extraction (the fiber is doped in the derivatizing reagent and then exposed to the sample). This latter approach is considered the most interesting and potentially useful technique, since it allows high efficiencies.

There are currently several commercially available fiber coatings, which have found widespread analytical applications, particularly in the field of toxicology. The selection of the polarity and thickness of the fiber coating depends on the analyte being analyzed. The most widely used coating is the apolar polydimethylsiloxane (PDMS), often used in the extraction of nonpolar compounds. On the other hand, to extract polar compounds, the higher polar polyacrylate coating is recommended. However, the problem with those fiber coatings is that they lack specific surface area due to their linear structure. By contrast, mixed coatings are available and these are blended with porous solid particles of polydivinylbenzene (DVB), carboxen (CAR) or templated resin; examples of those mixed coatings are PDMS/DVB, carbowax/DVB, PDMS/CAR, carbowax/templated resin and DVB/CAR/PDMS. Those coatings present large specific surface areas and can be used to extract volatile low molecular mass and polar analytes [1,4,8,11].

Most nonpolar drugs in biological specimens can be extracted by means of PDMS, polyacrylate, DVB or PDMS/DVB fibers, and analyzed in combination with GC–MS procedures. These fibers are quite fragile, and breaking of the fiber is unfortunately relatively common. However, the StableFlex type of fiber has a flexible fused-silica core and is therefore less breakable. In

addition, newly developed and commercialized metal fiber assemblies have provided enhanced durability and robust performance. Fiber coatings are available in increasing thicknesses (usually from 7 to 150 µm); this increase leads to an increase in the partitioning ratio of the target analytes, but also increases the time needed to reach equilibrium [1,4].

Nevertheless, new fiber coatings are being developed to meet special needs for particular analytes [17]. For instance, polypyrrole [18] coatings seem adequate for the efficient extraction of polar, aromatic and anionic compounds, since they are positively charged intrinsic conducting polymers. This fiber coating presents inclusively higher extraction efficiencies than commercial SPME coatings. Other promising fiber coatings are based on principles of immunoaffinity. These coatings contain covalently immobilized antibodies on the surface of the fiber, and may be used for the selective and sensitive extraction of specific analytes [19,20]. In addition, molecularly imprinted polymers (MIPs) [21–24] are stable, selective and crosslinked synthetic polymers, synthesized by the copolymerization of a monomer with a crosslinker in the presence of a template molecule and are used to coat SPME fibers, inner surfaces of capillaries and sorbent particles. This approach seems promising in forensic analysis. A testosterone-imprinted SPME fiber was developed for the selective extraction of anabolic steroids in urine samples, followed by GC–MS analysis [25].

Restricted access materials (RAM) are biocompatible sample preparation supports that enable the direct injection of biological fluids into a chromatographic system [26]; the same principle has been adopted for direct SPME extraction from blood [27]. This RAM-based SPME approach was able to simultaneously separate proteins from a biological sample, while directly extracting the active components from a natural drug. MIP and RAM particles have also been used for syringe and tip SPME techniques [1,4]. An online procedure using RAM has been published for the determination of bile acids in serum [28].

Trace amounts of methamphetamine were extracted from human saliva using a pencil lead fiber with a custom-designed unique extraction phase [29].

A new SPME method using an ionic liquid (IL)-based fiber was developed for the determination of methamphetamine and amphetamine in human urine [30]. The authors have concluded that, despite the fact that extraction efficiency of the IL fiber was lower than that of the 100 µm PDMS, the method was simple, fast and sensitive due to its ability to select a wider number of both cations and anions. Furthermore, it had advantageous physical and chemical properties, including hydrophobicity, viscosity, thermal stability, selectivity and low vapor pressure.

The use of monolithic materials has also been described and have shown to be highly efficient for the extraction of basic analytes from aqueous matrices [31–33]. These monolithic capillaries showed excellent reusability and high stability at extreme pH values. Furthermore, an imprinted porous polymer monolith fiber coated with homemade capillary glass and poly(ST-DVB) carbon monolith fiber showed higher extraction efficiency than other commercially available SPME fibers [34].

Another promising approach appears to be the use of aptamers, which are oligonucleotides (DNA or RNA) that bind with high affinity and specificity to a wide range of target molecules, such as drugs, proteins and other organic or inorganic molecules. Aptamers show a very high affinity for their targets, with dissociation constants typically ranging from the micromolar to low picomolar, comparable to those of some monoclonal antibodies, sometimes even better [35]. The use of these molecules has been reported for the selective isolation of some compounds, for example, cocaine, from biological fluids by means of high-selectivity binding [26,35]. This appears to be a future trend in sample preparation, despite the need to further improve the capacity of the highly selective supports [26]. Forensic toxicology applications of these new SPME approaches are scarce at the moment, but their capability to extract basic or weakly basic analytes makes them good candidates for future applications in this area. Further reading concerning these new SPME coatings can be found elsewhere [4,17,26,36–39].

The number of publications on SPME applications has been increasing exponentially year after year, leading to about 1500 publications since SPME was first developed [10]. Concerning forensic toxicology, SPME has been used for the quantitative analysis of several compounds in a variety of biological specimens. For instance, Paradis *et al.* have successfully detected sufentanil in human plasma by GC–MS [40]. They have optimized the method concerning the sample pH and ionic strength, and a 65 µm PDMS/DVB fiber was used in the

direct-immersion mode. Using a sample volume of 1 ml, the method quantitation limit was 6 ng/ml. A method using a 100 µm PDMS fiber and its direct immersion in the sample has been reported for the quantitative determination of cocaine and cocaethylene in plasma samples by GC–MS [41]. Quantitation limits of 25 ng/ml were obtained for both compounds in a sample volume of 0.4 ml.

Sha *et al.* have determined tramadol in human plasma by GC–MS using a 65 µm PDMS/DVB fiber, yet using the HS approach [42]. Using a sample amount of 0.5 ml, a detection limit of 0.2 ng/ml was reported. Ephedrine and pseudoephedrine have been successfully determined in urine and serum samples via extraction using a monolithic MIP fiber and capillary electrophoresis (CE) [43]. Despite the quite large amount of biological sample needed to accomplish the analysis (5 ml of serum or urine), detection limits as low as 0.96 ng/ml were obtained, demonstrating the high selectivity of this approach.

SPME, mainly using the HS approach, has also been successfully used for the analysis of biomarkers of ethanol consumption, both in hair [44–46] and meconium [47,48] samples, as well as for the determination of opiates in hair [49]. These samples usually need some kind of pretreatment before SPME, since the analyzed drugs need to be liberated from the matrix. For instance, Agius *et al.* extracted ethyl glucuronide from hair matrix with water, cleaned up the sample by means of SPE and performed derivatization with heptafluorobutyric anhydride [44]. Only after those procedures, SPME (75 µm CAR/PDMS fiber) was applied in the HS mode, and a detection limit of 0.6 pg/mg using only 10 mg of hair was obtained. On the other hand, Moller *et al.* performed an overnight incubation at 56°C with methanol to extract opiates from hair [49]. After evaporation to dryness, the analytes were derivatized by sylilation and were analyzed by GC–MS after concentration on a 100-µm PDMS fiber. By using this approach, the authors were able to detect as low as 2 pg/mg of the compounds (using only 10 mg of hair), an impressive sensitivity, taking into consideration that a single quadrupole GC–MS instrument was used.

Roehsig *et al.* successfully analyzed eight fatty acid ethyl esters in meconium samples, using a 100-µm PDMS fiber and GC–MS [50]. Although very low absolute recoveries were obtained (3–33%, depending on the analyte), the authors have obtained detection limits of 5–100 ng/g, using only 100 mg of sample. This highlights the superior selectivity of HS-SPME.

In addition, other types of substances have been analyzed in forensic scenarios. For instance, organophosphorous insecticides have been determined by Gallardo *et al.* [51–53] in postmortem blood and urine samples using the direct immersion approach, while Musshoff *et al.* [54] have used the HS approach for their determination in blood. This sample preparation technique has also been used for the determination of the alkaloid strychnine in blood [55].

In-tube SPME

In-tube SPME using a capillary column was developed aiming the miniaturization, automation, high-throughput performance, online coupling with analytical instruments and reducing solvent consumption [56,57]. In contrast to what happens with fiber SPME, in-tube SPME typically uses a short inner-wall coated fused-silica capillary (Figure 1B), but fiber-packed, sorbent-packed and rod-type monolith capillaries are also available, improving efficiency and specificity [4]. In those formats, analytes are either absorbed or adsorbed onto the outer surface of the packing material. The fiber-packed device consists of a capillary tube packed with fibrous rigid-rod heterocyclic polymers, while sorbent-packed and rod-type monolith formats consist of micro-LC capillary columns packed with the extracting phase [58].

In-tube SPME may operate as a flow-through extraction system, in which solutions are passed continuously in one direction through an extracting capillary column or as draw/eject extraction systems, in which the sample solution is repeatedly aspirated into and dispensed from an extracting capillary column [4]. After desorption of the extracted analytes by a stream of mobile phase or a static desorption solvent, analysis can be performed offline or online, either by GC, LC or CE. In-tube SPME can be automated to directly extract target analytes from aqueous matrices by using a column-switching device [4].

This microextraction technique can overcome some of problems usually associated with conventional fiber SPME, namely fragility, low sorption capacity and bleeding of fiber coatings [1]. The details of online in-tube SPME techniques for sample preparation are described and well-documented elsewhere [1,3, 8,37,56,57,59–61].

Several commercial GC capillary columns are currently available for in-tube SPME [57], namely RAM, MIP or polypropylene.

Recent applications of in-tube SPME include the use of monolithic capillaries for the analysis of illicit drugs in urine samples [62–64]. An automated online in-tube SPME/LC–MS method was developed to determine seven anabolic steroids in human urine [65]. Twenty draw/eject cycles were performed on a sample size of 40 µl, and the analytes were readily desorbed by a flow of the mobile phase. Using this approach, no carryover was observed and low LODs were obtained.

An in-tube SPME method based on a poly(methacrylic acid-ethylene glycol dimethacrylate) monolithic capillary column was developed for the extraction of amphetamine, methamphetamine and their methylenedioxy derivatives from urine samples. Recovery values near 100% were obtained for all analytes, and quantitation limits as low as 4.6 ng/ml were achieved using a HPLC–UV instrument [63]. In addition, a method has been described for the determination of butyrophenone derivatives in human plasma [66]. The authors have used an automated online in-tube SPME procedure, and have obtained detection limits of 0.2 ng/ml for most compounds. Only 0.1 ml of sample was used, and chromatographic analysis was performed by LC-MS/MS. However, efficiencies of only about 15% were obtained.

TFME

TFME was recently developed to increase the mass uptake rates and sensitivities of SPME [67]. Indeed, increasing the surface area will lead to a much higher extraction phase volume. The membrane can be attached to a holding rod for its better introduction in the analytical instrument; after extraction, the membrane can be rolled around the rod and introduced into the injection system for analyte desorption. This approach is especially applicable to hydrophobic and semivolatile components with high distribution constants. A TFME method using LC–MS/MS was developed to measure both free and conjugated testosterone and epitestosterone in urine samples for clinical diagnosis and therapy, obtaining a detection limit of 1 pg/ml for both compounds in 5 ml of sample [68].

SBSE

Introduced by Baltussen *et al.* in 1999 as a novel solventless sample preparation method [69], SBSE has become a popular analytical technique for the preconcentration of organic compounds into a stir-bar coated by a thick layer of PDMS (0.3–1 mm) (**Figure 1C**) [70], particularly for the enrichment of analytes from aqueous samples. It is compatible with both GC and LC chromatographic systems. The extraction process is based on the PDMS–water equilibrium, and its applications have been thoroughly reviewed [69–75]. In SBSE, sample volume and stirring speed greatly influence extraction efficiency, and it is common to have extraction times between 30 and 60 min [61]. The extraction mechanism is similar to that of SPME based on PDMS sorption; however, SBSE uses a much higher sample capacity. Nevertheless, the apolar PDMS phase is not suitable for extracting polar compounds, unless a derivatization step is used.

SBSE is, in theory, more sensitive than SPME fibers, at least for certain applications; however, it requires a special desorption unit and, as such, automation is difficult [61]. Similarly to what occurs in SPME, the typical extraction parameters that need optimization include sample pH, temperature, extraction time, stirring speed and salting-out effect [72].

Antidepressant drugs have been determined in 1 ml plasma samples diluted with borate buffer. A 10-mm rod was used and analytes were extracted in 45 min. Using a HPLC–UV, limits of quantitation of 15–40 ng/ml were obtained [76]. Still concerning medicines, a method for the determination of barbiturates in 5 ml urine samples was described, using a 20-mm PDMS-coated rod. The extraction time was 30 min and analytes were thermally desorbed and analyzed by GC–MS, obtaining a LOD of 12 ng/l [77].

Concerning forensic analysis, Crifasi *et al.* have evaluated the performance of several thermal desorption systems for the detection of a number of basic drugs in human blood by GC–MS [78]. Anabolic steroids have been detected in hair and urine samples using a 30-min extraction with a new self-assembled hollow fiber solvent-stir bar microextraction device and analysis by GC–MS. The LODs were lower than 0.1 ng/ml, and the recoveries were between 74 and 94% [79]. LC–MS methods have also been used with SBSE, namely the sensitive detection of the antidepressant drug fluoxetine in 1 ml human plasma, with a detection limit of 3 ng/ml [80].

A SBSE-thermal desorption GC–MS method was developed for the analysis of amphetamine derivatives (amphetamine, methamphetamine, 3,4-methlenedioxy-amphetamine [MDA] and 3,4-methlenedioxy-methamphetamine [MDMA]) in urine, but further details are

not available [81]. Only one publication deals with the determination of ketamine in urine [82]. Using HPLC–UV, a recovery higher than 90% was obtained, with a detection limit of 27 ng/ml.

As stated before, the most important limitations of SBSE are related to the coating of stir-bars, being limited to non-polar PDMS. For this reason, recovery of polar analytes is very poor, with *in situ* derivatization often required. New coating materials are therefore required to extend the range of applications. New materials, namely poly(phthalazine ether sulfone ketone), polypropylene, PDMS/polypropylene, monolithic materials, have been also used as SBSE coatings. RAM- and MIP-based coatings have been evaluated as well [70].

A new technique called bar-adsorptive microextraction [83] appears to be adequate for the analysis of polar analytes. Indeed, it has been developed for trace analysis of polar analytes in aqueous samples and was evaluated for the extraction of morphine and codeine from water samples. However, the high volume of sample used (30 ml) makes this approach somewhat difficult to apply in the case of biological specimens in forensic situations, at least using a HPLC–UV instrument. Perhaps this high sample volume could be further reduced using MS detection.

MEPS

MEPS is a recent sample preparation technique developed by Abdel-Rehim at the laboratories of AstraZeneca in 2004 [84]. This is considered a new technique for miniaturized SPE packed bed columns, which can be connected online to GC or LC systems without any modification of the device [84–88] as well as to CE systems [89].

MEPS can be also regarded as a short LC column embedded in a syringe [26,87] and performs the same function as the standard sorbent-phase extraction, specifically the concentration of the analytes of interest followed by the removal of interfering substances, leaving the former dissolved in an eluate. MEPS consists of two parts, the MEPS syringe and the barrel insert and needle assembly (BIN) containing the SPE phase. Approximately 2 mg of the sorbent is packed inside the syringe (typically of 100–250 µl) or pipette-tip as a plug, or between the barrel and the needle as a cartridge, not in a separate column (Figure 1E). The plug is tightly fixed in the syringe to prevent it from moving [84,87].

The same reasons that made conventional SPE so attractive to analyze drugs in biofluids are also applicable to MEPS because both techniques are based on the same sorbent chemistry [87]. Commercially available BINs include silica-based sorbents (SIL, C_2, C_8, C_{18}, M_1 [mixed-mode with C_8 and cation exchange]), but also RAM or MIPs [3,26,87,88,90,91].

MEPS is based on multiple extractions in which the sample flows through a bed of solid sorbent. To allow this, the extractant phase and its particle size must be as small as possible in order to speed up the mass transfer of analytes from the liquid sample to the sorbent. Very close contact between the aqueous sample and the surface is also very important and, therefore, a balance between the amount of the sorbent, the loading volume and the volume of the elution is necessary to avoid exceeding the method's capacity [84,87].

At the first stage, the material is activated with an organic solvent such as methanol, thus facilitating analyte retention. Then, the syringe withdraws the sample and the analytes are retained. This process can be repeated several times, concentrating the analytes inside the syringe, forcing an increase in sample response and volume. The packing material is then rinsed with water (usually 50 µl) to remove proteins and other interferences present in the sample. Afterwards, the analytes are eluted with 20–50 µl of an organic solvent (e.g., methanol or mobile phase) and directly injected into the chromatographic system. The multiple extraction cycles can be made from the same aliquot (draw/eject in the same vial) or by draw-up from aliquot and discarded in waste (extract/discard) [87].

The procedure can be performed automatically by an autosampler and even connected online with a GC injector, provided that large-volume injection techniques are used. However, most current MEPS applications involve online connection with LC, rather than GC, since it is not easy to dry the SPE material before automatic elution, and small amounts of water would be introduced in the GC instrument. In addition, the elution is typically performed with relatively polar solvents, which may be less compatible with GC procedures [88].

Packed pipette tips are typically used in offline mode. In these offline procedures, more than one washing step can be carried out and drying can be done by applying vacuum, or by using a drying agent to remove water from the final extract [92]. In GC, the entire eluate can be injected using a programmable temperature vaporizing technique, increasing sensitivity [93].

Many factors, such as volumes and composition of washing and elution solutions, sorbent amount and sorbent type, may affect the MEPS performance [87]. However, the selection of SPE material is the most critical parameter in optimizing the extraction.

This approach to sample preparation is very promising [85,88]. In fact, compared with SPE or LLE, MEPS reduces sample preparation time, organic solvent consumption and the cost of analysis is minimal [87,88], as well as a possibility of full automation. Compared with SPME, MEPS reduces sample preparation time (<1 min), sample volume (10–1000 µl) and presents in general a much higher absolute recovery (>50%) [3,32,85,90]. Furthermore, the packed syringe can be used several times, and more than 50–100 extractions from plasma or 400 extractions from water samples are possible, whereas a conventional SPE column is used only once [26,38,85,88,94].

MEPS has been applied for the extraction of many drugs and metabolites from various biological specimens. A study has been published on the quantitative analysis of risperidone and 9-hydroxyrisperidone in human plasma, urine and saliva using LC–UV. The authors have used a C_8 sorbent and a sample volume as low as 0.05 ml (for urine). The detection limits were low (~1 ng/ml for both compounds) and an extraction yield higher than 90% was obtained [95]. A method for the determination of remifentanil in 0.02 ml plasma samples by LC–MS/MS has been published and a detection limit of 0.02 ng/ml was obtained [96]. Somaini *et al.* have developed a method for the determination of methadone, buprenorphine, norbuprenorphine and naloxone in plasma (0.1 ml) using HPLC with coulometric detection [97]. Absolute recoveries of about 90% were obtained and the method's detection limits were 0.08 ng/ml for most analytes.

As stated before, the factors that influence the extraction usually need to be optimized during method development. This optimization is often time-consuming and laborious, due to the high number of factors that need to be studied. Moreno *et al.* have used the factorial design approach for the optimization of a MEPS method for the quantitation of four selected piperazines in low volume urine samples (0.1 ml), with chromatographic analysis by HPLC–DAD [98]. This approach allows reducing the total number of experiments and, in this particular study, five factors were optimized using only 16 experiments. A mixed-mode sorbent was used and the detection limits were between 50 and 100 ng/ml, while analyte recoveries ranged from 52 to 100%. The same authors have also studied a C_{18} sorbent for the same purpose [99]. However, extraction efficiencies were lower (10–70%), and quite higher detection limits were obtained (500 ng/ml). Nevertheless, those limits were found adequate, since the expected concentrations of the analytes in urine samples usually fall within the dynamic range of the assay.

■ SPDE

SPDE is an inside-needle technique for vapor and liquid sampling [100–102]. It uses stainless steel needles coated with a film of PDMS and 10% activated carbon. Dynamic sampling is performed by passing the HS through the tube using a syringe, and the analytes are concentrated onto the stationary phase (FIGURE 1F) [61]. This is accomplished by pulling in and pushing out a fixed volume of the HS for an appropriate number of times, and this allows operation under dynamic conditions while keeping the HS volume constant. The trapped analytes are then thermally desorbed in the injection port of a GC instrument. This technique has a great advantage over SPME, that is, the robustness of the capillary. In contrast to the fragile SPME fibers, the SPDE device is not easily mechanically damaged. However, the possibility of carryover is of concern because the analytes tend to remain in the inside needle wall after thermal desorption in the GC injection port [61,102]. Another disadvantage of SPDE is the length of the coating, which results in possible desorption problems, if the GC injector shows a considerable temperature profile [86].

This sample preparation approach has been used for the determination of several drugs of abuse in biological specimens. For instance, Musshoff *et al.* have determined cannabinoids in hair samples by GC–MS and obtained detection limits of approximately 0.1 ng/mg in a 10 mg sample [103]. However, the extraction efficiency was extremely low (0.6–8.4%). The authors concluded about the superiority of SPDE over SPME, taking into account the number of extractions using the same device (more than 350 vs 90–100). The same research group has published a paper on the determination of amphetamines and synthetic designer drugs in hair, again using GC–MS [104]. The obtained detection limits were in the range of

0.04–0.19 ng/mg in a 10 mg sample. However, extraction efficiency was higher for these analytes (10.2–16.7%) than for cannabinoids. Again, the authors concluded about the superiority of SPDE sampling over SPME, because of the higher extraction rate, as well as a faster automated operation.

Another interesting paper uses GC–MS/MS for the detection of several drugs in human hair [105]. The obtained detection limits were in the range of 6–52 pg/mg hair, while extraction efficiencies were between 0.5 and 24%.

■ Polymer monolithic microextraction

Although less known than the aforementioned syringe SPME procedures, several applications of polymer monolithic microextraction have been described. This sampling technique was first introduced in 2006 as an alternative to SPME. The extraction device consists of a regular plastic syringe, a polymer monolithic capillary and a plastic pinhead connecting both components [106,107]. The analytes are extracted and desorbed by driving the sample and desorption solution through the monolithic capillary using a syringe infusion pump [106]. This technique shares some advantages with SPME, but provides greater extraction efficiency because of its higher surface area. Furthermore, the monolithic column can be easily prepared and the extraction material is not expensive [107,108].

Polymer monolith microextraction has been used for the analysis of opiates and a method coupled with capillary-zone electrophoresis and UV detection was developed for the determination of those compounds in human urine. The limits of detection were between 6.6–19.5 ng/ml, while extraction efficiencies higher than 90% were obtained [108]. Saliva samples have also been analyzed using this sampling approach, namely for the determination of cannabinoids by GC–MS. Analyte recoveries near 90% were obtained and the method presented a detection limit of 2.26 ng/ml [109].

■ In-tip SPME

To minimize the required volume of solvents and samples, this type of SPE is performed in the form of a pipette tip (Figure 1D). This micro-SPE procedure is now a routine tool in the purification, concentration and selective isolation of proteins and peptides in the fields of genomics, proteomics and metabolomics [110–112]. From the several packing materials commercially available, mixed-mode cation exchange and C_{18} moieties are the most popular [113,114]. These extraction tips are now commercially available as ZipTip (Millipore; MA, USA), Omix (Varian; CA, USA); and NuTip and MonoTip C_{18} tip (GL Sciences; Tokyo, Japan) [4].

The procedure for drug extraction with this SPE tip is basically the same as that for the conventional C_{18} SPE. The difference is that in the former, all manipulations are carried out by aspirating and dispensing through a single pipette tip using a micropipette [32,113–116]. The advantage over traditional SPE is that the extraction is more easily and rapidly performed, and disposable materials are used [4,32,116,117]. Moreover, the small bed volume and sorbent mass within the tip enable using reduced solvent volumes, both in the conditioning and eluting steps. This speeds up the evaporation step and provides higher throughput, minimizing costs [113].

A number of pipette tip methods have already been published for the extraction of peptides and proteins from biological samples. Concerning drug analysis, methods were described for the detection of several compounds in biofluids, namely methamphetamine, amphetamines [118,119] and phenothiazines [116], tricyclic antidepressants [120] and dextromethorphan [115]. A method for the determination of several stimulants, hallucinogens, ketamine and phencyclidine in oral fluid was also published [121]. In this method, LC–MS/MS was used and detection limits were in the range of 0.3 to 4.9 ng/ml. Supplementary Table 1 summarizes the most recent publications regarding SPME procedures for forensic toxicology purposes [30,43–50,65,66,95–99,107,109,115,118–151].

Liquid-phase microextraction

Liquid-phase microextraction (LPME) was developed by Dasgupta and Cantwell in 1996 [152,153] and uses minimal amounts of solvent. It is rapid and inexpensive, and the operator's exposure to toxic organic solvents is kept to a minimum [2,152–156]. The extraction takes place by partition of the analytes between an aqueous phase containing them and a small amount (usually a drop in the microliter or submicroliter range) of a water-immiscible solvent. This latter acceptor phase can be immersed directly in the sample, or suspended above it for HS sampling. This allows high enrichment factors, due to the high sample volume-to-acceptor phase volume ratio [61,157]. LPME is usually categorized into single-drop microextraction (SDME) using a single droplet, membrane-assisted LPME

using a HF, membrane bag or flat-sheet membrane module [61,155,156–160], solidification of floating organic droplet (LPME-SFO) [161,162] and dispersive liquid–liquid microextraction (DLLME) [156,157,162].

■ SDME

SDME uses a microdrop (1–10 µl) of a water-immiscible extracting solvent and is based on the principle that the equilibrium ratio of the concentration of solute between the organic phase and the aqueous phase is constant. Briefly, a microdrop is exposed to an aqueous sample and the analyte is extracted into the drop. This drop is formed with a regular GC or LC syringe. After extraction, the microdrop is retracted back into the syringe and directly injected into chromatographic instruments for further analysis [61,160,163–165]. Since the drop volume matches up with the volume required for chromatography or MS analysis, the coupling efficiency of the technique is often high [163–165] (Figure 2A).

Several parameters, including the nature of the organic solvent, exposure time, agitation, salt concentration and drop volume and stability strongly influence the process, and therefore optimization is deemed necessary prior to application of SDME to a particular matrix [163–165].

Compared to traditional LLE, solvent consumption is reduced by 99% in SDME, turning this simple, fast and cheap technique into an environmentally friendly approach for sample preparation. Furthermore, there is no need for solvent evaporation and/or reconstitution prior to instrumental analysis. However, some drawbacks should be considered when using SDME. For instance, the volume of the extractant microdrop is small, which limits the amount of extracted analyte, affecting efficiency. On the other hand, the microdrop is unstable and can be easily displaced, which is more critical for long extraction times. In addition, reproducibility is often poor due to significant dissolution of organic extractant, which has a small volume and a large contact area with the sample [160,163–165]. Concerning the extracting solvent, n-octyl acetate, isoamyl alcohol, undecane, octane, nonane and ethylene glycol are often used in SDME [155,163–165].

Depending on the number of phases coexisting at equilibrium, LPME procedures can be classified into two-phase or three-phase techniques. In the two-phase systems, usually involving analyte partitioning between an aqueous sample and an organic solvent, there are direct immersion (DI), continuous flow, drop-to-drop and directly suspended droplet modes. On the other hand, in three-phase systems, the analyte is extracted from an aqueous sample solution to an organic solvent and then transferred from the organic solvent to an aqueous solution, consisting of HS, liquid–liquid–liquid (LLL) and a combination of LLL and directly suspended droplet [160,165].

In DI-SDME, the extracting solvent is withdrawn into a syringe whose needle pierces the vial septum and is immersed in the liquid sample [61,160,165]. Subsequently, the plunger is depressed and a solvent drop is formed, being suspended from the needle tip and in contact with the sample [164,165]. After extraction, the drop is retracted back into the syringe needle and injected into the chromatographic system [61,163,164,166]. This is highly convenient, since extraction and injection are performed using a single device.

As already stated, this solvent must be immiscible with water, since it is in direct contact with the aqueous sample. Therefore, nonpolar or very slightly polar solvents need to be used and the analytes must be more soluble in the extracting phase than in the sample solution [160,163,164,166]. For this reason, this sampling mode is adequate for the extraction of nonpolar to moderately polar volatile and semivolatile analytes from relatively clean matrices.

The major advantages of this approach are the simplicity of the equipment needed and its low cost. Concerning the disadvantages, these are mostly related to the ease of dislodgment of the microdrop hanging from the tip of the needle, especially when high stirring rates or temperatures are used, or the analyzed sample matrix is dirty [61,160,165].

Drop-to-drop microextraction (DDSME) (Figure 2B) is a miniaturized version of DI-SDME, in which the volumes of both the sample and the extractant are in the range of microliters. This makes this technique useful when sample amount is limited (e.g., blood), and this is a problem usually associated with forensic analysis. The main advantage of this sampling mode, besides the already mentioned reduction in sample size, is its high selectivity, due to the extensive sample cleanup [160,165,166].

An interesting microextractive approach does not need a syringe or hollow fiber to hold the extraction solvent. Instead, in directly suspended droplet microextraction, an organic drop

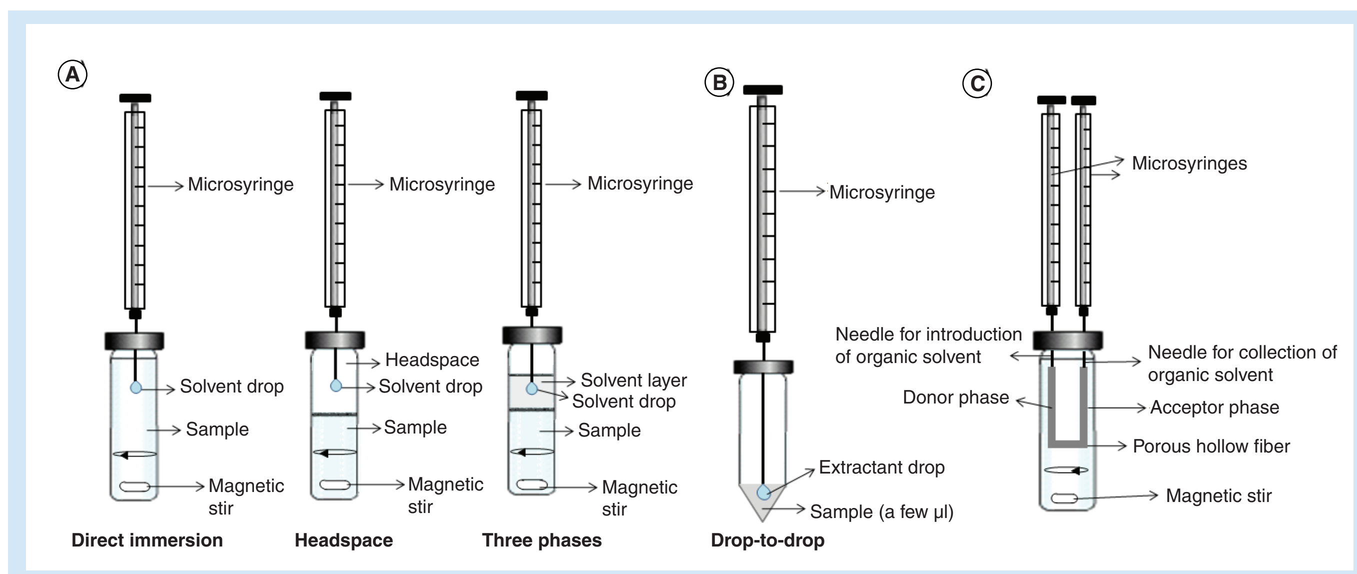

Figure 2. Liquid-phase microextraction procedures. (A) Single-drop microextraction; **(B)** drop-to-drop microextraction; **(C)** hollow-fiber liquid-phase microextraction.

is placed directly on a stirred aqueous sample [166]. After extraction is completed, a syringe is used to take an aliquot of the solvent drop and inject it into a LC or GC instrument [164,167].

Three-phase single-drop microextraction, also called LLLME or LPME by back-extraction (LPME-BE) is also possible (Figure 2A) [156,168]. This process involves the extraction of analytes from aqueous samples into an organic layer or membrane and afterwards back-extracted into an aqueous acceptor phase, suspended from the tip of a microsyringe in the organic membrane [26,160,169]. The pH of the aqueous solution and the aqueous microdrop can be adjusted to obtain, in the first stage, the neutral form of the analyte (which is better extractable by the organic solvent), followed by ionization for back-extraction by the aqueous drop [26,160]. If compared with other forms of SDME, this approach is more difficult to perform, but has the advantage that it is more convenient for coupling with reverse-phase-HPLC or CE [26,159,166]. In addition, higher selectivity can be obtained [170].

However, the HS approach is the most commonly used solvent microextraction method, allowing the preconcentration of both polar and nonpolar volatile or semi-volatile analytes [160]. The advantage is that pre-concentration and derivatization can be performed in just one step [26]. The working procedure of HS-SDME is similar to that of DI-SDME, but the microdrop is not directly immersed in the sample, remaining in the HS above it [160,164]. Equilibrium times may, however, be longer than in DI-SDME. Nevertheless, extraction times in the HS approach can be substantially reduced by increasing the HS capacity (i.e., the amount of analyte contained in the HS) [160]. The use of HS-SDME is not easy, since the choice of suitable solvents is limited, particularly if the vapor pressure of the solvent is the primary concern [26,61].

A number of advantages are associated to HS-SDME, including the elimination of interference of dirty and/or complex matrix constituents and the possibility of stirring the sample without restrictions on the rate [160,163–165].

A method has been described for the determination of tobacco alkaloids in urine and saliva by means of SDME and analysis by GC–flame ionization detector (FID) [171]. The authors have previously optimized several parameters, such as extraction solvent, stirring rate, salting out, extraction time and sample pH. An extraction time of 30 min was used and detection limits of approximately 0.4 µg/ml were obtained, using 0.5 and 0.1 ml of saliva and urine, respectively. In addition, extraction efficiency was higher than 70% for all analytes.

Concerning drugs of abuse, a method by HPLC–UV was described for the determination of methamphetamine and amphetamine in urine samples [172]. The method used a three-phase LLLME approach and n-hexane was selected as the organic extractant phase. The analytes were back-extracted into a 5 µl aqueous drop and then

directly injected in the chromatographic system. Enrichment factors higher than 500-fold and detection limits of 0.5 ng/ml were obtained for both analytes, using a sample volume of 6 ml. The same research group used an aqueous drop and HPLC–UV for the extraction and analysis of the same compounds in urine samples [173]. This procedure eliminated the use of organic solvents, and detection limits of 0.3 ng/ml were obtained. However, and unfortunately, no data related to sample volume is given.

Opioid analgesics, namely fentanyl [169] and tramadol [174], have also been determined in biological specimens, both by means of the LLLME approach. The extracting organic solvent was the same for both analytes (n-octane) and the obtained detection limits were similar (~0.1 ng/ml) despite the differences in sample volumes (3.6 ml for fentanyl vs 2 ml for tramadol). The total extraction times were 50 min for fentanyl and 40 min for tramadol; fentanyl was back-extracted into a 5 µl 1 mM perchloric acid drop, while back-extraction of tramadol involved a 3.5 µl drop of phosphoric acid/sodium hydrogen phosphate buffer. For fentanyl, the enrichment factors were between 130 (plasma) and 300 (urine), while for tramadol an overall enrichment factor of 366 was reported. The approach has shown to be more efficient for tramadol (64% recovery) than for fentanyl (49%).

Es'haghi *et al.* determined MDMA in human hair samples [175]. The authors used 1-octanol as extracting solvent and the analyte was back-extracted by a droplet of deionized water (with its pH adjusted to 12), which was afterwards recovered with a syringe and directly injected on a HPLC–DAD system. A detection limit of 0.1 ng/ml was obtained in 50 mg of sample, and the authors have calculated an enrichment factor of 98 for the procedure.

A method using HS SDME with in-drop derivatization and CE has been described for the determination of cyanide in urine and saliva samples [176]. The LOD was 0.08 nmol/ml, and the authors report an enrichment of 58-fold in only 20 min of extraction. The method was successfully used to determine free cyanide in both smokers and nonsmokers saliva and urine.

HF-LPME

In order to improve stability and reliability of SDME, an approach using a HF (HF-LPME) was introduced in 1999 [177], and has recently found wide application in the fields of both environmental and drug/pharmaceutical analysis [26,156]. This technique is based on traditional LLE, but the extracting solvent volume is of only a few microliters and an additional solvent evaporation step is not necessary [156,177]. The extractant solvent is not in direct contact with the sample, and as such the samples may be stirred or vibrated vigorously [61,165,178,179]. The hollow fiber is placed in the sample solution and analytes passively diffuse into the intermediary organic phase (represented by the supported liquid membrane) and then into an acceptor solution inside the lumen [61,156,165,178,179]. After extraction, the analytes are back-extracted into the receiving phase and then injected into chromatographic systems [2,159,179,180] (Figure 2C). Similarly to what happens in SDME, also in HF-LPME two-phase or three-phase modes are possible [156,157,181–183]. In the two-phase mode, the analytes are extracted from the aqueous matrix to a water-immiscible solvent placed within the lumen of the fiber [156,181,182,184], while when three phases are used (HF-LLLME), analyte extraction occurs first to the organic extractor phase and finally back-extracted to the receiving aqueous phase [26,156,159,182].

This approach presents advantages, such as lack of carryover due to the fact that the hollow fiber is intended for single use. Furthermore, the small pore size prevents large molecules and particles present in the sample from entering into the acceptor phase, yielding very clean extracts [183,185]. The use of HF-LPME has been increasing in the last few years due to its simplicity and efficiency [156,181]; in addition, the process is cheap, consumes little organic solvent and enrichment factors of more than 100-fold have been reported [61,165]. The automation of this approach has been published, although with no application to forensic toxicology [186,187].

Ghambarian *et al.* have determined tramadol in plasma and urine samples using GC–MS [188] using this approach. An enrichment factor of 546 was obtained and a detection limit of 0.08 ng/ml was achieved. Furthermore, the authors report an extraction efficiency of 68%.

Cui *et al.* have reported the use of the HF-LPME approach to determine flunitrazepam from plasma and urine samples by GC–MS/MS [189]. Using sample volumes of 4 ml, detection limits of 0.001 and 0.025 ng/ml were obtained for urine and plasma, respectively. The authors have also observed that method recovery was much higher for urine samples than for plasma (84 vs 6%), which was attributed to the fact that the compound binds to plasma proteins, and is

therefore not capable of crossing through the pore of the hollow fiber.

This approach has also been used for the determination of cannabinoids in human hair by GC–MS/MS [190]. After alkaline hydrolysis using sodium hydroxide, the analytes were extracted in 20 min. Using 10 mg of hair, detection limits between 0.5 and 15 pg/mg were obtained, and extraction efficiencies ranged from 4.4 to 8.9%.

■ DLLME

Another approach is DLLME, involving an interaction between fine droplets of a disperser solvent with sample matrix containing the analytes [191]. In this technique an appropriate mixture of the extraction and the disperser solvents is injected into the aqueous sample and forms a cloudy solution, which results from the formation of fine droplets of the extraction solvent that disperse in the sample. This cloudy solution is then centrifuged and the droplets sediment at the bottom of the test tube, being determined by analytical instruments [162,191].

Owing to the large surface area, equilibrium state is achieved quickly and the extraction is time independent, which is the most important advantage of this method. Also, its speed, high enrichment factor, high extraction recovery, simplicity of operation and low cost are other advantages [162].

The extraction solvents used should be dense and immiscible with water, in order to support the phase separation, always by centrifugation, after the extraction [191]. However, high density organic solvents, such as chlorobenzene, chloroform and carbon tetrachloride, are typically highly toxic [156,158,162]. ILs appear to be a good alternative for extraction, due to their unique physicochemical properties [156,162]. Indeed, they have negligible vapor pressure, thermal stability and high viscosity, which enables using larger and more stable extractant drops. However, their extremely low volatility makes it somewhat difficult to couple with the GC instruments, being more suitable for HPLC or CE-based procedures. Nevertheless, interfaces that prevent the IL from reaching the capillary GC column have been described [192].

Despite being widely applied to the preparation of environmental water samples, the application of DLLME for drug analysis in complex biological specimens is still scarce. Of course, matrix effect must be reduced, which is achieved by sample dilution. However, and in spite of sample dilution, extraction efficiencies are still low [156,162].

The antidepressant drugs amitriptyline and nortriptyline have also been determined by means of DLLME methods, namely in water [193] and plasma and urine [194] samples. In the former paper, the authors have used the DLLME approach with a GC–FID instrument, and methanol was used as the disperser solvent. Using carbon tetrachloride as extracting solvent, LODs of 5 and 10 ng/ml were obtained for amitriptyline and nortriptyline, respectively, with extraction efficiencies between 55 and 74%. Furthermore, the authors have reached the conclusion that extraction efficiency was independent of the extraction time. In the latter study, a disposable pipette tip was used to hold the microextraction drop, and toluene was used as the extraction solvent. An atmospheric pressure MALDI-MS procedure was used, and the extraction was performed by toluene for 5 min. The method's LODs were 47.5 and 94.9 nM for urine and plasma, respectively.

Moradi *et al.* have determined cannabinoids in urine samples using this approach [195]. After a 3 min extraction, analysis was performed by HPLC–UV, and detection limits between 0.1 and 0.5 ng/ml were obtained.

DLLME has been also used for the determination of opium alkaloids in urine samples by HPLC–UV [196]. Extraction was performed in less than 4 min, and the obtained detection limits were between 0.2 and 10 ng/ml in a 2 ml sample.

Supplementary Table 2 summarizes the most recent publications regarding LPME procedures for forensic toxicology purposes [169,171,173–175,185,188,189,194–223].

A general overview of the discussed SP- and LPME procedures, concerning their main advantages and drawbacks, is provided on Supplementary Table 3.

Conclusion & future perspective

The field of forensic toxicology has undoubtedly taken benefit from the use of microextraction procedures. Indeed, and also due to an increase in analytical instrumentation sensitivity, these techniques allow the use of less sample volumes, which is important, since sample availability is often limited.

In addition, miniaturized procedures usually provide more rapid and cleaner extractions when compared with conventional sampling techniques, such as LLE and SPE. Furthermore, relatively high amounts of organic solvents are needed when the latter are used, which may be

Executive summary

Miniaturized sampling techniques

- There is a growing trend in the use of miniaturized techniques, due to their speed, simplicity and organic solvents reduction.

Solid-phase microextraction

- Fiber solid-phase microextraction (SPME):
  - Concentrates extraction and enrichment of the analytes into one solventless step;
  - The fiber may be directly exposed to the sample or to the headspace above it;
  - Absolute recoveries are in general low, and therefore comprehensive method optimization is often deemed necessary.
- In-tube SPME:
  - A capillary column is used as the extracting device;
  - Some of the problems usually associated to conventional fiber SPME can be overcome.
- Thin-film microextraction:
  - Developed to increase the mass uptake rates and the sensitivities of SPME, due to the substantial increase in the extracting surface area.
- Stir-bar sorptive extraction:
  - Organic compounds are concentrated into a polydimethylsiloxane-coated stir bar;
  - Sample volume and stirring speed greatly influence extraction efficiency;
  - Automation of stir-bar sorptive extraction procedures is difficult.
- Microextraction by packed sorbent:
  - Based on multiple extractions in which the sample flows through a bed of solid sorbent;
  - Many factors such as volumes and composition of washing and elution solutions, sorbent amount and sorbent type may affect the method's performance;
  - Reduced sample preparation time, organic solvent consumption and cost of analysis.
- Solid-phase dynamic extraction:
  - The headspace is passed through the tube using a syringe and the analytes are concentrated onto the stationary phase;
  - The possibility of carryover is of concern.
- Polymer monolithic microextraction:
  - The analytes are extracted and desorbed by driving the sample and desorption solution through the monolithic capillary using a syringe infusion pump;
  - It provides greater extraction efficiency when compared to fiber SPME.
- In-tip SPME:
  - All manipulations are carried out by aspirating and dispensing through a single pipette tip using a micropipette;
  - The advantage over traditional SPE is that the extraction is more easily and rapidly performed, and disposable materials are used.

Liquid-phase microextraction

- Single-drop microextraction:
  - Uses a micro-drop of a water-immiscible extracting solvent;
  - The nature of the organic solvent, exposure time, agitation, salt concentration, and drop volume and stability strongly influence the process;
  - Compared to traditional LLED, solvent consumption is reduced by 99% in single-drop microextraction;
  - The volume of the extractant microdrop is small, affecting efficiency;
  - Reproducibility is often poor due to significant dissolution of organic extractant;
  - Both the direct immersion and headspace approaches are possible.
- Hollow fiber liquid-phase microextraction:
  - The extractant solvent is not in direct contact with the sample and as such the samples may be vigorously stirred;
  - After extraction, the analytes are back-extracted;
  - This approach presents no carryover, since the hollow fiber is intended for single use;
  - The small pore size prevents large molecules and particles present in the sample from entering into the acceptor phase, yielding very clean extracts.
- Dispersive liquid–liquid microextraction:
  - Involves an interaction between fine droplets of a disperser solvent with the sample matrix;
  - The extraction is time-independent, which is the most important advantage of this method. However, extraction efficiencies are still low.

toxic to the operator and need to be properly discarded; increasing the costs of analysis as well causing environmental pollution.

These miniaturized techniques may be roughly categorized into SP- and LPME. Fiber SPME was first introduced in the late 90s by Pawliszyn and coworkers, and thousands of applications have been published ever since. At the moment, new approaches on SPME, as well as new fiber coatings, have been introduced into routine practice, some of them with the potential for application in forensic toxicology. From these, both the MEPS approach and the use of MIPs appear to be good future trends for sample preparation. First, MEPS enormously reduces sample volume and already has been applied for the analysis of several substances in biological specimens in a forensic context. On the other hand, MIPs provide greater selectivity, which is relevant for the unambiguous identification of the analytes, since a positive result for a particular compound may have legal consequences to the individual's life or freedom. However, more developments are still needed to broaden the number of analyzed substances. A promising area of research is *in vivo* sampling procedures, which may be used, for instance, to study the degree of protein-binding of a given drug, since SPME usually extracts only the unbound drug. However, it is much more demanding than conventional SPME, since the devices must be biocompatible and sterilizable. Perhaps for these reasons, its application in forensic toxicology has not yet been reported.

In LPME procedures, the consumption of organic solvents is reduced to a minimum, as it allows analyte extraction into a droplet that is directly injected into chromatographic systems. This feature would make this sampling approach interesting to forensic toxicological analysis. However, more developments are still needed concerning drop stability, and this approach still requires high sample volumes for analysis, which may make its application to forensic toxicology quite difficult.

Some applications using ILs have been reported in both SP- and LPME procedures and appear to be good candidates in the application of analytical sample preparation in the future, particularly when their compatibility with GC instruments is improved.

Supplementary data

Supplementary data accompanies this paper and can be found at www.future-science.com/doi/full/10.4155/BIO.12.139

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Chapter II

1. Theme justification and global objectives

The new psychoactive substances and their consumption patterns, although initially used in restricted social groups and circumscribed geographical areas, have led to serious social and public health problems [1]. Currently, European drug markets continue to change and evolve rapidly, as a result, the availability of these new compounds over the past decade has also been increasing rapidly [2]. Notifications made by Member States to the early warning mechanism of the European Union indicate that new psychoactive substances on the European market continue to increase in both variety and quantity. This evolution is demonstrated not only by the increasing number of seizures reported each year but also by the number of new substances detected. Only in 2014 were detected for the first time 101 new substances, an increase of 25% compared to 2013 [2].

This large number is due to the fact that new compounds are synthesized so as to be different from prohibited substances, thereby creating a legal vacuum which allows their marketing. Therefore, the systematic search of these new substances is hampered [3,4]. This is the case of drugs covered in this study, *Salvia divinorum* (salvinorin A), ketamine and norketamine, and methoxetamine. These substances are not regulated in some Member States of the European Union, are relatively easy to acquire, for instance in the Internet, and often regarded as legal alternatives, cheaper and safer than traditional drugs. Nevertheless, their reputation as safe drugs has been questioned by some cases of fatal poisoning, particularly with methoxetamine in Poland [5] and ketamine in Canada [6].

These new drugs, in contrast to those whose metabolism and toxicity are generally well-known, have a limited toxicological profile. Therefore, it is virtually impossible to identify and quantify these compounds, predict potential drug interactions, side effects and prevent severe intoxications. In the absence of known dosage and identification and/or quantification methods, it is not surprising that drug abuse leads to various poisonings with fatal and non-fatal results [3].

Many of these poisoning cases are due to wrong diagnostics carried out in hospital emergency services, when individuals are attended with poisoning by ketamine or methoxetamine, for

example. When this happens, they may be mistaken for poisoning by other drugs (e.g. alcohol) and may be erroneously treated according to the symptoms [3].

In order to avoid these situations, forensic toxicology laboratories are often requested by the authorities to assess the possible consumption of these substances by an individual. Furthermore, as scientific information is still scarce on these new drugs, the development of new, faster and more efficient methods for their identification and quantification in biological samples is particularly important in toxicology, both in the clinical and forensic settings, allowing thus a better monitoring and characterization of both drugs as consumers.

Therefore, this work consisted of detection and identification of a number of those emerging drugs (*Salvia divinorum* (salvinorin A), ketamine and norketamine, and methoxetamine) in biological samples of toxicological and forensic interest, namely plasma and urine, classically used in the context of toxicology.

The specific objectives of this study were:

- To develop sensitive and easy to use analytical methods, enabling the identification and accurate quantification of these drugs using gas chromatography coupled to tandem mass spectrometry;

- To develop and optimize extractive methods (MEPS) in urine and plasma samples;

- To validate the developed methodologies according to internationally accepted standards of bioanalytical methods validation, including those of the Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH) to ensure reliable and reproducible results;

- To analyze real samples by means of the developed and validated methodologies.

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Chapter III

Article II

Analysis of Salvinorin A in urine using microextraction in packed syringe and GC-MS/MS

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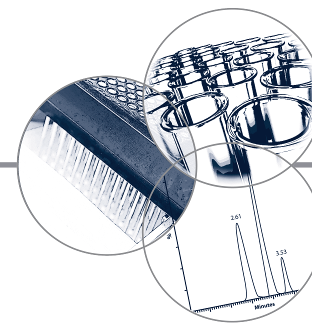

Research Article

Analysis of Salvinorin A in urine using microextraction in packed syringe and GC–MS/MS

Background: The aim of this work was to develop and validate a method for the determination of Salvinorin A in human urine using microextraction by packed sorbent (MEPS) and GC–MS/MS. **Results:** The technique uses a sample volume as low as 0.2 ml, and the analyte was extracted using a C18 sorbent. The method showed to be linear between 20 and 1000 ng/ml and presented a LOD of 5 ng/ml. Intra- and inter-day precision and accuracy were acceptable. Absolute recoveries ranged from 71 to 80%. **Conclusions:** GC–MS/MS with MEPS showed to be a fast and simple procedure for the quantification of Salvinorin A in urine. This is the first time that GC–MS/MS with MEPS was used for the determination of this compound in biological fluids. Furthermore, the device could be reused for up to 80 extractions, which accounted for a lower cost of analysis.

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The plant ***Salvia divinorum***, a powerful psychoactive herb, is a rare member of the *Lamiaceae* (mint) family [1–6]. Originally, the plant was used by Mazatec Indians of Mexican state of Oaxaca [3,7–9] for medical purposes, including headaches, rheumatism, abdominal swelling or diarrhoea, as well as for non-medical practices [2–6,8,10–12]. This herb is also called 'magic mint', 'diviner's sage,' 'mystic sage', 'ska Maria', 'ska Pastora', 'hierba de Maria', 'hojas de la Pastora', all referring to the Mazatec belief that *S. divinorum* is the incarnation of the Virgin Mary [4,5,11]. Today, *S. divinorum* has become increasingly popular among young adults and adolescents as a recreational drug due to its hallucinogenic effects, and it is available to users in the so-called 'smart shops' (which are widespread in Europe, and sell hemp food, dietary supplements and plant extracts with supposed nutritional and healthy properties) or websites [13]. The use of *S. divinorum* is not banned in most countries, because neither the plant nor any of its constituents are listed in the controlled substances lists [11,12]. The main psychoactive component has been identified as **Salvinorin A** [1–4,7,10,12,13] primarily in the leaves, and to a lesser extent in the stems [1,7]. It is an extremely potent and non-nitrogenous selective κ-opioid receptor agonist [1,3–7,10,11,13,14]. This receptor may be an important therapeutic target for analgesia and neuroprotection, among others [15]; however, Salvinorin A does not activate the serotonin 2A receptor, which mediates the effects of other scheduled hallucinogens [1–4,7].

S. divinorum also contains other structurally related compounds, including Salvinorins B–G, which occur in the plant at lower concentrations than Salvinorin A [1]. The plant is typically consumed by smoking the dried leaves, although chewing or ingesting teas are also used [4,11]. The onset of action is relatively rapid, on the order of 30 s for smoking and 5–10 min for buccal absorption. Inhaled doses of 200–500 µg in humans produce profound hallucinations lasting up to 1 h [4,11]. Its effect is reported to be qualitatively distinct in structure and mechanism of action from that of both naturally-occurring (*N,N*-dimethyltryptamine, psilocybin and mescaline) and synthetic (lysergic acid diethylamide, 4-bromo-2,5-dimethoxyphenylisopropylamine and ketamine) hallucinogens [2–4,12]. Salvia is often compared with cannabis due to its similar effects, which include perceptions of bright light, vivid colors and shapes, body and object distortions, dysphoria, uncontrolled laughter, a sense of loss of body, overlapping realities, hallucinations, incoordination, dizziness, slurred or incoherent speech, depersonalization and unconsciousness [5,7,11,14].

GC–MS [1,9,11] or GC×GC–TOF-MS [8] and LC–MS [10,14] or LC–MS/MS [16] have been applied to the quantitative analysis of Salvinorin A from biological fluids. SPE has been utilized with some success [14]; however, this sample preparation technique is time-consuming and requires relatively large amounts of organic solvents. For these reasons, the use of microextraction techniques, such as **microextraction**

Key Terms

***Salvia divinorum*:** Hallucinogenic plant from the mint family that has been used in traditional spiritual practices for its psychoactive properties.

Salvinorin A: Psychoactive ingredient of *Salvia divinorum*. It is a highly potent κ-opioid receptor agonist, and the most potent naturally occurring hallucinogen isolated so far.

Microextraction by packed sorbent: New technique for miniaturized SPE that can be connected online to analytical instruments, such as gas or liquid chromatographs.

by packed sorbent (MEPS) seems appealing. This technique can be regarded as a miniature version of conventional SPE, and uses a gas-tight syringe for extraction [17–19]. MEPS is very promising because the device can be reused several times (over 100 extractions using plasma or urine samples have been reported) [19–21], is fast (1–3 minutes), with lower consumption of organic solvents, being therefore more environmentally friendly. Simultaneously, the costs of analysis are reduced when compared with conventional SPE procedures. This extraction technique has been used in bioanalysis, namely for the quantification of antidepressants [22], risperidone [23], piperazines [17,19], methamphetamine and amphetamine [18], local anesthetics [24] and neurotransmitters [25] in several biological specimens.

In this paper we describe a novel and sensitive method for the quantification of Salvinorin A in human urine using a combination of MEPS and GC–MS/MS.

Experimental

Reagents & standards

The analytical standard of Salvinorin A was purchased from LGC Promochem (Barcelona, Spain). Methanol (Merck Co., Darmstadt, Germany), 2-propanol (Panreac, Barcelona, Spain), acetonitrile (Fisher Scientific, Leicestershire, UK) and formic acid (Merck Co.) were of HPLC grade. The internal standard (quinalphos; IS) was obtained from Sigma–Aldrich (Sintra, Portugal). Deionized water was obtained from a Milli-Q System (Millipore, MA, USA). MEPS 250 µl syringe and MEPS C18 BIN (Barrel insert and Needle) (SGE Analytical Science, Victoria, Australia) were purchased from ILC (Porto, Portugal). Stock solution of salvinorin A was prepared at 1 mg/ml by weighing 1 mg of the compound to a 1 ml volumetric flask, and filling up to volume with methanol. Working solutions were prepared by proper dilution of the stock solution with methanol to the final concentrations of 4, 0.4 and 0.04 µg/ml. A working solution of the IS at 10 µg/ml was prepared also in methanol. All those solutions were stored in the absence of light at 4°C.

Biological specimens

Drug-free urine samples used in all experiments were provided by laboratory staff and stored at 4°C. An informed consent was obtained from the volunteers involved in the study.

Authentic urine samples in which there was a suspicion of consumption of *S. divinorum* were also analyzed. Those samples were obtained from hospital services following clinical observation of eventually intoxicated patients.

GC & MS conditions

For chromatographic analysis, a HP 7890A GC system (Agilent Technologies, Waldbronn, Germany), equipped with a model 7000B triple quadrupole mass spectrometer (Agilent Technologies), a MPS2 autosampler and a PTV-injector from Gerstel (Mülheim an der Ruhr, Germany) was used. Separation of the analytes was achieved using a capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness) with 5% phenylmethylsiloxane (HP-5 MS), supplied by J & W Scientific (CA, USA).

The oven temperature started at 70°C for 3 min, followed by an increase of 30°C/min to 300°C and held for 10 min. The temperatures of the injection port and the ion source were set at 250°C and 280°C, respectively. Helium was used as carrier gas with a constant flow rate of 0.8 ml/min.

The mass spectrometer was operated with a filament current of 35 µA and electron energy 70 eV in the positive electron ionization mode. Nitrogen was used as collision gas at a flow rate of 2.5 ml/min. Data were acquired in the SRM mode, using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies).

The transitions were chosen based on selectivity and abundance in order to maximize the signal-to-noise ratio in matrix extracts. The transitions chosen for Salvinorin A were 272.1 > 121.1 (quantitative transition) and 93.0 (qualitative transition) using a collision energy of 10 eV, and its retention time was 16.26 min. For IS the transition 296.8 > 156.0 (collision energy of 10 eV) was used, and the retention time was 10.32 min.

Sample preparation

Urine samples (0.2 ml) were diluted with 0.3 ml of deionized water and spiked with 20 µl of the IS working solution. The mixture was homogenized by rotation/inversion movements for 15 min and stored light-protected until extraction by MEPS.

Before using for the first time, the sorbent was activated by aspirating and eliminating 5 × 0.25 ml of methanol through the device, and then conditioned likewise with 4 × 0.25 ml of water. The samples were afterwards withdrawn

and passed through the device six times (at a flow rate of 10 µl/s) without discarding them. Endogenous interferences were removed with 0.15 ml of 8% 2-propanol in 2% formic acid; the analyte was finally eluted with 50 µl of methanol:acetonitrile (7:3, v/v). Carryover was evaluated by analyzing a blank sample after an extraction of the highest concentration. Following this procedure, no carryover was observed. Nevertheless, after each extraction, the sorbent was washed sequentially with 5 × 0.25 ml methanol and 4 × 0.25 ml water, in order to prevent an eventual carryover. The extracts were evaporated to dryness, and were dissolved in 50 µl of methanol, transferred to autosampler vials and an aliquot of 2 µl was injected into the GC–MS/MS instrument in the splitless mode.

■ Validation procedure

The procedure was validated in terms of selectivity, linearity and limits, intra- and inter-day precision and accuracy, absolute recovery and stability.

Selectivity was evaluated by analyzing blank urine samples of ten different origins (laboratory staff). Each of these samples was extracted and analyzed by the described procedure, in order to assess potential interferences from endogenous components. QC samples were prepared and analyzed contemporaneously. Identification criteria included an absolute retention time within 2% (±0.1 min) of the retention time of the analytes in the control samples and the existence of two transitions for each compound. The maximum allowed tolerances for the relative ion intensities between the two transitions (as a percentage of the base peak) were as follows: considering the relative ion intensity in the control sample, if this value was higher than 50%, between 25 and 50% or 5 and 25%, or lower than 5%, tolerances of ±10, ±20, ±5 or ±50%, were accepted, respectively [101]. The method would be considered selective if no analyte could be identified in the blank samples by means of those criteria.

Calibration data were generated by spiking blank urine samples in concentrations ranging from 20 to 1000 ng/ml (six calibrators evenly distributed, with no replicates: 20, 100, 200, 600, 800 and 1000 ng/ml), which were analyzed by the described protocol. Five calibration curves were prepared, the acceptance criteria included a R^2 value equal or higher than 0.99, and accuracy within a ±15% interval, except at the LLOQ, for which ±20% was considered acceptable. Together with each calibration curve, a zero sample (blank sample spiked with IS) and two QC samples at low (LQC: 50 ng/ml) and medium (MQC: 400 ng/ml) concentrations (n = 3) were also analyzed.

The LLOQ was defined as the lowest analyte concentration that could be measured both precisely (%CV of less than 20%) and accurately (RE within ±20% of the nominal concentration), and was determined by analyzing five replicates of spiked samples (prepared separately from those used for calibration). The LOD was defined as the lowest concentration that yielded a minimum signal-to-noise ratio of three.

Intra-day precision was evaluated in terms of RSD (%) by analyzing five replicates of spiked urine samples at four different concentrations (20, 100, 400 and 1000 ng/ml) on the same day. Interday precision was evaluated at six concentrations (20, 100, 200, 600, 800 and 1000 ng/ml) during 5 days. The accuracy of the method was characterized in terms of the mean RE between the concentrations measured using the calibration equation and the spiked concentration; the accepted limit was 15% for all concentrations, except at the LLOQ, where 20% was considered acceptable. Intermediate precision and accuracy (combined intra- and inter-day precision and accuracy) were calculated using the LQC and MQC samples (a total of 15 measurements were performed for each concentration).

Absolute recovery was determined by analyzing spiked samples at 50, 400 and 800 ng/ml (n = 6). Simultaneously, blank extracts were spiked with the same amount of analyte. In all these samples, the IS was added only after extraction to the elution solvent, in order to allow for peak area ratio comparison between extracted and nonextracted (neat standard) samples.

Stability of Salvinorin A was evaluated using QC samples, spiked at the above-mentioned concentrations of LQC and MQC, which were extracted using the MEPS procedure (n = 3). To study the stability in processed samples, the extracts were stored at room temperature in the autosampler for 24 h. These samples were analyzed and the peak–area ratios compared with the ones obtained by analysis of freshly prepared samples. Short-term stability was assessed at the same concentrations (n = 3). Urine samples were spiked and left for 24 h at room temperature, after which they were extracted and compared with freshly spiked samples. Freeze–thaw stability was assessed as follows. Urine samples

were spiked at the intended concentrations, and stored 24 h at -20°C, after this period, they were thawed at room temperature, unassisted. After completely thawed, the samples were frozen once again for 12–24 h under the same conditions. This freeze–thaw cycle was repeated three times for each sample, after which the samples were analyzed. The obtained peak areas were compared with those obtained by analysis of freshly prepared samples. For each stability study, the analyte was considered stable if the %CV between the two sets of samples were below 15%.

Results & discussion

Method validation

The method was fully validated following a 5-day validation protocol. The evaluated parameters included selectivity, linearity and LOD and LOQ, intra- and inter-day precision and accuracy, recovery and stability. The entire validation was performed according to the guiding principles for bioanalytical method validation of the US FDA [102] and ICH [103].

Selectivity

Based on the above-mentioned criteria for positivity, the analyte was successfully and unequivocally identified in all the QC samples, whereas in the blank samples no interfering peaks could be detected and/or misidentified as being the analyte. Therefore, the method was considered selective for Salvinorin A determination in urine. FIGURE 1 shows a representative ion chromatogram of a spiked urine sample, and FIGURE 2 represents a chromatogram obtained by the analysis of a blank urine sample.

Calibration curves & limits

Calibration curves were obtained by plotting the peak–area ratio between the analyte and the IS versus the analyte concentration. Since the adopted calibration range was wide and in order to compensate for heterocedasticity, weighted

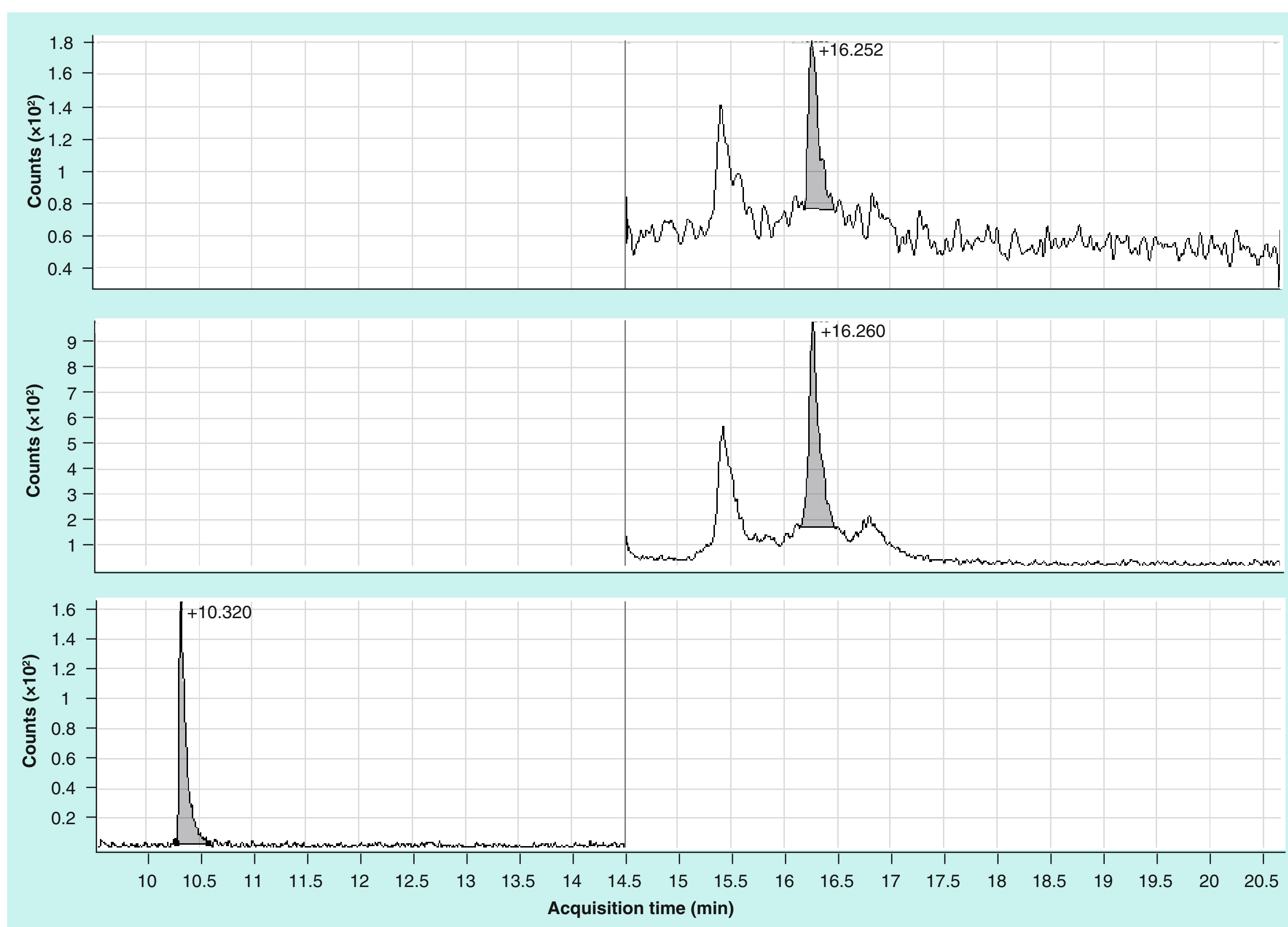

Figure 1. Ion chromatogram of a spiked sample. Salvinorin A (5 ng/ml) and IS (1 µg/ml).

least squares regression had to be adopted. Six weighting factors were evaluated (1/√x, 1/x, 1/x^2, 1/√y, 1/y, 1/y^2), and based on the data obtained during the assessment of interday precision and accuracy, the factor that originated the best result was chosen [26]. This choice was performed by calculating the mean RE for each factor, and summing the absolute value. The factor that showed the lowest sum of errors and presented simultaneously a mean R^2 value of at least 0.99 was chosen (Table 1). Using this weighting factor, linear relationships were obtained, and the calibrators' accuracy (mean relative error [bias] between measured and spiked concentrations)was within the above-mentioned criteria (within ±15% for all calibrators, except at the LLOQ, where ±20% was considered acceptable). Calibration data is shown in Table 1.

The obtained LLOQ for the analyte was found to be 20 ng/ml. The method's LOD was 5 ng/ml.

The obtained limits are satisfactory, especially if compared with those obtained by other authors. For instance, Barnes *et al.* have obtained the same values (5 ng/ml), however using a higher sample volume (20 ml) and GC × GC–TOF-MS [8]. Pichini *et al.*, using GC–MS, were able to detect 5 ng/ml, with a LLOQ of 15 ng/ml, yet using a larger sample volume (1 ml) [11]. This fact shows the high selectivity of MS/MS, allowing the reduction of the background noise normally observed in bioanalysis. This also leads to an increase in sensitivity, and lower amounts of the analytes can be detected.

Intra- & inter-day precision & accuracy

Regarding intraday precision and accuracy the obtained CVs were typically below 11% at all concentrations, while the relative errors lower than ±9% (Table 2). In what concerns interday precision and accuracy, the obtained CVs were lower than 8% for all concentrations, while accuracy was lower than ±8%. These data are shown in Table 2. Concerning intermediate precision using QC samples, the obtained CVs were typically below 10%, while the measured

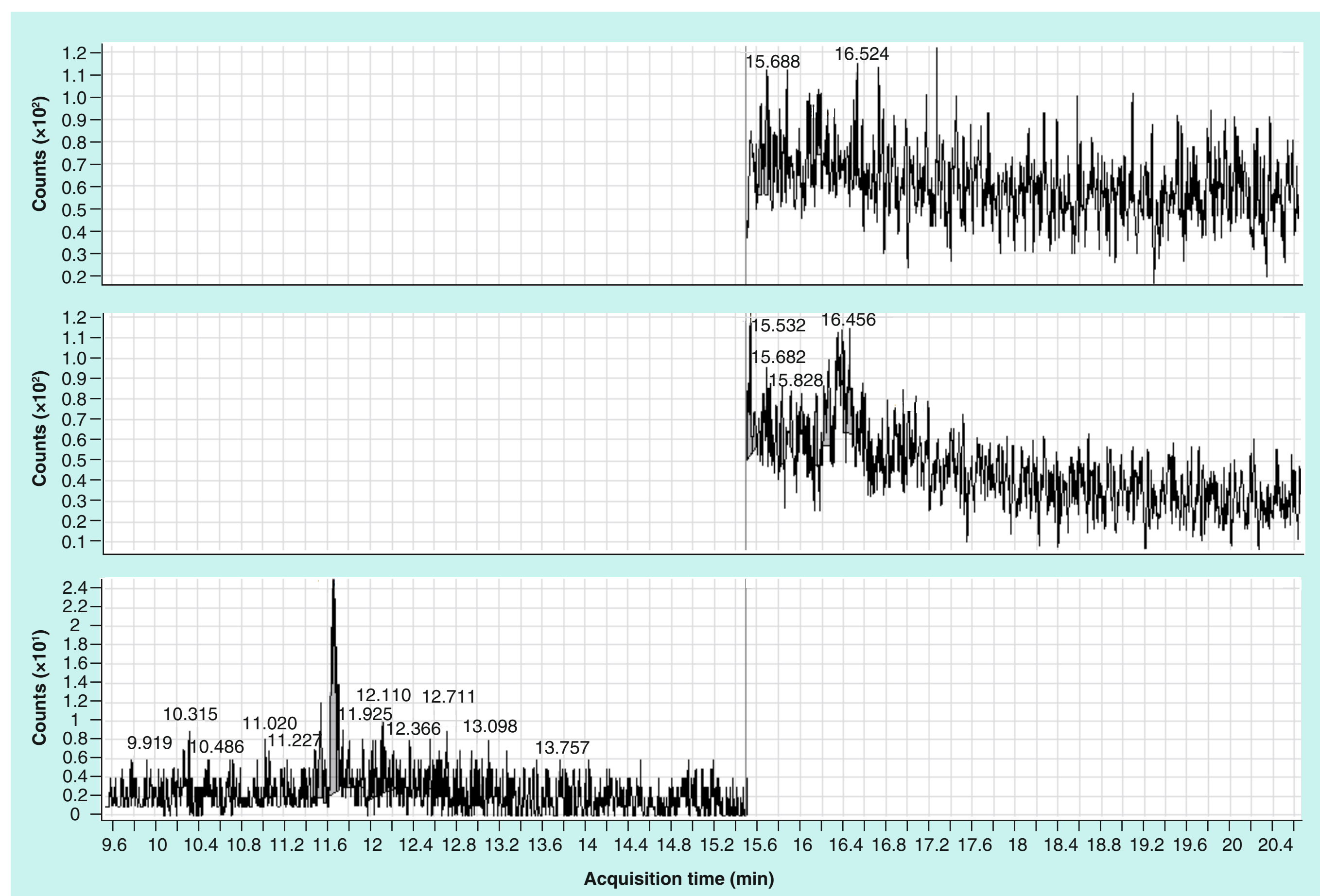

Figure 2. Ion chromatogram of a blank sample.

Table 1. Linearity data (n = 5).

Weight	Linear range (ng/ml)	Linearity		R^2	LOD (ng/ml)
		Slope	Intercept		
1/y	20–1000	0.0002 ± 3.44E-05	-0.0003 ± 0.0002	0.9979 ± 0.0009	5

Table 2. Intra- and inter-day precision and accuracy (n = 5)[†].

Spiked	Measured		%CV		RE[‡] (%)	
	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day
20	21.13 ± 1.61	20.52 ± 1.61	7.60	7.83	4.88	2.05
100	94.40 ± 6.87	92.43 ± 4.08	7.27	4.41	-6.35	-8.36
200	192.17 ± 11.92	–	6.20	–	-0.16	–
400	–	365.28 ± 11.18	–	10.40	–	-5.38
600	599.59 ± 9.81	–	1.64	–	0.85	–
800	799.48 ± 9.41	–	1.18	–	0.92	–
1000	999.13 ± 10.10	1025.08 ±3.43	1.01	4.72	0.24	4.78

[†]*All concentrations in ng/ml.*
[‡]*(measured concentration - spiked concentration/spiked concentration) × 100.*

concentrations were within a ±12% interval from the target concentration for both studied levels.

Extraction recovery

Using the aforementioned approach the absolute recoveries at 50, 400 and 800 ng/ml were respectively 80.15% ± 0.01; 73.11% ± 0.24 and 71.91% ± 0.06.

Schmidt *et al.* reported recoveries ranging 104–106% for Salvinorin extraction from urine using SPE [14], while Pichini *et al.* reported 93% using liquid–liquid extraction [11]. However, an adequate comparison with our results cannot be done since the determination of Salvinorin A in urine samples with MEPS has not yet been reported in literature.

Stability

Concerning processed samples stability, the obtained CVs were lower than 6%, indicating that the analyte is stable at least for 24 h in the autosampler under room temperature.

The obtained CVs concerning short-term stability were lower than 11%, indicating that the analyte is stable at least for 24 h in the samples at room temperature.

Regarding teh stability of freeze–thaw samples, after comparison of the analyzed samples with freshly prepared ones we found that Salvinorin A was stable for at least 3 freeze–thaw cycles since the obtained CVs were lower than 7%.

Those data related to stability allow for sample analysis to be carried out within a comfortable time window (e.g., since the arrival at the laboratory), since the analyte is not significantly affected by the storage conditions.

Method applicability

The developed method is being used routinely for the determination of the analyte in suspected intoxicated individuals. Five urine samples have been analyzed so far, but none were positive for Salvinorin A. Taking into consideration our detection limits, it is likely that the individuals didn't consume Salvinorin A, or it had been consumed a long time before urine collection.

Method performance

The combination of MEPS as extraction technique with GC–MS/MS has shown to be adequate for the determination of this compound in urine samples, achieving good limits of detection and high recoveries. Indeed, good results were obtained when compared with other published papers on Salvinorin A determination, using only a small volume of sample. In addition, when compared with other conventional extraction techniques, MEPS is faster and uses much lower amounts of organic solvents, minimizing environmental problems. Another advantage is that the extracting adsorbent can be reused several times, minimizing the costs of analysis.

Conclusion

A simple and fully validated method is described for the determination of Salvinorin A in human urine samples, by means of MEPS and GC–MS/MS. The method was found to

be linear within the adopted range, and presented adequate precision and accuracy. The low sample volume provides a considerable advantage, mainly when small amounts of sample are available, allowing the use of the same sample for further analysis. Therefore, this method is suitable for laboratories performing regular urine analysis in the field of forensic toxicology. Moreover, this is the first study describing the identification and quantification of Salvinorin A in urine samples by means of MEPS and GC–MS/MS.

Future perspective

In recent years the use of the so-called 'legal-highs' has increased among young people due to the hallucinogenic properties of these drugs and to the fact that the acquisition of preparations containing the active ingredients is legal in many countries. This has led to the widespread use of 'smart shops' (also on the internet) that make the compounds available. From the most used hallucinogenic compounds, *S. divinorum* plays an important role, and its use is likely to increase in the next years. Therefore, forensic toxicology laboratories must always be one step ahead and must develop new methods for the detection of the compounds in biological samples. In this paper we report a novel and rapid method using MEPS and GC–MS/MS for the identification and quantification of the plant's major active compound Salvinorin A in human urine samples, which can be successfully used in forensic scenarios where the compound is involved.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Executive summary

First use of microextraction by packed sorbent (MEPS) coupled with GC–MS/MS method for the quantitative analysis of Salvinorin A in human urine samples

- Rapid sample preparation using MEPS for the GC–MS/MS quantification of Salvinorin A in urine samples.
- Useful method for application in laboratory routine for rapid assessment of situations where the compound is involved, both in clinical and forensic scenarios.

Method validation

- The method was fully validated according to internationally accepted guidelines for bioanalytical method validation, including selectivity, linearity and limits, intra- and inter-day precision and accuracy, recovery and stability.
- The obtained values for the different studied parameters were considered adequate, and therefore the method was found suitable for application in routine analysis.

Method performance

- Combination of MEPS as extraction technique with GC–MS/MS has shown to be adequate for the determination of this compound in urine samples, achieving good LOD and high recoveries.
- MEPS is faster than conventional extraction procedures, and uses much lower amounts of organic solvents, thus minimizing environmental problems. Furthermore, the extracting adsorbent can be reused several times, thereby minimizing the costs of analysis.

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Article III

Determination of ketamine and its major metabolite, norketamine, in urine and plasma samples using microextraction by packed sorbent and gas chromatography-tandem mass spectrometry

Ivo Moreno, Mário Barroso, Ana Martinho, Angelines Cruz, Eugenia Gallardo

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Determination of ketamine and its major metabolite, norketamine, in urine and plasma samples using microextraction by packed sorbent and gas chromatography-tandem mass spectrometry

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ABSTRACT

Ketamine is a club drug widely abused for its hallucinogenic effects, being also used as a “date-rape” drug in recent years. We have developed an analytical method using gas chromatography-tandem mass spectrometry (GC–MS/MS) for the identification and quantification of ketamine and its major metabolite in urine and plasma. No derivatization step is needed to accomplish analysis. The compounds were extracted from 0.25 mL of sample using microextraction by packed sorbent on mixed mode (M_1) cartridges. Calibration curves were linear in the range of 10–250 ng/mL for urine and 10–500 ng/mL for plasma, with determination coefficients higher than 0.99. The limit of detection (LOD) was 5 ng/mL for both compounds in both specimens. Recoveries ranged from 63 to 101%, while precision and accuracy were below 14% and 15%, respectively. These low limits of detection and the quite high recoveries obtained, in very low sample amounts, allow detecting small quantities of the compounds, making this procedure suitable for those laboratories performing routine analysis in the field of forensic toxicology. Compared with existing methods, the herein described procedure is fast, since no derivatization step is required, and cost effective for the quantification of ketamine and norketamine in biological specimens by gas chromatography.
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1. Introduction

Club drugs are generally used at nightclubs and music festivals to increase sensory stimulation and social intimacy [1,2]. Ketamine (K), one of the most widely used club drugs and a parenterally administered anesthetic agent, possesses sedative, amnesic and analgesic properties at sub-anesthetic doses [1–8]. Due to the rapid onset of effects and short duration of action, it has been used primarily as a veterinary anesthetic and in short-term surgical procedures in humans [2,6,9–16]. Ketamine is metabolized in the liver by microsomal cytochrome P450 system, yielding at least two compounds of pharmacological interest: norketamine (NK) and dehydronorketamine (DHNK). NK, the main metabolite, is produced via *N*-demethylation of K [7,9,17], and is converted to dehydronorketamine by dehydrogenation. The latter is then conjugated with glucuronic acid and eliminated in urine [3,6,9,17]. These two metabolites may contribute to the pharmacological effect of K, especially NK [3,6], which has shown anesthetic potency equal to 20–35% of that of K, probably contributing to the long-lasting anesthetic effects [4,7,16]. Moreover, ketamine can block the *N*-methyl-D-aspartate receptor, originating several post-hypnotic emergence reactions, for instance prolonged hallucinations and delirium [15,16]. K has been initially abused by medical personnel for its hallucinogenic effects, becoming gradually popular on the European party scene in the early 1990s, spreading afterwards to other parts of the world [9,18]. Ketamine is abused by an increasing number of teenagers as a “club drug”, and is often available at “raves” and parties [16,17,19]. It is also easily accessed in the black market, appearing as a “safe” and “clean” drug, being available as either a powder or a liquid [2,20,21]. Furthermore, since K is flavor- and odorless, it can be used to induce amnesia and facilitate sexual assaults after unknowingly spiked into a victim’s drink [22–24]. At higher doses, K produces narcotic effects, similar to those of phencyclidine, and hallucinogenic effects, mimicking LSD use [18,25]. It also produces kind of out-of-body or near-death experiences, as well as vivid dreams and

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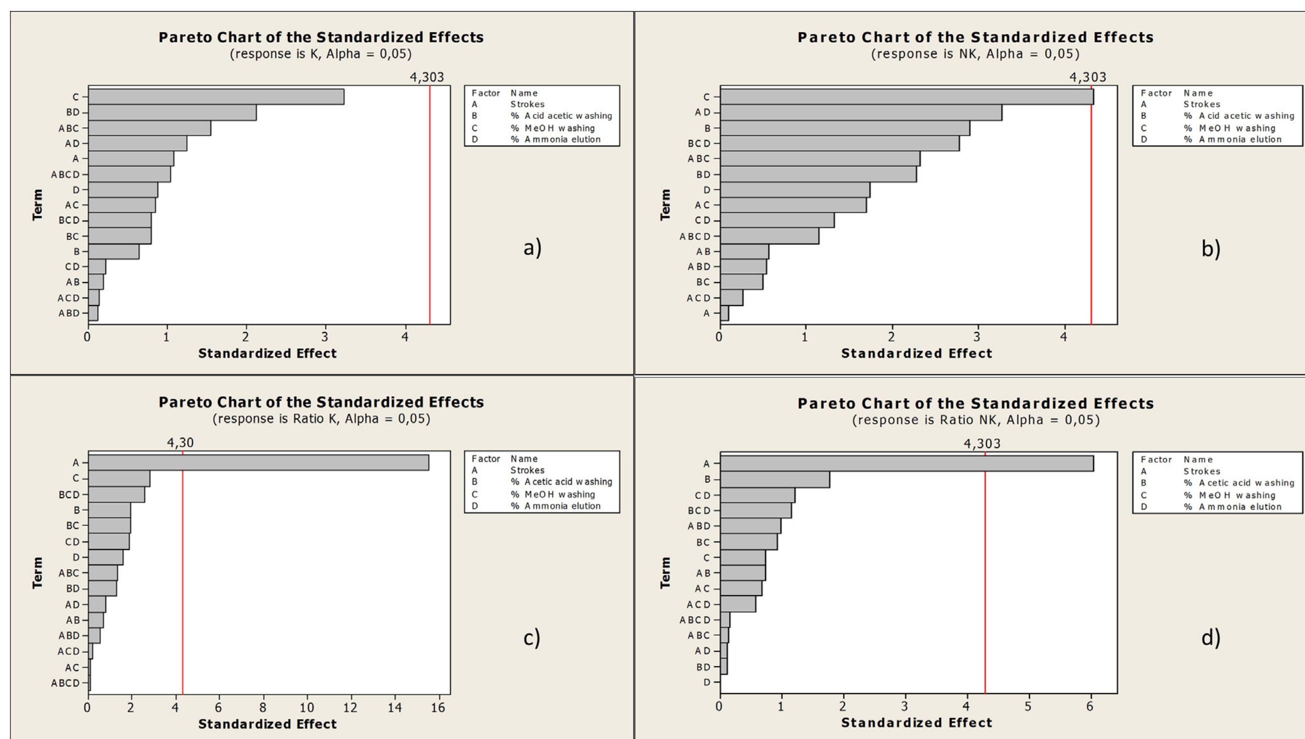

Fig. 1. Pareto Chart illustrating the factors that influence the extraction process for each compound individually, (a) ketamine and (b) norketamine in urine; (c) ketamine and (d) norketamine in plasma.

delirium [10,16]. The recreational use of K as a "rave", party, and nightclub drug has led to an increased public concern about its potential hazards [17,23]. In order to increase the ability and scope of drug testing, effective screening and confirmation methods for the determination of K and metabolites in biological specimens are needed. This would be of benefit not only for clinical research, but also for forensic analysis. Several methods have been reported for the determination of K and metabolites in various biological fluids, namely plasma [3,7,18,26] and urine [2,17,18,24,25,27,28–30], using gas chromatography (GC) coupled to either mass spectrometry (MS) [2,18,24,30,31], tandem mass spectrometry (MS/MS) [11,12] or flame ionization detector (FID) [32]; or liquid chromatography (LC) with UV [3,5], MS [6] or MS/MS [22,23,25,27,33,34] detection. Sample preparation is usually performed by means of liquid–liquid extraction (LLE) [5,10,35] or solid-phase extraction (SPE) [7,17,23] procedures. However, those sample preparation techniques are often time-consuming, laborious and expensive; moreover, they present deleterious effects on the environment as well, due to the high amounts of organic solvents that need to be used and discarded. For these reasons, microextraction techniques, such as microextraction by packed sorbent (MEPS), appear to be a good alternative, helping overcoming those problems [36–38]. This recent approach for sample preparation is based on the miniaturization of conventional SPE, and can easily be connected on-line to either gas or liquid chromatographic systems, without the need of modifying the extracting device [37–39]. Another advantage is the reduction of sample amount and organic solvent consumption. In addition, the device can be reused several times, and more than 100 extractions have been reported using plasma or urine samples; by comparison, conventional SPE cartridges are recommended for single use only [36,39]. MEPS has been used in bioanalysis, namely for the quantification of antidepressants [40], risperidone [41], piperazines [37,38], methamphetamine and amphetamine [42], local anesthetics [43], neurotransmitters [44], antipsychotic drugs [45] and salvinorin A [46] in various biological specimens.

This paper describes a method for the identification and quantification of K and its major metabolite NK in human plasma and urine by means of GC–MS/MS. A microextraction method was used for sample preparation, yielding cleaner extracts, thus minimizing matrix effects and prolonging the life of analytical instrumentation.

2. Materials and methods

2.1. Chemicals and materials

The reference standards, K and NK, as well as their deuterated analogues (K-*d4*, NK-*d4*) were purchased from LGC Promochem (Barcelona, Spain) as methanolic solutions at 1 (K, NK) and 0.1 mg/mL (K-*d4*, NK-*d4*). Acetic acid (50% purity) was acquired from Sigma–Aldrich (Lisbon, Portugal), methanol from Merck (Darmstadt, Germany), and ammonium hydroxide (analytical grade) from J.T. Baker (Holland). Deionized water was obtained from a Milli-Q System (Millipore, Billerica, MA). MEPS 250 μL syringe and MEPS BIN (Barrel insert and Needle) M_1 (4 mg; 80% C_8 and 20% SCX) from SGE Analytical Science were purchased from ILC (Porto, Portugal).

2.2. Stock and working solutions

Working solutions at 1, 0.4, and 0.1 μg/mL of K and NK were prepared by direct dilution of stock solutions with methanol. A working solution of the internal standards (IS) (K-*d4*, NK-*d4*) at 1 μg/mL was prepared also in methanol. All those solutions were kept in amber glass vials and stored light protected at 4 °C.

2.3. Biological specimens

Fresh human plasma was obtained from the excess supplies of the Instituto Português do Sangue (outdated transfusions) and

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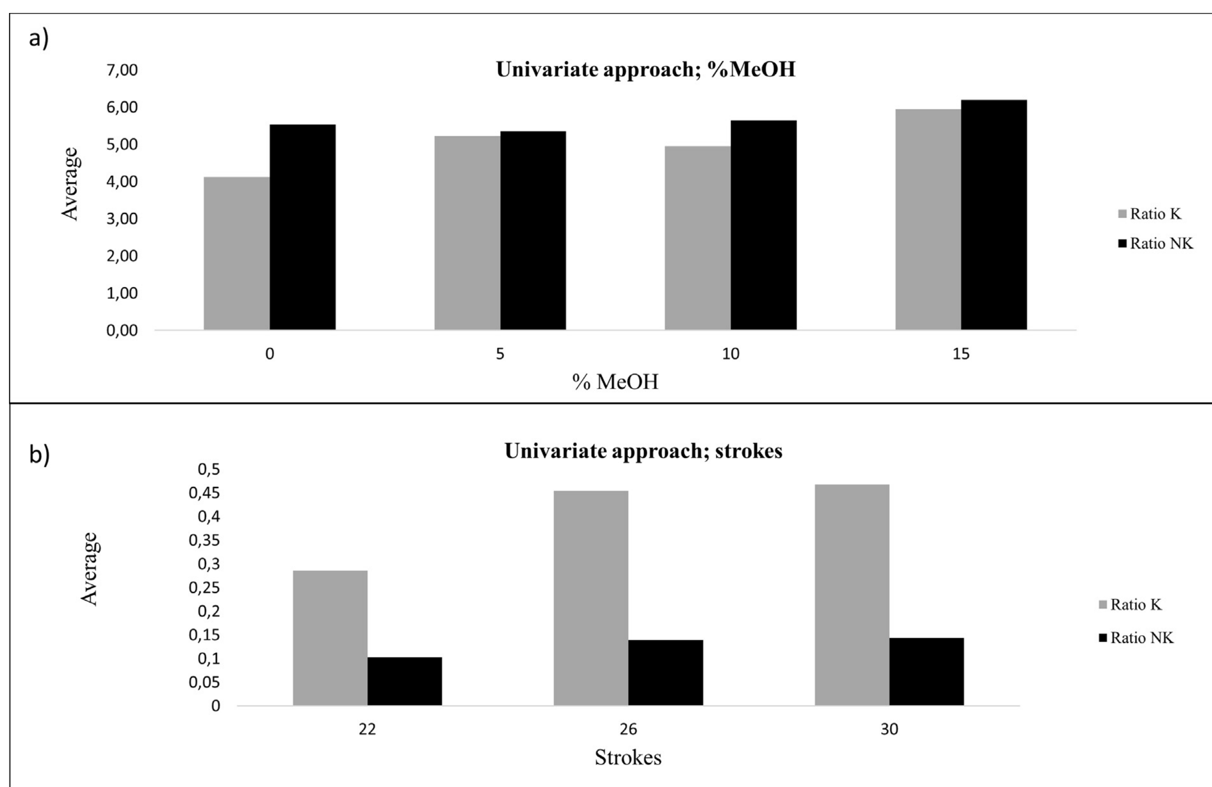

Fig. 2. Univariate approach to (a) % MeOH in urine and (b) strokes for plasma.

drug-free urine samples used in all experiments were provided by laboratory staff. All samples were stored at −20 °C until analysis.

2.4. Sample preparation

Frozen urine and plasma samples were allowed to thaw at room temperature, and were centrifuged at 3500 rpm for 15 min before analysis. The extraction sorbent was activated by aspiration and elimination of 5 × 0.25 mL of methanol through the device, and then conditioned likewise with 4 × 0.25 mL of water before its first use. The extraction of the analytes was previously optimized (see Section 3), and the final extraction conditions were the following (conditions for plasma are presented in square brackets): Ten microliters of the IS solution (1 μg/mL) was added to 0.25 mL of urine [0.25 mL of plasma] previously diluted with 0.25 mL of deionized water [7 mL of phosphate buffer] in a glass tube, and the sample was slightly vortex-mixed for 30 s. Urine samples were afterwards aspirated and passed through the device 8 times [26 times] at an approximate flow rate of 10 μL/s. The number of aspirations for plasma samples was justified because these specimens should be diluted at least 20 times in order not to obstruct the extraction mechanism, and 26 aspirations would be necessary to allow the whole sample to pass through the device. Then the sorbent was washed with 0.25 mL of 5.25% acetic acid [0.1 mL of 0.1% acetic acid] and 0.1 mL of 5% methanol in water [10% methanol] to remove matrix-borne interferences. Finally the analytes were eluted with 0.1 mL of 6% ammonia in methanol [3% ammonia in methanol]. The eluates were evaporated to dryness under a gentle nitrogen stream at room temperature, re-dissolved in 50 μL of methanol, transferred to autosampler vials and an aliquot of 2 μL was injected in the GC–MS/MS instrument in the splitless mode. Carryover was evaluated by analyzing a blank sample after extraction of the highest concentration, and no memory effects were observed. Even though, after each extraction, the sorbent was washed sequentially with 5 × 0.25 mL methanol and 4 × 0.25 mL water, preventing carryover and conditioning the sorbent for the next extraction.

2.5. Gas chromatographic and mass spectrometric conditions

For chromatographic analysis, an HP 7890A GC system Agilent Technologies (Soquimica, Lisbon, Portugal), equipped with a model 7000B triple quadrupole mass spectrometer (Agilent Technologies, (Soquimica, Lisbon, Portugal)), a MPS2 autosampler and a PTV-injector from Gerstel (Soquimica, Lisbon, Portugal) was used. The separation of the studied compounds was performed on an HP-5MSfused-silica capillary column with 30 m × 0.25 mm id, and 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). Helium (purity ≥99.999%) was set at 0.8 mL/min as the carrier gas. A programmed oven temperature was performed as follows: 100 °C (0.5 min), 25 °C/min to 280 °C (7.2 min) and held for 7 min. The total separation time was 14.7 min. The temperatures of the injection port and the ion source were set at 250 and 280 °C, respectively. The mass spectrometer was operated with a filament current of 35 μA and the ionization energy was 70 eV. Multiple reactions monitoring (MRM) mode with electron impact ionization was used in this investigation, using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies). The transitions were chosen based on selectivity and abundance, in order to maximize the signal-to-noise ratio in matrix extracts. Nitrogen (purity ≥99.999%), at a flow rate of 2.5 mL/min, was used as the collision gas. Table 1 summarizes MS data for both analytes.

2.6. Validation procedure

The described method was fully validated according to the guiding principles of the Food and Drug Administration (FDA) [47] and the International Conference on Harmonization (ICH) [48]. The validation was performed following a 5 day validation protocol, and

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Table 1
GC–MS/MS parameters (quantitation transitions underlined).

Compound	Retention Time (minutes)	MRM transition, m/z (collision energy, eV)	Dwell time(μs)
Norketamine	6.78	165.7 → 131.0 (10)	26.8
		165.7 → 148.7 (10)	82.3
Norketamine-d4		169.8 → 135.2 (10)	34.4
Ketamine	6.92	179.7 → 150.8 (10)	22.4
		179.7 → 115.9 (15)	29.9
Ketamine-d4		183.7 → 155.0 (5)	20.8

included selectivity, linearity and limits, intra- and inter-day precision and accuracy, recovery and stability.

The method's selectivity was evaluated by analyzing blank urine/plasma samples of ten different origins, to investigate the potential interferences at the retention times and selected transitions of the studied compounds. Samples were pooled and separated in 20 aliquots (ten were analyzed as blanks and ten were spiked with all the analytes); all of the 20 samples were spiked with the IS. Quality control (QC) samples were prepared and analyzed contemporaneously. Identification criteria for positivity included an absolute retention time within 2% or ±0.1 min of the retention time of the same analyte in the control sample and the presence of two transitions per compound. To guarantee a suitable confidence in identification, the maximum allowed tolerances for the relative ion intensities between the two transitions (as a percentage of the base peak) were as follows: if the relative ion intensity in the control sample was higher than 50%, then an absolute tolerance of ±10% was accepted; if this value was between 25 and 50%, a relative tolerance of ±20% was allowed; if it was between 5 and 25%, an absolute tolerance of ±5% was accepted; and, finally, for relative ion intensities of 5% or less, a relative tolerance of ±50% was used [49]. The method would be considered selective if no analyte could be identified in the blank samples by applying those criteria. The linearity of the method was established using spiked samples, prepared and analyzed using the described extraction procedures, in the range of 10–250 ng/mL for urine and 10–500 ng/mL for plasma (five replicates). Calibration curves were obtained by plotting the peak area ratio between each analyte and the IS against analyte concentration. The acceptance criteria included a correlation coefficient (R^2) value of at least 0.99 and the calibrators' accuracy within ±15% (except at the lower limit of quantification (LLOQ), where ±20% was considered acceptable). Together with each calibration curve, a zero sample (blank sample with IS) and three QC samples at low [LQC: 15 ng/mL (urine); 20 ng/mL (plasma)], medium [MQC: 125 ng/mL (urine); 250 ng/mL (plasma)] and high [HQC: 215 ng/mL (urine); 450 ng/mL (plasma)] concentrations (n = 3) were also analyzed. The LLOQ was defined as the lowest concentration that could be measured with adequate precision and accuracy, i.e. with a coefficient of variation (CV, %) of less than 20% and a relative error (RE, %) within ±20% of the nominal concentration. The limits of detection (LOD) were determined as the lowest concentrations that showed a discrete peak clearly distinguishable from the blank with a signal-to-noise ratio of at least 3, in which the analytes could be unequivocally identified using the above-mentioned criteria for positivity, and were determined by analyzing five replicates of spiked samples. Intra-day precision was evaluated by analyzing in the same day 6 replicates of blank samples spiked with the studied analytes at 3 concentration levels (50, 100 and 200 ng/mL) for urine and 5 concentration levels (10, 50, 100, 400 and 500 ng/mL) for plasma. Inter-day precision was evaluated at a minimum of six concentration levels within a 5-day period. The accuracy of the method was characterized in terms of the mean RE between the measured and the spiked concentrations; the accepted limit was 15% for all concentrations, except at the LLOQ, where 20% was accepted. For recovery studies, blank samples were spiked with both analytes at three concentrations for urine (50, 100 and 200 ng/mL) and four concentrations for plasma (50, 100, 200 and 500 ng/mL) and analyzed by the described method (n = 3), after which the IS was added. At the same time, blank extracts were spiked with the same quantity of the analytes and IS after elution. The obtained peak area ratios were compared between extracted and non-extracted samples (the latter were used as neat standards). In order to study stability in processed samples, blank specimens were spiked at the above-mentioned LQC, MQC and HQC levels, and extracted using the MEPS procedure (n = 3); extracts were then stored at room temperature in the autosampler for 24 h. Short-term stability was evaluated at the same concentration levels (n = 3); blank samples were spiked and left at room temperature for 24 h. To study freeze/thaw stability, samples were spiked at the above-described concentrations, and stored at −20 °C for 24 h; after this period they were thawed unassisted at room temperature, and then refrozen for 12–24 h under the same conditions. This freeze/thaw cycle was repeated twice more, and after the third cycle the samples were analyzed. The analyzed samples were compared to freshly prepared samples during the entire stability procedure. For each stability study, the analyte was considered stable if the CV between the two sets of samples was below 15%.

3. Results and discussion

3.1. Optimization of the extraction parameters

Using the M_1 sorbent (C_8 and SCX), the factors susceptible of influencing the extraction efficiency (and their respective levels) were studied using the full factorial design approach. These factors included: number of sample aspirations through the device (strokes) (urine: 4 and 10; plasma: 6 and 26), sorbent activation with acetic acid (urine: 0.5 and 10%; plasma: 0.1 and 3%, v/v), amount of methanol in the washing step (urine: 0 and 20%; plasma: 0 and 10%, v/v) and amount of ammonia in the elution solvent (urine: 2 and 10%; plasma: 0.5 and 3%, v/v). This multivariate approach is a powerful statistical tool which allows eliminating those factors with little or no significant influence on the response and analyses the interactions between factors, reducing the number of experiments (which would be much higher if a univariate approach was used). Therefore, less effort is spent to obtain the same amount of data, saving time and money. These results were interpreted considering the Pareto Chart of main effects and linked interactions, which graphically displays the magnitudes of the effects. At the studied levels, the amount of methanol in the washing step (for norketamine in urine) and the number of strokes (for both compounds in plasma) were the only parameters with significant influence on the response (Fig. 1). This allowed us to set all the other factors at the most convenient values (those that originated a better apparent response), and hence to study the influencing factors using a univariate approach. The percentage of methanol in the washing step (for urine) varied from 0 to 15; and the number of strokes (for plasma) varied from 22 to 30. The best results were obtained using 15% methanol (for urine) and 26 strokes [for plasma, since 30 strokes did not significantly improve recovery (Fig. 2)]; therefore, those were chosen as the final optimized conditions for analyte extraction.

3.2. Method validation

3.2.1. Selectivity

Using the above-mentioned criteria for positivity, all the analytes were successfully and unequivocally identified in all the spiked samples, whereas in the blank samples no interfering

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Table 2
Linearity data ($n = 5$).

Sample	Compound	Weight	Linear range (ng/mL)	Linearity		R^{2a}	LOD(ng/mL)
				Slope[a]	Intercept[a]		
Urine	Norketamine	1/x	10–250	0.015 ± 0.001	0.039 ± 0.001	0.996 ± 0.002	5
	Ketamine		10–250	0.037 ± 0.078	0.037 ± 0.112	0.998 ± 0.009	5
Plasma	Norketamine	1/x	10–500	0.018 ± 0.001	0.057 ± 0.066	0.996 ± 0.002	5
	Ketamine		10–500	0.048 ± 0.010	0.005 ± 0.389	0.997 ± 0.001	5

[a] Mean values ± standard deviation.

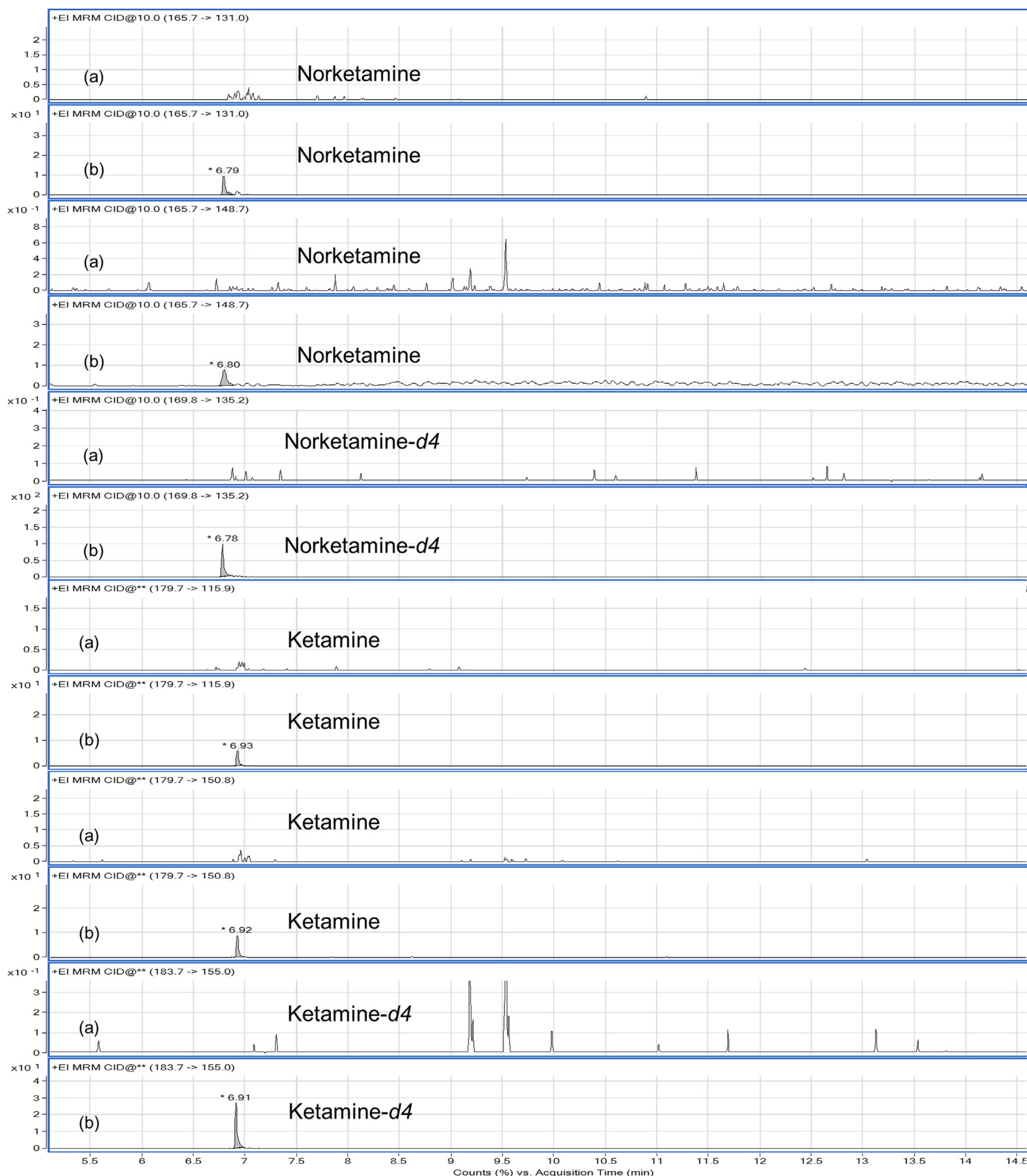

Fig. 3. Comparison between ion chromatograms of a blank urine sample (a) and a spiked urine sample at LOD (b).

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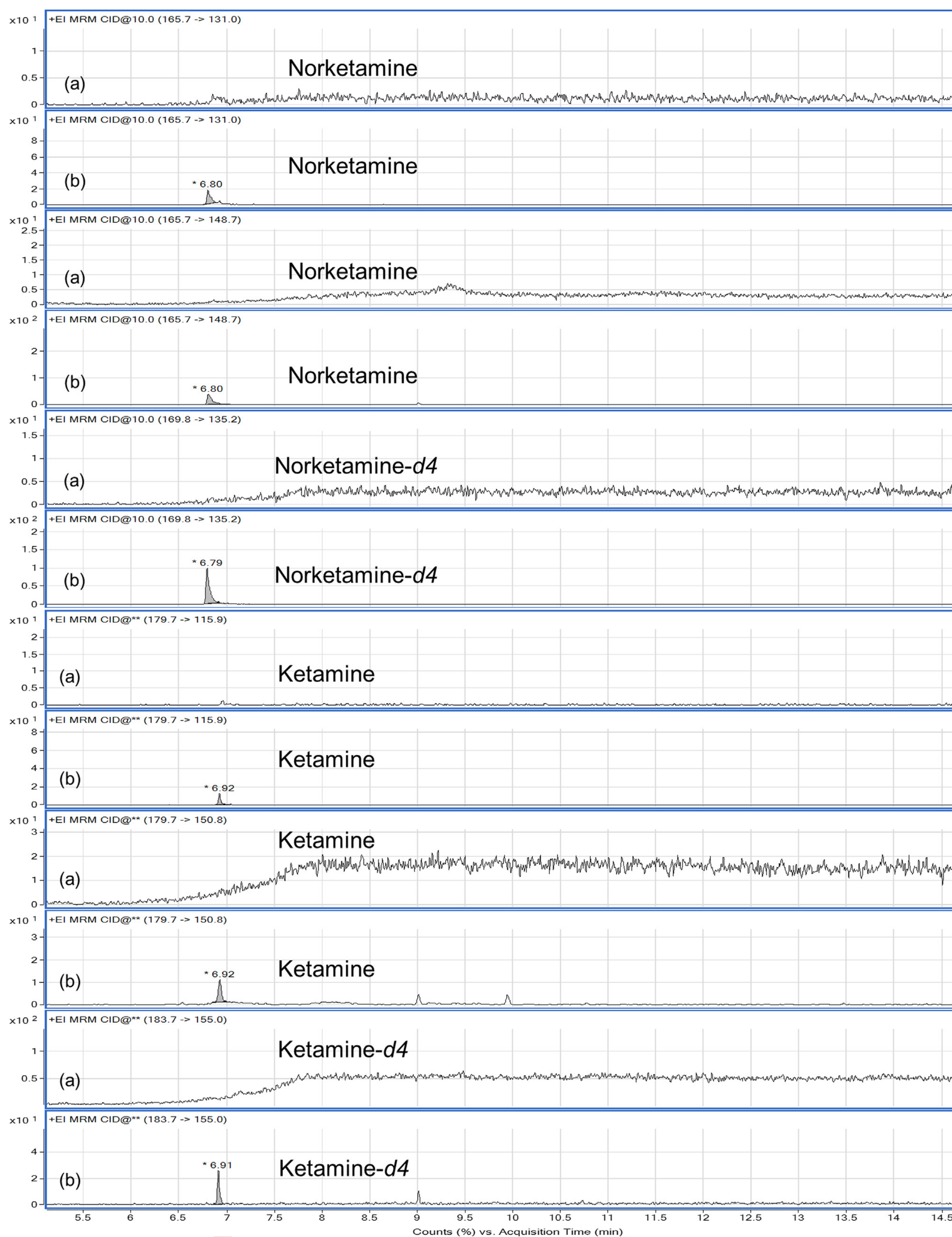

Fig. 4. Comparison between ion chromatograms of a blank plasma sample (a) and a spiked plasma sample at LOD (b).

peaks could be detected and/or misidentified as being the analyte. Therefore, the method was considered selective for K and NK determination. Representative ion chromatograms of a spiked (at the LOD) and a blank sample, for urine and plasma matrices, are shown in Figs. 3 and 4.

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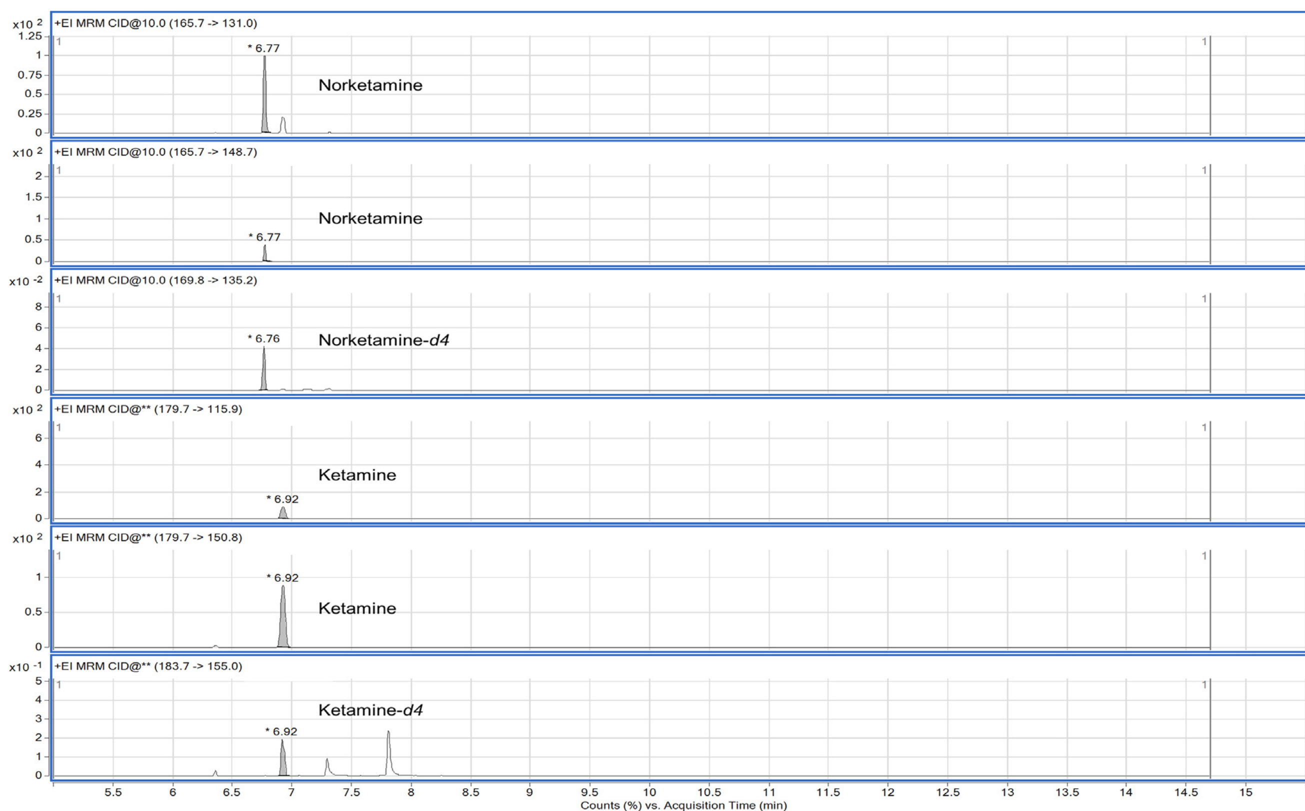

Fig. 5. Ion chromatogram of an authentic urine sample (ketamine 348 ng/mL and norketamine 809 ng/mL).

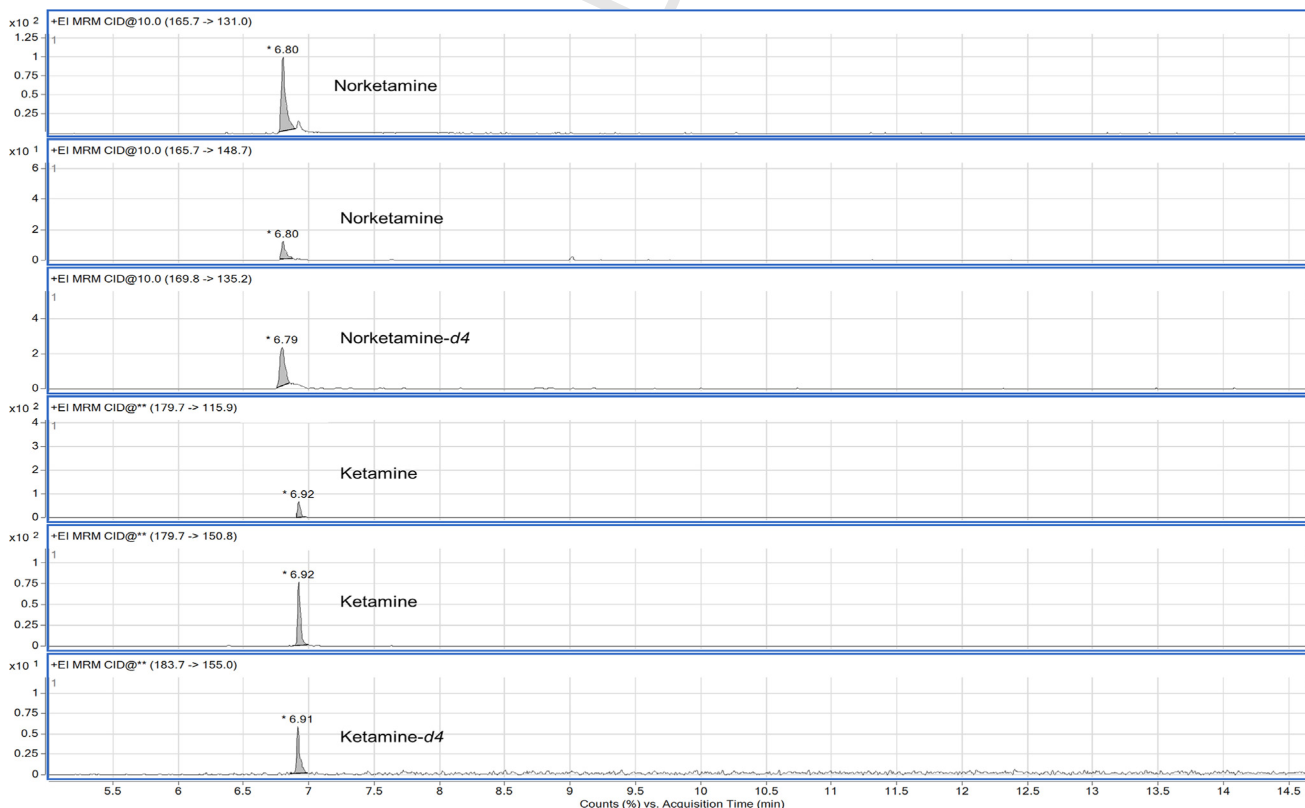

Fig. 6. Ion chromatogram of an authentic plasma sample (ketamine 292 ng/mL and norketamine 211 ng/mL).

3.2.2. Calibration model and limits

The method was linear within the adopted calibration ranges for both analytes; however, since the adopted calibration range was wide and in order to compensate for heteroscedasticity, weighted least squares regressions had to be used. Six weighting factors

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Table 3
Comparison of methods for the determination and quantification of ketamine and metabolites in urine and plasma samples.

Compounds	Matrix	Matrix volume (mL)	Matrix preparation	Detection mode	LOD; LOQ (ng/mL)	Recovery (%)	Ref.
K, NK	UrinePlasma	0.25	MEPS (M1 column)	GC–MS/MS	5; 10	72.5–10.7 (Urine) 63.1–88.9 (Plasma)	This work
K, NK	Urine	1	SPE (DAU columns)	GC–MS	10; 25 (K) 30; 30 (NK)	91.5 (K) 89.8 (NK) 63.4 (DHNK)	[2]
K, NK	Urine	2	SPE (SPEC DAU)	GC–MS (EI)	15; 15 (K) 5; 20 (NK)	71.4–96.5 (K) 68.4–90.1 (NK)	[4]
K, NK	Urine	1	SPE (ZSDAU020)	LC–MS/MS (ESI)	0.6; 1.9 (K) 0.6; 2.1 (NK)	98.3–113.4 (K) 97.9–102.1 (NK)	[17]
K	UrinePlasma	2	LLE	GC–MS (EI)	10; 40	96.0–98.4 (Urine) 94.8–97.4 (Plasma)	[18]
K, NK	Urine	4	SPE (Bond Elut Certify I)	UHPLC–MS/MS (ESI)	0.03; (K) 0.05; (NK)	68.0–72.0 (K) 62.0–65.0 (NK)	[23]
K, NK, DHNK	Urine	2	HF-LPME (polypropylene fiber)	GC–MS	0.25; 0.5 (K) 0.1; 0.5 (NK,DHNK)	85.2–101.0 (K) 86.9–94.3 (NK) 64.6–69.7 (DHNK)	[24]
K	Urine	0.20	SALLE	LC-HR-QTOFMS (ESI)	6; 17	52.0–60.2	[28]
K, NK, DHNK	Urine	0.50	Filtered (0.22-μm filter)	LC–MS/MS	25; 25 (K) 10; 25 (NK) 10; (DHNK)	–	[29]
K	Urine Blood	1	HF-LPME	GC–MS (EI)	2.5; 7.5 (Urine) 2.5; 7.5 (Blood)	81.3–98.6	[30]
K	Urine Blood	1	UA-LDS-DLLME	GC–MS (EI)	1.5; 4.5 (Urine) 2.5; 7.5 (Blood)	89.3–103.4	[30]
K	Urine	3	HF-LPME (polypropylene fiber)	GC-FID	8; 30	82.6–110.4	[32]
K	Plasma	1	SPE (Chromabond Drug)	LC–MS/MS (ESI)	2.5; –	89.0	[51]

DHNK—dehydronorketamine; EI—electron ionization; ESI—electrospray ionization; FID—flame ionization detector; GC—gas chromatography; HF-LPME—hollow fiber liquid phase microextraction; HR-QTOF—high-resolution quadrupole-time-of-flight; K—ketamine; LC—liquid chromatography; LLE—liquid–liquid extraction; LOD—limit of detection; LOQ—limit of quantification; MEPS—microextraction by packed sorbent; MS—mass spectrometry; MS/MS—tandem mass spectrometry; NK—norketamine; SALLE—salting- out liquid–liquid extraction; SPE—solid-phase extraction; UA-LDS-DLLME—ultrasound-assisted low-density solvent dispersive liquid–liquid microextraction; UHPLC—Ultra high performance liquid chromatography.

309 were evaluated for each analyte ($1/\sqrt{x}$, $1/x$, $1/x2$, $1/\sqrt{y}$, $1/y$, $1/y2$), 310 and the one which originated the best results was selected tak- 311 ing into account the data obtained during the assessment of the 312 inter-day precision and accuracy [50]. This choice was performed 313 by calculating the mean RE for each weighting factor and sum- 314 ming the absolute values. The factor that showed the lowest sum 315 of errors and presented simultaneously a mean R^2 value of at least 316 0.99 was chosen (Table 2). This factor was $1/x$ for both analytes. By 317 means of these weighted least squares regressions, linear relation- 318 ships were obtained, and the calibrators' accuracy [mean relative 319 error (bias) between the measured and spiked concentrations] was 320 within a ±15% interval for all concentrations, except at the LLOQ 321 (±20%). Calibration data are shown in Table 2. The method's LOQ 322 and LOD were 10 ng/mL and 5 ng/mL, respectively, for both ana- 323 lytes in both matrices. These limits were considered satisfactory, 324 especially when compared to those obtained by other authors. 325 Table 3 summarizes published methods for the determination of 326 K and NK in plasma and urine. As it can be observed, the LODs 327 obtained using our method are lower than those reported by some 328 authors [2,4,18,28,29,32], but higher than others [17,23,24,30,51]. 329 Among these, the lowest LOQ using GC–MS was reported by Bair- 330 ros et al. [24] (0.5 ng/mL for both K and NK), while Parkin et al. [23] 331 achieved a LOD of 0.03 ng/mL for K and 0.05 ng/mL for NK in urine, 332 yet using UHPLC–MS/MS. Moreover, Harun et al. [17] also reported 333 lower LODs; nevertheless, they hydrolyzed the samples, turning 334 the whole process more laborious and time consuming. In general, 335 in the papers that report better LODs [17,23,24,30,51], higher sam- 336 ple volumes (1–4 mL) were used, and/or more laborious methods 337 were described, including derivatization steps [24]. Liquid chro- 338 matography has been successfully applied in the quantification of

Table 4
Intra- and inter day precision and accuracy for plasma.

Compound	Spiked	Measured		CV%		RE%	
		Inter-day[a] (n = 5)	Intra-day[a] (n = 6)	Inter-day (n = 5)	Intra-day (n = 6)	Inter-day (n = 5)	Intra-day (n = 6)
Norketamine	10	11.50 ± 0.53	9.93 ± 1.34	4.63	13.45	14.97	−0.70
	50	47.83 ± 3.52	45.03 ± 0.72	7.35	1.60	−4.34	−9.94
	100	92.09 ± 5.22	92.67 ± 7.20	5.66	7.77	−7.91	−7.33
	200	187.72 ± 9.76		5.20		−6.14	
	300	287.59 ± 4.94		1.72		−4.14	
	400	418.06 ± 16.46	439.83 ± 7.16	3.94	1.63	4.51	9.96
	500	515.22 ± 10.40	458.11 ± 39.70	2.02	8.67	3.04	−8.38
Ketamine	10	10.67 ± 1.40	10.55 ± 1.13	13.14	10.73	6.68	5.47
	50	47.49 ± 3.30	45.91 ± 2.84	6.95	6.18	−5.02	−8.18
	100	97.78 ± 11.30	98.55 ± 5.58	11.55	5.66	−2.22	−1.45
	200	197.42 ± 9.29		4.71		−1.29	
	300	301.59 ± 16.06		5.33		0.53	
	400	406.22 ± 23.60	424.34 ± 31.53	5.81	7.43	1.56	6.09
	500	498.83 ± 14.55	449.99 ± 21.29	2.92	4.73	−0.23	−10.00

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

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Table 5
Intra- and inter day precision and accuracy for urine.

Compound	Spiked	Measured		CV%		RE%	
		Inter-day[a] ($n=5$)	Intra-day[a] ($n=6$)	Inter-day ($n=5$)	Intra-day ($n=6$)	Inter-day ($n=5$)	Intra-day ($n=6$)
Norketamine	10	10.59 ± 0.74		7.05		5.92	
	50	45.70 ± 4.13	52.80 ± 6.14	9.05	11.63	−8.59	5.60
	100	97.92 ± 6.71	99.86 ± 5.73	6.85	5.74	−2.08	−0.14
	150	153.22 ± 3.99		3.90		2.15	
	200	204.76 ± 1.90	183.15 ± 8.18	1.63	4.81	2.38	−8.43
	250	247.68 ± 1.15		1.06		−0.93	
Ketamine	10	10.10 ± 0.67		6.68		0.97	
	50	48.57 ± 3.42	46.52 ± 5.78	7.03	12.43	−2.85	−6.96
	100	101.79 ± 2.42	105.35 ± 5.54	2.38	5.26	1.79	5.34
	150	146.81 ± 4.01		2.73		−2.13	
	200	201.61 ± 5.51	197.97 ± 20.63	2.73	10.42	0.81	−1.02
	250	251.02 ± 7.48		2.97		0.41	

All concentrations in ng/mL; CV—Coefficient of variation; RE—Relative error [(measured concentration-spiked concentration/spiked concentration) × 100].

Table 6
Absolute recovery ($n=3$).

Sample	Compound	Recovery[a] (%)			
		50 ng/mL	100 ng/mL	200 ng/mL	500 ng/mL
Urine	Norketamine	76.68 ± 7.42	72.54 ± 2.45	73.12 ± 0.99	–
	Ketamine	100.68 ± 10.70	91.51 ± 8.53	89.03 ± 6.24	–
Plasma	Norketamine	75.26 ± 13.21	73.42 ± 8.84	66.37 ± 9.56	63.13 ± 1.47
	Ketamine	88.92 ± 11.36	87.78 ± 10.96	84.05 ± 3.06	73.15 ± 10.86

[a] Mean values ± standard deviation.

K and metabolites in biological samples; however, its application potential is restricted to some extent due to higher solvent consumption and to the fact that the process is more time-consuming. By comparison, GC is widely used in forensic drug testing because of its easy operation, high separating efficiency, selectivity and sensitivity. Furthermore, and to the best of our knowledge, GC–MS/MS has not been used yet for K determination in biological specimens.

3.2.3. *Intra- and inter-day precision and accuracy*

Concerning intra-day precision and accuracy, the obtained CVs were typically below 14% for both compounds at all tested concentrations, presenting a mean relative error within a ±10% interval. Regarding inter-day precision and accuracy, the obtained CVs were generally lower than 14% for both analytes at all concentration levels, while accuracy was within a ±15% interval. These data are presented in Tables 4 and 5.

3.2.4. *Extraction recovery*

Using the aforementioned approach, the absolute recovery values ranged from 73 to 101% for the studied analytes in urine and 63–89% in plasma (Table 6). Lee et al. [2] reported recovery values of 113.4% for K and 102.1% for NK in urine using solid-phase extraction, while Lian et al. [18] obtained 97% and 95% for K in urine and plasma respectively using liquid–liquid extraction. It is not possible to compare adequately our results to those obtained by other authors because the determination of K and metabolites using MEPS has not been published yet, but in general our results can be considered adequate. Besides the good recoveries achieved, the suggested method, when compared to those normally used in drug abuse analysis, reveals many practical advantages, namely minimal solvent consumption, small amount of sample required, simple device, easy operation and low-cost.

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Table 7
Q3 Identification of K and NK in urine.

Ketamine

Control	MRM transition	Area	Relative area (%)	Tolerance	Lower limit (%)	Upper limit (%)
Transition #1	179.7 → 150.8	19950	100.0			
Transition #2	179.7 → 115.9	4742	23.8	±5% abs.	18.8	28.8
RT (min)	6.92			±0.1 min	6.82	7.02
RT-IS (min)	6.91	RRT	1.00	±1% rel.	0.99	1.01

Sample	MRM transition	Area	Relative area (%)	Conformity
Transition #1	179.7 → 150.8	5547	100.0	Yes
Transition #2	179.7 → 115.9	1249	22.5	Yes
RT (min)	6.92			Yes
RT-IS (min)	6.92	RRT	1.00	Yes

Norketamine

Control	MRM transition	Area	Relative area (%)	Tolerance	Lower limit (%)	Upper limit (%)
Transition #1	165.7 → 131.0	21869	100.0			
Transition #2	165.7 → 148.7	8548	39.1	±20% rel.	31.3	46.9
RT (min)	6.79			±0.1 min	6.69	6.89
RT-IS (min)	6.78	RRT	1.00	±1% rel.	0.99	1.01

Sample	MRM transition	Area	Relative area (%)	Conformity
Transition #1	165.7 → 131.0	14161	100.0	Yes
Transition #2	165.7 → 148.7	4604	32.5	Yes
RT (min)	6.77			Yes
RT-IS (min)	6.76	RRT	1.00	Yes

IS—Internal standard; RT—Retention time; RRT—Relative retention time.

Table 8
Identification of K and NK in plasma.

Ketamine

Control	MRM transition	Area	Relative area (%)	Tolerance	Lower limit (%)	Upper limit (%)
Transition #1	179.7 → 150.8	139941	100.0			
Transition #2	179.7 → 115.9	23548	16.8	±5% abs.	11.8	21.8
RT (min)	6.92			±0.1 min	6.82	7.02
RT-IS (min)	6.91	RRT	1.00	±1% rel.	0.99	1.01

Sample	MRM transition	Area	Relative area (%)	Conformity
Transition #1	179.7 → 150.8	30587	100.0	Yes
Transition #2	179.7 → 115.9	6144	20.1	Yes
RT (min)	6.92			Yes
RT-IS (min)	6.91	RRT	1.00	Yes

Norketamine

Control	MRM transition	Area	Relative area (%)	Tolerance	Lower limit (%)	Upper limit (%)
Transition #1	165.7 → 131.0	307944	100.0			
Transition #2	165.7 → 148.7	86865	28.2	±20% rel.	22.6	33.8
RT (min)	6.80			±0.1 min	6.70	6.90
RT-IS (min)	6.79	RRT	1.00	±1% rel.	0.99	1.01

Sample	MRM transition	Area	Relative area (%)	Conformity
Transition #1	165.7 → 131.0	61243	100.0	Yes
Transition #2	165.7 → 148.7	18519	30.2	Yes
RT (min)	6.80			Yes
RT-IS (min)	6.79	RRT	1.00	Yes

IS—Internal standard; RT—Retention time; RRT—Relative retention time.

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3.2.5. *Stability*

Regarding processed samples stability, the obtained CVs values were lower than 6%, indicating that the analytes are stable for at least 24 h in the autosampler at room temperature. The obtained CVs for short-term stability were lower than 11%, meaning that the analytes are stable in the samples for at least 24 h at room temperature. Finally, K and NK are also stable for at least three freeze/thaw cycles since the obtained CVs were lower than 7%. Taking into account the stability of the analytes, sample analysis can be carried out within a comfortable time window since they are not significantly affected by the storage conditions.

3.2.6. *Identification and quantification of K and NK in urine and plasma*

In order to demonstrate the applicability of the developed method, it was applied to real samples of suspected consumers of recreational drugs. However, neither K nor NK were detected in these samples. Nevertheless, the fact of not having obtained

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any positive for K and/or NK in the analyzed samples does not mean that the present method is not sensitive enough to detect the subject compounds in patients, since the concentrations obtained following consumption or administration are usually higher than our limits of detection and quantification.

We believe that this situation is due to the lack of knowledge and information about these new drugs; indeed, specific symptoms of ketamine abuse remain unnoticed when these consumers arrive at hospital emergency services, and therefore, they are treated taking in consideration the more commonly used drugs, such as cocaine, opiates or alcohol; for this reason, K and NK would probably be no longer in the body at the time of sampling. To evaluate the methodology in real samples, and since we did not obtain positives in human samples, a single dose of K (50 mg/kg) was administered intraperitoneally (i.p.) to one rat (Wistar han), and urine and plasma samples were collected 1 h after administration. These samples were analyzed by the described procedures, and K and NK were detected at concentrations of 348 and 809 ng/mL in urine, and 292 and 211 ng/mL in plasma, respectively (the urine sample had to be diluted 5 times because the concentration was higher than 250 ng/mL), proving that the method is appropriate to detect K and NK in authentic urine and plasma samples. Figs. 5 and 6 show the chromatograms obtained by analysis of these samples after K administration, and the maximum allowed tolerances for retention times and relative ion intensities are shown in Tables 7 and 8.

4. Conclusions

When individuals attend hospital emergency services with ketamine intoxication, they may be mistaken for poisoning by other drugs (e.g. alcohol) and may be erroneously treated according to these symptoms. Ketamine intoxication should also be tested in case of suspected drug-facilitated sexual assault. So, the development of new methods for the detection of those compounds in biological samples is mandatory for forensic toxicology laboratories, in order to be one step ahead of those practices. In this paper, and for the first time, a fast, simple and fully validated method is described for the qualitative and quantitative determination of K and its main metabolite NK in urine and plasma, combining MEPS with GC–MS/MS. The method was found to be linear within the studied calibration ranges with adequate precision and accuracy. MEPS in combination with chromatographic analysis by GC–MS/MS has shown to be adequate for the determination of these compounds in urine and plasma samples, achieving good limits of detection and generally high recoveries. Furthermore, no derivatization step was needed to accomplish the analysis, making the procedure less laborious and time-consuming. The analyses were carried out using reduced sample volumes (0.25 mL), which provides a significant advantage, particularly when there is little sample availability, enabling further exams to be performed if necessary. The low detection and quantification limits enable detecting small amounts of the compounds, making this procedure suitable for those laboratories performing regular urine and plasma analysis in the field of forensic toxicology.

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Article IV

Analysis of methoxetamine in urine and plasma samples using microextraction by packed syringe and gas chromatography-tandem mass spectrometry

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ANALYSIS OF METHOXETAMINE IN URINE AND PLASMA SAMPLES USING MICROEXTRACTION BY PACKED SORBENT AND GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

Methoxetamine, a ketamine analog, is a new designer drug which was synthesized for its longer lasting and favorable pharmacological effects over ketamine. The aim of this work was to develop and validate a method for the identification and quantification of methoxetamine in human urine and plasma samples by using microextraction by packed sorbent (MEPS) and GC–MS/MS without derivatization. The technique uses a sample volume as low as 0.25 mL, extracting the analyte with a mixed-mode sorbent (M_1). The method showed to be linear between 10 and 600 ng ml^{-1} for urine, and between 2 and 600 ng ml^{-1} for plasma, with determination coefficients higher than 0.99, and presenting a LOD of 1 ng ml^{-1} for both matrices. Intra- and inter-day precision and accuracy were below 15% and 14%, respectively, fulfilling the criteria normally accepted in bioanalytical method validation. Under the optimized conditions, extraction efficiency ranged from 80 to 110% for urine and from 81 to 88% for plasma. GC–MS/MS in combination with MEPS showed to be a fast and simple procedure for the determination of this compound in urine and plasma, since no derivatization step is required. Moreover, MEPS device could be reused for up to 100 extractions, allowing minimizing the handling time and costs usually associated to this type of analysis. Furthermore, the fact that only 0.25 mL of sample is required, makes this method a valuable and powerful tool for drug monitoring in human urine and plasma in situations where methoxetamine is involved, for instance in forensic scenarios. This is the first time that GC–MS/MS with MEPS was used for the determination of this compound in biological fluids.

Keywords: Methoxetamine; MEPS; Biological samples; GC-MS/MS

1. Introduction

In the last years, an extensive amount of novel psychoactive substances (NPS), frequently characterized as "designer drugs", "research chemicals" or "legal highs", emerged on the recreational drug market [1]. Their sale through internet sites at low cost, and the uncontrolled production and distribution have elevated concerns about their potential damages, like poisoning and addiction. Methoxetamine (MXE), one of these substances [2–4], and chemically 2-(3-methoxyphenyl)-2-(N-ethylamino) cyclohexanone (3-MeO-2-Oxo-PCE) [2,4–7], is an analogue of ketamine (K) [1,2,6,8–10], that belongs to the arylcyclohexylamine class [11–13] (Fig. 1). Nonetheless, there are two main structural modifications between MXE and K, causing differences in the intensity and duration of effects [1,3]. The first modification involves the replacement of the chlorine atom at position 2 in the aromatic ring by a 3-methoxy group [1,3,5,7,12,14], which leads to weaker analgesic and anesthetic effects than K [3,5,8,12]; the second alteration comprises the substitution of the N-methyl group on the amine portion of the molecule, by an N-ethyl group [5,7,12,14], yielding MXE with more potency and longer lasting effects than K [5,8,12,15].

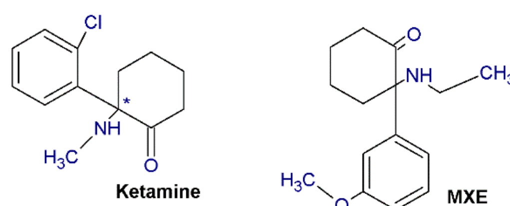

Figure 1. Chemical structure of MXE and K.

Probably, MXE was specifically synthesized with the aim of providing a safer alternative to ketamine, mainly in respect to urinary tract complications common with ketamine use (ketamine bladder syndrome) [4]; and also, as a legal high, to be a legal alternative to the established banned drugs, namely ketamine and phencyclidine (PCP), avoiding the law, but holding the psychoactive properties of the parent compounds [4,5,9,13].

Recreational use of MXE was first informed to the EMCDDA by the UK, on 9th November 2010 [16]. MXE is available for sale over the Internet, marketed as "legal ketamine", without any information about safety, side-effects or toxicity [4]. Notwithstanding being labeled as "not for human consumption", this substance is consumed for its recreational and psychedelic effects [5,11,12], mostly between young people, and more precisely in the group of males aged 16 to 25 [4]. It was traded in the form of a bright white powder, and under different street names such as "MXE", "M-ket", "Mexxy", "Special M", "Kmax", "MXE-powder", or "METH-O" [2,5,10,11,13,14]. Concerning the routes of administration, MXE is usually taken by nasal insufflation (snorting), oral ingestion or sublingual application, but it can also be injected (intramuscularly or intravenously), or inserted rectally [6,8,10,11,17]. Typical doses vary from

5 to 100 mg [8,13], depending on the route of administration. Acute intoxications have been related with dosages up to 500 mg [6].

Although there are few studies demonstrating the mechanism of action of MXE, and since it is structurally similar to ketamine, it has been supposed that it acts as an N-methyl-D-aspartate (NMDA) receptor blocker, as a dopamine reuptake inhibitor [1,3,8–10,14,17] and also interacting with 5-HT2 receptors and muscarinic cholinergic receptors [5]. There are scarce reports in the scientific literature about the effects of MXE, and these are mainly based in observation of patients arriving at hospitals with acute MXE-related toxicity, or even on Web forum debates, where customers describe their own experiences [6,10,13]. Effects start 10 to 20 minutes after consumption and usually last for 2 to 3 hours [5,6,8,13]. Customers describe pleasurable effects such as mild euphoria, agitation, hallucinations [3,6,8,13,14], and a dissociative catatonic state, called "M-Hole", a subjective state of dissociation from the body [2,5,11]. Nonetheless, some side effects like tachycardia, vomiting, confusion, nystagmus, paranoid reactions, hyperthermia, dizziness, diarrhea and numbness were also described [2,3,6,10,11,13,14]. The UK reported on 21st October 2010 the first overdose by MXE [16], and since then some cases of fatal intoxication with MXE have been published [5,6].

As a recent drug of abuse, little is known about MXE's metabolism. In 2013, Meyer *et al.* [11] identified phase I and II metabolites in rat and human urine. The authors claimed that MXE was extensively metabolized, mainly by CYP2B6 and CYP3A4 enzymes [5,11]. This study showed that phase I metabolic pathways included N-deethylation to normethoxetamine (probably the most abundant metabolite of MXE in humans [11]), oxidative metabolism to dehydronormethoxetamine, hydroxylation to 3-hydroxymethoxetamine, O-demethylation to O-desmethylmethoxetamine and reactions via sulfation or glucuronidation, producing phase II metabolites, like O-desmethylmethoxetamine glucuronide, O-desmethylnormethoxetamine glucuronide and hydroxynormethoxetamine glucuronide [1,11,16].

Regarding the legal situation, until now MXE is still not a controlled substance in Canada and is not scheduled under U.S. Controlled Substances Act [5,7,9]. In the UK, on 5th April 2012, MXE was subjected to a temporary class drug control (TCDO), that prohibited importation and sale for a 12 months period [1,11,16], but authorized possession and use [5]. However, on 26th February 2013, as a result of the recommendation from the Advisory Council on the Misuse of Drugs (ACMD), MXE became a Class B drug under the Misuse of Drugs Act [1,16]. In Portugal, since April 2013, MXE is under the legislation of the Decree-Law n. º 54/2013 of 17th April, that prohibits the production, importation, exportation, advertisement, distribution, sale, possession, or availability of new psychoactive substances [18]. MXE is also a controlled substance in Germany, Switzerland, Russia and Japan [5].

Due to the lack of information about the toxicity of MXE, its rapid availability on the Internet and the scarce legal control in most countries, it is plausible that its consumption increases and constitutes a great challenge to public health care in the next years. Consequently, it is

important to perform strict investigations to better understand its epidemiology and pharmacology, sustained by toxicological analytical screening [4,10]. GC–MS [11] and LC–MS [11] or LC–MS/MS [10,19,20] have been applied to the analysis of MXE from plasma [10] and urine [11,19,20], being mass spectrometry the chosen analytical technique for drugs of abuse, due to its capability to offer unequivocal identification of compounds [21,22]. In order to isolate the compound of interest from biological samples, eliminating the interferences, a good sample preparation approach is required. Miniaturized techniques, such as microextraction by packed sorbent (MEPS), appear to be a good choice, because they provide some advantages when compared to the traditional liquid-liquid extraction (LLE) and solid-phase extraction (SPE). One of the greatest improvements is the diminution of the sample volume and the organic solvent consumption, and the possibility of being reused several times, as more than 100 extractions have been reported using plasma or urine samples. By comparison, the conventional SPE cartridges are recommended for single use only [23,24]. This paper describes for the first time the development and validation of a method for the identification and quantification of MXE in human urine and plasma by means of MEPS and GC-MS/MS.

2. Experimental

2.1. Chemicals and materials

The analytical standard, MXE, as well as deuterated ketamine (K-*d4*), used as internal standard (IS), were purchased from LGC Promochem (Barcelona, Spain) as methanolic solutions at 1 mg mL^{-1} (MXE) and 0.1 mg mL^{-1} (K-*d4*). Formic acid (99% purity) and methanol were acquired from Merck (Darmstadt, Germany), and ammonium hydroxide (analytical grade) from J.T. Baker (Holland). Deionized water was obtained from a Milli-Q System (Millipore, Billerica, MA). MEPS 250 µL syringe and MEPS BIN (Barrel insert and Needle) M_1 (4 mg; 80% C_8 and 20% SCX) from SGE Analytical Science were purchased from ILC (Porto, Portugal).

2.2. Stock and working solutions

Working solutions at 2.5, 0.25, and 0.025µg mL^{-1} of MXE were prepared by direct dilution of stock solutions with methanol. A working solution of the IS at 1 µg mL^{-1} was prepared also in methanol. All those solutions were kept in amber glass vials and stored light protected at 4 °C.

2.3. Biological specimens

Fresh human plasma was obtained from the surplus of the Instituto Português do Sangue (outdated transfusions) and drug-free urine samples used in all experiments were provided by laboratory staff. All samples were stored refrigerated at -20 °C until analysis.

2.4. Sample preparation

Frozen urine and plasma samples were allowed to thaw at room temperature. Previously to analysis, biological matrices were centrifuged at 3500 rpm for 15 minutes. Concerning sample preparation, 0.25 mL of urine was diluted with 0.5 mL of phosphate buffer, while plasma samples (0.25 mL) were mixed with acetonitrile for protein precipitation, and afterwards centrifuged at 3500 rpm for 15 min. The supernatant was transferred into a glass tube and evaporated under a gentle stream of nitrogen at room temperature. Then the residue was dissolved with 0.5 mL of 0.1 M phosphate buffer (pH 6), ten microliters of the IS solution (1 μg mL^{-1}) was added, and the sample was slightly vortex-mixed for 30s. Before its first use, the extraction sorbent was activated by aspiration and elimination of 5×0.25 mL of methanol through the device, and then conditioned similarly with 4×0.25mL of water. The extraction technique was previously optimized (see Section 3), and the final extraction conditions, for urine and plasma (in square brackets), were the following. Urine samples were aspirated and passed through the device 10 times [20 times for plasma] at an approximate flow rate of 10 μL s^{-1}. To remove matrix-borne interferences, the sorbent was washed with 0.1 mL of 0.55% formic acid [0.1 mL of 1.45% formic acid for plasma]. Finally the retained analytes were eluted from the sorbent with 0.3 mL of 3% ammonia in methanol [1 mL of 3% ammonia in methanol for plasma]. The eluates were evaporated to dryness under a gentle nitrogen stream at room temperature, re-dissolved in 50 μL of methanol, transferred to autosampler vials and an aliquot of 2 μL was injected into the GC–MS/MS instrument in the splitless mode. Moreover, after each extraction, the sorbent was cleaned sequentially with 4×0.1 mL of 1% ammonia in methanol:acetonitrile (50:50, v/v) and 1% formic acid in 2-propanol, in order to decrease carryover. The absence of carryover was confirmed when no analyte was observed in a blank sample injected immediately after the analysis of a sample containing a high concentration of the target analytes.

2.5. Gas chromatographic and mass spectrometric conditions

Analyses were carried out using a HP 7890A GC system Agilent Technologies (Soquimica, Lisbon, Portugal), equipped with a model 7000B triple quadrupole mass spectrometer (Agilent Technologies, (Soquimica, Lisbon, Portugal)), a MPS2 autosampler and a PTV-injector from Gerstel (Soquimica, Lisbon, Portugal). The separation of the compounds was performed on an HP-5MS fused-silica capillary column with 30m × 0.25mm id, and 0.25μm film thickness (J&W Scientific, Folsom, CA, USA). Helium (purity ≥99.999%) was set at 0.8 mL min^{-1} as the carrier gas. A programmed oven temperature was performed as follows: 100 °C (0.5 min), 20 °C/min to 280 °C (9 min) and held for 5 min. The total separation time was 14.5 min. The temperatures of the injection port and the ion source were set at 250 and 280 °C, respectively. The mass spectrometer was operated with a filament current of 35 μA and the ionization energy was 70eV. Multiple reactions monitoring (MRM) mode with electron impact ionization

was performed in this investigation, using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies). The transitions were chosen based on selectivity and abundance, in order to maximize the signal-to-noise ratio in matrix extracts. Nitrogen (purity ≥99.999%) was used as the collision gas at a flow rate of 2.5 mL min^{-1}. Table 1 summarizes MS data for MXE.

Table 1. Retention time and GC-MS/MS parameters (quantification transitions underlined).

Compound	Retention Time (minutes)	MRM transition, m/z (collision energy, eV)	Dwell time (µs)
K-*d4*	8.12	<u>183.7→120 (20)</u>	70
MXE	8.28	189.6→115.0 (20)	70
		189.6→147.1 (10)	70
		<u>189.6→189.6 (10)</u>	70

2.6. Optimization of MEPS technique

In order to decrease the number of interferences and get better efficiency in the process, we proceeded to the optimization of extraction technique. Taking into account the characteristics of the analyte under study, experiments were performed using a mixed-mode sorbent (M_1) column. The parameters (independent variables) that could possibly affect the extraction efficiency of the MEPS procedure, as well as their interactions, were screened by means of a fractional factorial design methodology with control by intermediate point (in triplicate) and studied at two levels, low (-1) and high (+1). This multivariate approach is a powerful statistical tool capable of eliminating factors with little or no significant influence on the response; this allows assessing the interactions between all the factors as well, and reducing the number of experiments, which would be much higher if a univariate approach was used. So, less effort is spent to obtain the same amount of data, saving time and money. The studied factors and respective levels (low; high) were number of sample aspirations through the device (strokes) (5; 15), amount of formic acid in washing step (0.1; 1%, v/v), amount of ammonia in the eluting solvent (methanol) (0.5; 3%, v/v) and volume of eluting solvent (100; 500, µL). These experiments were carried out in a random order, to avoid the influence of noise factors, minimizing systematic errors. The experimental designs matrices were performed and evaluated using the MINITAB® statistical software, version 15 [23].

2.7. Validation procedure

The described method was fully validated according to the guiding principles of the Food and Drug Administration (FDA) [25] and International Conference on Harmonization (ICH) [26]. The validation was performed following a 5 days validation protocol and included selectivity, linearity and limits, intra- and inter-day precision and accuracy, recovery and stability.

The method's selectivity was evaluated by analyzing blank urine/plasma samples of ten different origins, to investigate the potential interferences at the retention time and selected transitions of MXE. These samples were pooled and separated in 20 aliquots (ten analyzed as blanks and ten spiked with the analyte), all spiked with the IS. Quality control (QC) samples were prepared and analyzed simultaneously. Identification criteria for positivity included an absolute retention time within 2% or ±0.1 min of the retention time of the same analyte in the control sample, and the presence of two transitions per compound. To guarantee a suitable confidence in identification, the maximum allowed tolerances for the relative ion intensities between the two transitions (as a percentage of the base peak) were as follows: if the relative ion intensity in the control sample was higher than 50%, then an absolute tolerance of ± 10% was accepted; if this value was between 25 and 50%, a relative tolerance of ±20% was allowed; if it was between 5 and 25%, an absolute tolerance of ± 5% was accepted, and, finally, for relative ion intensities of 5% or less, a relative tolerance of ± 50% was used [27]. The method would be considered selective if no analyte could be identified in the blank samples by applying those criteria. Linearity of the method was established on spiked samples prepared and analyzed using the described extraction procedure in the range of 10-600 ng mL^{-1} for urine, and 2-600 ng mL^{-1} for plasma (five replicates). Calibration curves were obtained by plotting the peak area ratio between each analyte and the IS, against analyte concentration. The acceptance criteria included a determination coefficient (R^2) value of, at least, 0.99 and the calibrators' accuracy within ± 15% (except at the lower limit of quantification (LLOQ), where ± 20% was considered acceptable). Together with each calibration curve, a zero sample (blank sample with IS) and three QC samples at low [LQC: 30 ng mL^{-1} (urine); 5 ng mL^{-1} (plasma)], medium (MQC: 250 ng mL^{-1}) and high (HQC: 500 ng mL^{-1}) concentrations (n=3) were also analyzed. The LLOQ was defined as the lowest concentration that could be measured with adequate precision and accuracy, i.e. with a coefficient of variation (CV, %) of less than 20% and a relative error (RE, %) within ± 20% of the nominal concentration. The limits of detection (LOD) were determined as the lowest concentrations that showed a discrete peak clearly distinguishable from the blank with a signal-to-noise ratio of at least 3, in which the analytes could be unequivocally identified, and were determined by analyzing five replicates of spiked samples. Intra-day precision was evaluated by analyzing in the same day 6 replicates of blank urine/plasma samples spiked with MXE at 4 concentration levels [(10, 100, 300 and 600 ng mL^{-1}) for urine and (2, 50, 300, and 600 ng mL^{-1}) for plasma]. Inter-day precision was evaluated at a minimum of six concentration levels within a 5-day period. The accuracy of the method was characterized in terms of the mean RE between the measured and the spiked concentrations;

the accepted limit was 15% for all concentrations, except at the LLOQ, where 20% was accepted. For recovery studies, two sets of samples (n= 3) were prepared at low, medium and high concentrations. Set 1 was spiked with MXE after extraction of a blank sample (representing 100% recovery), and set 2 consisted of spiked analyte in a blank sample before extraction. The IS was added to the two sets of sample only after elution. The recovery results were obtained by comparison of the relative peak areas of sample set 2 with those of the corresponding peaks in sample set 1. This parameter was studied for both type of samples applied in this study. The stability of MXE was studied in both types of samples, spiked at the above-mentioned LQC, MQC and HQC levels and extracted using the MEPS procedure (n=3), under specific conditions and time intervals (processed samples, short-term and freeze/thaw stability). To study the stability in processed samples, the extracts that were previously analyzed were re-analyzed after stored at room temperature in the autosampler for 24h. Short-term stability was evaluated at the same concentration levels (n=3); blank samples were spiked and left at room temperature for 24h. To study freeze and thaw stability, samples were spiked at the previously described concentrations, and stored at -20 °C for 24h; after this period they were thawed unassisted at room temperature, and then refrozen for 12-24h under the same conditions. This freeze/thaw cycle was repeated twice more, and after the third cycle the samples were finally analyzed. During the entire stability procedure the analyzed samples were compared with freshly prepared samples and analyzed in the same day. For each stability study, the analyte was considered stable if the CV between the two sets of samples was below 15%.

3. Results and discussion

3.1. Fractional factorial design

One of the goals of the statistical tool Design of Experiments (DOE) is the screening of the factors that are likely to influence the response. Indeed, if there are a large number of factors that can affect the recovery of MXE, this screening procedure will identify the most important ones. The comparative analysis between these factors, evaluating which have a greater influence, will permit the optimization of the selected extraction procedure. Using a cationic exchange sorbent M_1 (C_8/SCX), the parameters susceptible of influencing the extraction process (and their respective levels), were studied using a fractional factorial design approach (Table 2).

Table 2. Experimental array from DOE extractions for urine and plasma.

Strokes (n)	% Formic acid washing	% Ammonia elution	Elution solvent amount (µL)
10	0,55	1,75	300
5	0,1	0,5	100
10	0,55	1,75	300
15	1	3	500
5	1	0,5	500
15	0,1	0,5	500
5	1	3	100
10	0,55	1,75	300
15	1	0,5	100
5	0,1	3	500
15	0,1	3	100

In the above array, each row represents an experimental run.

The results were interpreted considering the Pareto Chart of main effects and linked interactions, which graphically displays the magnitudes of the effects. Those charts show each of the estimated standard effects in decreasing order of magnitude, and the length of each bar is proportional to the standardized value of the estimated effect, which is expressed as the ratio between the effect and the standard error [23]. Those plots also include a vertical red line that corresponds to the 95% confidence level. Any effect or interaction which exceeds this reference line is considered statistically significant as regards the response [23]. For urine, we conclude that none of the factors have statistically significant influence on response at the studied levels (Fig. 2-A). Therefore, the factors could be fixed in the most suitable levels, and those that originated a better apparent response were chosen: strokes (10), amount of formic acid in wash solution (0.55%), amount of ammonia for eluting the analyte (1.75%) and volume of elution solvent (300 µL) (Fig. 2-B).

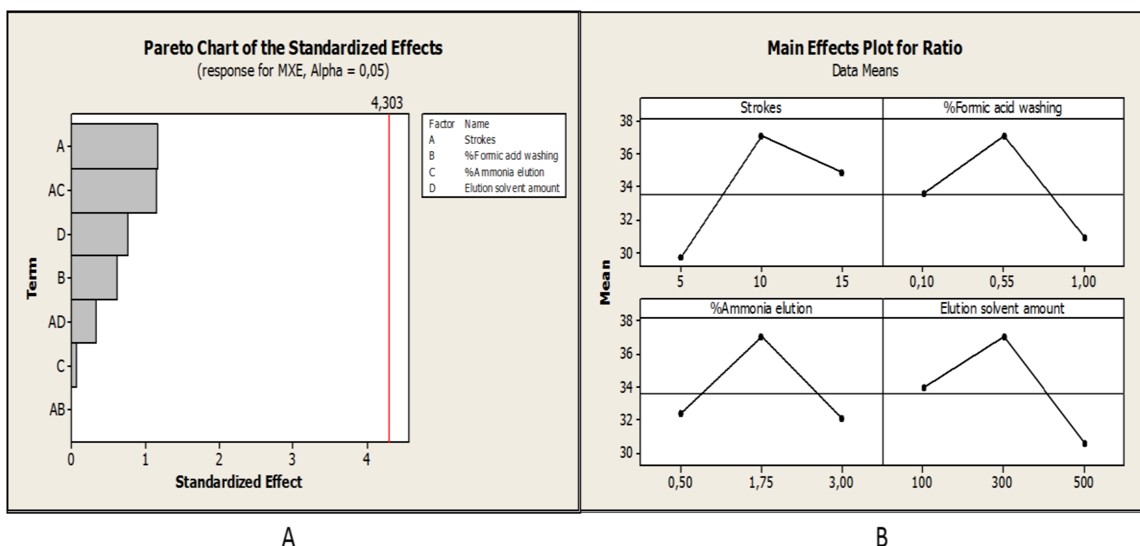

Figure 2. Pareto Chart (A) and diagram of main effects plot (B), illustrating the factors that influence the extraction process for MXE from urine.

Concerning plasma, by analyzing the Pareto Charts (Fig. 3), we can state that all parameters under study have statistical influence (95% confidence level), and, more specifically, that the amount of ammonia in the elution solvent, the volume of elution solvent and the strokes are isolated factors affecting the recovery of MXE. In turn, the interaction between the strokes and the percentage of formic acid in the wash solution also has statistically influence in the final response.

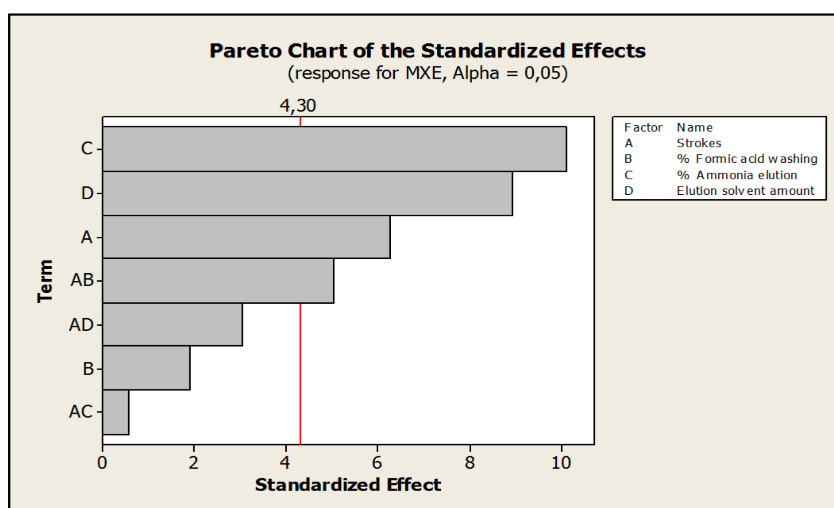

Figure 3. Pareto Chart illustrating the factors that influence the extraction process for MXE from plasma.

As the experimental design showed inconclusive results, we have used a response surface model (RSM). This statistical tool is employed in situations where the response is influenced by several factors, in order to optimize it. As any factor could not be excluded by the previous method (DOE), we used again Minitab v.15 program for the creation of a new array. This was established with the same four variables from DOE, but in this case, we applied a full factorial

planning. The univariate optimization was hence performed varying the number of strokes from 5 to 40 (5, 10, 20, 30, and 40) (n=3) and volume of elution solvent from 100 to 1000 µL (100, 300, 500, 700, 1000 µL) (n=3). The best results were obtained using 20 strokes, as number of sample aspirations through the device (Fig.4-A), and 1000 µL, as volume of elution solvent (Fig. 4-B); therefore, those settings were chosen as the final optimized conditions for MXE extraction.

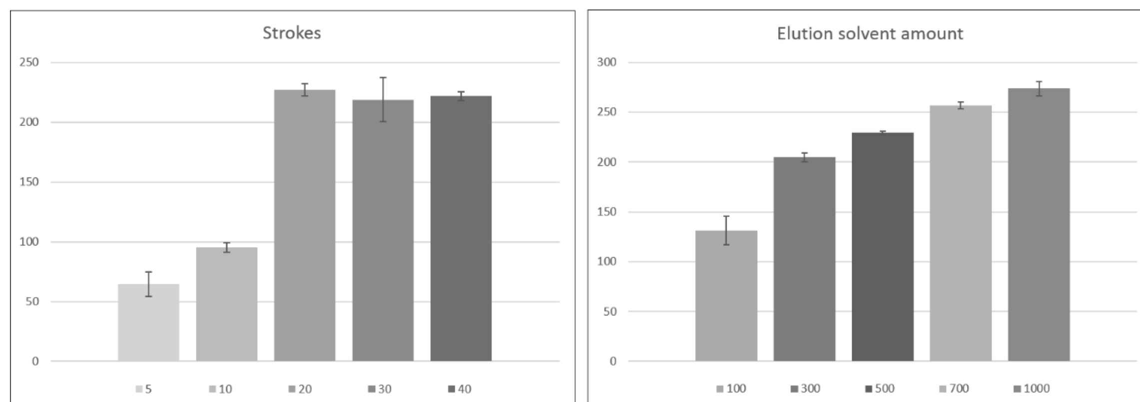

Figure 4. Univariate approach to strokes (A) and elution solvent amount (B) in urine.

3.2. Method validation

3.2.1. Selectivity

Using the above-mentioned criteria for positivity, MXE was successfully and unequivocally identified in all the spiked samples, whereas in the blank samples no interfering peaks could be detected and/or misidentified as being the analyte. Therefore, the method was considered selective for MXE determination in urine and plasma. Representative ion chromatograms of a spiked (at the LOD) and a blank sample, for urine and plasma matrices, are shown in Figs. 5 and 6, respectively.

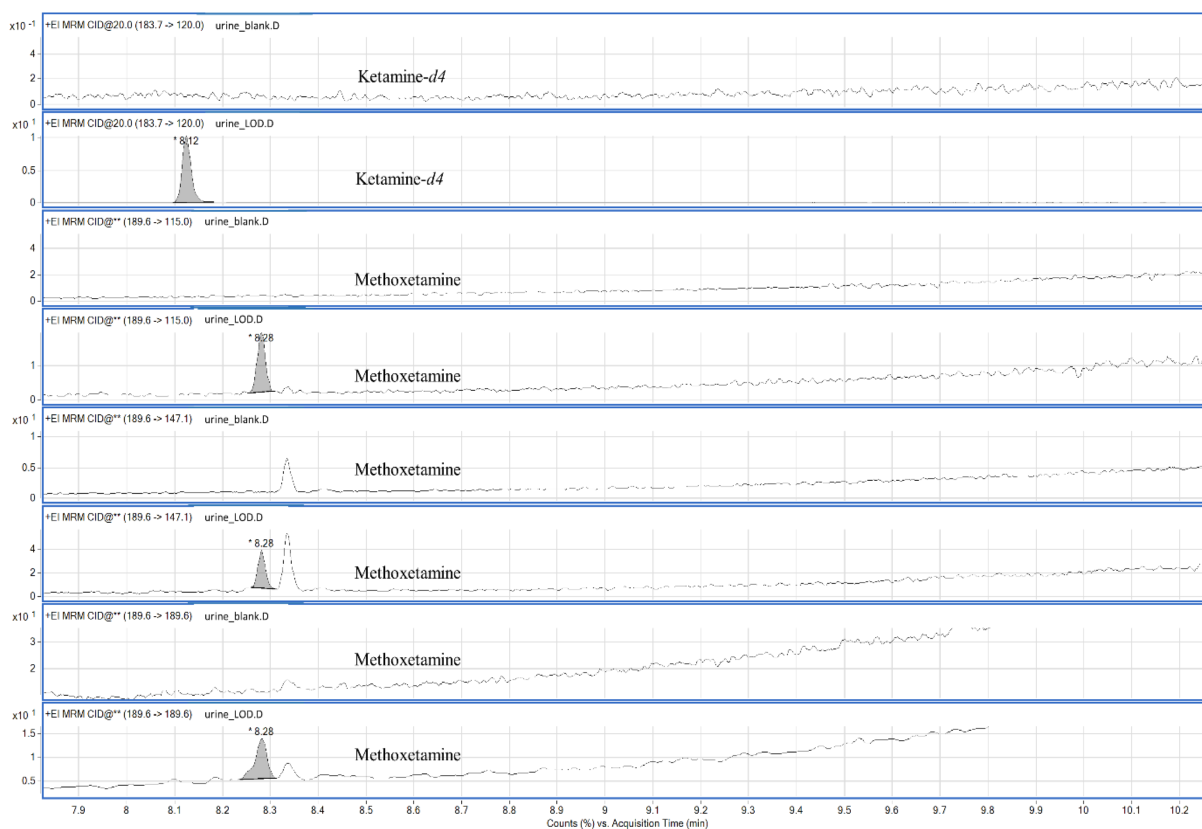

Figure 5. Comparison between ion chromatograms of a blank urine sample and a spiked urine sample at LOD.

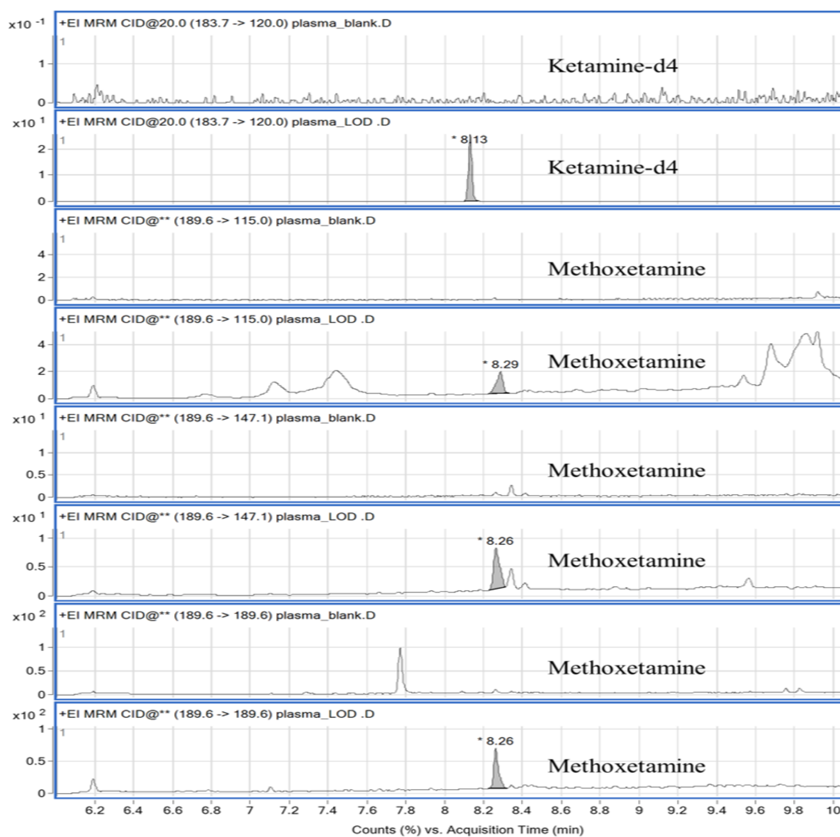

Figure 6. Comparison between ion chromatograms of a blank plasma sample and a spiked plasma sample at LOD.

3.2.2. Calibration model and limits

The method was linear within the adopted calibration ranges for MXE; however, since the adopted ranges were wide and in order to compensate for heteroscedasticity, weighted least squares regressions had to be adopted. Six weighting factors were evaluated for each analyte (1/√x, 1/x, 1/x2, 1/√y, 1/y, 1/y2), and the one which originated the best results was selected taking into account the data obtained during the assessment of the inter-day precision and accuracy [28]. This choice was performed by calculating the mean RE for each factor, and summing the absolute values. The factor that showed the lowest sum of errors and presented simultaneously a mean R^2 value of at least 0.99 was chosen (Table 3), and was 1/x. By means of these weighted least squares regressions, linear relationships were obtained, and the calibrators' accuracy (mean relative error (bias) between the measured and spiked concentrations) was within a ± 15% interval for all concentrations, except at the LLOQ (±20%). Calibration data are shown in Table 3. The method's LOD was 1 ng mL^{-1} for both matrices and LOQ was 10 ng mL^{-1} and 2 ng mL^{-1} for urine and plasma, respectively. These limits were considered satisfactory, especially when compared to those obtained by other authors. As can be seen in Table 4, there are few published methods for the detection or quantification of MXE in biological samples, and in only two methods [10,19] MXE is quantitated. As it can be observed, the LOD/LOQ obtained by our method are higher than those obtained by Saffar *et al.* [19], however these authors used a software (MassLynx) to estimate these limits. Comparing with Abe *et al.* [10], our LOD and LOQ are similar, nonetheless, they used liquid chromatography, whose application is restricted to some extent due to higher solvent consumption and the process being more time-consuming. By comparison, GC is extensively used in forensic drug testing because of its ease of operation, high separating efficiency, selectivity and sensitivity. Furthermore, and to the best of our knowledge, GC-MS/MS has not been used yet for MXE determination in biological specimens.

Table 3. Linearity data (n=5).

Matrix	Weight	Linear range (ng mL^{-1})	Linearity		R^{2*}	LOD (ng mL^{-1})
			Slope*	Intercept*		
Urine	1/x	10-600	0.094±0.150	0.032±0.011	0.996±0.002	1
Plasma	1/x	2-600	-0.262±0.050	0.015±0.149	0.997±0.001	1

*Mean values ± standard deviation

Table 4. Methods for the detection and quantification of methoxetamine in urine and plasma samples.

Matrix	Matrix volume (mL)	Matrix preparation	Detection mode	LOD ; LOQ (ng mL^{-1})	Recovery (%)	Ref.
Urine Plasma	0.25	MEPS (M_1 column)	GC-MS/MS	1 ; 10 1 ; 2	80.8 – 109.1 (Urine) 81.9 – 87.9 (Plasma)	This work
Plasma	0.1	Turbulent flow on-line extraction	LC-MS/MS	1 ; 2	86.0 – 91.0	[10]
Urine	0.05	-	LC-MS/MS	0.5 ; 0.5	-	[19]
Urine	-	SPE LLE	LC-MS/MS	-	-	[20]
Urine	-	SPE LLE Protein Precipitation	GC-MS LC-HR-MS	-	-	[11]

GC- gas chromatography; HR-high- resolution; LC- liquid chromatography; LLE- liquid-liquid extraction; LOD- limit of detection; LOQ- limit of quantification; MEPS- microextraction by packed sorbent; MS- mass spectrometry; MS/MS- tandem mass spectrometry; SPE- solid-phase extraction

3.2.3. Intra- and inter-day precision and accuracy

Concerning intra-day precision and accuracy, the obtained CVs were typically below 15% at all tested concentrations, presenting a mean relative error within a ±14% interval. Regarding inter-day precision and accuracy, the obtained CVs were generally lower than 13% at all concentration levels, while accuracy was within a ± 13% interval. These data are presented in Table 5.

Table 5. Intra- and inter day precision and accuracy for plasma

Matrix	Spiked	Measured		CV%		RE%	
		Inter-day * (n=5)	Intra-day* (n=6)	Inter-day (n=5)	Intra-day (n=6)	Inter-day (n=5)	Intra-day (n=6)
Urine	10	9.97±0.96	10.16±1.45	9.59	14.29	3.16	1.61
	50	50.06±3.77	51.22±0.25	7.53	0.49	-4.34	-0.04
	100	103.32±10.21		9.88		3.03	
	200	189.26±5.02	192.76±16.11	2.65	8.36	-5.42	-3.62
	300	297.15±18.81		6.33		-1.62	
	400	406.50±16.17		3.98		1.62	
	600	606.07±15.43	605.03±26.50	2.55	4.38	1.01	-1.83
Plasma	2	2.21±0.16	1.84±0.20	7.32	11.14	10.65	-8.14
	10	8.73±0.56		6.39		-12.69	
	50	49.45±6.36	45.84±5.77	12.86	12.60	-1.09	-8.33
	150	157.42±9.47		6.01		4.95	
	300	306.64±22.17	302.49±37.71	7.23	12.47	2.21	0.83
	450	450.62±17.02		3.78		0.14	
	600	589.05±16.69	517.91±30.49	2.83	5.89	-1.83	-13.68

All concentrations in ng mL^{-1}; CV – Coefficient of variation; RE – Relative error [(measured concentration-spiked concentration/spiked concentration) x 100; *Mean values ± standard deviation.

3.2.4. Extraction efficiency

Absolute recovery values ranged from 80 to 110% in urine, and from 81 to 88% in plasma (Table 6). Abe *et al.* [10] reported recovery values between 86-91% for MXE from plasma by means of turbulent flow on-line extraction. However, an adequate comparison with our results cannot be done since the determination of MXE in urine and plasma samples with MEPS has not yet been reported in literature, but in general our results can be considered adequate. Besides the good recoveries achieved, the suggested method, particularly when compared to those normally used in drug abuse analysis, reveals many practical advantages, namely minimal solvent consumption, small amount of sample required, simple device, easy operation and low-cost.

Table 6. Absolute recovery (n=3).

Matrix	Concentration (ng mL^{-1})	Recovery* (%)
Urine	10	109.05±11.72
	150	82.99±8.30
	450	80.81±3.18
Plasma	50	87.95±4.88
	200	81.92±1.08
	400	83.84±0.78

*Mean values ± standard deviation

3.2.5. Stability

Regarding processed samples stability, the obtained CVs values were lower than 8%, indicating that the analytes are stable at least for 24h in the autosampler at room temperature. The obtained CVs for short-term stability were lower than 10%, meaning that the analytes are stable in the samples for at least 24h at room temperature. Finally, MXE is also stable for at least three freeze/thaw cycles since the obtained CVs were lower than 7%. Therefore, sample analysis can be carried out within a comfortable time window, since the analytes are not significantly affected by the storage conditions.

4. **Conclusions**

A simple and fully validated method was described for the determination of methoxetamine in human urine and plasma samples, by means of MEPS and GC–MS/MS. The method was found to be linear within the studied calibration range, and presented adequate precision and accuracy. The combination of MEPS as extraction procedure with GC-MS/MS has shown to be suitable for the determination of this analyte in urine and plasma samples, achieving goods limits of detection and high recoveries. Furthermore, no derivatization step was needed to accomplish the analysis, making the procedure less laborious and time-consuming. Moreover, when compared to other conventional extraction processes, MEPS is faster and uses much lower amounts of organic solvents, minimizing environmental problems. Another advantage is that the sorbent can be reused several times, reducing the costs of analysis. The analyses were carried out using reduced sample volumes (0.25 mL), which provides a considerable advantage, mainly when small amounts of sample are available, enabling further exams to be performed if necessary. For that reason, this procedure is suitable for laboratories performing

regular urine and plasma analysis in the fields of clinical and forensic toxicology, who are lacking for established analytic methods. Furthermore, this is the first time that the combination of MEPS and GC-MS/MS was used for the identification and quantification of MXE in urine and plasma samples.

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Chapter IV

1. Discussion

Although each of the experimental articles covering the research work performed has its own discussion (Chapter III), this section is intended to discuss in a more integrated and wider way all the investigation studies presented in this thesis.

With regard to this type of substances, traffickers always go one step ahead of the authorities. The continual search for new synthesis processes and the indiscriminate consumption of these drugs usually result in an increasing number of reports of seizures and poisoning. Therefore, the use of these substances remains a serious public health issue and still accounts for a large proportion of the overall health and social costs associated with drug use. For these reasons, the assessment of NPS use and abuse scenarios is extremely important and as such sensitive analytical methods are needed for consistent determination of these compounds in biological samples. Indeed, analytical methods for the measurement of drugs and metabolites concentrations in biological matrices play a decisive role, by the quality of the data provided in the evaluation and interpretation of results arising from preclinical and/or biopharmaceutics and clinical pharmacology studies [1,2]. In addition, the reliability of the analytical results is a matter of great significance in forensic and clinical toxicology [3]. Indeed, imprecise or unreliable analytical data could not only be questioned in court, but could also lead to unfounded legal consequences for the suspect or to wrong diagnosis or treatment of the patient [3,4]. To counteract these undesirable problems and to obtain reliable results which can be satisfactorily interpreted, it is essential that the applied bioanalytical methods are well characterized, fully validated and documented [1–3,5].

Thus, before a bioanalytical method can be employed for routine use, it must be validated in order to demonstrate its reliability and reproducibility for the determination of an analyte of interest in a particular biological sample [1,2,6].

The validation of an analytical method is the systematic process that purposes to demonstrate that the methodology is acceptable for the intended analytical applications and ensures a better confidence of the collected data [2,6]. Therefore, the following fundamental parameters must be evaluated: calibration model (linearity), limits of detection and quantification, accuracy, precision, selectivity, sensitivity, recovery, and stability of the analytes [1,2]. The success of the validation process comprises, however, the prior existence of an analytical method properly developed [7]. Therefore, it is not possible that the validation procedure of analytical methods is staked out of the relevant conditions of development, once the validation studies determine if the state of development of the

method is fairly acceptable or if changes are necessary to improve the analytical procedures and subsequent revalidation [4].

In the development and validation of analytical procedures, all of the variables of the method should be considered, e.g. the biological sample used and its pre-treatment, chromatographic separation of the analyte, detection system and evaluation of results [8,9]. The development of the analytical method allows an analyte of interest to be identified and quantified in a sample. This analyte can be regularly measured by various methods and the choice of an analytical method includes some concerns, such as matrix selection, chemical properties of the compound, quantitative or qualitative measurement, required precision, necessary equipment, speed and cost of the analysis [6]. Accordingly, the development of a novel analytical method is the utmost critical step of the process and can implicate only the adaptation of an existing method, with slight variations suitable for the new application, or conversely may comprise original ideas and procedures that require harder work, being often unclear whether the required level of development will be achieved [10].

Assumed these considerations about bioanalytical methods validation and the general scope of the work to be performed, the first assignment was the development of chromatographic methods to identify salvinorin A, ketamine and norketamine, and methoxetamine. This was accomplished by means of GC-MS/MS.

1.1. Gas chromatography-tandem mass spectrometry (GC-MS/MS)

GC was chosen to identify salvinorin A, ketamine and norketamine, and methoxetamine, since the studied substances are volatile and they are best separated by gas chromatography, as it enables higher resolution, shorter analysis time and higher selectivity compared to other chromatographic techniques such as LC [11]. Another important choice in the development of the method is the detector. The detector must be chosen taking into account the sensitivity, linearity and specificity required. GC can be combined with different detection systems, it is one of the most widely used analytical techniques and, consequently, of better performance [11]. The coupling of gas chromatography to mass spectrometry has become the "gold standard” technique for clinical and forensic substances identification and quantification, since it tests for specific substances and not for a general composition or identification. This combination is very sensitive, i.e., low levels can be detected in a precise, specific and rapid way allowing the identification of all types of drugs of abuse in any body fluid [12,13]. GC separates mixtures of chemicals into individual components and the components are fragmented into ionized species which are then separated based on their mass-to-charge ratio (m/z). This is the great advantage of the combination of GC as the first separation step and MS/MS as the qualitative detector. GC-MS/MS is also the preferred method to use in terms of costs and operation. As regards the detection of the compounds, the use of mass spectrometry is essential, as only this type of technology allows unambiguous identification of

the toxic substances present in the sample – "fingerprint" - which is of vital importance in forensic contexts; in addition, there is also increased sensitivity and the possibility of using deuterated internal standards.

1.2. GC-MS/MS development

GC-MS methods offer several ways of analyzing compounds, including full scan mode, selected ion monitoring (SIM), product ion (PI) and multiple reaction monitoring (MRM). In the full scan mode, the mass spectrometer is programmed to analyze all masses of its operation spectrum within a certain range of *m/z*, which is essential for the identification of the characteristic ions of a compound (precursor ions). In the SIM mode, after ionization, only specific ions belonging to the compound of interest are monitored, thereby increasing the sensitivity and allowing lower limits of detection [11]. The PI mode allows identifying the resulting ions from the fragmentation of the precursor ions. MRM mode works like a double mass filter, which extensively decreases noise and increases selectivity, monitoring one precursor ion (parent mass) and two daughter ions [14]. To choose the appropriate precursor and product ions for the studied compounds, a solution of each analyte in study at 100 µg/mL was prepared in methanol and injected in full scan mode (50–500 *m/z*) to obtain the retention time of each analyte. Analytes were then identified by comparing the obtained mass spectrum with those available in the mass spectra library of the instrument. Once identified the analytes, the characteristic ions from the scan mass spectrum (i.e., the precursor ions - most abundant ions) were selected. PI mode was used to identify the ions resulting from the fragmentation of the precursor ions also called product ions or daughter ions. In the PI mode, the selected ion enters a high vacuum chamber - collision cell - where they are exposed to an ionization source [collision energy (CE)] that breaks apart the compound into a number of ionized fragments. These new fragments are analyzed and separated into their individual masses, creating a reproducible fragmentation pattern (mass spectrum) – fingerprint of the drug [15]. To optimize the voltage in collision-induced dissociation, values of 5, 10, 15 and 20 V were selected. Nitrogen (purity ≥99.999%) was used as the collision gas, at a flow rate of 2.5 mL/min. Each precursor ion with each of its product ion represents an exclusive transition for each compound. Selected transitions were chosen based on selectivity and abundance, in order to maximize the signal-to-noise ratio in matrix extracts. In the obtained mass spectrum, the most abundant product ion was selected to be the quantitative ion, allowing compound quantification, while the next most abundant product ion was chosen to be the qualitative ion for MRM transition for each analyte. Qualitative ion is a confirmatory ion in analyte identification. Only if the two product ions are present the analyte can be successfully identified and quantified.

After choosing these ions for all the analytes, an injection was carried out in MRM mode, at a considerably lower concentration in order to exclude eventual interferences between the

analytes and the biological matrix. In this mode of analysis, the instrument searches only for the previously chosen transitions, highlighting the greater selectivity of the approach. The dwell time, which describes the time taken to analyze a particular transition, was also optimized. It can be adjusted so that specified transitions can be measured for longer periods, increasing the sensitivity of detection [16]. Table 3 summarizes the selected dwell times, as well as the collision-induced dissociation energies, the transitions and the retention times for each analyte.

Table 3. GC-MS/MS parameters (quantification transitions underlined).

Compound	Retention Time (minutes)	MRM transition, *m/z*	Collision energy (eV)	Dwell time (µs)
Quinalphos (IS)	10.32	296.8→156.0	10	93.8
Salvinorin A	16.26	272.1→121.1		56.3
		272.1-> 93.0		44.9
NK	6.78	165.7→131.0	10	26.8
		165.7→148.7		82.3
NK-*d4* (IS)		169.8→135.2		34.4
K	6.92	179.7→150.8	10	22.4
		179.7→115.9	15	29.9
K-*d4* (IS)		183.7→155.0	5	20.8
MXE	8.28	189.6→115.0	20	36
		189.6→147.1	10	26.6
		189.6→189.6	10	74.5
K-*d4* (IS)	8.12	183.7→120	20	21.8

Owing to the relatively high polarity and thermal instability of many compounds in GC, they tend to be decomposed at high column temperatures, leading to a bad peak shape [17]. Moreover, the similar fragmentation patterns between the target analytes and consequently poor diagnostic ions in the mass spectrum makes their analysis a difficult task [18]. Therefore, to avoid this kind of situation, a chemical derivatization step (ChD) is required. Derivatization is a procedure that modifies mainly an analyte’s functionality, leading to new compounds with altered polarity and volatility properties [17,19,20]. It also allows for more characteristic mass spectrum fragment ions and improves peak symmetry by reducing tailing [17,20,21]. This enables the compounds to become volatile enough for analysis, improving sensitivity and selectivity [17]. Despite the advantages of derivatization, we opted not to

derivatize our extracts, since the equipment response for the studied compounds was high enough for identification and quantification. Furthermore, derivatization is a tedious process that usually requires long reaction times, hazardous chemicals and produces many side reactions [21].

1.3. Development and optimization of the extraction technique

The complexity of the biological matrices, which may contain several types of interferences with similar properties to the compounds of interest, requires the pretreatment of the sample before chromatographic analysis, in order to obtain a clear and free of contaminants extract, increasing also sensitivity and selectivity. The MEPS pretreatment technique has several advantages over other preparative techniques, including the low consumption of organic solvents, shorter extraction times, lower volume of sample used and ease of automation. Despite the fact that information on the use of this approach in sample pretreatment for our compounds, it was decided to use this technique in our study, improving the analytical performance and detection capability. MEPS theory was previously described in Chapter I, so the following lines specifically state the development and optimization procedure of the technique.

The development and optimization of the MEPS procedures were performed using the DOE approach, a statistical tool applied to decision making process that evaluates in a multivariate form all the factors involved in the extraction.

The factors capable of influencing MEPS, and mentioned with detail in **articles II** to **IV** were systematically optimized by DOE to improve the extraction performance (recovery, repeatability and selectivity). These parameters included the kinds of sorbents, number of sample aspirations through the device (strokes), amount of acid and organic solvent in the washing step (in order to eliminate interferences), amount of organic solvent in the elution step and volume of eluting solvent. In **article II**, due to the non-polar nature of the salvinorin A, we decided to use a non-polar sorbent (hydrophobic C_{18}), since C_{18} phases are suitable for non-polar analytes using hydrophobic interaction mechanisms. Using the C_{18} sorbent, the final conditions were as follows: strokes (6), the endogenous interferences were removed with 0.15 mL of 8% 2-propanol in 2% formic acid and the analyte was finally eluted with 50 µL of methanol:acetonitrile (7:3, v/v). For the determination of K and NK (**article III**), a mixed-mode polymeric sorbent M_1 suitable for the extraction of these basic compounds was selected for sample preparation. The selectivity of this MEPS support is increased due to the combination of cation exchange and hydrophobic interactions. Using this cartridge, the samples were withdrawn and passed through the device 8 times in urine and 26 times in plasma, then the sorbent was washed with 0.25 mL of 5.25% acetic acid [0.1 mL of 0.1% acetic acid for plasma] and 0.1 mL of 5% methanol in water [10% methanol for plasma] to

remove matrix-borne interferences. Finally the analytes were eluted with 0.1 mL of 6% ammonia in methanol [3% ammonia in methanol for plasma]. In **article IV** two sorbents were evaluated (C_{18} and M_1). The mixed-mode column gave the best results regarding recovery and absence of interfering substances. The optimized final conditions were: 10 (urine) and 20 (plasma) strokes; amount of formic acid in wash solution 0.55% (urine) and 1.45% (plasma); amount of ammonia for eluting the analyte 1.75% (urine) and 3% (plasma); volume of elution solvent 300 µL (urine) and 1000 µL (plasma). The difference in the number of strokes between urine and plasma relates to the fact that plasma samples should be diluted more times, in order not to obstruct the extraction mechanism, so a higher number of aspirations should be performed to allow the whole sample to pass through the device.

After development and optimization of the chromatographic and extraction techniques, the methodologies were fully validated according to the mentioned internationally accepted criteria. In this manner, it was possible to provide a new and innovative bioanalytical tool using for the first time MEPS as sample preparation technique. The methods proved to be selective, sensitive enough, precise and accurate, and linear in a wide concentration range, making them important analytical tools for laboratories performing regular urine and plasma analysis in the field of forensic toxicology. In general, the obtained limits of detection (LOD), 5 ng/mL for salvinorin A, K and NK, and 1 ng/mL for MXE, were satisfactory when compared to those obtained by other authors. For example, Barnes *et al.* [22] and Pichini *et al.* [23] obtained a LOD of 5 ng/mL for salvinorin A, Harun *et al.* [24] reached a LOD of 0.6 ng/mL in urine for K and NK using LC-MS/MS and Abe *et al.* [25] reported similar LOD to ours for MXE. However these limits were obtained, for example, by using higher sample volumes (20 mL) or after hydrolyzing the samples, becoming the process more laborious, time-consuming and with higher organic solvent use. The choice of an initial volume so small of biological samples demonstrated to be a considerable advantage, mainly when reduced amounts of sample are available, allowing further exams to be performed if necessary, not compromising the research of other compounds in a systematic toxicological analysis. It also enabled compliance with the requirements as regards the number of analyses to be performed on the quantification of the analyte (in triplicate). Using the aforementioned approaches, absolute recoveries ranged from 71 to 80% for salvinorin A, from 73 to 101% for K and NK in urine and 63-89% in plasma, and from 80 to 110% (urine) and 81-88% (plasma) for MXE. Our results were considered adequate when compared to those obtained by other authors that reported recovery values ranging from 89-97% for K and NK [17,24] or 86-91% for MXE [25] or 93% for salvinorin A [23] using other extraction techniques. However, an adequate comparison with our results cannot be adequately done since the determination of these compounds by MEPS, and in the studied biological samples, has not yet been reported in literature. It can be concluded that the exhaustive and thorough optimization of the various sample preparation

techniques has originated suitable recovery values, and consequently in quite low limits of detection and quantification.

1.4. Method applicability

The developed methods were applied to real samples of suspected consumers of recreational drugs. However, none of the analyzed samples was positive for the other studied drugs of abuse. We believe that the difficulties of positivity for NPS are related with the lack of knowledge and information about these new drugs; indeed, when these consumers arrive at hospital emergency services with specific symptoms of abuse of these substances, they may be mistaken for poisoning by the more commonly used drugs, such as cocaine, opiates or alcohol and may be erroneously treated according to these symptoms. Consequently, the drugs would not be present anymore in body fluids at the time of sampling. Taking into account our limits of detection, which would allow drug detection at low concentrations (the concentrations reported in the literature are usually higher than our limits), it is likely that either the individuals didn’t consume these drugs, or in the case that they did, consumption occurred a long period of time before sample collection.

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Chapter V

Conclusions

The experimental work presented in this thesis consisted in the development and full validation of bioanalytical methods for the identification and quantification of salvinorin A, ketamine and its major metabolite norketamine and methoxetamine in biological samples.

Concisely, the most pertinent achievements and conclusions obtained through the work subjacent to the present thesis are the following:

1. Several methodologies using gas chromatography coupled to tandem mass spectrometry were developed, allowing the identification and quantification of some new psychoactive substances in urine and plasma samples, with prior extraction of compounds by means of microextraction by packed sorbent.

2. Combination of MEPS as extraction technique with GC-MS/MS showed to be adequate for the determination of these compounds in urine and plasma samples, achieving good LOD and high recoveries.

3. The methods were successfully validated in accordance to internationally accepted guidelines, and showed to be selective, linear within the studied ranges, precise and accurate, presenting in addition low detection (5 ng/mL for salvinorin A, K and NK and 1 ng/mL for MXE) and quantification limits, using sample amounts as low as 0.25 mL.

4. The choice of a low sample amount demonstrated to be a considerable advantage, which is of merit, particularly in those situations where sample availability is an issue, as often occurs in forensic scenarios. Furthermore, more tests can be performed on the same sample is necessary.

5. The major parameters capable of influencing the extraction were initially optimized with the objective of maximizing recovery, thereby allowing low limits of detection and quantification to be obtained. In this context, the design of experiments has

proven to be a useful tool in optimizing the process, allowing reducing the number of experiments and permitting significant cost and time savings.

6. The use of a mixed-mode stationary phase (M_1) for K, NK and MXE and C_{18} for salvinorin A led to clean chromatograms and high recovery values, which ranged from 71 to 80% for salvinorin A, 63-77% for NK, 73-101% for K and 80 to 101% for MXE.

7. The use of GC-MS/MS showed to be extremely useful in the determination of those drugs of abuse, mainly concerning the use of mass spectrometry, because this kind of detection allows the unequivocal identification of the studied compounds, which is of vital importance both in the clinical and forensic contexts.

8. Thus, given the lack of screening methods for this type of compounds in those matrices with MEPS, the proposed methodologies seem to be appropriate for implementation in routine laboratories, where fast bioanalytical assays are essential whenever a large number of samples have to be analyzed.

Annexes

List of publications

Articles included in the thesis:

Article I – Role of microextraction sampling procedures in forensic toxicology

Mário Barroso, Ivo Moreno, Beatriz da Fonseca, João António Queiroz, Eugenia Gallardo

Bioanalysis (2012) 4:1805-1826.

Article II – Analysis of Salvinorin A in urine using microextraction in packed syringe and GC–MS/MS

Ivo Moreno, Beatriz da Fonseca, David Oppolzer, Ana Martinho, Mário Barroso, Angelines Cruz, João António Queiroz, Eugenia Gallardo

Bioanalysis (2013) 5:661-668.

Article III – Determination of ketamine and its major metabolite, norketamine, in urine and plasma samples using microextraction by packed sorbent and gas chromatography-tandem mass spectrometry

Ivo Moreno, Mário Barroso, Ana Martinho, Angelines Cruz, Eugenia Gallardo

Journal of Chromatography B (accepted for publication; doi: 10.1016/j.jchromb.2015.09.032; 2015).

Article IV – Analysis of methoxetamine in urine and plasma samples using microextraction by packed syringe and gas chromatography-tandem mass spectrometry

Ivo Moreno, Mário Barroso, Angelines Cruz, Eugenia Gallardo

Submitted for publication to the Journal of Chromatography A (2015).

List of scientific communications

Oral scientific communications during the Doctoral work:

I- **Microextracção em seringa empacotada: aplicação na determinação de novas drogas de abuso**

I Moreno, B da Fonseca, D Oppolzer, M Barroso, S Costa, A Cruz, J A Queiroz, E Gallardo

XI Congresso Nacional de Medicina Legal e Ciências Forenses, Évora, Novembro 2012.

Poster presentations during the Doctoral work:

I- **Analysis of Salvinorin A in human urine using microextraction by packed sorbent and gas chromatography-tandem mass spectrometry**

IED Moreno, BM da Fonseca, M Barroso, JA Queiroz, E Gallardo

22nd Conference of the International Academy of Legal Medicine, Istambul, Turquia, Julho 2012.

II- **Análisis de ketamina y norketamina en muestras de orina por cromatografia de gases/espectrometria de masas en tándem**

Moreno I, da Fonseca B, Barroso M, Cruz A, Queiroz J, Gallardo E.

XX Congresso Español de Toxicologia y IV Iberoamericano, Salamanca, Junho 2013.

III- **Gas chromatography-tandem mass spectrometry analysis of ketamine and norketamine in urine using MEPS.**

Moreno Ivo, da Fonseca Beatriz, Costa Suzel, Barroso Mário, Cruz Angelines, Queiroz João A, Gallardo Eugenia

The 51st Annual Meeting of The International Association of Forensic

Toxicologists (TIAFT), Madeira, Setembro 2013.

IV- **Determinação de ketamina e norketamina por cromatografia gasosa acoplada à espectrometria de massa e com recurso à microextracção em seringa empacotada**

I.E.D. Moreno, B.M. da Fonseca, M. Barroso, S. Costa, A.C.Landeira, J.A. Queiroz, E. Gallardo

12.º Congresso Nacional de Medicina Legal e Ciências Forenses, Porto, Novembro 2013.

V- **Aplicação da microextracção em seringa empacotada para a determinação de ketamina e norketamina em amostras de urina**

I.E.D. Moreno, B.M. da Fonseca, M. Barroso, A. Cruz, J.A. Queiroz, E. Gallardo

8.º Encontro Nacional de Cromatografia, Covilhã, Dezembro 2013.

VI- Desenvolvimento e validação de um método analítico para a determinação da metoxetamina em amostras de urina

I Moreno, M Barroso, JA Queiroz, A Cruz, E Gallardo

II Jornadas Ibéricas de Toxicologia, Covilhã, Novembro 2014

VII- Identificação e quantificação de ketamina e norketamina em amostras biológicas por cromatografia gasosa acoplada a espectrometria de massa em tandem

I Moreno, M Barroso, JA Queiroz, A Cruz, E Gallardo

II Jornadas Ibéricas de Toxicologia, Covilhã, Novembro 2014

VIII- Analysis of methoxetamine in biological samples by using microextraction by packed syringe and gas chromatography-tandem mass spectrometry

Moreno I., Barroso M., Cruz A., Gallardo E.

X Annual CICS-UBI Symposium, Covilhã, Portugal, July 2015

Collaboration in other projects:

Research Fellowships:

- **Fellowship UBI/Santander-Totta**
  Universidade da Beira Interior, Covilhã, 2011-2013

- **Research project: "Therapeutic drug monitoring on age related diseases", financed by Regional Center Operational Programme "Mais CENTRO-07-ST24-FEDER"** Universidade da Beira Interior, Covilhã, 2013-2015.

Articles:

- **Determination of seven selected antipsychotic drugs in human plasma using microextraction in packed sorbent and gas chromatography-tandem mass spectrometry**
  B.M. da Fonseca, I.E.D. Moreno, Cruz B, M. Barroso, J.A. Queiroz, E. Gallardo

  Analytical and Bioanalytical Chemistry 2012, 405 (12):3953-63.

Oral scientific communications:

- **Análise de neurolépticos em fluido oral por SPE e GC/MS/MS**
  D. Oppolzer, B. Fonseca, I. Moreno, J. Queiroz, M.Barroso, E. Gallardo

  XI Congresso Nacional de Medicina Legal e Ciências Forenses, Évora, Novembro 2012.

- **Determinación de antipsicóticos en plasma por cromatografía de gases-espectrometría de masas en tándem: comparación de dos metodologías de análisis**
  da Fonseca B, Moreno IM, Oppolzer D, Barroso M, Queiroz JA, Gallardo E
  XX Congresso Espanõl de Toxicologia y IV Iberoamericano, Salamanca, Junho 2013.

- **Desenho experimental: Uma ferramenta importante no desenvolvimento de uma metodologia para determinação de anticoagulantes orais e antiagregantes plaquetários em sangue**

A. Pires, D. Figueirinha, T. Rosado, I. Moreno, A. Martinho, M. Magalhães, D. Ferreira, A.P. Duarte, L. Breitenfeld Granadeiro, G. Alves, S. Silvestre, F. Domingues, E. Gallardo

IV Encontro Nacional em pós-graduação em Ciências Biológicas, Aveiro, 30 de Março a 02 de Abril de 2015.

Posters presentation:

- **Novel methodology using microextraction in packed sorbent for the determination of antipsychotic drugs in plasma by GC-MS/MS**
  BM da Fonseca, IED Moreno, M Barroso, JA Queiroz, E Gallardo
  22nd Conference of the International Academy of Legal Medicine, Istambul, Turquia, Julho 2012.

- **Desenvolvimento e validação de uma metodologia para determinação de antipsicóticos em plasma por GC-MS/MS**
  B da Fonseca, I Moreno, D Oppolzer, M Barroso, J A Queiroz, E Gallardo
  XI Congresso Nacional de Medicina Legal e Ciências Forenses, Évora, Novembro 2012.

- **Determinación de antipsicóticos en fluido oral por cromatografia de gases/espectrometria de masas en tandem**
  da Fonseca B, Moreno I, Oppolzer D, Barroso M, Varela S, Oliveira V, Leitão C, Queiroz JA, Gallardo E
  XX Congresso Español de Toxicologia y IV Iberoamericano, Salamanca, Junho 2013.

- **Analysis of selected antipsychotics in water using microextraction in packed sorbent and GC-MS/MS**
  Lourenço C., da Fonseca B., Moreno I., Barroso M., Queiroz J.A., Araújo R.T.S.A., Gallardo E.
  VIII CICS Symposium, Covilhã, Julho 2013.

- **Determination of selected antipsychotics in oral fluid using solid-phase extraction and GC-MS/MS**
  da Fonseca Beatriz, Moreno Ivo, Oppolzer David, Fonseca Suzana, Barroso Mário, Queiroz João A, Gallardo Eugenia
  The 51st Annual Meeting of The International Association of Forensic Toxicologists (TIAFT), Madeira, Setembro 2013.

- **Triagem e confirmação de 13 antidepressivos em plasma por microextracção em seringa empacotada e cromatografia gasosa acoplada à espectrometria de massa**
  I. Moreno, A. Gonçalves, S. Silva, A. Martinho, L. Breitenfeld, S. Silvestre, F. Domingues, G. Alves, AP Duarte, E. Gallardo
  12.º Congresso Nacional de Medicina Legal e Ciências Forenses, Porto, Novembro 2013.

- **Screening of 13 antidepressant drugs in human plasma using microextraction in packed sorbent and gas chromatography-mass spectrometry**
  A. Gonçalves, I. Moreno, A. Martinho, S. Silva, AP Duarte, L. Breitnfeld, S. Silvestre, F. Domingues, G. Alves, E. Gallardo
  8.º Encontro Nacional de Cromatografia, Covilhã, Dezembro 2013.

- **Simultaneous determination of selected antipsychotic drugs in oral fluid using GC-MS/MS**
  B.M. da Fonseca, I.E.D. Moreno, D. Oppolzer, M. Barroso, J.A. Queiroz, E. Gallardo
  8.º Encontro Nacional de Cromatografia, Covilhã, Dezembro 2013.

- **Monitorização terapêutica de antidepresivos: desenvolvimento de um método analítico com recurso à microextracção em seringa empacotada** T. Rosado, I. Moreno, A. Gonçalves, A. Martinho, S. Silva, A.P. Duarte, S. Silvestre, G. Alves,F. Domingues, L. Granadeiro, E.Gallardo
  II Jornadas Ibéricas de Toxicologia, Covilhã, Novembro 2014.

- **Comparação de duas técnicas de extração para quantificação de anticoagulantes e antiagregantes plaquetários em sangue por UHPLC-DAD**

  D. Figueirinha, A. Pires, T. Rosado, I. Moreno, A. Martinho, M. Magalhães, D. Ferreira, A.P. Duarte, L. Breitenfeld Granadeiro, G. Alves, S. Silvestre, F. Domingues, E. Gallardo

  IV Encontro Nacional em pós-graduação em Ciências Biológicas, Aveiro, 30 de Março a 02 de Abril de 2015.