UNIVERSIDADE DA BEIRA INTERIOR

Ciências

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Cláudia Isabel Reis Margalho

Tese para obtenção do Grau de Doutor em
Bioquímica
(3º ciclo de estudos)

Orientador: Prof. Doutor Manuel Lopez-Rivadulla Lamas
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Resumo Alargado

A principal razão para o desenvolvimento do presente estudo residiu essencialmente no facto de que nele se reuniram duas áreas de crescente interesse no campo da toxicologia forense: as amostras alternativas postmortem e as novas substâncias psicoativas.

Em primeiro lugar, é possível verificar que as matrizes alternativas têm tido um papel importante no campo de toxicologia forense ao longo da última década, principalmente devido às vantagens que apresentam quando usadas em complementaridade com o sangue e/ou urina ou em sua substituição, quando as suas colheitas são inviabilizadas por diversos fatores (carbonização, hemorragia extensa, politraumatizados, avançado estado putrefativo etc.).

O recurso a diferentes matrizes biológicas postmortem, na condução de uma análise toxicológica, constitui um verdadeiro desafio para a toxicologia forense pois são necessárias metodologias suficientemente sensíveis para proceder ao isolamento dos diferentes analitos que eventualmente estejam presentes em baixas concentrações nas amostras a analisar. No entanto, entende-se que uma análise toxicológica conduzida em distintas matrizes biológicas permite uma interpretação mais completa do caso, já que cada uma delas poderá fornecer uma perspetiva particular sobre a anterior exposição a determinadas substâncias. Mais problemático, e não menos raros, são os casos em que a quantidade de sangue é insuficiente ou mesmo inexistente, para realizar a investigação pericial. Nestes casos, o humor vítreo (VH) e o líquido pericárdico (PF) poderão ser usados como alternativa viabilizando, deste modo, a realização da análise.

Apesar de escassos, os estudos realizados em VH na determinação de anfetaminas e metanfetaminas e de novas substâncias psicoativas, demonstram que existe uma boa correlação entre a concentração das substâncias encontradas e as correspondentes no sangue. Esta situação poderá ser justificada pelo facto de o VH ser maioritariamente composto por água (99%), possuir um pH de 7.5 (semelhante ao do sangue) e não ter atividade metabólica significativa. A maior desvantagem desta matriz é o limitado volume de amostra (1 a 3 mL) que não permite uma análise extensiva a várias substâncias.

No caso do líquido pericárdico, o volume de amostra acessível para análise é de 5 a 10 mL, no entanto esta matriz tem sido pouco estudada. Os poucos trabalhos existentes demonstram que esta matriz poderá ser uma útil alternativa ao sangue uma vez que apresenta uma boa correlação das concentrações das substâncias encontradas nestas amostras. A maior desvantagem que esta matriz apresenta é a possibilidade de ser contaminada por fenómenos de difusão postmortem, resultantes de uma elevada concentração das drogas eventualmente presentes no estômago.

Uma vez que, em Portugal e por todo o mundo se tem assistido a uma nova tendência emergente em relação ao consumo das "novas substâncias psicoativas" (NSP) que se encontra

associada a diversos perigos na saúde pública, afigurou-se relevante realizar este estudo. O presente trabalho foi direcionado para substâncias pertencentes aos grupos mais representativos em termos de consumo e grau de toxicidade: catinonas, fenetilaminas e *salvia divinorum*.

Em Portugal, esta realidade alastrou-se de forma discreta, sem receber a atenção devida por parte de todas as entidades competentes, estando estreitamente associada à abertura das lojas designadas por "smartshops". Consequentemente, entre a abertura da primeira loja (que ocorreu em 2007) e 2013, foram registadas cerca de 40 lojas especializadas e dedicadas exclusivamente ao comércio destas NSP. Com a entrada em vigor do Decreto-lei nº 54/2013 de 17 Abril de 2013, estes locais foram encerrados por motivo da proibição da venda destas substâncias. No entanto, a comercialização e consumo destas substâncias não cessou o que é confirmado por estudos de monitorização da venda destas substâncias no espaço europeu, promovidos pelo Observatório Europeu da Droga e da Toxicodependência (OEDT) e Europol. Novas substâncias continuam em ascendente proliferação o que é indicador da existência de redes de larga escala de produção e distribuição.
Deste modo, este trabalho pretendeu contribuir para o desenvolvimento de metodologias analíticas para determinações quantitativas de várias destas novas substâncias psicoativas em distintas matrizes biológicas tais como: sangue total, plasma, humor vítreo e líquido pericárdico. As substâncias sobre as quais realizámos o nosso estudo foram: salvinorina a (principal componente ativo da planta *Salvia divinorum*); algumas catinonas como: metcatinona, PMA, mefedrona e metedrona; e algumas fenetilaminas tais como: catina, efedrina, DOM, DOB e as pertencentes à série 2C-X: 2C-H, 2C-B, 2C-I, 2C-T-2, 2C-T-4 e 2C-T-7.

Para a preparação de volumes de 250 µL de amostras de sangue, plasma e líquido pericárdico e de 100 µL de humor vítreo foi usada a extração em fase sólida (SPE). Atualmente, a SPE é uma das técnicas mais usadas na extração e/ou concentração de amostras complexas, que viabiliza a deteção de compostos em concentrações reduzidas por técnicas cromatográficas especializadas, como a cromatografia de gases e a líquida acopladas à espectrometria de massa (GC-MS e LC-MS).

Neste estudo, os extratos foram obtidos por GC-MS em modo SIM. Para habilitar a deteção e a separação das substâncias, por esta técnica cromatográfica, foi previamente aplicado um procedimento de derivatização através do uso de micro-ondas. Este procedimento, com duração de apenas 90 segundos, foi otimizado de forma a minimizar o consumo de tempo (superior a 30 minutos) que é geralmente despendido nas técnicas clássicas de derivatização para a análise de drogas de abuso. Tratando-se de uma técnica analítica pouco estudada na área da toxicologia forense, oferece-nos significativas vantagens em termos de rapidez na

derivatização completa dos compostos. O método foi aplicado a todas as substâncias, com exceção da salvinorina a, tendo sido obtidos derivados trifluoroacetilados estáveis.

Dado que a confiança na interpretação dos resultados analíticos é um pré-requisito em toxicologia forense e clínica, foi necessário garantir que os métodos desenvolvidos eram adequados para os fins pretendidos. Assim, as duas metodologias desenvolvidas foram validadas, em todas as matrizes biológicas estudadas de acordo com normas preconizadas internacionalmente para a validação de métodos bioanalíticos. No decurso da validação foram estudados os seguintes parâmetros analíticos: seletividade, intervalos de linearidade, fenómenos de arrastamento entre amostras de maior concentração para outras de concentração mais baixa, limites de deteção e de quantificação (LOD e LLOQ), precisão (intra-dia e intermédia) e exatidão, eficiência do processo extrativo e estabilidade (permanência dos extratos no amostrador em condições ambientais por 48 h, permanência das amostras na bancada de trabalho por períodos de 3h, ciclos de congelação e descongelação ao longo de 7 dias).
Os métodos foram considerados seletivos para todos os compostos estudados e em todas as matrizes analisadas. Foi obtida linearidade dentro das gamas de trabalho estudadas (5-100 ng/mL para a salvinorina a e 5-600 ng/mL para as catinonas e fenetilaminas) com coeficientes de correlação superiores a 0.99. Os valores obtidos de recuperação variaram entre 77% (2C-T-4 a 20 ng/mL) e 113% (2C-H a 500 ng/mL) para cada composto analisado nas diferentes matrizes biológicas estudadas. Verificou-se que todos os resultados obtidos para os parâmetros de repetibilidade e precisão intermédia apresentaram valores de CV<20% e que a exatidão se situou dentro do intervalo de ±20% dos valores nominais, em todos os níveis de concentrações estudados. Os métodos apresentaram valores de LOD e LLOQ de 5 ng/mL para cada um dos compostos, em todas as matrizes biológicas. As amostras não processadas que permaneceram no laboratório à temperatura ambiente durante 3h (para todas as substâncias analisadas) e os extratos deixados no amostrador (nas condições a que se encontra o sistema de GC-MS) por períodos até 24 h (salvinorina a) e 48 h (catinonas e feniletilaminas) revelaram-se estáveis. Por último, não foi verificada degradação dos compostos após três ciclos de congelação e descongelação completa ao longo de sete dias.

As metodologias desenvolvidas foram aplicadas a amostras resultantes de autópsias realizadas no serviço de Clínica e de Patologia Forenses e nos Gabinetes médico-legais pertencentes à delegação do centro do Instituto Nacional de Medicina Legal e Ciências Forenses, I.P. Estes procedimentos poderão ser uma ferramenta bastante útil em laboratórios de toxicologia forense. Até ao momento, estas substâncias não tinham sido simultaneamente estudadas em amostras postmortem de humor vítreo e líquido pericárdico.
As metodologias validadas foram implementadas nas análises de rotina do serviço de Química e Toxicologia Forenses da delegação do centro do Instituto Nacional de Medicina Legal e Ciências Forenses, I.P.

Palavras-chave

Salvinorina a, catinonas e fenetilaminas, matrizes biológicas convencionais e alternativas, derivatização conduzida por micro-ondas, cromatografia de gases acoplada à espectrometria de massa.

Abstract

The main reason for this study's development lies essentially in the fact that it gathers two areas of great interest and increasing progress in the forensic toxicology field: the postmortem alternative matrices and the new psychoactive substances.

Firstly, it is possible to verify that alternative matrices have been playing an important role in the forensic toxicology field throughout the last decade. This relates with the fact they can be used as either a blood/urine complementary or in substitution when their collection is unavailable by several factors.

In addition, Portugal and the rest of the world experienced a new emergent trend regarding the consumption of "new psychoactive substances". This phenomenon brought about major public health injuries.

The objective of the presented work was a contribution to the development of new analytical methods for the quantitative determinations of several new psychoactive substances in various biological matrices such as whole blood, plasma, vitreous humor and pericardial fluid. The substances on which we conducted our study were: salvinorin a (main active component of the plant *salvia divinorum*); some cathinones such as cathine, ephedrine, methcathinone, PMA, mephedrone, methedrone and some phenethylamines belonging to the D series, like the DOM and DOB and those that belong to the 2C-X series like 2C-H, 2C-B, 2C-I, 2C-T-2, 2C-T-4 and 2C-T-7. The mixed-mode was used (cathinones and phenethylamines) and reversed-phase (salvinorin a) adsorbents in the solid phase extraction for preparing samples of 250 µL of blood, plasma and pericardial fluid and 100 µL for vitreous humor. The obtained extracts were further analysed by gas chromatography coupled to mass spectrometry in the SIM mode. To enable the detectability and the separation of the studied cathinones and phenethylamines a fast microwave derivatization procedure was previously applied, using the MBTFA reagent. Thus, in order to minimize the time required for classical techniques of derivatization in the analysis of drugs of abuse (over 30 minutes) a microwave procedure was optimized which lasts only 90 seconds. The method was applied to all substances with the exception of salvinorin a. Stable trifluoroacetyl derivatives were obtained with this optimized methodology.

Once confidence in the interpretation of the analytical results was attained (as a prerequisite in forensic and clinical toxicology), it was necessary to ensure that the methods developed were suitable for its intended purposes. Thus, the proposed methodologies were fully validated, in all biological matrices studied according to internationally accepted recommendations.

The methods were found to be selective for all tested compounds in all matrices studied. Linearity was achieved from 5-100 ng/mL for salvinorin a and from 5-600 ng/mL for cathinones and phenethylamines. The extraction efficiencies were between 80-100% for salvinorin a and 77-113% for cathinones and phenethylamines, in all the biological matrices studied. All results obtained for the parameters repeatability and intermediate precision presented CV<20% and accuracy were within the acceptance interval of ±20% of the nominal values at all concentration levels. The methods presented limits of quantitation of 5 ng/mL for each compound, in the all biological matrices analysed.

The developed methodologies have been applied to authentic samples from autopsies performed in the Clinical and Forensic Pathology Service and in the medico-legal Offices belonging to the delegation of the centre for the National Institute of Legal Medicine and Forensic sciences, I.P. These procedures can be a useful tool in forensic toxicology laboratories. The major contributions are the complementation of the information possibly provided by each one of those fluids regarding drugs' consumption and the prevention of situations where the blood is not available due to several factors of each individual case.
In addition, it was also intended to implement the developed methodologies in routine analysis on the Laboratory of Chemistry and Forensic Toxicology of the centre branch of the National Institute of Legal Medicine and Forensic Sciences.

Keywords

Salvinorin a, cathinones and phenethylamines, conventional and unconventional biological specimens, microwave fast derivatization, gas chromatography-mass spectrometry.

Thesis Overview

This thesis is organized into fifth chapters:

The first chapter gives general information concerning the new concept of drugs, highlighting the situation in Portugal and Europe and the important role of vitreous humor and pericardial fluid as alternative postmortem biological matrices in forensic toxicology. A review article was included in this chapter (Paper I), regarding toxicological analysis in human biological specimens of *Salvia divinorum* and its active substance salvinorin a.

The second chapter presents a general description and the functionality of pericardial fluid and vitreous humor as alternative biological fluids in analytical toxicology.

The third chapter consists of the original research and is structured in two papers:

Paper II – A validated procedure for detection and quantitation of salvinorin a in pericardial fluid, vitreous humor, whole blood and plasma using solid phase extraction and gas chromatography–mass spectrometry.

Paper III - Determination of "new psychoactive substances" in postmortem matrices using microwave derivatization and gas chromatography-mass spectrometry.

The fourth chapter covers a general discussion.

Lastly the fifth chapter compiles the conclusions of the study.

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List of abbreviations

2C-B	4-Bromo-2,5-dimethoxyphenethylamine
2C-H	2,5-Dimethoxyphenethylamine
2C-I	4-Iodo-2,5-dimethoxyphenethylamine
2C-T-2	2-[2,5-Dimethoxy-4-(ethylthio)phenyl]ethanamine
2C-T-4	2,5-Dimethoxy-4-isopropylthiophenethylamine
2C-T-7	2-[2,5-Dimethoxy-4-(propylthio)phenyl]ethanamine
5-HT	5-Hydroxytryptamine (Serotonin)
ATS	Amphetamine-type stimulants
BBB	Blood-brain barrier
BL	Blood
CNS	Central Nervous System
CV	Coefficient of Variation
DA	Dopamine
DEA	Drug Enforcement Agency
DOB	4-Bromo-2,5-dimethoxyamphetamine
DOM	2,5-Dimethoxy-4-methylamphetamine
EI	Electronic impact
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EU	European Union
Europol	European Police Office
GC-MS	Gas chromatography-mass spectrometry
HPLC-UV	High-performance liquid chromatography-ultraviolet detector
INMLCF, I.P.	Instituto Nacional de Medicina Legal e Ciências Forenses, I.P.
IUPAC	International Union of Pure and Applied Chemistry
khat	*Catha edulis*
LC–ESI-MS/MS	Liquid-chromatography–tandem- pneumatically assisted electrospray ionisation mass spectrometry
LC-HRMS	Liquid chromatography–high resolution mass spectrometry
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
LSD	Lysergic acid diethylamide
MAO	Monoamine oxidase
MBTFA	N-Methyl-bis(trifluoroacetamide)
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine

MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
NA	Noradrenaline (norepinephrine)
NIDA	National Institute on Drug Abuse
NPS	New Psychoactive Substances
OEDT	Observatório europeu da droga e da toxicodependência
PCP	Phencyclidine
PF	Pericardial fluid (Líquido pericárdico)
pKa	Acid dissociation constant
PMA	1-(4-methoxyphenyl)-propan-2-amine
PMMA	*p*-methoxymethamphetamine
QC	Quality control
SA	Salvinorin a
SD	*Salvia divinorum*
SERT	Serotonin
SIM	Selected Ion Monitoring
SPE	Solid-Phase Extraction
SQTF-C	Serviço de Química e Toxicologia Forenses da Delegação do Centro
TMS	Trimethylchlorosylane
UBI	Universidade da Beira Interior
UHPLC-MS/MS	Ultrahigh-performance liquid chromatography-mass spectrometry
UN	United Nations
UNODC	United Nations Office on Drugs and Crime
UPLC-QTOF/MS	Ultrahigh-performance liquid chromatography-time-of-flight mass spectrometry detector
UPLC-QTRAP/MS	Ultrahigh-performance liquid chromatography-triple quadrupole-linear ion trap mass spectrometer detector
VH	Vitreous humor (Humor vítreo)
VMAT2	Vesicular monoamine transporter 2
WHO	World Health Organization

Justification and aims of the study

Since the turn of the 21st century, there has been a significant increase in consumption of "new psychoactive substances", already targeted as a worldwide concerning phenomenon. The entire issue is mainly based on the false concept that none of these substances actually carry any hazardous consequences to one's health due to their legalization.

These compounds known as "legal highs" have become progressively accepted among diverse age levels. Presented in many varied formulas as slight variations to the chemical structure of older traditional stimulant substances, they are already revolutionizing the market for drugs of abuse. This market's efficiency is mainly characterized by how fast its suppliers provide new alternative products in reaction to the strict measures of products' control imposed by the authorities.

These products are sold in places commonly known as "smartshops" or "headshops" and are widely distributed on the Internet in various forms, labelled "not for human use" and without any information regarding safety, interactions with other substances and side-effects. Nowadays, the internet plays a crucial role by encouraging consumption and providing easy access to these substances. There is a great variety of psychoactive synthetic chemicals, plants or fungal products which are intended to induce a psychoactive response, similar to well-known controlled drugs. The evolution of the different drug types has resulted in a continuous analytical challenge for the forensic toxicologists in the detection and identification of these substances. Their consumption has been associated with several intoxication events and deaths throughout recent years, which motivated the introduction of major improvements on the legislative drugs-related board in Portugal and many other countries all over the world. So, by aiming to develop analytic methodologies that allow the identification and quantification of these emergent drugs by gas chromatography–mass spectrometry/electronic impact (GC-MS/EI), it is clear that this work comes up as a reaction to this concerning phenomenon, consequently becoming a valuable asset to fight this global scale issue.

In Portugal, this "new psychoactive substances" issue only began to be publically debated at the end of 2010 and it initially came up as a subject not grounded on scientific facts and mainly based on speculations. Since it would not be possible to approach all the market available psychoactive substances in one single study, we directed this project at the ones responsible for major controversial issues during this work's initiation period and at those from which it was possible to acquire analytical reference standards.

The *Salvia divinorum*, despite being neither a new substance (it was consumed for centuries by Mazatec Indians in religious rituals) nor synthetic, it is a plant with hallucinogenic properties that aroused great interest among young people. In Portugal, it was a major topic in the social media, since its consumption was associated with several cases of hospital emergency.

In addition, the synthetic cathinones and phenethylamines (d-cathine (d-norpseudoehedrine), ephedrine, methcathinone, 1-(4-methoxyphenyl)-propan-2-amine (PMA), mephedrone, methedrone, 2,5-Dimethoxy-4-methylamphetamine (DOM), 4-Bromo-2,5-dimethoxyamphetamine (DOB), 2,5-Dimethoxyphenethylamine (2C-H), 4-Bromo-2,5-dimethoxyphenethylamine (2C-B), 4-Iodo-2,5-dimethoxyphenethylamine (2C-I), 2-[2,5-Dimethoxy-4-(ethylthio)phenyl]ethanamine (2C-T-2), 2,5-Dimethoxy-4-isopropylthiophenethylamine (2C-T-4) and 2-[2,5-Dimethoxy-4-(propylthio)phenyl]ethanamine (2C-T-7) were also included in the study, because of their presence, in various combinations, among the most commonly consumed substances throughout Europe. The relevance of studying these compounds mainly lies with the high incidence of their recreational use and subsequent psychoactive effects associated with great lack of knowledge regarding medium and long term effects, toxicity, and abuse potential.

According to the 2016 report of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the prevalence of the consumption of new psychoactive substances in Europe continues to be difficult to determine, due to the differences in the legal status of these substances between the various countries. On one hand, there were countries that have taken steps to restrict direct access to these substances by closing the stores where these products were being sold (such as Portugal). On the other hand, the Internet and social networks became increasingly powerful in the marketplace for the sale of these new psychoactive substances. This new reality allows direct access to online stores, increased ease for producers to access both pharmaceutical and research data and the implementation of online forums exclusively intended for information exchange between potential consumers. Despite the increasing awareness of the public and competent authorities regarding consumption of the new psychoactive substances, the knowledge of risks associated with that consumption did not follow this evolution.

It is widely known that assessing the toxicological significance of a substance in the cause of a death is an extremely difficult task. This difficulty lies in the fact that, in the majority of the drug-induced deaths, several substances were consumed. With the new psychoactive substances this is even more evident because the amount and type of compounds that are present in the formulations vary considerably.

As a consequence, the existence of simultaneously fast and sensitive methods for routine use in forensic toxicological laboratories becomes fundamental so that searching for these substances in biological samples is possible. Considering that forensic toxicology focuses mainly on postmortem analysis, it is crucial to study alternative specimens to assist death investigations.

CHAPTER 1
INTRODUCTION

1.1. The "new" concept of drug

The term "drug" has been generally used for a long time in the academic and scientific discussion and also by the World Health Organization (WHO), which abandoned the use of the expressions "narcotics" and "toxic substances". Similarly, the Single Convention on Narcotic (1961) and the Convention Against Illicit Traffic in Narcotic Drugs and Psychotropic Substances (1988) refer to "toxic substances" or "narcotics" simply by the term "drug" [1]. The word "drug" was adopted worldwide, being important to keep in mind that since the 1970's Portuguese legislation on use and illicit trafficking of drugs, has been based on the recommendations of these two international conventions [2].

According to the WHO, a drug is any substance that affects the normal functioning of the body and/or the brain. What varies is how it acts in the body of each individual, as well as the purpose of its use. When the drug is used for therapeutic purposes, it is called medicine. Therefore, all medicines are drugs, but not all drugs are medicines.
Illegal drugs are drugs that are so harmful that countries across the world have decided to control them. Countries have passed several international laws, in the form of United Nations conventions, that specify which drugs are controlled [3].

Drugs have been used and abused all over the world, since ancient times, because humankind always had a desire to smoke, drink or eat substances that make them feel relaxed, stimulated, or euphoric [4].
Historically, the use of psychoactive drugs dates back to ancient times where mind-altering substances were used to create states of consciousness so intense and meaningful that religions have traditionally considered them a spiritual and sacred phenomenon [4].

Drug abuse is a common problem and a growing concern worldwide. Over the past decade, "new atypical drugs" have emerged and expanded in popularity. The consumption of these substances induces widely differing effects and risk profiles resulting in many challenges for toxicologists and health care providers.

Drug types are categorized depending on origin and effect. They can either be *naturally occurring, semi synthetic* substances (chemical manipulations of substances extracted from natural materials) or *synthetic* compounds (created entirely by laboratory manipulation). For example, drugs derived from the opium poppy (*Papaver somniferum*) such as opium, morphine and codeine, the coca (*Erythroxylum coca*), marijuana (the dried flowering tops of the *Cannabis sativa* plant) [4] and khat (*Catha* edulis) are naturally derived psychoactives; heroin, lysergic acid diethylamide (LSD) and phencyclidine (PCP) are a semi-synthetic substances and methadone, buprenorphine, pethidine and fentanyl are synthetic products [5].

Much of what is known about this new wave of drugs in Europe comes from the EU Early Warning System. This comprehensive early warning assessment allows the Member States to directly report data to the EMCDDA and Europol on events such as law enforcement seizures, acute intoxications and deaths. The EMCDDA collects information on these substances from various national sources including police, customs, forensic laboratory networks, health care systems, event organisers, drug checks, and Internet test purchase samples. Then, the two agencies analyse and assess the data to detect signals of harm, allowing the agencies and the Member States to react in a timely manner in order to reduce that harm. Such measures include public health alerts, law enforcement operations and risk assessments; in some cases, control measures across the EU may be applied by the Council of the European Union [6].

1.2. "New" and Psychoactive Substances (NPS)

By definition the "new psychoactive substances" are a new narcotic or psychotropic substances, in pure form or in preparation, that are not controlled by the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 United Nations Convention on Psychotropic Substances, but which may pose a public health threat comparable to that posed by substances listed in these conventions (Council Decision 2005/387/JHA, 10 of May 2005) [7,8].

"New psychoactive substances" (NPS) are a range of compounds that have been created to mimic classic illicit drugs such as cannabinoids, cocaine, ecstasy and LSD [9,10]. Several terms have been used to describe these new emerging drugs, including "legal highs", "herbal highs", "party pills", "bath salts", "plant fertiliser", "research chemicals", "drug analogues", "emerging psychoactive substances", "novel psychoactive substances", "synthetic drugs", "designer drugs", "smart drugs" or simply "new psychoactive substances" [11]. This last expression (NPS) is currently the most used and includes both the emergent substances of synthetic origin (resulting from minor structural modifications of existing substances) and those of natural origin [9,10,12].
Although use of these substances has recently been widely publicized as new, a large number of them have been used recreationally for centuries, such as cathinone [13,14].

The use of psychoactive substances is as old as the history of the civilization, the first human experiences occurred through the consumption of plants [12]. Since the 19th century, man isolated active compounds of plants such as morphine, cocaine and ephedrine. However, it was at the end of the last century, with the emergence of amphetamines, that a psychoactive substance was fully synthesized in laboratory. With the emergence of synthetic drugs in the 1980's, the popularization of designer drugs occurred [15]. The dissemination of the designer drugs arose with the appearance of the synthetic drugs 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) in the nineteen sixties and eighties,

respectively [16,17]. These drugs have as their essential characteristic laboratory modification and manipulation, in order to enhance or create psychoactive effects, to prevent undesirable effects and to circumvent drug laws [18]. In addition, the availability and the reduction of technological cost allowed such drugs to be rapidly synthesized in clandestine domestic laboratories [15]. In the 1990's, with the disclosure of the internet, there was a huge dissemination and distribution of the NPS [12]. However, it was in the first decade of the 21st century that the number of new users of these substances had increased significantly, especially in recreational use, with reports of seizure of various designer drugs worldwide [12]. Consequently, the urgent need for the detection, confirmation and quantification of these compounds for clinical and forensic purposes is understood. From an analytical view this constitutes a major challenge for the toxicology laboratory due to the diversity and versatility of the changes that occurred in the molecules and the speed at which they have appeared on the market. The appearance of new substances on the market is faster than the development of appropriate methods for their determination.

The phenomenon of NPS consumption depends both on the social context and the social representations of its consumers. Even though the same substances are scattered throughout the world their abuse depends on local legal control. As has occurred in other countries, in Portugal this phenomenon only received proper attention after having reached alarming proportions [12].

The consumption of these substances suffered an exponential increase especially amongst the younger age groups, coinciding with the opening of the so called "smartshops" where these products were freely sold camouflaged under many guises (incense, pills, powders, bath salts, decorative snow, plant feeder), often containing small amounts of illegal drugs, sometimes labelled "not for human use", "not for sale to minors", "keep out of reach of children" or "not tested for hazards or toxicity" " [13,19–24]. In the vast majority of cases consumers are unaware of the actual content of these products [12].

The first "smartshop" opened in the Netherlands (Amsterdam) in 1994.The first store to open in Portugal was in 2007 (Aveiro). Numerous stores have emerged across Europe and via the internet [12,25]. By this time, the products sold on these shops were a variety of natural origin such as herbs (e.g. *salvia divinorum* and kratom), seeds (e.g. hawaiian baby woodrose), fungi (e.g. hallucinogenic mushrooms) and cacti (e.g. peyote), designated by "herbal highs" [12,26,27]. Posteriorly, a second wave of products with the same designation, were sold in those stores, composed of various naturally occurring and new semi-synthetic or synthetic compounds with emphasis on both cathinones and its derivatives, synthetic cannabinoids and piperazines [11,25]. Thus, if initially the products that were sold in such stores were natural, currently there is no guarantee regarding the type and origin of the products that the consumer acquires, which further increases their exposure to unknown hazards [12].

One of the greater and most important groups of psychostimulant drugs are the phenethylamine derivatives. These newer alternative drugs have significant differences from traditional amphetamines. Despite the similarity in chemical structure among many of these synthetic derivatives, small changes can have a great impact in potency, hallucinogenic effects and in undesirable symptoms in humans [28–31].

In recent years we have witnessed an unprecedented growth in the number of NPS on the illicit drug market. In the European Union, 41 novel psychoactive substances were identified for the first time in 2010, 49 in 2011, 73 in 2012, 81 in 2013, and 101 in 2014 for the European Early Warning System [32]. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), between the years 2000 and 2005 five NPS's emerged per year, and that number doubled by the end of 2008. From 2009 to the end of 2015, there has been a clear exponential increase with more than 560 NPS's being currently monitored in Europe [33]. Currently, more than one new substance is identified in one of the EU countries every week [33]. However, synthetic cathinones and synthetic cannabinoids were the two groups with the most prominent place in the number of notifications [32,33]. Purity and composition of products containing NPS are often not known, which places users at high risk as evidenced by hospital emergency admissions and deaths, sometimes as a result of polysubstance abuse. NPS's have become a global phenomenon with over a hundred countries and territories from all regions of the world having reported one or more NPS's (Figure 1). Up to December 2015, more than 600 substances have been reported to the United Nations Office on Drug Crime (UNODC) Early Warning Advisory (EWA) on New Psychoactive Substances (NPS) [34] by governments, laboratories and partner organizations. The NPS available on the market have similar effects to substances under international control such as cannabis, cocaine, heroin, LSD, MDMA (ecstasy) or methamphetamine [34].

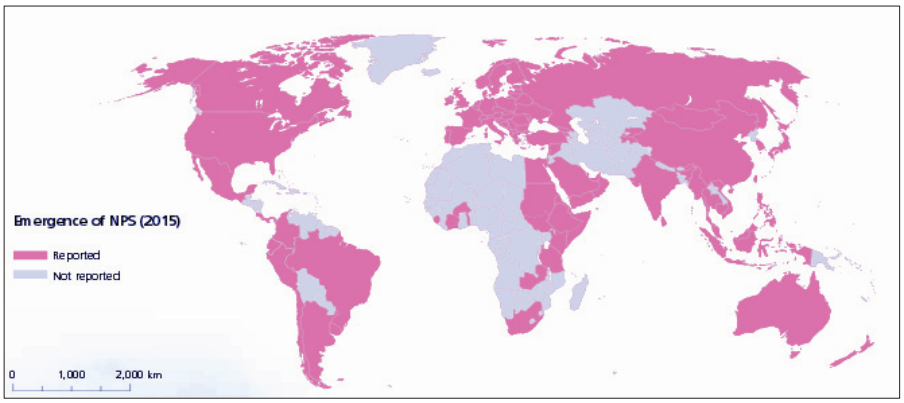

Figure 1. Global emergence of NPS up to December 2015.
(Source: United Nations Office on Drugs and Crime, Early Warning Advisory on NPS, 2015: in https://www.unodc.org/LSS/Page/NPS).

In Portugal, as an attempt to control the disturbing commercialization and consumption of these substances, in April of 2013 a list of NPS [35] was published prohibiting the production, importation, exportation, advertising, distribution, sale, possession and availability of those drugs. Currently, that list includes a total number of 159 NPS that comprise phenethylamines, cathinones, piperazines, cocaine derivatives, plants and respective active compounds and synthetic cannabinoids [36].

The Decree-Law nº 54/2013, 17 April defines the legal regime of prevention and protection against advertising and trade of NPS. This law defines NPS as substances not specifically classified and controlled under legislation but which in pure form or in a preparation, can pose a threat to public health comparable to that posed by controlled drugs, due to effects on the central nervous system, possible significant changes in motor function, as well as mental functions, including reasoning, critical judgment and behaviour, often with delirium states, hallucinations or extreme euphoria, with the ability to cause dependence and, in certain cases, produce long-term or even permanent damage to consumers health. Considering any substance that is suspected to constitute a serious risk to human health, this legislation also included the closure of the shops where these substances were freely sold (smartshops) [36].

In summary, we believe it is legitimate to say that the novelty of these psychoactive substances is related to:

- Legality;
- Sale in specialized stores;
- Prices;
- Method of reaching consumers (new and sophisticated forms of dissemination and marketing);
- Diversity and high potency;
- Motivation and consumption;
- Polydrug use;
- Synthesis from molecular changes of existing substances in order to mimic the effects of illegal drugs and to escape legal regulation;
- Globalisation of drugs market through the internet [9,12].

As we can see the NPS have been developed, promoted and commercialized for decades. More recently, which clearly has changed, and perhaps the most unsettling, is the way it is marketed via the internet.

Although most of the NPS consumers believe that they are less addictive and less harmful to health, what has been observed is that their effects seem to be as devastating as those of classic illicit drugs of abuse [12,33].

Nowadays, we are beginning to fathom the future implications of these developments in public health and drug control [13,24,28,37–39]. A significant amount of research is still needed in order to fully quantitate the short- and long-term effects of this substances and its interaction with other drugs of abuse.

1.3. Paper I

Salvia divinorum: toxicological aspects and analysis in human biological specimens

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Review

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Bioanalysis

Salvia divinorum: toxicological aspects and analysis in human biological specimens

The identification and quantitation of the main psychoactive component of *Salvia divinorum* (salvinorin A) in biological specimens are crucial in forensic and clinical toxicology. Despite all the efforts made, its uncontrolled abuse has increased quickly, exposing its users' health to serious risks both in the short and long term. The use of alternative biological matrices in toxicological analyzes can be advantageous as complementary postmortem samples, or in situations when neither blood nor urine can be collected; they may be useful tools in those determinations, providing important information about prior exposure. The aim of this article is to present a brief summary of legal aspects of *Salvia divinorum* and salvinorin A, including the methods used for the determination of the latter in biological matrices.

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Keywords: analytical methodologies • *Salvia divinorum* • salvinorin A

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Background

Over the past years, new psychoactive substances have appeared on the market and quickly spread worldwide. The subsequent abuse of these drugs with stimulant and psychoactive activity is affecting both health and safety of consumers, which can be proved by all recent deaths throughout Europe (presumably related with the hallucinogenic and toxic properties of this type of drugs) [1,2]. This situation is largely associated with the false idea that neither of these substances carries any danger to health when compared with the classic illicit drugs. Similar to what happened in other countries, this phenomenon only received special attention in Portugal after reaching alarming proportions.

Salvia divinorum (SD) is among the ample diversity of existing plants with psychoactive properties. This plant contains an active component designated by salvinorin A (SA). Although not being new or a synthetic substance, it is considered the most frequently available prominent psychoactive drug with hallucinogenic effects at smart and web shops [3–6].

The world's first smartshop was allegedly opened in 1994 in Amsterdam (Holland), whereas the first webshop movements were only recorded later, in 1999. However, even before their creation, it was already possible to access products with psychoactive effects through stores dedicated to sell natural products. During the first period of smartshops, natural substances were sold in those shops, including SD, kratom (*Mitragyna speciosa*), Hawaiian baby woodrose (*Argyreia nervosa*) and hallucinogenic mushrooms (psilocybin and psilocin). Later on, in those same stores, a second wave of products arose. Even though these products had similar designations, they were a diverse group of naturally occurring and new semisynthetic or synthetic compounds, from which synthetic cannabinoids and cathinones and its derivatives may be highlighted [7,8]. Only a small number of cases were reported about the use of SD in combination with other psychoactive sub-

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stances, making it impossible to conclude about the existence of possible interactions [4,9]. Despite the fact that this plant is not the typical recreational drug, in contrast to alcohol or cannabis [10], its consumption gained some importance in the era of the synthetic psychoactive drugs, and the vast majority of its consumers are young adults and adolescents [4–6,11].

According to the annual report (2011) of the European Monitoring Centre for Drugs and Drug Addiction, it seems that consumption of SD is restricted or experimental, and not a party or social drug [12,13]. However, most published papers consider SD a recreational drug [5,14–29]. Objectively, though, in spite of being consumed in amused and joyful atmospheres, SD does not cause euphoria or good mood (more common in other psychoactive drugs that promote dancing and human interaction) [10]. There are several studies suggesting different motivations for SD use, including curiosity, relaxation, fun, 'getting high' or spiritual effects [6]. In general, SD is widely discussed in cyberspace and is seen as a nonsynthetic hallucinogen substance whose psychoactive effects are short but very intense [5]. It is fairly studied when compared with the other psychoactive substances (particularly in ethnographic, botanical and chemical terms), and there is ample information available, published especially on the internet (even if scattered), but the truth is that most of these studies are very recent with plenty to discover and explain.

SD is a perennial plant with a soft, oval, serrated and intense green colorful leaves and white corolla flowers surrounded by a violet blue calyx. It is about a meter high and produces almost no smell when fresh [13]. This plant, with unique characteristics, is variously known as Diviner's Sage, Lady Salvia, Magic Mint, Purple Sticky, Sally D, Sage of the Seers, Psychedelic Sage, Ska Maria, Ska Pastora, Hojas de Mariais, Hojas de Petora, Maria Pastora or, simply and most widely, *Salvia* [6,10,13,20,30–31].

It is a plant member of the *Lamiaceae* mint family that has been used for centuries by the Mexican Mazatec, both for traditional religious rituals and medical purposes [32]. In the 20th century, this plant entered in the western world (during the 1960s), when the demand for psychoactive products was intensified and the consumption of hallucinogenic substances, such as LSD and mescaline, was implemented. However, the consumption of this herbal plant gained popularity in the middle of the 1990s with the rise of synthetic drugs. After detailed experimental research and characterization of its psychoactive potential, Daniel Siebert proposed a scale of intensity (S-A-L-V-I-A) to evaluate the effects. Each one of the letters of the scale corresponds to a stage of different intensity: (S) subtle effects, (A) altered perception, (L) light visions, (V) vivid visions, (I) immaterial existence and (A) amnesic effects.

With the advent of the 21st century, SD started to be sold via internet and through smartshops, reaching new groups of consumers. The product is sold in packages that include small pieces of macerated leaf enriched with its own active ingredient (SA) in different concentrations labeled as '1X, 5X, 10X, 20X, 40X, 60X,' etc. [13]. There is a great ease in acquiring this drug with no reference to the adverse effects associated with its consumption. However, even being of reduced risk, SD seems to have the potential for tolerance since the consumer's dosage has to be increased each time, in order to achieve the same previous effects [20,33–34]. To what concerns the consequences of its consumption in the long term, there is a lack of available data.

Despite SD's secular consumption, there is a wide difference between the ancient Mazatec methods and the ones that currently take place in the occidental civilization, starting by the way of use (inhalation of smoke), frequency, context and expectations of consumers. There are authors who warn about mental problems which may derive from SD consumption, especially in people with a predisposition for this type of health disorders [5–6,35]. These hazard consequences to one's health may become enhanced when the plant is consumed in combination with other psychoactive substances [4,34]. The toxicity of SD is currently poorly understood. Despite the significant lack of information available concerning the real dangers of SD uses (including the scarce data about intoxications or deaths from overdose), this does not mean that its consumption is effectively safe [5,36]. Recent studies have suggested the existence of low risks of addiction, toxicity and psychiatric disorders with the use of SD [6]. Currently, ascertaining the prevalence of its consumption worldwide remains a problematic issue, mainly because its legal status varies between countries and, in the case of the USA, between states. According to the National Institute on Drug Abuse, the prevalence of SD in 2009 was 6.0% among high school seniors, a higher amount than the previous year prevalence for ecstasy. There is limited information on the extent of use of SD and its preparations in Europe. For instance, in Romania, a 2008 survey among Bucharest-based young people aged 15–34 showed that 0.3% had tried SD at least once in the past; and in an online survey involving UK club-goers (2009), 3.2% of respondents admitted a last month consumption of SD. These findings cannot, however, be considered representative of the wider population of club-goers due to the methodological limitations that online surveys present [13]. This lack of information is also due to the different legal status of SD. Despite the fact that a scientific consensual opinion on the regulation of SD uses is still inexistent,

this hallucinogenic plant started to be under law surveillance in several countries, due to the growth in its popularity. The first country to prohibit possession and sale of SD and its active metabolite SA was Australia in June 2002; even though, the Committee responsible for this prohibition admitted the absence of danger to public health related with its consumption. Later on, while many other countries such as Belgium, Denmark, Italy, Japan, Latvia, Lithuania, Romania, Sweden and a high number of states of the USA also banned the possession and sale of the plant and its active metabolite; others, such as Croatia, Poland, Germany and Spain maintain control over the sale of the plant. In the Republic of Ireland, Czech Republic and Switzerland, it is not legal to possess or sell leaves or its extracts containing SD. At July 2012, the government of Hong Kong included SD and SA at the amended Schedule I of the Dangerous Drugs Ordinance, prohibiting its trafficking, manufacturing, possession, supply, import and export. More recently (January 2014), South Korea included both SD and SA in the list of controlled drugs. In Chile and Russia, it is illegal to traffic the plant and its extract. In Estonia, Finland and Norway, SD falls under medical legislation and in Iceland a license from the Natural Health Products Regulation is required, in order to import and sell the drug. Last February, the Canadian government officially announced that SD and SA were added to Schedule IV of the Controlled Drugs and Substances Act. Finally, in Portugal, SD, SA and SB are regulated by legislation (Decree nº54/2013, 17 of April) that prohibits its production, importation, exportation, advertising, distribution, sale, possession and availability.

However, despite all the efforts made, the implementation of legal constraints regarding the farming, consumption, possession and marketing of this substance is still a very controversial subject. In order to prevent more restrictions on the SD use, support groups claim its control without criminalization and a '*Salvia Divinorum* Defense Fund' was founded by consumers [37,38].

PK & PD of SA

SD can be consumed by chewing the fresh or dried leaves, drinking an infusion or by inhaling or smoking dried leaves. It has been published that this plant induces intense hallucinations in humans with a typical time of action between 30 s for smoking and 5–10 min for buccal absorption after ingestion [5,13,16,19–23,39–43]. However, the doses of SA needed for hallucinogenic effects vary from one individual to another [6].

The SD's main active ingredient, SA, is a nonnitrogenous neoclerodane diterpene and is considered to be the most potent naturally occurring hallucinogen known with an unique mode of action and pharmacology [13,39]. This compound is a powerful and selective κ-opioid receptor (KOR) agonist with no affinity for the 5-HT_{2A}, the main molecular target responsible for the action of classical hallucinogens (DOB, LSD, psilocybin, *N,N*-dimethyltryptamine, mescaline and ketamine) [12–13,21,31,39,44–45].

Other SA-related compounds were identified at much lower concentrations in the SD plant [22,46], some of which include salvinorins B-J [12,22,44,46–51]; salvinicins A-E and divinatorins A-F [48,50,52]. All the pharmacological studies carried out on these substances have shown that only SA is responsible for the psychoactive properties of the plant [15,46].

There are few studies on the metabolism of SA, but it was not fully investigated in humans, and therefore needs to be better understood and known [12,20–21,23,29,34,39,41,43,53–58]. Some studies report that SA is rapidly metabolized by deacetylation to a much less powerful compound, Salvinorin B (SB) with no significant affinity to KOR. PK studies showed a relatively fast elimination of SA with a half-life ($t_{1/2}$) of 75 min and a clearance (Cl/F) of 26 l/h/kg [22,29,53,58]. A lingering contact of leaves or infusions of the plant with the mouth allows its active substance to be greatly absorbed through the oral mucosa. If this contact is minimized, the only route of absorption becomes the gastrointestinal system where SA is deactivated before entering into the blood stream [29,39,41]. When doses of 200–500 µg are smoked, the effects of the drug are much more intense: breathing in the fume of pure SA seems to be not recommended, since the inhaled amount cannot be controlled. The effects arising from the use of SA can range from mild to profound, including sensations that can vary from pleasure and well-being (much more rarely) to fear and terror [12,22,29,49]. At present, it is known that the short duration of the psychotropic effects of SD is due to the rapid hydrolysis of SA into SB.

Despite its availability for science over the last few decades, the investigations about the use of this psychoactive substance are still under development [12,19–20,22–23,39,44,59].

Toxicological analysis in biological specimens

Drugs determination in different biological specimens provides a perspective of an individual's drug exposure history and is a precious tool to help answering some questions that are difficult to solve through the analysis of a unique specimen. In clinical toxicology, blood and urine are the specimens commonly analyzed. Nevertheless, in forensic toxicology a diversity of biological matrices are used in addition

to blood and urine. A large number of studies have been published describing the relevance of the developed methodologies for the analysis of drugs of abuse in various biological specimens, highlighting some review articles [60–63]. In this review, on the basis of reports published between 2004 and 2015 describing the analysis of SA in human biological specimens, different analytical methodologies in conventional and unconventional matrices (blood, plasma, urine, vitreous humor, pericardial fluid, sweat, oral fluid and hair), with special attention to its advantages and disadvantages, are introduced [15,24–26,54–56]. The biological fluids that are most commonly analyzed for SA are blood, plasma (or serum) and urine.

Blood, plasma & serum

Whole blood is the most complex fluid to be analyzed. This matrix consists of a buffered clear solution (plasma) containing soluble proteins, dissolved fats and salts, and suspended cells. However, the major constituent (erythrocytes) can be separated from the plasma simply by centrifugation. Serum is similar to plasma except for the presence of soluble factors that originate the coagulation phenomena. Serum and plasma have large amounts of proteins [64]. Thus, adequate techniques (SPE and liquid–liquid extraction [LLE]) are usually required to extract and concentrate the drugs from the matrix [65]. During an autopsy postmortem blood is usually collected, both from peripheral vessels and cardiac cavity. However, cardiac blood may be more affected by the phenomenon of postmortem redistribution of the unabsorbed drugs from the stomach and tissues, and so it is recommended that blood is sampled preferably from peripheral vessels [66–68]. Despite all these concerns, blood (serum or plasma) is still the best specimen to assess intoxication situations in toxicology. This is due to the main advantage that blood has over other specimens: the possibility of correlation between concentrations and observed effects. Peripheral blood is the matrix of choice for estimating toxicity because it is usually less affected by postmortem changes in drug concentrations [64]. However, blood is not the sample of choice for SA determinations in clinical toxicology because patients under the effect of this drug do not present physical conditions for blood collection without putting themselves and health professionals at risk, due to the invasive collection procedure involving needles [55,56]. Otherwise, in forensic toxicology, whole blood could be a very useful specimen to confirm the consumption of SD if death occurs in a short period after exposure, in order to be able to detect the active ingredient SA [20,24,29,39,54–55].

Urine

Urine is mostly constituted by water and is generally free of protein, lipids and other large molecular-weight compounds due to the renal filtration mechanism, which means it has a low content of endogenous interferences. The urine's pH values can vary widely depending on the diet and medications. This matrix can be collected in a noninvasive manner, it is easily tested, and drugs and metabolites are usually found at high concentrations following recent drug use. The major drawback is related with the possibility of specimen substitution or adulteration (e.g., dilution). If collected at autopsy, urine can provide relevant information about antemortem drug consumption. Usually, drugs and their metabolites are present in high concentrations facilitating their detection. Depending on the drug, the detection time in this specimen can vary from 24 h to 1 month. Thus, it is an excellent specimen for the detection of a wide variety of substances [64,65]. However, positive results in this matrix indicate only past drug use but it is not possible to attribute accurately an observed effect to the detected values. If death occurred rapidly after drug ingestion, a negative urine drug result is consistent with high-drug blood concentrations. Due to its characteristics, this specimen does not require complex methodologies to extract the substances [64].

Urine is an adequate specimen to confirm the consumption of SD up to 1.5 h after an extremely low dosage (0.58 mg) was consumed [55]. In addition, this matrix may be helpful in identifying other metabolites in which SA can be converted [12,18,20,22,40,49,51,53–54,69]. This specimen could be quite useful, and perhaps the most appropriate, in clinical and forensic toxicology analysis to detect and confirm SD consumption [15,25,54–56].

Vitreous humor

Vitreous humor is located in the eye and is composed of 99% water with a pH of 7.5. The major components of this matrix are the structural protein collagen and hyaluronic acid. This is the matrix with less endogenous interfering compounds, and toxicological analysis does not require complex preparation. Substances that are highly protein bound would not have a significant concentration in vitreous humor, and compounds not highly protein bound would be expected to have significant concentrations in this matrix [63,64]. The absence of significant metabolic activity in the eye suggests that the concentration of the drug found in vitreous humor correlates well with the corresponding concentration in blood. This specimen is often the most suitable (or even the only available) for toxicological analysis, particularly in situations of putrefaction or vastly

traumatized. The main drawback of this specimen is the low volume that can be collected during a forensic autopsy (at best about 5 ml). A large number of compounds can be detected in this specimen, being of great interest in forensic toxicology, as has been demonstrated by many authors [63]. Actually, this specimen can be useful in forensic toxicology for SA analysis as a complementary sample to blood and/or urine when considering intake of SD, even though the studies of SA disposition regarding vitreous humor are limited to a publication [24]. Further studies should be done to evaluate the correlation of SA concentration to blood, since VH and blood concentrations do not correlate for all substances.

Pericardial fluid

Pericardial fluid is a plasma ultrafiltrate and, therefore, it is very similar in its electrolyte composition. However, when compared with plasma, this specimen contains about half the protein concentration, about a third of cholesterol and triglycerides, and less than a fifth of the amount of white blood cells. Some studies have shown that this matrix can be useful for drug determinations, since pericardial fluid and femoral blood have a good correlation [70,71]. Another advantage of this matrix is the larger volume that can be collected during a forensic autopsy (about 10 ml), without significant contamination, enabling the various toxicological analyzes usually made. This fluid can be obtained in large amounts even from a widely exsanguinated corpse. However, caution must be taken during its collection to prevent contaminations from stomach and adjacent organs. The extraction of the drugs from this fluid does not require complex methodologies. Thus, this specimen seems to be useful to evaluate situations of intoxication in forensic toxicology. Similarly to vitreous humor, it has the disadvantage of the limited database in the analysis of SA that can support the interpretation of the results obtained [24]. Pericardial fluid has been poorly studied and further investigations are needed to supplement the results obtained in the analysis of SA. The inclusion of this sample in routine toxicological analysis may be helpful to investigate the influence of the mechanisms of postmortem redistribution and diffusion of the drugs in the death process and the importance of this specimen in forensic toxicology.

Sweat

Sweat is the moisture lost through the surface of the skin and is secreted from eccrine and apocrine glands. This specimen is mostly constituted by water (99%), and sodium chloride is the most concentrated solute. As the blood, sweat also contains albumin, gamma globulins and many other substances. It can be useful as a means of monitoring drug use over a period of several weeks. It can be collected noninvasively with a specialized collection patch. However, the amount of sweat excreted is highly variable both within a single person and between individuals. The maximum excretion volume produced by healthy subjects was reported to be about 2 l/h and 4 l/h in trained athletes. Sweat can be easily and noninvasively collected with patches over a long period. The mechanisms of incorporation of drugs into this fluid include passive diffusion from blood and transdermal passage of drugs across the skin [62,72]. There is a strong correlation between the pKa of a substance and the amount found in the sweat. Some authors reported that a few drugs can be detected in this fluid. Sweat is usually not suitable for quantitative analysis of drugs because it is difficult to estimate the volume of sample that was actually collected. In addition, the possibility of contamination must be taken into account and adequately avoided. However, this fluid has the great advantage of the longer detection windows when compared with urine and plasma. The drugs can be detected in sweat between 1 and 4 weeks [72]. The existing study for the determination of SA in sweat was inconclusive due to the fact that it was collected only by 2 h after its administration and the nonpolar nature of the substance does not favor the excretion through it [55].

Oral fluid

Oral fluid is a complex matrix composed of 99% water, mucins (oligomeric and monomeric mucous glycoproteins), enzymes (mostly amylase) and electrolytes. This matrix is secreted by three main glands: parotid, submandibular and sublingual. Most substances enter into oral fluid by passive diffusion through cell membranes, and this depends on the physicochemical properties of the compounds, such as molecular weight, liposolubility, pH and pKa, protein binding and ionization state [62,73]. Oral fluid concentrations of these substances are related to blood/plasma concentrations of the drug unbounded to plasma proteins or lipophilic metabolites. This alternative specimen has a window of detection comparable to that of blood, and therefore is appropriate to confirm a recent consumption. However, with drugs that can be taken orally (ingested or smoked), it has the disadvantage that they can be detected in high concentrations following recent use. In these situations, the drug concentration found in oral fluid may not reflect the existing concentration in the blood. It has the advantage that can be easily accessible and the collection procedure is minimally invasive [74]. For sample pretreatment, SPE or LLE is recommended to reduce matrix interferences. The main handicap of this specimen is the limited volume available for analy-

sis and the low drug concentration. The disadvantage of the short window of detection is the inability to detect some substances within 24 h. In addition, oral fluid has the advantage of indicating recent use of the drug. Several studies have been published in the analysis of distinct compounds in this specimen, demonstrating its usefulness in both clinical and forensic toxicology [73,74]. Oral fluid was considered an useful tool as an alternative specimen for determinations of SA, taking into account the existing published work, where it was possible to quantitate this substance in two volunteers in amounts consistent with that which was consumed [55]. However, further studies in this alternative specimen must be done to support the existing data.

Hair

Hair consists of five morphological and chemical different components (cuticle, cortex, medulla, melanin granules and cell membrane complex). It is constituted essentially by keratin protein (65–95%), water (15–35%), lipids (1–9%) and minerals (<1%) [75]. The hair grows in cycles that alternate between phases of anagen (active growing), catagen (transition) and telogen (resting) [62]. It is generally accepted that the mechanisms of incorporation of the drugs into the hair occur by either adsorption (followed by absorption) from the external environment, from blood supplying the hair follicle during its growth and from the excretion by sweat and sebum. The possibility of drug detection in hair following environmental exposure is actually the most important drawback in hair testing, since the presence of a drug in hair does not necessary mean that it has been actively consumed. The Society of Hair Testing has published a few guidelines on the issues of hair collection, analysis and results interpretation to be used by laboratories in clinical and forensic context, in order to minimize the possibility of reporting false positives [76]. Taking into account the extensive published data of hair analysis, a large number of compounds can be detected in this specimen, making it of great interest in forensic and clinical toxicology. Unlike to what happens with other biological specimens, hair analysis starts with drug extraction from within the matrix (since the drugs are bound to inner hair constituents), usually followed by a sample cleanup procedure, in order to minimize interferences caused by exogenous and endogenous substances. This matrix has the advantage of being easy to obtain, not invasive, and difficult to adulterate [74,77]. From the several advantages that hair presents in what concerns toxicological analysis, the possibility to collect a similar sample (or more accurately, representing an equivalent time frame) in case of suspicions or breach in the chain of custody or its extended detection window are clearly the most relevant. Segmental hair analysis allows detecting exposure to drugs for a period of up to 12 months, provided that the collected sample is sufficiently long. This way, it is an important matrix in the confirmation of chronic consumption of drugs [77]. For SA determinations, hair may be an important specimen as a complement to the blood and/or urine in order to confirm the existence of a past consumption as clearly stated in only documented existing study [26]. Similar to what was mentioned toward other studies performed on human biological samples, all the developed work on hair is in its very beginning, to which proper relevance and expansion must be given.

As previously stated, there is a lack of studies describing the determination of SA in biological specimens, and some of the used matrices in the field of toxicology (e.g., specimens allowing assessing *in utero* drug exposure) have not been tested for this drug yet.

In addition, and concerning the most used specimens of blood and urine, one major advantage is the possibility of using immunoassay screening procedures for the more common drugs-of-abuse. However, to date there is no such technology in what SA is concerned, and therefore chromatographic analysis is still deemed necessary for screening purposes. The same problem occurs with *in situ* devices for oral fluid testing, widely spread for drug analysis on the roadside and assessment of driving-under-the-influence situations.

Methodologies for the analysis of SA in human biological specimens

Bioanalytical methodologies for the analysis of SA in different human biological specimens have been playing an imperative role in the forensic and clinical toxicology field. In order to obtain a more accurate review, considering the extraction procedures in human biological matrices and chromatographic analysis, a comprehensive literature search was accomplished using the bibliographic databases PubMed and Google Scholar with the following searching terms: 'analysis and salvinorin A and salvia divinorum'; 'salvia divinorum and salvinorin A'; 'biological specimens and salvonorin A'; 'body fluids and salvia divinorum and salvinorin A'; 'human and salvia divinorum or salvinorin A' and 'chromatographic analysis and salvinorin A.'

Concerning SA determination in human biological specimens, some new approaches have been published over the last 10 years. It should be noted that to date there are few published studies on the determination of SA in biological fluids and their toxicological implications.

SA was first quantitated in human plasma and urine using SPE for sample pretreatment, and negative-ion LC–MS/APCI for chromatographic separation [54].

The negative-ion mode of LC–MS/APCI was chosen in order to obtain the parent masses of the functional group of interest and to be able to use deuterated salvinorin A-d3 as internal standard. Excellent LODs and LLOQs (2 ng/ml) were obtained using 0.5 ml of sample. During the same year, it was published the first analytical procedure to determine SA in 1 ml of conventional (plasma and urine) and unconventional (oral fluid and sweat) human biological specimens using LLE with chloroform/isopropanol and GC-MS for the detection and quantitation of the substance [55]. With this nonautomated technique of sample preparation, an emulsion can occur, in which case it will lead to a poor separation of the substances of interest and, as consequence, to an expansion of the time of the analysis. The same LODs (2 ng/ml) were obtained by [54] but the LLOQs were higher than those previously obtained.

Three years later, McDonough *et al.* reported a method to detect and quantitate SA in urine using SPE associated with LC–ESI–MS [56]. The method was merely validated in urine and the results obtained were applied to the blood matrix. The LOD and LLOQ achieved (2.5 and 5 ng/ml, respectively) were adequate using 1 ml of urine.

In 2012, Barnes and Snow published a procedure that combined solid-phase microextraction with analysis by 2D gas chromatography-time of flight mass spectrometry (GC × GC–ToFMS) for the determination of SA in urine [15]. Even though proper LOD (5 ng/ml) and LLOQ (8 ng/ml) were obtained, the volume of urine required was very high (20 ml).

Moreno *et al.* reported the quantitative determination of SA in urine using microextraction by packed sorbent (MEPS) combined with chromatographic analysis by GC–MS/MS [25]. The LOD (5 ng/ml) and LLOQ (20 ng/ml) achieved were adequate taking into account the lower volume of the sample used (0.2 ml). MEPS is a technique with great potential due to its enhanced speed and decreased volume of organic solvents. In addition, it brings along major advantages such as the fact that the device can be reused several times and it requires a rather rapid sample preparation.

Also in 2013, Margalho *et al.* published a procedure for the detection and quantitation of SA in conventional (whole blood and plasma) and unconventional (pericardial fluid and vitreous humor) human biological matrices [24]. In this procedure, an SPE combined to GC–MS was used, and excellent LOD and LLOQ (5 ng/ml) in low volumes of samples (0.1 ml of vitreous humor and 0.25 ml of whole blood, plasma and pericardial fluid) were obtained. The LLOQ in blood were similar to those obtained by McDonough *et al.* [56], and the LOD in plasma was similar to that obtained by Pichini *et al.* [55], yet using smaller volumes of samples. One of the great advantages of this methodology was the possibility of analyzing SA in alternative postmortem matrices (vitreous humor and pericardial fluid) that are useful for application in forensic toxicology routine analysis as complementary to blood (to provide information about SD consumption) or as substitute (when blood and/or urine are not available for collection).

Table 1. Analytical methodologies in different biological specimens for salvinorin A determinations.

Specimens	Extraction procedure	Sample amount (ml)	LOD/LOQ (ng/ml)	Instrumental analysis	Ref.
Blood	SPE (Oasis® HLB)	1.0	2.5/5	LC–MS/ESI	[56]
Whole blood	SPE (Oasis HLB)	0.25	5/5	GC–MS	[24]
Plasma	LLE (chloroform/isopropanol)	1.0	5/15	GC–MS	[55]
	SPE (Oasis HLB)	0.5	2/2	LC–MS/APCI	[54]
	SPE (Oasis HLB)	0.25	5/5	GC–MS	[24]
Pericardial fluid	SPE (Oasis HLB)	0.25	5/5	GC–MS	[24]
Vitreous humor	SPE (Oasis HLB)	0.1	5/5	GC–MS	[24]
Urine	SPE (Oasis HLB)	0.5	2/2	LC–MS/APCI	[54]
	SPE (Oasis HLB)	1.0	2.5/5	LC–MS/ESI	[56]
	LLE (chloroform/isopropanol)	1.0	5/15	GC–MS	[55]
	SPME (chloroform)	20	5/8	GC×GC–ToF/MS	[15]
	MEPS	0.2	5/20	GC–MS/MS	[25]
Sweat	LLE (chloroform/isopropanol)	1.0	5/15	GC–MS	[55]
Oral fluid	LLE (chloroform/isopropanol)	1.0	3/10	GC–MS	[55]
Hair	LLE (VMA-TM3 acidic aqueous buffer)	25 mg	0.02/0.05 ng/mg	UHPLC–MS/MS	[26]

Finally, SA was analyzed in hair using ultra-high-pressure LC–MS/MS (UHPLC–MS/MS) after hair washing with methyl alcohol and diethyl ether and subsequent treatment with VMA-TM3 reagent [26]. The LOD (0.02 ng/mg) and LLOQ (0.05 ng/mg) were adequate for the determination of SA in 25 mg of hair from SD consumers. Considering this as a preliminary study, it was possible to conclude that SA can be found in the hair of SD consumers, evidencing past consumption.

In Table 1, an overview of the analytical techniques studied in the various human biological matrices for SA determinations is presented.

Conclusion & future perspective

Since almost all published papers about this plant (SD) highlighted the lack of data on the long-term consequences of its consumption, there is still a long line of work to do in this area. Further studies are required in order to determine more accurately the frequencies and patterns of use of the SD.

Even though the majority of intoxication events related with SD consumption have not been confirmed, several consumers were assisted at various emergency services of hospitals, with no knowledge of the consequences and sequels to their health. This means that despite lacking records of confirmed deaths related to SD use, it is already known that the substance is still being commercialized in several formulations associated with other synthetic drugs, and the consequences of their interactions are still unknown. Unfortunately, the unpredictability of how these substances enter and leave the illicit market makes them more difficult to track.

There are several published studies on the extraction of SA from the plant material. However, only a few are available to detect and quantitate the substance in human biological specimens, as described in this paper.

Executive summary

***Salvia divinorum* & salvinorin A**

- Psychoactive plant can induce visions and other altered and spiritual experiences.
- Its native habitat is in cloud forest in the isolated Sierra Mazateca of Oaxaca, Mexico.
- The known active constituent of *Salvia divinorum* (SD) is a transneoclerodane diterpenoid, known as salvinorin A (SA).
- Other terpenoids have been isolated from this plant, including other salvinorins and related compounds named divinatorins and salvinicins.
- It can be taken by chewing the fresh or dried leaves, drinking an infusion or by inhaling or smoking dried leaves.
- The use of SD as a new psychoactive substance dates back to the 1990s.
- The scientific consensual opinion about the regulation of SD uses is still inexistent, for this reason this plant is legal in many countries. However, in recent years both SD and its active principle SA have become controlled under drugs legislation.

Pharmaceutical/toxicological actions of SA

- SA is a potent natural hallucinogen.
- This compound is a powerful and selective κ-opioid receptor agonist with no affinity for the 5-$HT_{2A.}$
- Some studies report that SA is rapidly metabolized by deacetylation to a much less powerful compound, Salvinorin B (SB), with no significant affinity to κ-opioid receptor.
- The effects can range from mild to profound, including sensations that can vary from pleasure and well-being (though in much rarer cases) to fear and terror.

Analysis of SA in biological specimens

- Only a few studies have been published on the determination of SA in biological fluids and their toxicological implications.
- SA was first quantitated in 2005 in human plasma and urine using SPE, for sample pretreatment, and negative-ion LC–MS/APCI for chromatographic separation of the substances.
- In the same year, the first analytical procedure to determine SA in oral fluid and sweat was published. Liquid–liquid extraction with chloroform/isopropanol and GC–MS were used.
- Microextraction techniques were also applied to determine SA in biological fluids, namely microextraction by packed sorbent (MEPS). The advantage of this technique was the rapid sample preparation and the fact that the device can be reused several times.
- The determination of SA in postmortem alternative specimens (pericardial fluid and vitreous humor) has been also published. Excellent limits were obtained using low volumes of samples (0.1 ml of vitreous humor and 0.25 ml of pericardial fluid).
- More recently, SA was analyzed in hair samples using ultra-high-pressure–LC–MS/MS after liquid–liquid extraction.

So, it is of high relevance to continue the researches aiming to determine the primary metabolite of SD in different biological matrices. This will enable specialists to prepare and anticipate the absence of appropriate methodologies in the toxicological and clinical laboratories, and subsequently establish whether an exposure to the substance occurred.

In addition, in recent years SA has been object of scientific research due to its unique chemical characteristics, particularly in the pharmaceutical field; some researchers believe that this KOR agonist has significant potential to combat pain without producing dependence and contribute to the development of short-acting anesthetics, as well as drugs against schizophrenia, Alzheimer's disease or bipolar disorder, among others [43,78–80]. This substance may also have potential application in the treatment of cocaine addiction [6].

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1.4. Cathinones and Phenethylamines

The NPS, including a wide diversity of cathinones and phenethylamines, have continuously emerged in the recreational drug market over the past 10 years. Cathinones are ß-ketophenethylamines structurally related to cathinone or other naturally occurring alkaloids, whereas phenethylamines are substances structurally related to phenethylamine or amphetamine [24,40]. Cathinone can be considered as the prototype from which a range of synthetic cathinones have been developed. Cathinones are derivatives of the active phenylpropylamino alkaloids found in young leaves of the *Catha edulis* plant, commonly known as khat. The main active principles of khat are believed to be, primarily (-)-cathinone [(S)-α-aminopropiophenone], (+)-cathine [(1S)(2S)-norpseudoephedrine], and (-)-norephedrine [(1R)(2S)-norephedrine] [41,42]. Cathinone concentration declines during leaf maturation due to biochemical reduction to cathine (nor-pseudoephedrine) and norephedrine [43]. Khat is a perennial shrub whose young leaves are chewed for their psychostimulats and anorectics properties, a practice that has been in existence for centuries and continues today in several Middle Eastern and East African countries [44]. This plant originated in Ethiopia and has been traditionally used at cultural and religious ceremonies, but its recreational and sacramental use has spread it through much of Africa, the Arabian Peninsula, and the Middle East (Figure 2). More recently, as khat also made its appearance in the United States and several European countries with the improvement of transportation and distribution, its availability ceased to be exclusive to the native regions. Khat use is sporadically reported in Europe as a substance of choice among immigrants from Somalia, Ethiopia, Kenya and Yemen [45–47]. Some East African indigenous populations used it as a traditional medicine, but its therapeutic use has not been recognized globally [48].
Khat leaves are typically wrapped as a bundle in banana leaves (Figure 2).

Cathinone is an alkaloid, similar in structure and action to amphetamine, differing only in the presence of the carbonyl group in ß-position of the side ring (Figure 3) [47].
Cathinone derivatives are characterised by the presence of a ß-keto group on the side chain of the phenethylamines. They are synthesized by adding different substituents at distinct positions of the cathinone molecule. The synthetic cathinones or substituted cathinones are phenylalkylderivatives, structurally similar to amphetamine and methamphetamine (but with lower potency) and are derived from cathinone. The chemical structures and synthesis of some synthetic cathinones have long been known but only recently abused. This chemical class of “legal highs” is a large family of synthetic ß-ketophenethylamine (2-amino-1-phenyl-1-propanone) derivatives, often referred as ßk-amphetamines [49]. The general structure of a cathinone derivative (Figure 3) shows substitution patterns at four different locations of the cathinone molecule (R, R1, R2 and R3). These various substituents may include alkyl, alkoxy, alkylenedioxy, haloalkyl , halo and phenyl groups. However, differences between substituted cathinones may be limited to a simple addition of a methyl group [47,49] .

Figure 2. *Catha edulis* (khat) plant and growing regions. (Source: https://www.flickr.com/photos/nostri-imago/sets/72157610816054899/ http://summer2011botany01.providence.wikispaces.net/Khat)

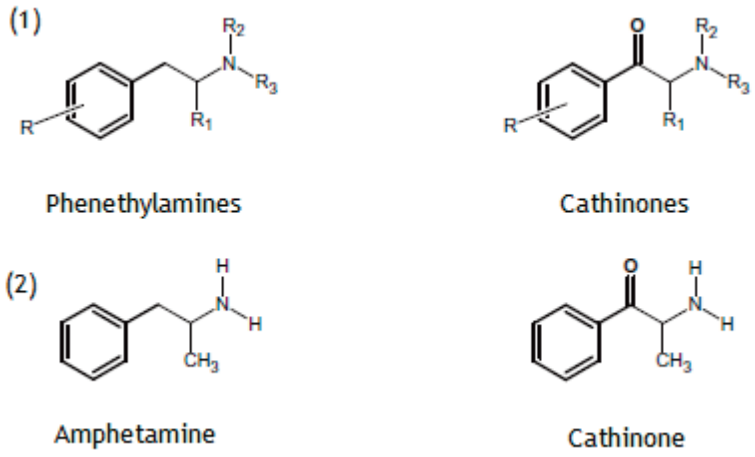

Figure 3. (1) General structures of phenethylamines and cathinones and (2) Comparison of the representative compounds of each group. (Source: [47])

The first cathinone to be synthesized was the methcathinone in the 1920 for therapeutic purposes (as an anti-depressant) [50] and it was first reported as a drug of abuse in the early 1990s. Later, other derivatives of cathinone appeared, such as 4-methylmethcathinone (mephedrone, 1929), 3,4-methylenedioxyprovalerone (MDPV, 1967), and more recently 3,4-methylenedioxymethcathinone (methylone, 1996). However, in the beginning of the 21st century these synthetic substances gained public attention as recreational drugs most likely due to their low cost and easy purchase and also due to decreased purity and availability of other classic drugs of abuse. [28].

Nowadays, they are the most frequently encountered substances in the "legal highs" products [14]. These drugs are typically used in parallel or associated with other drugs. The synthesis of new cathinone derivatives, by small chemical modifications of the molecular structure, has seriously increased the diversity of these compounds, in response to the market trends and inadequate legal control [51].
Ephedrine, pseudoephedrine and pharmaceutical products containing these chemical substances are used as precursors to produce methamphetamine and methcathinone [52–54].

According to the EMCDDA the number of substances officially notified, between 1997 and 2009 was highest for phenethylamines followed by tryptamines, cathinones and piperazines. The popularity of these substances was motivated mainly by their low cost, accessibility of their precursors and ease of synthesis. However, after the publications by Shulgin, Alexander and Shulgin, Ann (PiHKAL: A Chemical Love Story, 1991 and TiHKAL: The Continuation, 1997), the consumption of these compounds as recreational drugs had an increase.
Also according to the EMCDDA 2015 [55], synthetic cathinones and synthetic cannabinoids are the groups of NPS most commonly seized, reflecting the relatively high demand of stimulants and cannabis on the illicit drug market. This means that the number of NPS detected continues to grow, synthetic cathinones being the largest category of new drugs identified in Europe in 2014, followed by synthetic cannabinoids.
The latest data presented in the EU Drug Markets Report 2016 [33], revealed that a wide range of NPS were reported through the EU Early Warning System including synthetic cathinones, phenethylamines, synthetic cannabinoids (mainly synthetic cannabinoid receptor agonists), opioids, tryptamines, benzodiazepines, arylalkylamines and a range of other substances. In 2015, 100 new substances were detected for the first time, bringing the total number of new substances monitored to more than 560 — with more than 380 (70 %) of these detected in the last 5 years alone (Figure 4). There are now more than twice as many new uncontrolled substances on the market as there are drugs controlled under international drug control conventions [6].
Cathinones and its derivatives are under international control and substances such as cathinone, methcathinone, cathine and pyrovalerone are controlled under the 1971 Convention on Psychotropic Substances [56].

Phenethylamines are also known as amphetamine-type stimulants (ATS), although many derivatives do not stimulate the central nervous system. Amphetamine, metamphetamine and MDMA are considered phenethylamine derivatives, as they are indeed phenylpropylamines [47].
Phenethylamines are extremely simple-structured substances with a molecular combination that includes a benzene ring attached to a chain that is connected to a nitrogen atom. Phenethylamine is strictly related to the vital brain neurotransmitter, dopamine (Figure 5) [57].

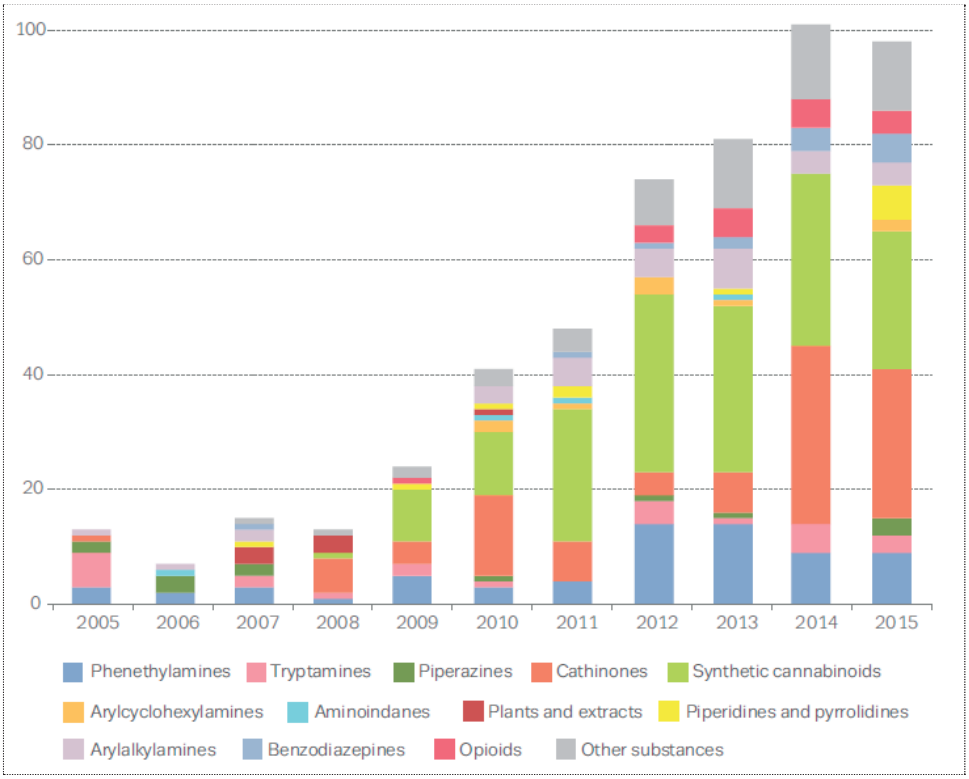

Figure 4. Number of NPS reported to the EU Early Warning System for the first time, 2005–2015 (EMCDDA-Europol 2015).
(Source: http://www.emcdda.europa.eu/system/files/publications/2880/TDAS16001ENN.pdf)

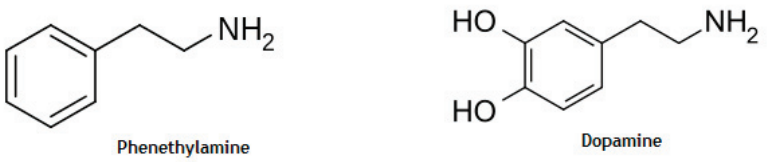

Figure 5. Chemical structures of phenethylamine and dopamine. (Source: [57])

Phenethylamines refer to a class of substances with documented psychoactive and stimulant effects and include amphetamine, methamphetamine and MDMA, all of which are controlled under the 1971 United Nations Convention on Psychotropic Substances [58]. The phenethylamines also include ring substituted substances such as the "2C-X series", ring substituted amphetamines such as the "D series" (e.g. DOM, DOB, DOI, DOC), benzodifurans (e.g. Bromo-Dragonfly, 2C-B-Fly) and others (e.g. *p*-methoxyamphetamine (PMA) *p*-methoxymethamphetamine (PMMA)). The "2C series" differs from the "D series" only by a slight modification in the chemical structure, and their psychoactive effects have been

reported to be dose dependant, ranging from mere stimulant effect at lower doses to hallucinogenic and entactogenic effects at higher doses [10,57,58].

Phenethylamines include the most popular "legal highs" of this class of substances, those being the group 2C-X series, where X represents an atom or a functional group replaced at position 4 of the benzene ring of the 2,5-dimethoxyphenethylamine molecule and the group of the amphetamine derivatives, where replacement of one or two methoxy groups may occur at positions 2 and/or 5 of the benzene ring, and/or replacement of the hydrogen at position 4 by a halogen or an alkyl group [47,59] (Figure 6).

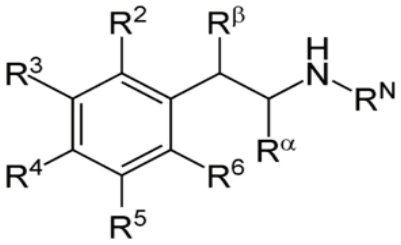

Figure 6. The structure of substituted phenethylamines. Phenethylamine is obtained when $R^2=R^3=R^4=R^5=R^N=R^\alpha=R^\beta=H$.

(Source: http://www.h1n1.ro/Phenethylamine.html#Substituted_phenethylamines)

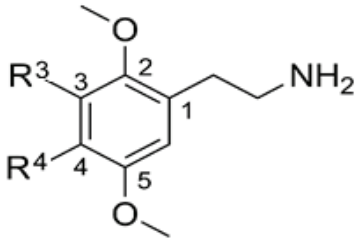

Figure 7. General structure of a 2C-X compound. (R^α= R^β= R^N=H; R^2= R^5=OCH_3). (Source: https://en.wikipedia.org/wiki/2C_(psychedelics)#/media/File:2C-general.png)

Most of the currently known 2C compounds were first synthesized by Alexander Shulgin in the 1970s and 1980s and published in his book [60]. The "2C" term means that there are two carbon atoms between the benzene ring and the amino group (Figure 7). The first compound from this group was 2C-B [59] and since it was scheduled by the Drug Enforcement Agency (DEA) in 1995, several new 2C compounds have been synthesized and introduced on the drug market, namely 25B-NBOMe (2C-B-NBOMe), 25C-NBOMe (2C-C-NBOMe), and 25I-NBOMe (2C-I-NBOMe; "N-bomb", "Smiles", "CIMBI-5") [61]. The most potent compound of this group is 25I-NBOMe and was first synthesized by Ralph Heim at the Free University of Berlin as one of a series of pharmacological tools to study 5-HT2A receptors [62].

In table 1 some examples of the substituted compounds referred to above are presented.

Table 1. Some examples of substituted phenethylamines.

Short name	R^N	R^α	R^β	R^2	R^3	R^4	R^5	IUPAC name	Biologic activity
2C-B	H	H	H	OCH_3	H	Br	OCH_3	2,5-dimethoxy-4-bromophenethylamine	Psychedelic, stimulant, entactogen, euphoriant
2C-H	H	H	H	OCH_3	H	H	OCH_3	2-(2,5-Dimethoxyphenyl)ethanamine	Unknown
2C-I	H	H	H	OCH_3	H	I	OCH_3	2,5-dimethoxy-4-iodophenethylamine	Psychedelic, stimulant
2C-T-2	H	H	H	OCH_3	H	$S\text{-}CH_2CH_3$	OCH_3	2,5-dimethoxy-4-ethylthio-phenethylamine	Psychedelic
2C-T-4	H	H	H	OCH_3	H	$S\text{-}CH(CH_3)_2$	OCH_3	2,5-dimethoxy-4-isopropylthio-phenethylamine	Psychedelic
2C-T-7	H	H	H	OCH_3	H	$S\text{-}CH_2CH_2CH_3$	OCH_3	2,5-dimethoxy-4-propylthio-phenethylamine	Psychedelic
DOM	H	CH_3	H	OCH_3	H	CH_3	OCH_3	2,5-dimethoxy-4-methylamphetamine	psychedelic
DOB	H	CH_3	H	OCH_3	H	Br	OCH_3	2,5-dimethoxy-4-bromoamphetamine	Psychedelic
PMA	H	H	H	H	H	H	OCH_3	1-(4-methoxyphenyl)propan-2-amine	Hallucinogenic, stimulant, entactogen, euphoriant

In order to circumvent controlled substances legislation, cathinones and phenethylamines are designed by replacing phenyl ring hydrogens with diverse substituents. The most common replacements are made by methyl, ethyl, methoxy, ethoxy groups and halogens such as fluoro or chloro. For each of the substituents in the ring, the *orto*, *meta* or *para* positional isomer is possible. However, multiple substitutions by other groups of substances may occur [40]. Most of the phenethylamine derivatives included in the "2C" and "D" series are controlled under the 1971 Convention on Psychotropic Substances, but some of new derivatives are not under international control [56].

1.4.1. Routes of administration and toxicokinetics

A great diversity of NPS is available online and in the illicit drug market in many European countries, in the United States and in other countries. These products are marketed in a variety of formulations such as bath salts, incense, plant food, fertilizer, pills or tablets, smoking mixtures, plant extracts, herbal products, powders, liquids, orodispersible strips, chewing gum and other products [63]. These drugs are typically used in parallel or associated with other drugs (e.g. alcohol, tobacco, ecstasy, cannabis, cocaine, etc.) and they can be taken orally, smoked, inhaled, by intravenous or intramuscular injection, and by rectal suppository [13,22,49]. All routes produce similar psychological and physiological effects although onset and duration may differ [23,49,64].

The doses of synthetic cathinones that are usually consumed may vary greatly from derivative to derivative, depending on the potency of their effects and the route of administration.

Ingestion is the most common route of administration of phenethylamines and the vast majority of synthetic cathinones [56]. The inhalation route promotes the development of lesions in the nasal mucosa and increases the risk of dependence, when compared with the oral route. Oral administration includes the ingestion of capsules, tablets or powder substances wrapped in paper (bombing) or diluted in water or other liquid. The intravenous, intramuscular and rectal routes are also possible due to the solubility of these substances in water [64,65].

Typical doses of mephedrone vary with the route of administration used according to user reports. The inhalation of 5-75 mg induces a rapid onset of effects (less than 30 min), which last less than 60 min [24]. When administered orally (100-250 mg) the onset of effects can range from 15 min to 2 h, depending on the gastric contents, with a duration of 2–5 h [24]. Intravenous and intramuscular administration (75-167 mg) have a faster onset of stimulation, peaking 10–15 min after injection, and lasting less than 30 min [24]. Rectal administration (about 100 mg) is unusual and the onset of action and potency of effects are similar to both intramuscular and intravenous routes. Some consumers use both oral and inhalation routes in order to achieve both rapid and long-lasting effects [66,67].

Cathinone derivatives tend to be more hydrophilic, which decreases their ability to cross the BBB and renders them less potent than the amphetamines. Also, the carbonyl group makes the cathinone molecule more planar, which may contribute to its toxicity [68]. Mephedrone, for example, has a keto group that makes it more water-soluble than the amphetamine and some derivatives. In general, the presence of the ß-keto group increases the polarity of the synthetic cathinones, resulting in a decrease in their ability to cross the BBB. So, a greater dose of mephedrone is required to produce the same effects as amphetamine and some derivatives [23].

Cathinone, mephedrone, methcathinone, and methylone have all been shown to strongly inhibit reuptake of dopamine (DA), serotonin (SERT), and noradrenaline (norepinephrine) (NA). These substances also increase presynaptic release of the same monoamines, but to a lesser extent [69]. There is limited data in the medical literature on the toxicokinetics and toxicodynamics of the synthetic cathinones in humans.

Studies of mephedrone metabolism have demonstrated demethylation to the primary amine, reduction of keto moiety to an alcohol, and oxidation of tolyl moiety [70].

In fact, the natural cathinone, like all synthetic cathinones, suffer phase I metabolism after absorption, namely a reduction of the ß-keto group to an alcohol catalysed by liver microsomal enzymes producing cathine (norpseudoephedrine) and norephedrine (Figure 8) [71]. Cathinone is rapidly metabolized and excreted as metabolites (norephedrine and cathine) [41,42]. Excretion occurs mainly through the urine [42,71].

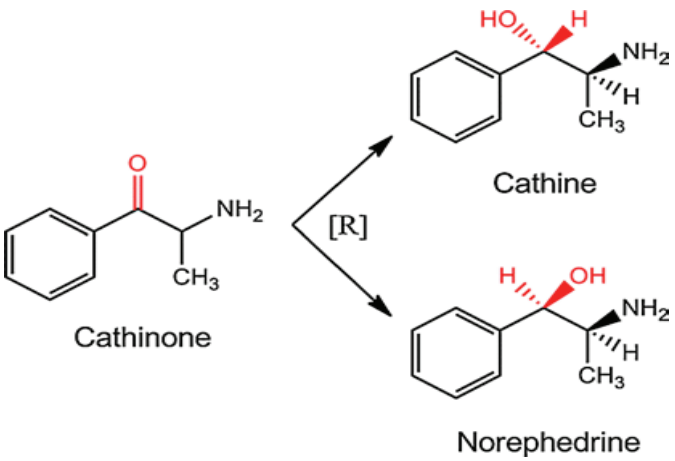

Figure 8. Metabolism of cathinone in humans (phase I). [R]: reduction. (Source: [72]).

Concerning mephedrone, several studies revealed that its metabolism occurs in three phase I routes, 7 metabolites being so far identified in human biological specimens (N-desmethyl-mephedrone, dihydromephedrone, N-desmethyl- dihydromephedrone, hidroxytolylmephedrone, N-desmethyl- hidroxytolylmephedrone, 4-carboxymephedrone and 4-carboxydihydromephedrone) [70,73,74]. Cytochrome P450, CYP2D6 was described as being the main enzyme involved in the phase I metabolism of mephedrone in human liver microsomes [74]. More recently, six phase I metabolites and four phase II metabolites were reported for human metabolism of mephedrone [75]. Phase I reactions included N-demethylation, reduction of the keto function, hydroxylations in C_3 and in benzylic carbon, and oxidation of C_3 and benzylic methyl to carboxylic acid. The presence of some derivatives that were previously described as mephedrone metabolites in humans was confirmed (e.g., M1, M3, M5, M6, M7, and M9). At phase II reactions, a conjugate with succinic acid (M4) and three metabolites conjugated with glucuronic acid (M8, M9, and M10) was reported [75] (Figure 9).

In spite of mephedrone being one of the most studied synthetic cathinones, more studies are needed to evaluate the behaviour of unchanged mephedrone and the 10 reported metabolites in urine samples to determine which should be the most appropriate biomarkers when investigating drug use in the context of forensic toxicology [75].

As regards the 2Cs compounds and according to the Drug Enforcement Agency, when they are inflated they originate faster and more intense effects [76]. For example, oral administration of 2C-T-7 has an onset of 1–2.5 h and duration of action of 5–7 h, whereas insufflation of this compound has an onset of 5–15 min and duration of action of 2– 4 h [57].

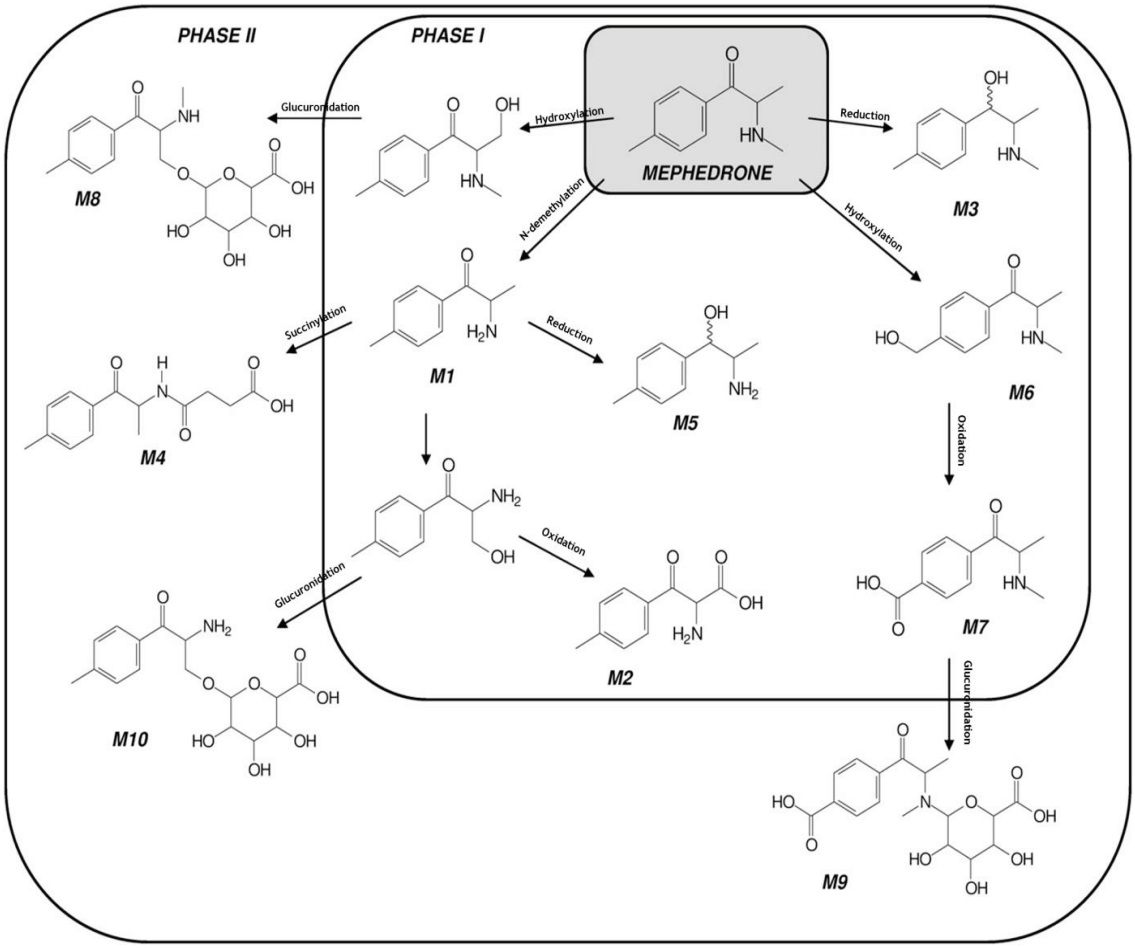

Figure 9. Metabolism of mephedrone (phases I and II). M1-M10: metabolites. (Source: [75])

Some studies reported that metabolism of 2C compounds occurs mainly by O-demethylation at positions 2 and 5 [59]. The compounds are deaminated by monoamine oxidase (MAO) and then oxidated by aldehyde dehydrogenase to either the corresponding acid or base. The principal enzymes involved in the deamination of this compounds are MAO-A and MAO-B [76]. Due to the involvement of MAO in 2C compounds metabolism, there are potential drug–drug interactions with MAO inhibitors, which can increase serum 2C concentrations and increase the risk of 2C toxicity [59]. The hepatic cytochrome CYP2D6 plays a minor role in the metabolism of the following compounds: 2C-D, 2C-E, 2C-T-2, and 2C-T-7 [76].Limited information suggests that 2C drugs also inhibit reuptake of monoamines including NA, DA, and SERT [59].

In table 2 dosages and duration of effects of 2C compounds, reported by Shulgin, are shown [58].

Table 2. Dose (mg) and duration of effects (h) of 2C compounds. (Source: [57,58]).

2C	Chemical name	Dosage	Duration (h)
2C-B	4-Bromo-2,5-dimethoxyphenethylamine	12–24 mg	4–8
2C-C	4-Chloro-2,5-dimethoxyphenethylamine	20–40 mg	4–8
2C-D	4-Methyl-2,5-dimethoxyphenethylamine	20–60 g	4–6
2C-E	4-Ethyl-2,5-dimethoxyphenethylamine	10–25 mg	8–12
2C-G	3,4-Dimethyl-2,5-dimethoxyphenethylamine	20–35 mg	18–30
2C-G-3	3,4-Trimethylene-2,5-dimethoxyphenethylamine	16–25 mg	12–24
2C-G-5	3,4-Norbornyl-2,5-dimethoxyphenethylamine	10–16 mg	32–48
2C-I	4-Iodo-2,5-dimethoxyphenethylamine	14–22 mg	6–10
2C-N	4-Nitro-2,5-dimethoxyphenethylamine	100–150 mg	4–6
2C-P	4-Propyl-2,5-dimethoxyphenethylamine	6–10 mg	10–16
2C-SE	4-Methylseleno-2,5-dimethoxyphenethylamine	~100 mg	6–8
2C-T	4-Methylthio-2,5-dimethoxyphenethylamine	60–100 mg	3–5
2C-T-2	4-Ethylthio-2,5-dimethoxyphenethylamine	12–25 mg	6–8
2C-T-4	4-Isopropylthio-2,5-dimethoxyphenethylamine	8–20 mg	12–18
2C-T-7	4-Propylthio-2,5-dimethoxyphenethylamine	10–30 mg	8–15
2C-T-8	4-Cyclopropylmethylthio-2,5-dimethoxyphenethylamine	30–50 mg	10–15
2C-T-9	4-(t)-Butylthio-2,5-dimethoxyphenethylamine	60–100 mg	12–18
2C-T-13	4-(2-Methoxyethylthio)-2,5-dimethoxyphenethylamine	25–40 mg	6–8
2C-T-15	4-Cyclopropylthio-2,5-dimethoxyphenethylamine	>30 mg	Several hours
2C-T-17	4-(s)-Butylthio-2,5-dimethoxyphenethylamine	60–100 mg	10–15
2C-T-21	4-(2-Fluoroehtylthio)-2,5-dimethoxyphenethylamine	8–12 mg	7–10

1.4.2. Mechanism of action

As has already been described, cathinone is a ß-ketophenethylamine structurally similar to amphetamine (Figure 3), while its metabolites (cathine and norephedrine) (Figure 8) are structurally related to dopamine and noradrenaline. Some studies showed that cathinone, cathine, and norephedrine are able to induce an amphetamine-like central nervous system (CNS) DA release, with cathinone being the most potent of the three alkaloids [44,77,78].

Cathinone derivatives share actions on central DA systems involved in the regulation of behavioural enforcement, motor coordination, and thermoregulation, after acute administration [79]. All of this substances cause increased locomotor activity, although different cathinones exhibit different potencies, stimulating capacity and duration of action [49].

Synthetic cathinones act predominantly as CNS stimulants mediating the actions of DA, NA and/or SERT, mimicking the effects of traditional drugs such as cocaine, amphetamine, methamphetamine, and ecstasy.

Cathinone analogues have high selectivity and strong activity for SERT receptors and monoamine transporters. However, their selectivity for these transporters varies

considerably. Synthetic cathinones are known to be potent inhibitors of monoamine transporters such as DA, SERT and NA, resulting in an increased concentration of these biogenic amines in the synaptic cleft, mostly by two mechanisms. First, these products interact with monoamine transporter proteins (namely NA, DA and SERT), inhibiting reuptake of NA, DA and 5-HT, respectively. Second, some of these compounds, when interacting with monoamine membrane transporters, also act as their substrates and promote the release of neurotransmitters from intracellular stores by reversal of the normal direction of transporter flux, and inhibition of the vesicular monoamine transport receptor, VMAT2 [80]. These compounds induce sympathomimetic effects and stimulation of the CNS, they easily cross the blood-brain barrier (BBB) and they are mainly concentrated in the kidneys, lungs, brain and cerebrospinal fluid. Despite most of these NPS being potent inhibitors of NA, there are significant differences in their inhibition profiles regarding DA and SERT reuptake transporters as well as their ability to release monoamines and antagonize monoamine reuptake [81]. This means that it becomes complicated to group these substances on mode of action or potency which can be responsible for the different effects and toxicities in the human subject. Each compound has a different affinity for the monoamine transporters. Mephedrone and methylone are examples of nonselective monoamine uptake inhibitors with affinity for DA and SERT, while cathinone and methcathinone are DA and NA uptake inhibitors and releasers [28].
As was also described previously, the phenethylamine-based structure is shared among such diverse compounds as catecholamines, amphetamines, cathinones, and so-called 2C group. They exert stimulating, hallucinogenic, and psychedelic effects, depending on the chemical structure and dose administrated. The 2C and D compounds have affinity for 5-HT2 and alpha-adrenergic receptors, but these compounds can be either agonists or antagonists, depending on the specific receptor subtype [59,82,83] . For example, 2C-B, 2C-T-2 and 2C-T-7 are partial agonists for 5-HT2 and alpha-adrenergic receptors and also act as both competitive 5-HT antagonists and non-competitive 5-HT antagonists, at small and high concentrations respectively [84]. In addition, DOB and DOM have a high affinity for 5-HT2 receptors. However, pharmacokinetics and pharmacodynamics of 2C products may vary between users and some may be more susceptible to toxicity than others [59,85].

1.4.3. Clinical effects and toxicity

Serotonergic drugs (entactogens and hallucinogens) are associated with acute serotonin syndrome, hyperthermia, seizures, and hyponatremia. Dopaminergic drugs are highly addictive and acute toxicity includes prolonged stimulation, insomnia, agitation, and psychosis [10].
From the clinical point of view there is a risk of cathinone- and phenethylamine- induced hepatotoxicity. Most of the known health effects come from clinical observations and users reports. Further research is needed to provide evidence of short and long-term health risks and the addiction potential associated with the use of these substances [86]. Synthetic

cathinones have stimulant and entactogenic effects. In recreational context, the most desired characteristic effects described by the users of these products include euphoria, intensification of sensory senses, increased sociability, increased sensory perception and energy, mental stimulation, empathy connection, openness, decreased inhibition and sexual arousal [23,87]. The most common adverse physical symptoms include a vast range of symptoms that require medical assistance and can be divided into the following main groups: cardiovascular, psychiatric, neurological, perceptual, gastrointestinal, and others more general. Clinical effects reported with synthetic cathinones intoxication are similar to sympathomimetic toxicity with amphetamine derivatives, agitation being the most common sign [23]. Other common effects include tachycardia, heart palpitation, arrhythmia, increased blood pressure, capillary dilatation, and haemorrhage. Users can also display headache, pupil dilatation, nausea, vomiting, suppressed appetite, bruxism, hyperthermia, dehydration, hot flushes, sweating, blue and cold extremities, and seizures. Studies revealed that in several patients admitted to the emergency service of hospitals, the pattern of clinical toxicity symptoms included anxiety, insomnia, fatigue, agitation, confusion, memory loss, myoclonus, paranoia, hallucinations, suicidal ideations, chest pain, tachycardia or bradycardia, nausea or vomiting, psychomotor agitation or retardation, mydriasis, respiratory distress, chest pain, cardiac arrhythmias, hyperthermia, perspiration or chills, and hypertension or hypotension. Toxic psychosis, psychiatric disease, sleep deprivation, renal failure and death can also occur in situations of repeat dosing. In overdose cases, physical manifestations range from severe hyperthermia, rhabdomyolysis, hyponatremia, acidosis, intense sweating, foaming from the mouth and seizures, to those as severe as stroke, cerebral oedema, cardiorespiratory collapse, myocardial infarction, multiple organ failure, and death [23,49,87,88].

Concerning 2C and D series compounds, reported effects are a combination of hallucinogenic and stimulating symptoms [89] ranging from mere stimulant effect at lower doses, to hallucinogenic and entactogenic effects at higher doses [8,84]. Phenethylamine effects included in the D series are described to be longer lasting, more potent and reportedly more liable to induce vasoconstriction than other members of the phenethylamine family [90] and PMA and PMMA are known to have a particularly high toxicity [8,91]. So, despite the lack of scientific studies concerning clinical and toxicological effects of these substances in humans, in general intoxication symptoms include excited delirium, hallucinations, severe agitation, aggression, violence, seizures, hyperthermia, tachycardia, hypertension, paranoid delusions, nausea, vomiting, nasal bleeding, pulmonary oedema, cardiac and pulmonary arrest, cerebral haemorrhage, hyperventilation, foaming from the mouth, multi-organ failure (such as the liver and kidney), coma and death [8,59,84,88].

The clinical toxicity of synthetic cathinones and phenethylamines are generally similar to that of other amphetamines including MDMA [10]. If a patient is significantly agitated and a diagnosis of NPS delirium is suspected, treatment efforts should first focus on preventing injury to the patient and clinicians. Once the patient has stabilized, continued medical evaluation is crucial to prevent complications such as hyperthermia, metabolic acidosis,

muscle damage and rhabdomyolysis [28,39,92]. Treatment is mainly supportive care but should include rapid sedation and aggressive treatment of severe hyperthermia with aggressive cooling [23,69]. Heart rate, blood pressure, and body temperature should be monitored and electrocardiogram and electroencephalogram might also be useful exams to help guide the clinical treatment [49,59,93].

Acute treatment of a sympathomimetic toxidrome primarily includes benzodiazepines and fluid replacement to control agitation, cardiovascular stimulation and hyperthermia. The use of haloperidol without benzodiazepines is generally not recommended because it may contribute to seizure, hyperthermia and dysrhythmias. As a general rule, the use of dopamine blocking (antipsychotic) agents that may potentially exacerbate thermoregulatory disturbances should be carefully managed in patients intoxicated with these kinds of substances. Hypertension should be treated primarily with nitrates and ß-blockers should be avoided because of unopposed α-adrenergic stimulation resulting in further increases in blood pressure [10,14,49,59].

Ultimately, management depends on an individual patient's signs and symptoms. Following resolution of an acute intoxication, the patient should be referred to psychiatric treatment [49,87,92].

1.4.4. Toxicological analysis

Ideally, the clinical toxicology tests should include: a basic metabolic panel with blood glucose levels, serum electrolyte concentrations (sodium and potassium), and serum osmolarity if hyponatremia is present, liver and kidney function tests (transaminases, alanine transaminase/aspartate transaminase, gamma-glutamyl transpeptidase, alkaline phosphatase, total and unbound bilirubin, creatinine, and urea); a complete blood cell count with coagulation studies (activated partial thromboplastin time, prothrombin time, platelet count, and p-dimer); cardiac markers (troponin and creatine kinase); total creatine kinase to exclude potential rhabdomyolysis; and myoglobin in urine if there are signs of rhabdomyolysis [49].

It is possible to identify specific synthetic cathinones and phenethylamines present in blood, urine, and other body fluids using gas-chromatography [19,28,70,86,94–100] and liquid-chromatography-mass spectrometry [19,20,28,31,42,86,94,101–105] confirmation methods.

Most NPS are not detected by standard immunoassay urine drug screens. Thus, intoxication would typically present as sympathomimetic or serotonergic toxidromes with a negative screening result for amphetamines [14,106,107]. Ideally, urine and blood should be sampled and sent to a laboratory for toxicological analyses by GC-MS. Usually, the detection and identification of the NPS will not be readily available for the management of acute intoxication. Nevertheless, the compounds should be identified to better document the substances and their associated toxicity.

The increased consumption of synthetic cathinones and phenethylamines in parallel or associated with other drugs continues to be underestimated and is a matter of concern for public health. One of the major points of concern relates to the inability of most current methods of toxicological screening of biological matrices to identify the new compounds that arise on the market [106]. This recent reality became a real challenge for the forensic toxicologist in the development of analytical procedures for the determination of these substances in various biological matrices [96]. Analytical methods that can follow the fast and numerous changes in chemical structures are required for routine analysis and screening of these NPS in biological specimens for forensic and clinical purposes [86]. Indeed, several methods have been used to monitor the NPS in non-biological specimens.

In recent years, analytical methods to determine different classes of NPS in biological specimens involving both gas and liquid chromatography coupled to mass spectrometry, have been published (Table 3).
The efforts that have been made in the development of more specific and sensitive chromatographic methods associated with mass spectrometry can now successfully identify a large number of NPS [86,96].

It is necessary that forensic toxicology laboratories are provided with suitable methods for detection of these substances in biological matrices. Once various human matrices are available to postmortem toxicological analysis, it is relevant to highlight the importance of studying the NPS in alternative specimens other than blood and urine in order to overcome situations where their collection is unavailable (e.g. exsanguination following severe traumatic injuries, advanced putrefaction, and carbonization). In these cases, the vitreous humor and pericardial fluid matrices could be useful to provide information about the intake of these drugs.

Table 3. Analytical methods for the determination of synthetic cathinones and phenethylamines in biological specimens.

Substance	Biological specimen	Analytical procedure	Sample preparation	Reference
Mephedrone	Blood	UHPLC-QTrap/MS	SPE	[108]
Cathinones; ephedrines		LC–ESI-MS/MS	LLE	[101]
Phenethylamines; Cathinones; ephedrines		LC-MS/MS	LLE	[20]
Phenethylamines; Cathinones; ephedrines		GC-MS	SPE	[109]
Cathinones		GC/MS; LC-MS/MS	LLE	[94]
Mephedrone		GC/MS	SPE	[100,110]
Cathinones and 2C series		UHPLC-QTOF/MS	LLE	[111]
Mephedrone	Plasma	UHPLC-QTrap/MS	SPE	[108]
Mephedrone		LC-MS/MS	LLE	[96]
Ephedrines		GC/MS	SPME	[97]
Ephedrines		HPLC-UV	SPE	[112]
Ephedrines		LC-MS/MS	LLE	[102]
Cathinones; ephedrines		LC-MS/MS	SPE	[113]
Cathinones	Serum	GC/MS	SPE	[114]
Cathinones, 2C and D series		LC-MS/MS	SPE	[115]
Mephedrone	Urine	LC-MS/MS	LLE	[108]
Mephedrone		GC/MS	SPE	[100,110]
2C and D series		LC-MS/MS	SPE	[31]
Cathinone; methcathinone		GC/MS	LLE	[98]
Ephedrines		HPLC-UV	SPE	[112]
Phenethylamines 2C and D series; Synthetic cathinones		LC-MS/MS	LLE	[20]
Phenethylamines 2C series		GC/MS	LLE	[99]
Phenethylamines; Synthetic cathinones		UHPLC-MS/MS	SPE	[105]
Cathinones; ephedrines		GC/MS	LLE	[116]
Mephedrone; butylone; methylone		GC/MS	SPE	[70]
Ephedrines		LC-MS/MS	LLE	[102]
Synthetic cathinones		LC-HRMS	SPE	[104]
Synthetic cathinones		LC-MS/MS	SPE	[103]
Synthetic cathinones		GC-MS	LLE	[96]
Synthetic cathinones	Hair	GC/MS	SPE	[114]
Cathinones		UHPLC-MS/MS	LLE	[117]
Mephedrone		GC/MS	LLE	[118]
Synthetic cathinones		GC/MS; LC-MS/MS	SPE; LLE	[95]
Synthetic cathinones		LC-MS; LC-MS/MS	SPE; LLE	[96]
Cathinones; ephedrines		GC/MS	LLE	[94]
Mephedrone		GC/MS	SPE	[100]
Synthetic cathinones	Oral fluid	UHPLC-MS/MS	SPE	[119]
Synthetic cathinones		LC-MS/MS	SPE	[120]
Cathinones	Tissues	GC-MS; LC-MS; LC-MS/MS	SPE; QuECHERS; LLE	[96]
Phenethylamines; Cathinones; ephedrines	Pericardial fluid	GC-MS	SPE	[109]
Phenethylamines; Cathinones; ephedrines	Vitreous humor	GC-MS	SPE	[109]

Abbreviations: LC–ESI-MS/MS: Liquid-chromatography–tandem- pneumatically assisted electrospray ionisation-mass spectrometry; UHPLC-QTrap/MS: Ultrahigh-performance liquid chromatography-hybrid triple quadrupole- linear ion trap mass spectrometer detector; UHPLC-MS/MS: Ultrahigh-performance liquid chromatography-mass spectrometry; UHPLC-QTOF/MS: Ultrahigh-performance liquid chromatography-time-of-flight mass spectrometry detector; HPLC-UV: High-performance liquid chromatography-ultraviolet detector; LC-HRMS: Liquid chromatography–high resolution mass spectrometry.

1.4.5. Legal status

According to the EU Drug Markets Report published in 2016, there are no signs suggesting a slowdown in the number, type or availability of NPS [6]. Conversely, a large number of substances are sold openly as legal substitutes for illicit drugs such as cannabis, methamphetamine, cocaine, MDMA, heroin and benzodiazepines. The EU Early Warning System currently monitors more than 600 substances [34], which is more than double the number of drugs controlled under UN conventions . The market supplies both recreational and, increasingly, chronic and marginalised drug users. What best characterizes this market is the producers' reaction and even anticipation of legal and regulatory controls by rapidly developing and introducing new substances. The sophisticated and aggressive marketing techniques related to NPS are also important sources of its distribution [33].

The legal status of NPS varies widely all over the world. Many substances are controlled in countries throughout the world, although the regulations are usually limited by their chemical structures. Many actions have been taken to ban these substances in Europe and in the United States, as has been published in annual reports of the EMCDDA [7]. Although many NPS are currently under legal control in Europe and in most states of the USA, criminalization of all NPS derivatives is difficult to attain [33]. Portugal has restrictive legislation through Decree-Law nº 54/2013, 17 April [36] which includes the illegality of the production, importation, exportation, publicity, distribution, sale and possession of NPS. This legislation also includes an extensive list of NPS as well as the derivatives, isomers, and salts of those substances, comprising all preparations associated with such compounds, in the Portaria nº 154/2013, of 17 April [35]. Recent years have witnessed a proliferation of NPS becoming available in Europe. The phenomenon has provoked a range of innovative legal responses geared towards controlling the open sale of these substances. Figure10 shows the map of the legal innovations made across Europe between 2009 and 2016. Each mark is colour-coded, depending on the type of innovation. Numbers are assigned chronologically (i.e. smaller numbers are for earlier innovations) [7].

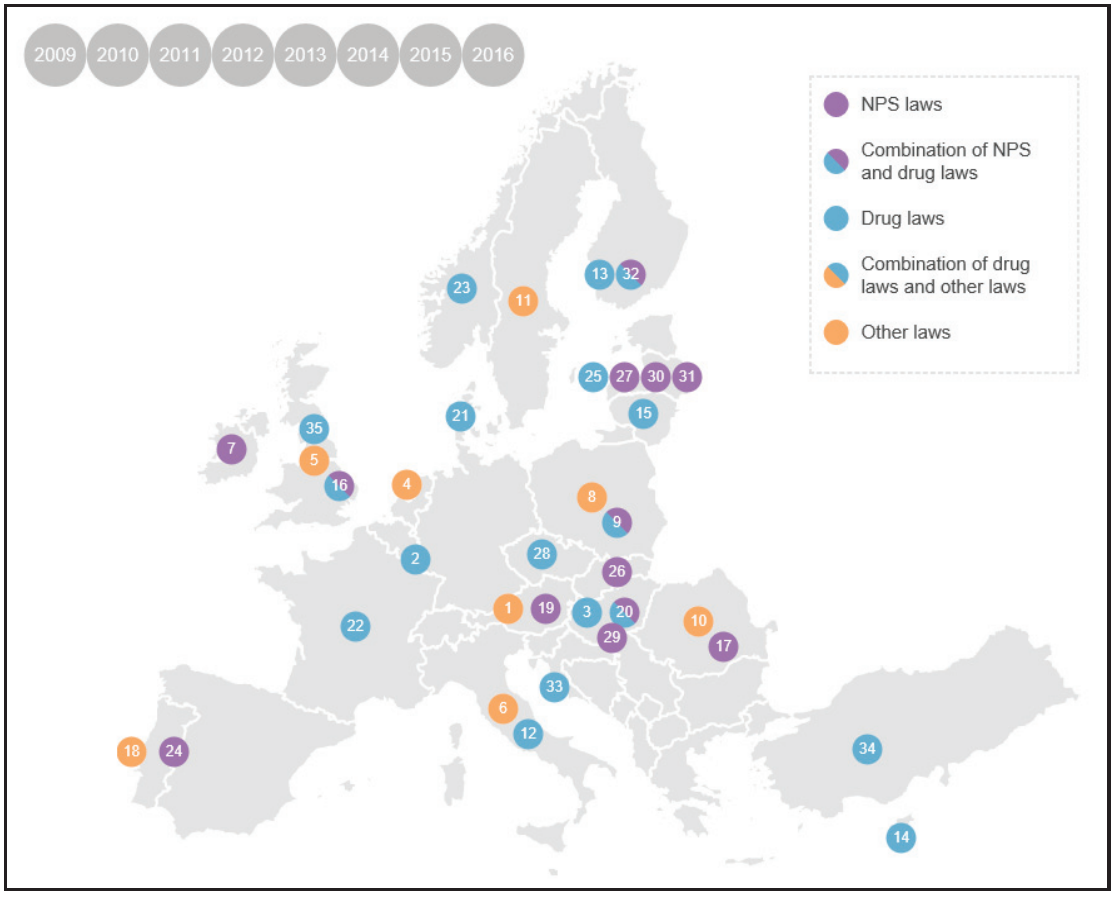

Figure 10. Legal approaches to controlling NPS. The map shows innovations from around Europe (2009-2016).
(Source: http://www.emcdda.europa.eu/topics/pods/controlling-new-psychoactive-substances)

CHAPTER 2
ALTERNATIVE POSTMORTEM MATRICES IN TOXICOLOGICAL ANALYSES

This chapter intends to highlight the postmortem alternative fluids studied throughout this work: vitreous humor (VH) and pericardial fluid (PF).

A biological fluid is a complex mixture containing a large number of components which can contribute to a diverse collection of interferences, as for example: masking the presence of a drug or increasing or decreasing its response due to degradation phenomena by enzymatic action or pH changes [121].

The specimens available in postmortem toxicology investigations can be numerous and variable, and may be selected based on the case history, requests, legal aspects and availability in a given case [122–124]. However, not all matrices are appropriate to the analysis of all drugs [125,126]. Usually, during an autopsy, fluids and tissue samples are collected for carrying out several complementary analyses, including forensic toxicology. The postmortem samples collected from the corpse and the requested analyses in these specimens are dictated by circumstances of the case and the condition of the body. So, although blood is the preferred reference matrix in the field of postmortem forensic toxicology, alternative matrices are required in case of limited volume, unavailable or unusable blood samples [123,126–128].

Blood, plasma and urine are the biological fluids most commonly analysed for detection, confirmation and quantitation drugs and their metabolites [123,129]. Concentrations of drugs in blood may be useful for establishing recent drug intake and to determine its effect on the deceased at the time of death, or at the time the blood was taken, in drug abuse situations. The drug concentration obtained in blood provides valuable information to assist in interpreting the toxicological results [123,125,130,131].

The toxicological analyses of drugs in biological specimens are complex processes requiring specific and sensitive methodologies, since the analytes are unknown in advance and various factors may interfere with their detection (e.g. presence of xenobiotics and endogenous biomolecules) [122,130].

In forensic toxicology a variety of biological matrices are analysed, in addition to blood and urine. This work began an investigation into the suitability of PF and VH as alternative matrices to characterize the consumption of the NPS studied and their influence on the occurrence of death. The use of other biological matrices as alternatives to blood has been described for decades and its importance in toxicological analyses has been reviewed by some authors [125,129,132–135]. The most traditional postmortem specimens used in toxicological analyses of drugs of abuse include liver and kidney, brain, bone and bone marrow, hair, nails and VH [109,136–138]. PF has also been documented but existing works are still scarce [109,138–144]. In clinical and workplace drug testing the most commonly used of these specimens are saliva/oral fluid, hair, sweat, breast milk, amniotic fluid, and meconium [135].

Although each of the published articles that support this investigation includes an approach to the advantages and disadvantages of the use of VH and PF in the analysis of NPS, when compared to other biological specimens, this section is intended to present only a brief

description of these alternative matrices. When blood is insufficient or has suffered changes by various postmortem factors, alternative matrices may be useful. An ideal alternative matrix should enable detection of the same xenobiotics as found in blood, at correlated concentrations, and without the postmortem effects [133]. The importance of toxicological results obtained from analysis of postmortem specimens is the substantial contribution to determining the cause of death and circumstances at the time of its occurrence.

2.1. Pericardial fluid

2.1.1. Human pericardium structure

The heart is inside the thoracic cavity and is connected to the great cardiac vessels within a thin fibroserous sac known as pericardium [145,146]. The pericardium is a structure that surrounds the heart and plays several important physiological roles. In a healthy person, this structure separates and isolates the heart from contact with the surrounding organs and tissues, allowing the freedom of cardiac movement within the confines of the pericardial space. This structure is located inside the middle mediastinum posteriorly to the sternum and the cartilages of the third to seventh left rib. Under normal conditions, it is not in contact with the frontal membrane of the thoracic cavity. Laterally, it is held together with the mediastinal parietal pleura. The pericardium is composed of two anatomical structures closely connected: the serous and the fibrous pericardium. The serous structure is an internal continuous sac formed by two layers: the parietal layer and the visceral layer or epicardium. The visceral structure is the interior surface of the pericardium which covers the surface of the heart and is intimately connected to it. The parietal structure is the exterior surface of the serous pericardium which is fused with the fibrous pericardium. Between the parietal and visceral layers of the serous pericardium exists the pericardial cavity, which contains a thin film of fluid, useful for lubrication of the heart during its cycles of contraction and relaxation, termed pericardial fluid [146,147]. The normal pericardium structure with its constituent layers is shown in figure11.

The arterial branches from the thoracic aorta, right and left pericardiophrenic artery (internal mammary artery branches), are responsible for the blood supply of the whole pericardium while the venous drainage is accomplished through the venae pericardial which drain into the azygos vein, to the superior vena cava or to the brachiocephalic [147]. The thickness of the pericardium increases proportionally to the size of the heart and the pericardial cavity (human 1–3.5mm) [146–148].

The parietal serous pericardium's lamina is formed of a monolayer of flattened mesothelial cells, which are based on a thin basement membrane supported by connective tissue stroma in a narrow submesothelial space. The connective tissue stroma contains several layers of collagen fibrils and small elastic fibres. The luminal surface of the mesothelial cells has a

well-developed microvillous border with occasional cilia, holding the friction and increasing the surface area for fluid transport. All of these morphological characteristics form permeability barriers [147].

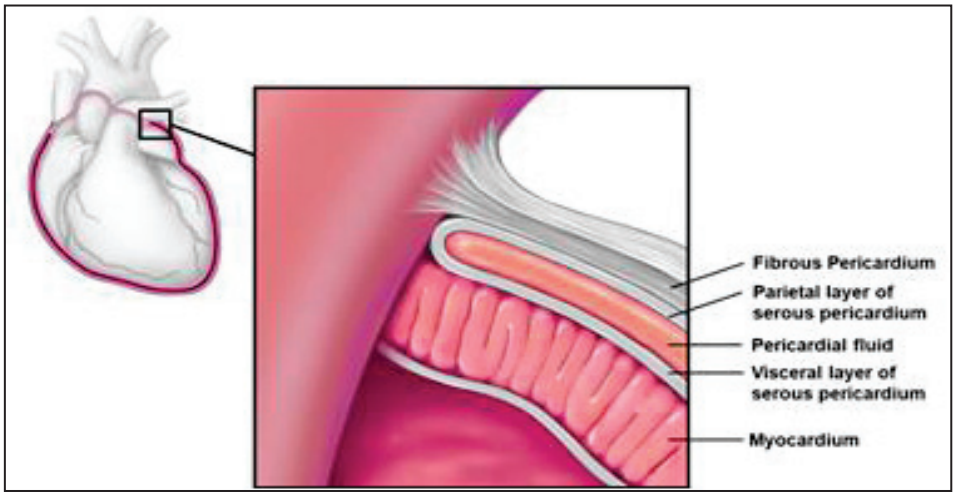

Figure 11. Human pericardium structure. (Source: [149]).

As previously said, the healthy pericardium contributes to important heart functions such as [146,147]:

1. Lubricating the movement of its surfaces;
2. Stabilizing its anatomic position, isolating it from the adjacent anatomical structures, prohibiting adhesion formation, inflammatory or neoplastic proliferation;
3. Limiting its dilatation during diastole, reducing the endomyocardial tension, preventing cardiac hypertrophy in pressure overload conditions;
4. Reducing the right ventricular impulse work in left ventricular overload conditions;
5. Preventing the ventriculoatrial blood retrogression during high end-diastolic ventricular pressures;
6. Preservation of the negative endothoracic pressure, which is crucial for atria blood filling;
7. Nervous stimulation response and regulation of cardiac frequency and arterial blood pressure;
8. Formation of a hydrostatic compensation system ensuring that end-diastolic pressure remains the same at all hydrostatic levels and the Frank–Starling mechanism is functional.

However, the physiology of the pericardial cavity needs to be better studied in order to provide new opportunities for further understanding the pharmacokinetics and pharmacodynamics of drugs and medicines in the PF [147,150].

2.1.2. Pericardial fluid composition

The healthy human pericardial space contains about 20-60 mL of PF [146,147,149,150]. This fluid is strongly considered an epicardial transudate derived by plasma ultrafiltration with electrolyte content similar to it, and is considered to be drained mainly by lymphatic capillary [147,151,152]. This fluid has specific characteristics with strong evidence that it also contains a small amount of interstitial fluid derived from the underlying myocardium and its volume is determined by the equilibrium between production/drainage. The fluid movement through the pericardial laminae is a hydrostatic/osmotic pressure equilibrium between the microvasculature and the cavity [147]. Some previous studies [146,153] have shown that the PF distribution inside its cavity is not uniform, the largest amount being stored inside the atrioventricular and the intraventricular sulcus [146]. The cell composition includes mesothelial cells, lymphocytes (53%), glanulocytes (31%), macrophages (12%), eosinophils (1.7%), and basophils (1.2%). When compared to the plasma, the fluid presents:

- Higher concentrations of K^+ and lower concentrations of Na^+, Ca^{2+}, Cl^- and Mg^{2+};
- Lower concentrations of protein, whose fractions vary in proportions, including albumin (in higher concentration), globulins, macroglobulins, and fibrinogen (in lower concentration) [146,147,151];
- Approximately half the protein concentration, about one-third of the cholesterol and triglycerides, and less than one-fifth of the amount of white blood cells;
- Slightly lower osmolality [147,151].

2.1.3. Pericardial fluid and forensic toxicology

Pericardial fluid can be sampled with a syringe (Figure 12), during a medico-legal autopsy, after opening the pericardium. In a relatively recent corpse 5-10 mL of this can be obtained [150]. Although some authors have developed methodologies to determine different drugs in this specimen [109,138,140–144,152,154–156], the potential of this matrix is not yet well explored in forensic toxicology.

Some studies have shown that this matrix can be useful for drugs determinations, since PF and femoral blood have a good correlation [140,157]. The authors Moriya F *et al.* [140] have compared the concentrations of acid, basic and neutral drugs in both PF and femoral blood taken from recent corpses almost without changes, and concluded that there was good correlation between these two specimens. Thus this specimen seems to be useful to evaluate situations of intoxication degree in forensic toxicology. Another advantage of this fluid is the larger volume that can be collected during a forensic autopsy (in cases without structural damage due to injury or medical intervention), without significant contamination, enabling the various usual toxicological analyses to be made [144]. This fluid can be obtained in large amounts even from a widely exsanguinated corpse. However, caution must be taken during its

collection to prevent contaminations from stomach and adjacent organs. The extraction of the drugs from this fluid does not require complex methodologies. Similar to VH, this fluid has the disadvantage of a limited database in the analysis of drugs for supporting the interpretation of the results obtained [143–146,154,157,160]. The inclusion of this sample in routine toxicological analysis may be helpful in investigating the influence of the mechanisms of postmortem redistribution and diffusion of drugs in the death process [160]. Pericardial fluid, then, can be a fairly good sample for quantitative confirmation of postmortem blood sample findings. In some cases, however, relatively large differences in drug concentrations were observed between PF and blood [142].

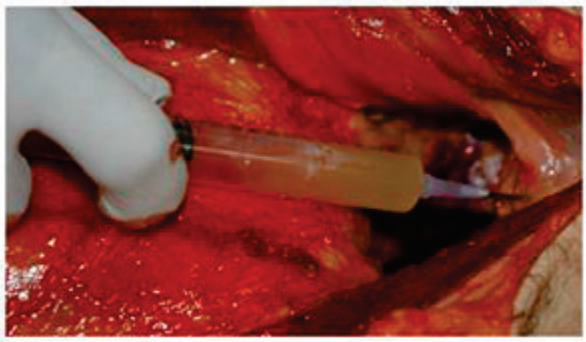

Figure 12. Pericardial fluid collection during a medico-legal autopsy (Source: kindly provided by Jerónimo Fontesanta, INMLCF 2011)

2.2. Vitreous humor

2.2.1. Structure of the human eye

The human eye is set into the bone orbit and is connected to the brain by the optic nerve. The eye is divided into three cavities: the anterior cavity (containing the aqueous humor), the posterior cavity, and the vitreous chamber (containing the vitreous humor) [126]. The eyeball provides protection for the retina, the photosensitive portion of the eye. Both the retina and the optic nerve are enclosed by dense fibrous tissue, the sclera, and the dura mater. The dura mater surrounds the optic nerve and merges with the sclera to occupy the posterior cavity which is five-sixths of the ocular globe; the remaining portion (anterior cavity) containing the cornea that refracts the incident light. As can be seen in Figure 13, the vitreous chamber is located between the lens and the retina and fills the centre of the eye. This cavity constitutes 80% of the eye with about 4 mL of capacity [126].

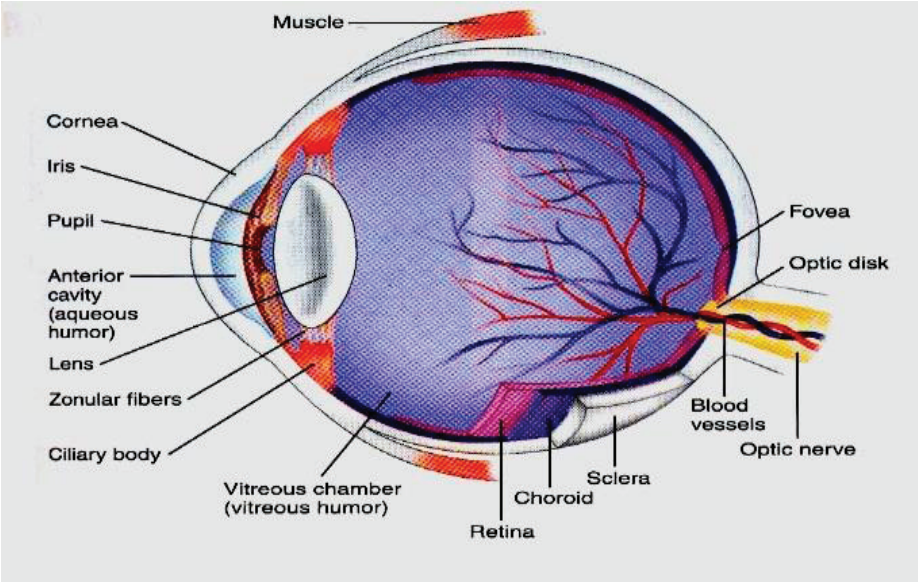

Figure 13. Structure of the human eye.
(Source: http://www.slideshare.net/medicmesirmansurah/refractory-media-of-eye-s4-march-10, Nisreen Abo-Elmaat, 2010)

2.2.2. Vitreous humor composition

Vitreous humor is a clear gelatinous fluid filling the vitreous body of the eyeball. This fluid weighs about 4 g, is composed mainly of water (99 %) and has a pH of 7.5. The VH has two major structural components: the structural protein collagen and the hyaluronic acid (a glycosaminoglycan carbohydrate). The VH is structured by arrangements of hyaluronic acid molecules and collagen fibrils. As many as 1205 proteins have been identified in this fluid. Substances that are highly protein bound would not have a significant concentration in vitreous humor, and compounds not highly protein bound would be expected to have significant concentrations in this matrix [133,158]. The VH also contains electrolytes such as sodium, chloride, calcium, glucose, urea and creatinine whose concentrations are similar to those found in the serum. This fluid is also avascular and contains very few cells. With the advancement of age a change in its composition occurs that leads to a gradual liquefaction [126,133].

After death, VH potassium concentrations increase rapidly, due to its output from the cells to the surrounding fluids. In non-diabetic cadavers glucose is rapidly broken down but remains high in non-medicated diabetics. During the early postmortem period urea and creatinine remains stable. Nevertheless, postmortem evolution involves dehydration, which some authors have assessed in terms of increased creatinine concentration [133].

The blood-retinal barrier is very selective. The movement of molecules across the blood-retinal barrier within the VH occurs by diffusion, active transport mechanism, hydrostatic pressure, osmotic pressure and convection. About 50% of the water is replaced every 10-15

min; substances with a high molecular weight and colloidal particles move by convection and those of low molecular weight move primarily by diffusion. Only free drugs with low molecular weight can move through the blood-retinal barrier by diffusion. Therefore, high protein-bound drugs are not expected to be present in the VH [126,159].

About 1–2 mL volume of this clear, avascular and gelatinous fluid can be obtained by puncture from each eyeball [133]. Obtained during an autopsy, VH is collected with a syringe by inserting the needle into the membrane at the lateral canthus (Figure 14). This fluid tends to liquefy according to postmortem interval and local conditions [133].

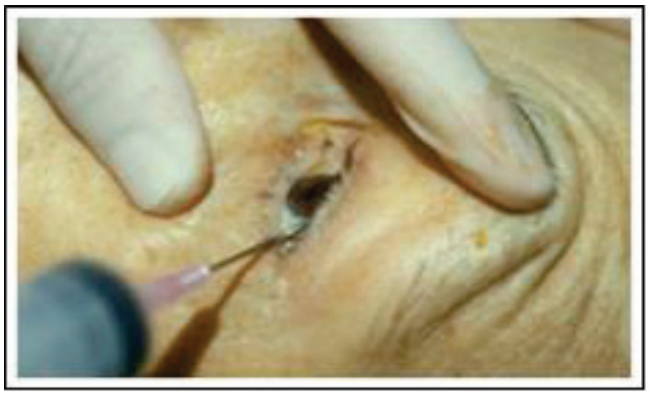

Figure 14. Vitreous humor collection during a medico-legal autopsy (Source: kindly provided by Jerónimo Fontesanta, INMLCF 2011)

Due to its environment being protected by the posterior cavity of the eye, its avascularisation and anatomic remoteness from viscera makes it a useful tool when blood cannot be sampled (e.g., extent exsanguinated body, after haemorrhagic shock or burns) or in cases of suspected postmortem redistribution, decomposition processes or contamination by chemicals (e.g. embalming). This specimen is more resistant to the bacterial decomposition and contamination that typically occur more rapidly in other biological fluids [126].

2.2.3. Vitreous humor and forensic toxicology

In forensic toxicology, VH has been used as an alternative matrix for more than 50 years [160,161]. It was first used for alcohol analysis in 1966 [162,163]. Since then, there have been numerous studies of VH in various forensic applications as reviewed by Fabien Bévalot *et al*. [133]. Many researchers developed methodologies for the determination of several abused and therapeutic drugs in that specimen, and studied the relationship between drug concentrations in VH and in BL [133]. The VH is a matrix of reduced complexity being easy to handle. This is the matrix with less endogenous interfering compounds and toxicological analysis does not require complex preparation. The analytical procedures for the analysis of drugs in VH are similar to those used in other postmortem fluids (e.g., blood or urine) [126,164]. Those methodologies utilize solid-phase [165–169] or liquid-liquid extraction [170–

178], depending on the characteristics of the compounds, and gas [166,179–181] and liquid [136,182,183] chromatography coupled to mass spectrometry for the detection and quantitation of the identified substances.

In short, the major advantages of VH over other biological specimens are its lower protein content, easy accessibility, low contamination, and its high stability. The absence of significant metabolic activity in the eye suggests that the concentration of the drug found in VH correlates well with the corresponding concentration in blood. Drugs and metabolites passively diffuse into VH and their concentration is often correlated to the drug concentration in the circulating blood. It has been reported that VH is a suitable specimen for the detection of drugs and their metabolites. Nevertheless, this unconventional biological matrix should not be regarded as a substitute for the traditional ones, but rather as a complementary matrix able to provide valuable and complementary information. This specimen is often the most suitable (or even the only available) for toxicological analysis, particularly in situations of putrefaction or vastly traumatized. This specimen is of particular toxicological importance in those cases where sufficient amounts of blood cannot be collected, as most compounds of forensic interest are also detected in VH [126,133].

It seems difficult to estimate the levels of a certain drug in a blood sample only comparing with the concentration of that same drug in VH, since concentrations obtained in both matrices do not correlate for all substances.

The major drawback of this fluid is the limited volume that can be collected (at best about 5 mL), and thus this specimen is not suitable for extensive analysis for many drugs [184].

VH is the matrix that probably comes closest to the ideal alternative to blood, considering the ability to detect the same substances as detected in the blood, and reduced postmortem effects. A large number of compounds can be detected in this specimen (e.g. ethanol, benzodiazepines, opiates, methadone, cocaine, paracetamol, LSD, arylcyclohexylamines), making it of great interest in forensic toxicology, as has been demonstrated by many authors [133]. Further studies should be done to evaluate the correlation of the studied substances concentration to blood, since VH and blood concentrations do not correlate for all substances.

CHAPTER 3

RESULTS

3.1. Paper II

A validated procedure for detection and quantitation of salvinorin a in pericardial fluid, vitreous humor, whole blood and plasma using solid phase extraction and gas chromatography–mass spectrometry

Cláudia Margalho, Eugenia Gallardo, Alice Castanheira, Duarte Nuno Vieira, Manuel López-Rivadulla, Francisco Corte Real

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A validated procedure for detection and quantitation of salvinorin a in pericardial fluid, vitreous humor, whole blood and plasma using solid phase extraction and gas chromatography–mass spectrometry

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ABSTRACT

The use of vitreous humor and pericardial fluid as alternative matrices to blood and plasma in the field of forensic toxicology is described to quantitate low levels of Salvinorin A using ethion as internal standard. The method was optimized and fully validated using international accepted guidelines. The developed methodology utilizes a solid phase extraction procedure coupled to gas chromatography mass spectrometry operated in the selected ion monitoring mode. The method was linear in the range of 5.0–100 ng/mL with determination coefficients higher than 0.99 in 100 μL of vitreous humor and in 250 μL of each matrix pericardial fluid, whole blood and plasma. The limits of detection and quantitation were experimentally determined as 5.0 ng/mL, intra-day precision, intermediate precision and accuracy were in conformity with the criteria normally accepted in bioanalytical method validation. The sample cleanup step presented mean efficiencies between 80 and 106% in the different biological specimens analyzed. According to the low volumes of samples used, and the low limits achieved using a single quadrupole mass spectrometer, which is available in most laboratories, we can conclude that the validated methodology is sensitive and simple and is suitable for the application in forensic toxicology laboratories for the routine analysis of Salvinorin A in both conventional and unconventional biological samples.

1. Introduction

In recent years, the number of stores called "*smartshops*" has increased, especially in major urban centers. In these shops products with psychoactive effects, commonly named "*legal highs*", are freely sold. *Salvia divinorum* is a plant with hallucinogenic effects that is among the substances sold in those stores. However, it should be kept in mind that "natural" and "legal" do not mean free from danger to health. Another problem related to this type of products refers to is fact that there are no sufficient data or studies on the long-term effects of these substances on the human body, which might expose its consumers to physical and psychological risks. According to the 2012 annual report of the European Monitoring Centre for Drugs and Drug Addiction [1], *S. divinorum* is among the three natural "legal highs" most frequently available at online shops.

S. divinorum is a plant member of the *Lamiaceae* mint family that has been used for centuries by the Mazatec in Oaxaca, Mexico, in traditional religious practices. Its main active metabolite is the neoclerodane diterpene Salvinorin A and the only known psychoactive terpenoid of *S. divinorum* [1,2]. Salvinorin A is a potent and selective *k*-opioid receptor agonist with no affinity for the 5-HT_{2A}, the principal molecular target responsible for the action of classical hallucinogens (DOB, LSD, psilocybin, N,N-dimethyltryptamine, mescaline and ketamine) [3–5]. This plant is growing in popularity in Portugal and many other countries as a powerful hallucinogenic recreational drug. Its acquisition is legal in most states of USA and several European countries, and its main consumers are adolescents and young adults. Its availability has been rapidly increasing, due to the spreading of the "*smartshops*" and also to its easy purchase in Internet websites. Since the beginning of this year, several Portuguese hospitals have notified the hospitalization of patients with symptoms including complete loss of contact

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with reality, uncontrollable laughter, short-term loss of consciousness, headaches, panic crisis, depression, tremor, nausea, hearing voices, unrealistic visions, sense of death, excitement, increased heart rhythm, potential self injuries without feeling pain, and, sometimes, coma, after the consumption of *S. divinorum* [6]. However, no published data is available concerning reported cases of salvinorin-related deaths. Nevertheless, postmortem analysis may be relevant, for instance in those cases where an individual died under the influence of the drug, but whose death was not directly caused by it.

This drug can be taken by smoking, chewing or drinking in a tea [7]. The active ingredient, Salvinorin A, has been reported to induce intense hallucinations in humans, with a typical duration of action between several minutes to an hour [3–10]. Mouth absorption is reduced and it is also poorly absorbed in the gastrointestinal tract. When smoked, the effects of salvinorin A are much more pronounced, inhaled doses of 200–500 μg produce profound hallucinations [3–8,11,12]. The complete metabolism of Salvinorin A is not well known [13]. Pharmacokinetic studies showed a relatively fast elimination of Salvinorin A, with a half-life (t1/2) of 75 min and a clearance (Cl/F) of 26 L/h/kg [14]. Concerning metabolism, studies using rhesus monkey blood have shown that Salvinorin A is deacetylated to Salvinorin B, a compound with no significant affinity to *k*-opioid receptors [15,16].

In 2005, two studies were published, in which Salvinorin A was determined in biological fluids: the first one was in human plasma, urine, saliva and sweat using liquid–liquid extraction coupled with gas chromatography-mass spectrometry [12]; and the second was in human and rhesus monkey plasma, human urine and in rhesus monkey cerebrospinal fluid, utilizing solid-phase extraction and high performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry [9]. Later, in 2008, Salvinorin A was studied in human urine and blood samples by solid-phase extraction and liquid chromatography – electrospray ionization mass spectrometry [17]. Finally, in 2012 and 2013, this compound was analyzed in human urine using either liquid–liquid extraction or solid-phase microextraction with comprehensive two–dimensional gas chromatography-time of flight mass spectrometry [18] and microextraction in packed syringe with gas chromatography-mass spectrometry [19]. As we can see, there are limited data and a few analytical methods available in the scientific literature for the determination of this compound in biological fluids, and data related to concentrations obtained in authentic samples are scarce.

The identification and quantitation of drugs in biological specimens is one of the most important objectives in forensic toxicology because in some postmortem cases, neither blood nor urine can be collected due to severe exsanguination or advanced putrefaction. In these situations vitreous humor and pericardial fluid can be useful. However, these biological matrices should not be seen as substitutes for blood but as complementary specimens that can provide important information about the intake of toxic substances. Vitreous humor is mainly composed of water (99%) and is anatomically protected from contamination and bacterial degradation due to the protected environment inside the ocular globe. A disadvantage of this matrix is the limited volume that can be collected during autopsy (1–2 mL per eye) [20]. Pericardial fluid, has several advantages as a matrix in forensic toxicology, such as the high volume that can be collected during autopsy (about 10 mL). This specimen is easily obtained from a closed cavity (pericardial cavity), and it is well protected from contamination and by postmortem changes [21,22]. One the other hand, sufficient amounts of this matrix can be obtained even from a completely exsanguinated body. A study realized with fresh cadavers demonstrated that there is a good correlation between pericardial fluid and blood of the femoral vein, suggesting that drug concentration in pericardial fluid is useful for estimation of intoxication degree [23–27]. However, care should be taken, since pericardial fluid can be contaminated by postmortem diffusion, if a large amount of a drug is present in the stomach [28].

The aim of this study was to develop and validate a sensitive and specific gas chromatography mass spectrometry (GC-MS-EI) method to determine Salvinorin A in pericardial fluid (PF), vitreous humor (VH), whole blood (BL) and plasma (PL) matrices, suitable for the application in forensic toxicology routine analysis.

2. Materials and methods

2.1. Reagents and standards

The analytical standards of Salvinorin A and ethion (internal standard) were purchased from LCG Promochem (Barcelona, Spain) and Sigma–Aldrich (St. Louis, USA), respectively.

Acetonitrile (LiChrosolv®), methanol (LiChrosolv®), 2-propanol, dichloromethane, *n*-hexane and potassium dihydrogen phosphate, all of analytical grade, were obtained from Merck (Darmstadt, Germany).

Oasis® HLB (3 mL, 60 mg) extraction cartridges were purchased from Waters (Milford, MA, USA).

A stock solution of Salvinorin A (1 mg/mL) was prepared in acetonitrile. Working solutions at 50, 5, 0.5 and 0.05 μg/mL were prepared by proper dilution of the stock solution with acetonitrile. Additional working solutions, at the same concentrations, were prepared to be used in the quality control samples. A working solution of the internal standard (ethion) at 2 μg/mL was prepared in methanol. All solutions were protected from light and stored at a temperature between 2 and 8 °C.

Potassium dihydrogen phosphate 0.1 M was prepared by dissolving 13.61 g of potassium dihydrogen phosphate in deionized water, obtaining a final volume of 1000 mL of buffer solution.

2.2. Biological samples

For calibration purposes and validation experiments, blank blood and plasma samples were obtained from a local blood bank. Vitreous humor, pericardial fluid and postmortem blank blood were collected during autopsies performed at the Medico-Legal Office of the National Institute of Legal Medicine and Forensic Sciences, Centre Branch, Aveiro, Portugal. These samples were free of drugs of abuse, as they were screened before being used for both calibrators and control samples. All samples were stored at −15 °C before analysis.

2.3. Gas chromatographic-mass spectrometric conditions

Chromatographic analysis was performed using a HP 6890 gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a 5973 mass-selective detector (Hewlett-Packard, Waldbronn, Germany) and a capillary column (30 m × 0.32 mm I.D., 0.25 mm film thickness) with 5% phenylmethylsiloxane (HP-5 MS) supplied by J&W Scientific (Folsom, CA, USA).

The gas chromatograph oven temperature program was as follows: 70 °C held for 3 min, which was increased by 30 °C/min to 300 °C and held for 6 min. The splitless injection mode (2 μL) was used with a constant flow rate (1.2 mL/min) of highly purified helium. The mass spectrometer was operated with a filament current of 300 μA at electron energy of 70 eV in the electron ionization (EI) mode. The temperatures of the injection port and detector were set at 250 and 280 °C, respectively. Quantitation was done in the selected ion monitoring (SIM) mode, and the ions were monitored at *m*/*z* 318, 359, 404 and 432 (quantitation ion) for Salvinorin A, and only one ion was monitored at *m*/*z* 231 for the internal standard,

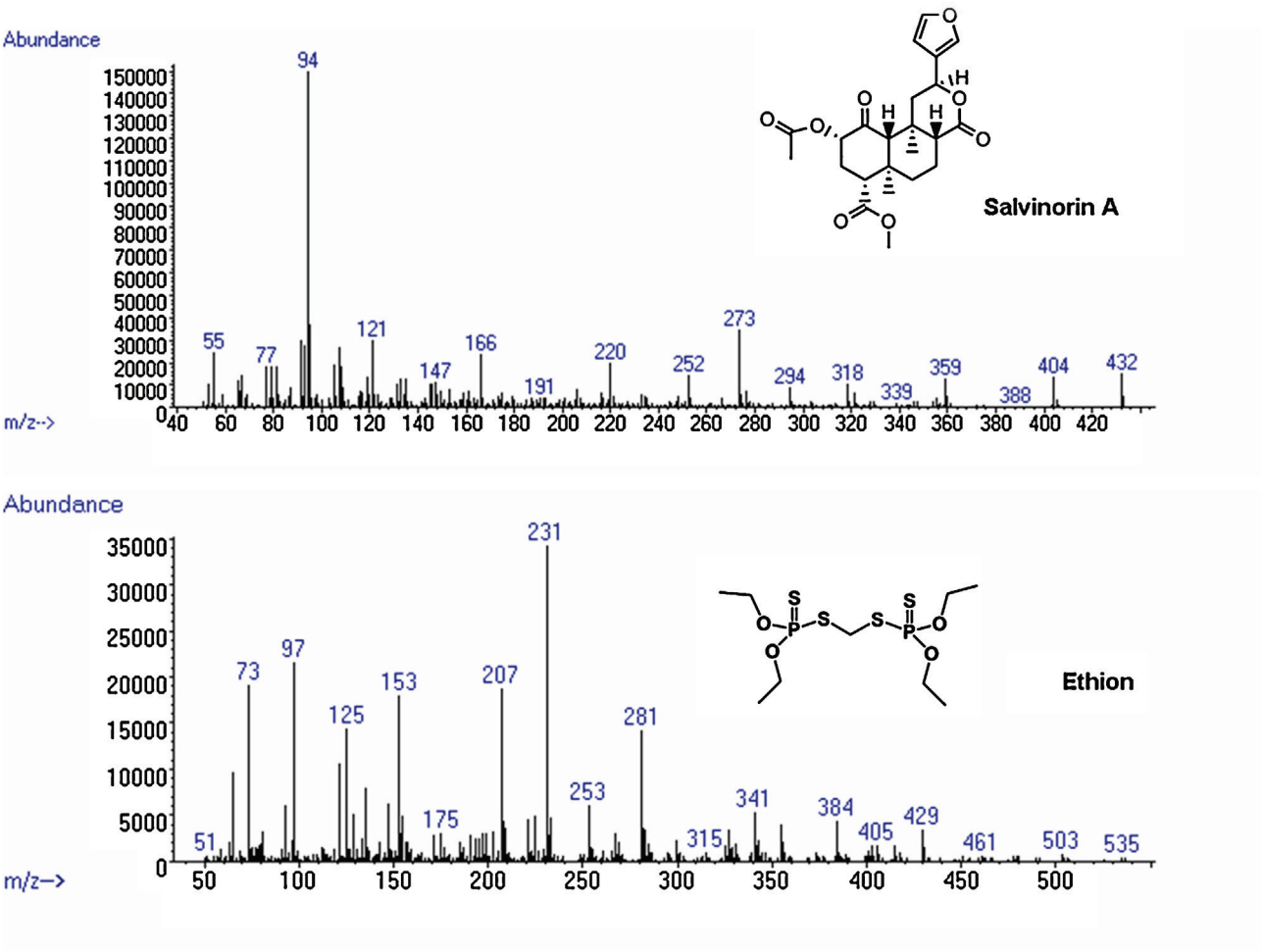

Fig. 1. Mass spectra and chemical structures of Salvinorin A and ethion.

ethion. A full-scan mass spectra of Salvinorin A and ethion, as well as their chemical structures are presented in Fig. 1.

2.4. Sample preparation and extraction

Samples of vitreous humor (100 μL), pericardial fluid (250 μL), blood (250 μL) and plasma (250 μL) were prepared by the addition of 3 mL of 0.1 M phosphate buffer (pH 4.4) and 25 μL of internal standard solution (ethion) and were homogenized and centrifuged at 3000 rpm for 5 min. The aqueous phases were added to the extraction cartridges, previously conditioned with 2 mL methanol and 2 mL of deionised water. After the samples had passed through, the cartridges were washed sequentially with 2 mL methanol 5% in deionised water and 2 mL of *n*-hexane. After drying under full vacuum the analytes were eluted with a 2 mL of a mixture of dichloromethane: isopropanol (75:25, v/v). The obtained extracts were evaporated to dryness at 30 °C under a gentle nitrogen stream, reconstituted with 50 μL of ethyl acetate and transferred to autosampler vials to be injected a 2 μL aliquot into the chromatographic system (GC-MS-EI).

2.5. Validation procedure

The described procedure was validated in terms of selectivity, linearity, limits of detection (LOD) and quantitation (LLOQ), precision (intra-day and intermediate) and accuracy, extraction efficiency and stability, according to international guidelines on bioanalytical method validation [29–38]. Validation data were obtained by preparing quality control samples (QC) with drug-free matrices spiked with Salvinorin A at three different concentrations (low, medium and high).

Selectivity was studied by analyzing 10 pools from different sources of blank samples of each matrix: vitreous humor, pericardial fluid, blood and plasma. They were checked for interferences at the retention times and monitored ions for the analyte of interest and the internal standard. Also, they were analyzed for potential interferences from other substances, namely the most commonly encountered in routine analysis in our laboratory (medical substances, pesticides and drugs of abuse, Table 1). From each pool, two sets of samples ($n = 10$) were prepared into 10 mL glass tubes, and they were spiked with the same concentration (100 ng/mL) of all the compounds presented in Table 1; in addition, 10 of these samples were further spiked with Salvinorin A (10 ng/mL). It was obtained 10 positive and 10 negative samples which were extracted and analyzed by the aforementioned procedure. The criteria for identification the compounds was established according to the recommendations of the World Anti-Doping Agency [38]. For chromatography, the relative retention time of the substance must be within a 1% window, or 0.2 min in absolute terms, from that of the same compound in a quality control sample prepared and analyzed contemporaneously. Mass spectrometric identification in the SIM mode, must include at least three diagnostic ions, and their relative intensities should not differ by more than a tolerated amount from those generated by the same compound in a quality control sample prepared and analyzed contemporaneously (if the relative intensity of the ion is within a 25–50% interval of the base peak

Table 1
Substances tested for interferences.

11-OH-THC	Clobazam	Fenarimol	Mirtazapine
6-Acetylmorphine	Clomipramine	Fentanyl	Morphine
7-Aminoclonazepam	Clonazepam	Fenthion	Naproxen
7-Aminoflunitrazepam	Clonazepam	Flunitrazepam	Nordazepam
Acetaminophen	Clorpromazine	Fluoxetine	Olanzapine
Acetylsalicylic acid	Clozapine	Flurazepam	Oxacarbamazepine
Alprazolam	Cocaine	Foxyme	Oxazepam
Amitryptiline	Codeine	Imipramine	Papaverine
Amphetamine	Cyalothrine	Ketamine	Paroxetine
Atrazine	Cyamemazine	Ketoprofen	Penconazole
Atrazine	Cyfluthrine	Lamotrigine	Phenacetin
Azinphos-ethyl	Cypermethrine	Levomepromazine	Phenobarbital
Azinphos-methyl	Deltamethrine	Lidocaine	Phenytoin
Bendiocarb	Demeton-S-methyl	Lindane	Propranolol
Bentazone	Demeton-S-methylsulphon	Lorazepam	Quetiapine
Benzoylecgonine	Desalquylflurazepam	Maprotiline	Quinalphos
Bitertanol	Diazepam	MBDB	Sertraline
Bromazepam	Diazinon	MCPA	Strychnine
Bupirimate	Dichlorvos	MDA	Sulphotep
Caffeine	Dimethoate	MDEA	Temazepam
Carbamazepine	Dinocap	MDMA	THC
Chlorenvinphos	DNOC	Methadone	THC-COOH
Chlorpyrifos	Ecgonine methyl esther	Methamphetamine	Topiramate
Chlorpyrifos-methyl	EDDP	Mianserine	Tramadol
Citalopram	Estazolam	Midazolam	Venlafaxine

in the control sample, a maximum relative tolerance of ±20% will be allowed for the same ion in the sample; if this intensity is less than 25% or higher than 50% in the control sample, then absolute tolerances of ±5 and ±10%, respectively, will be allowed for the ion in the sample).

In order to determine calibration curves, a linear range was established between 5 and 100 ng/mL (5, 8, 10, 15, 20, 30, 50, 60, 80, 100 ng/mL) for Salvinorin A, in each biological matrix. The calibration curves were obtained by plotting the peak area ratio between Salvinorin A and ethion (IS) against theoretical concentrations of the compound of interest. The criteria for acceptance included a R^2 value of at least 0.99, and the calibrator's accuracy [mean relative error (bias) between measured and spiked concentrations] within a ±15% interval, except at the LLOQ, for which ±20% was accepted.

The potential for carryover was analyzed by injecting extracted blank, immediately after analysis of the highest calibrator from each calibration curve.

The LLOQ was defined as the minimum concentration of Salvinorin A that could be measured with adequate precision (coefficient of variation <20%) and accuracy (±20%). The LOD was defined as the lowest concentration yielding a signal-to-noise ratio of at least three. The intra-day precision was determined by the analysis of five QC samples at each concentration level (low, medium and high) in each matrix on one day. It was characterized in terms of coefficient of variation (CV, %). The intermediate precision and accuracy were assessed by the analysis of three QC samples at each concentration level (low, medium and high) in each matrix on five different days. Accuracy was calculated in terms of mean relative error (RE, %) between the measured and the spiked concentrations for all QC samples; 15% was the limit of the acceptable variability for all concentrations.

The extraction efficiency was evaluated by analysis of six QC samples at each concentration level (low, medium and high) in each matrix, in which the IS was added after extraction. After that, the obtained peak area ratios were compared to those obtained by spiking blank extracts with the same concentrations of Salvinorin A (100% recovery).

To study the stability of Salvinorin A was used three QC samples at each concentration level (low, medium and high). The stability of the processed samples was evaluated through analysis of the extracts under the conditions of GC-MS analysis during 24 h. For bench-top stability, samples of each matrix were spiked and left at room temperature for 3 h, after that they were extracted and compared with freshly spiked samples. To evaluate freeze/thaw cycles, the samples were spiked and stored 24 h at −15 °C, after this period, they were completely thawed and then frozen once again under the same conditions (a total of three cycles was studied). Storage periods were one day, three days, and seven days, and the samples were analyzed after the third cycle. Comparisons between the means concentrations obtained in the control and in the stability samples were made against an acceptance interval of 90–110%. Furthermore, the 90% confidence interval has to be within 80–120% of the control mean [34–37].

3. Results and discussion

3.1. Selectivity

No matrices interferences were observed at the retention times and at m/z values of the monitored ions, by the analysis of the negative blank pools (see Figs. 2 and 3). This indicates that neither endogenous matrix constituents nor the substances in Table 1 interfere with the Salvinorin A or the IS. Furthermore, the analytes were successfully identified in spiked samples. These results indicated that the described method is selective for the determination of Salvinorin A in vitreous humor, pericardial fluid, blood, and plasma samples.

3.2. Linearity, limit of detection and limit of quantitation

The linearity, LOD and LLOQ are shown in Table 2. Linear calibration curves were obtained for all matrices with $R^2 > 0.99$. The calibrator's accuracy was within the acceptance criteria.

The analysis of extracted blank vitreous humor, pericardial fluid, blood and plasma, immediately after injection of the highest calibrator from each calibration curve did not present any traces of carryover.

The LOD and LLOQ were both determined at 5 ng/mL. These values are comparable to those obtained in previous studies in plasma and blood [9,12,17], but were obtained with lower sample volumes (100 μL of vitreous humor and 250 μL of pericardial fluid, whole blood and plasma), while plasma and blood volumes of 1 and 0.5 mL

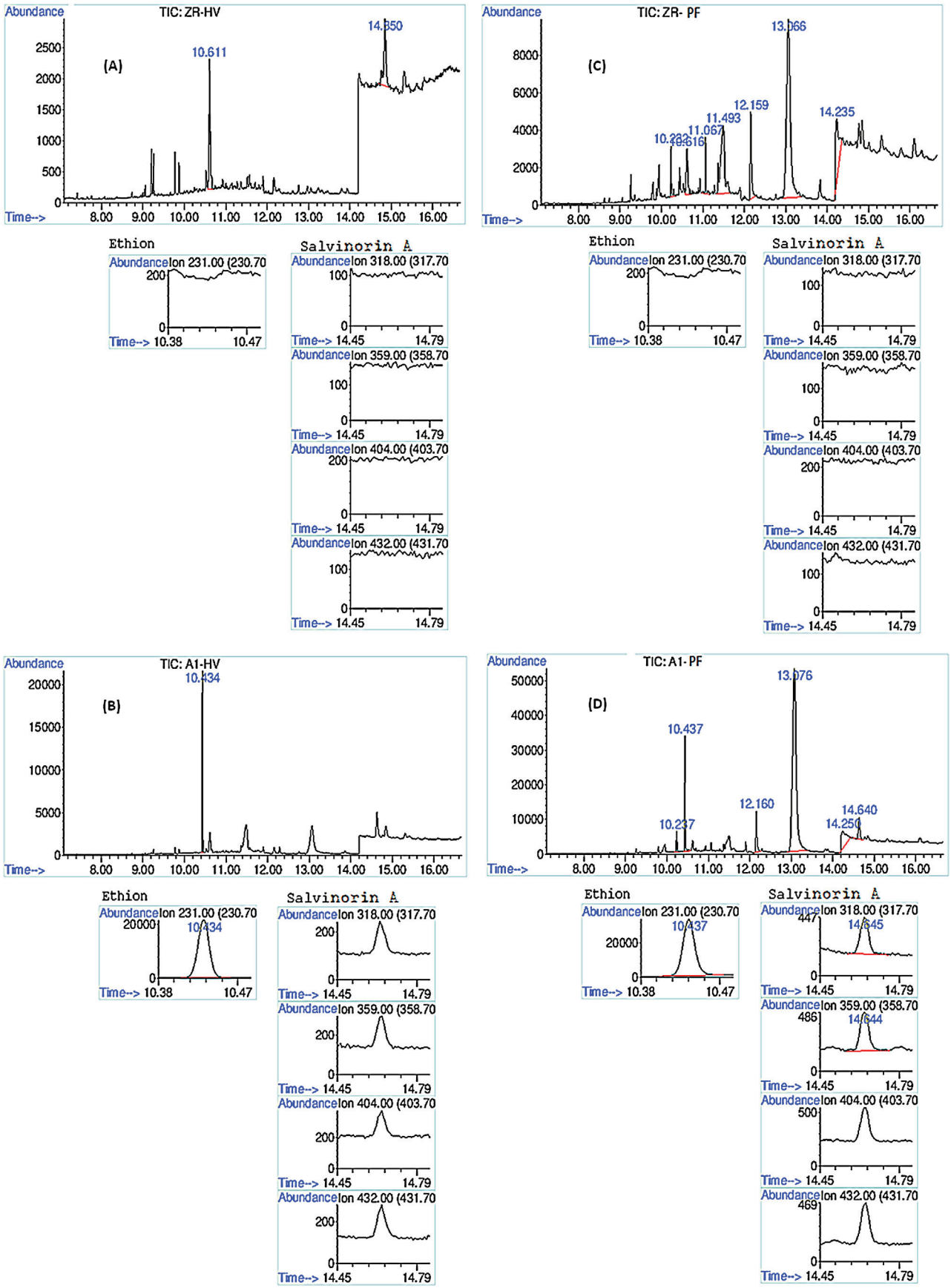

Fig. 2. Ion chromatograms of blank and spiked samples (5 ng/mL) of VH (A and B) and PF (C and D) for Salvinorin A at the monitored ions *m/z* 318, 359, 404 and 432 and at 231 for the internal standard (ethion).

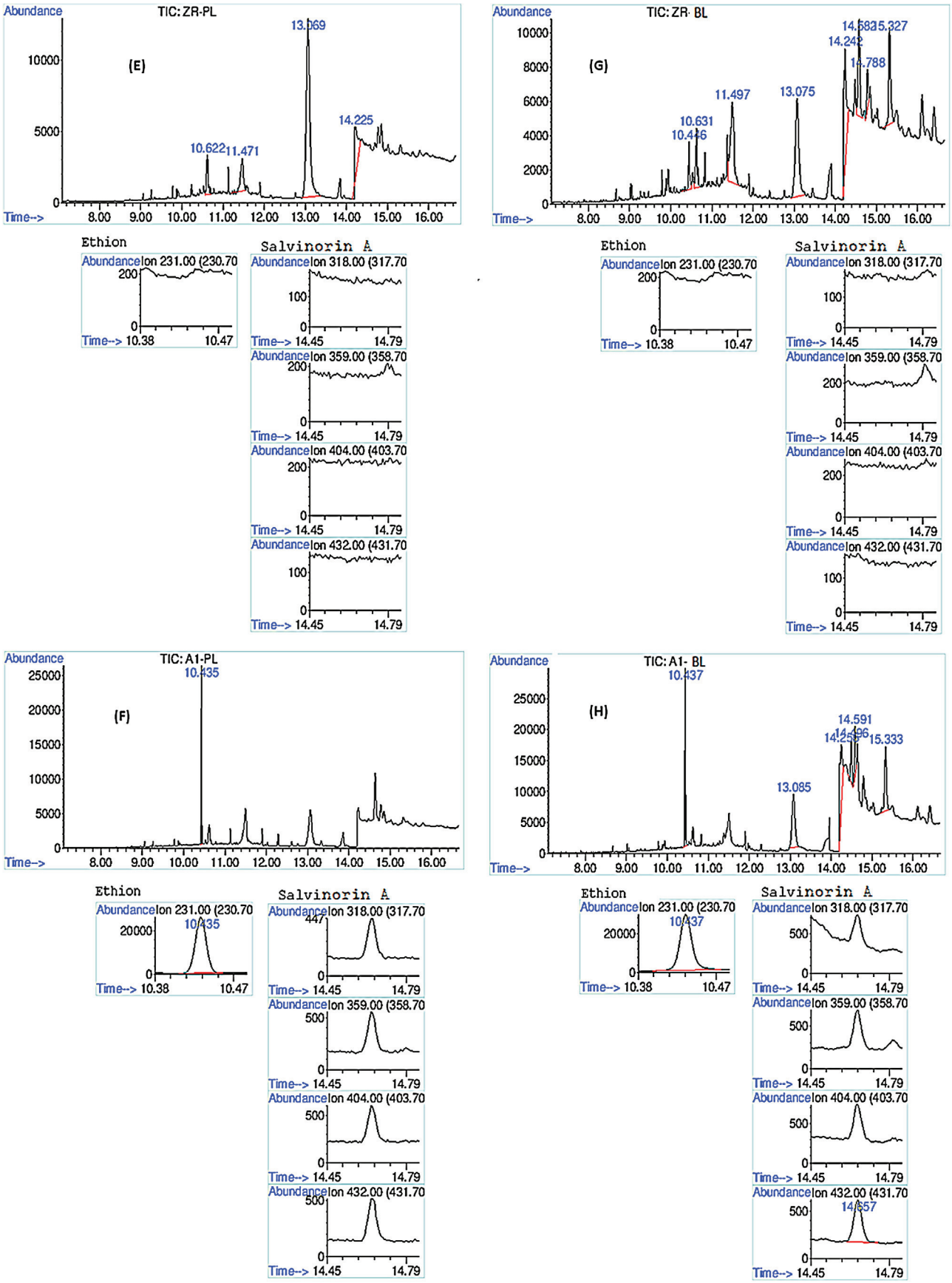

Fig. 3. Ion chromatograms of blank and spiked samples (5 ng/mL) of PL (E and F) and BL (G and H) for Salvinorin A at the monitored ions *m/z* 318, 359, 404, 432 and at 231 for the internal standard (ethion).

Table 2
Calibration and limits (LOD/LLOQ) data ($n = 5$).

Biological specimens	Linear range (ng/mL)	Linearity			LOD/LLOQ
		Slope[a]	Intercept[a]	R^{2a}	(ng/mL)
VH	5–100	8.8E−03 ± 1.1E−02	11.4E−03 ± 6.6E−02	0.9972 ± 1.9E−03	5
PF	5–100	1.2E−03 ± 1.1E−03	1.5E−03 ± 5.7E−03	0.9980 ± 0.7E−03	5
BL	5–100	8.7E−03 ± 1.5E−02	26.1E−03 ± 3.3E−02	0.9973 ± 2.3E−03	5
PL	5–100	0.3E−03 ± 0.4E−04	0.7E−03 ± 0.9E−03	0.9973 ± 1.3E−03	5

[a] Mean values ± standard deviation.

Table 3
Intra-day precision ($n = 5$).

Biological specimens	Spiked concentration (ng/mL)								
	10			25			100		
	Concentration found (ng/mL)	CV (%)	RE (%)	Concentration found (ng/mL)	CV (%)	RE (%)	Concentration found (ng/mL)	CV (%)	RE (%)
VH	10.2	8.8	1.9	25.1	11.7	0.2	93.9	6.3	−6.1
PF	9.1	10.9	−9.3	25.4	5.7	1.7	97.8	5.4	−2.1
BL	10.6	7.5	6.2	26.9	6.8	7.8	94.7	7.1	−5.3
PL	10.0	1.7	−0.3	26.2	1.2	4.8	96.4	4.0	−3.6

Table 4
Intermediate precision and trueness data ($n = 15$).

Biological specimens	Spiked concentration (ng/mL)								
	10			25			100		
	Concentration found (ng/mL)	CV (%)	RE (%)	Concentration found (ng/mL)	CV (%)	RE (%)	Concentration found (ng/mL)	CV (%)	RE (%)
VH	10.1	3.8	0.5	24.3	6.6	−2.8	98.8	3.0	−1.2
PF	10.4	11.2	3.9	25.7	5.1	2.7	102.3	6.8	2.3
BL	10.0	2.3	0.4	24.9	4.6	−0.3	100.3	3.8	0.3
PL	10.0	1.1	−0.1	25.6	1.9	2.2	100.8	3.5	0.8

CV: coefficient of variation; RE: relative error [(concentration found-spiked concentration)/spiked concentration × 100].

Table 5
Extraction efficiency (%) (n = 4).

Biological specimens	Concentration (ng/mL)		
	5	25	100
VH	97.4 ± 9.9	100.6 ± 7.2	79.6 ± 4.6
PF	100.2 ± 6.4	93.4 ± 0.2	98.0 ± 3.1
BL	98.9 ± 10.8	88.8 ± 9.8	99.1 ± 4.7
PL	91.3 ± 5.9	88.3 ± 3.6	98.0 ± 4.6

Mean values ± standard deviation.

were used in previous published works. It should be stated that these limits were considered to be good values when compared with previously works, in which were required plasma and blood volumes of 1 [9,12] and the 0.5 mL [17]. Regarding the results in vitreous humor and pericardial fluid, it is not possible to compare limits, since the determination of Salvinorin A in these matrices is not published yet.

3.3. Intra-day precision, intermediate precision, and accuracy

The results of the precision and accuracy are presented in Tables 3 and 4. The intra-day and intermediate precision were below 12% at the studied concentrations for all matrices. The accuracy was ±9% and thus within the acceptance criteria.

3.4. Extraction efficiency

Extraction efficiencies were between 79.65 ± 4.62 and 99.09 ± 4.68 as shown in Table 5. The reported extraction efficiencies in human plasma [12] and in rhesus monkey plasma [9] ranged between 84.6 ± 4.1 and 99.8%, respectively. However, an adequate comparison with our results in vitreous humor and pericardial fluid, it is not possible to do, since the determination of Salvinorin A in these matrices is not published yet.

Table 6
Freeze/thaw stability (%).

Concentration (ng/mL)	Freeze/thaw stability (7 days)							
	VH		PF		BL		PL	
	%ofcontrols	90% CI	% of controls	90% CI	% of controls	90% CI	% of controls	90% CI
10	105	85–116	103	99–107	103	99–108	100	93–106
25	105	96–114	92	87–98	97	87–108	105	96–113
100	104	95–113	84	81–87	86	85–87	100	95–105

CI: confidence interval.

3.5. Stability during bench-top and freeze/thaw cycles

Stability of processed samples in the autosampler was guaranteed for 24 h. The study also revealed that Salvinorin A is stable in each matrix for 3 h at room temperature and for the freeze/thaw experiments the acceptance criteria were fulfilled as shown in Table 6.

3.6. Applicability

This study seems to us to be very important especially because the recent cases of people hospitalized with severe psychotic disorders and with serious self-inflicted injuries that occurred during the hallucinogenic episodes. On the other hand, several deaths associated with the consumption of *S. divinorum*, were reported in a certain area of Portugal. Despite this worrying situation is occurring, unfortunately the forensic toxicological service did not have any request to analyze Salvinorin A in the routine casework. However, we believe that this situation is due to lack of knowledge and information about this new reality which makes forensic pathologists less prone to make the requests for the analysis of these new substances, as well as to collect the studied matrices. These situations can be an obstacle in the development of new methodologies, so it is important to be prepared for the requests that will be made in the future.

Nevertheless, and even though no request to analyze the substance, the developed methodology is being used routinely, in presumably intoxicated individuals with drugs of abuse. So far, fourteen blood samples and three vitreous humor samples were analyzed for Salvinorin A, but none was positive. Taking into account the low limits of the method, it is expected that the individuals did not consume *S. divinorum*, or there was a long period between the consumption and the samples collection.

3.7. Conclusions

According to the low volumes of samples used, and the low limits achieved using a single quadruple mass spectrometer, which is available in most laboratories, the validated methodology proved to be sensitive and specific for the analysis of Salvinorin A in conventional and unconventional biological matrices. Furthermore, the results obtained indicate that the procedure is suitable for application in forensic toxicology laboratories for the routine analysis of Salvinorin A. The small volumes required for the validated procedure are extremely useful in situations when the available volume of the sample isscarce. To the best of our knowledge this is the first procedure developed for the determination of Salvinorin A in vitreous humor and pericardial fluid.

After all, the development of the presented methodology seemed to be very timely, especially due to the increase in the number of cases of intoxication with this type of substances registered in the emergencies of the Portuguese hospitals. So, it is very important that the pathologists are alerted to this new reality that is the consumption of these kinds of drugs, mainly by young people. We believe that this procedure, in the short term will be very useful for national and international application to authentic antemortem and postmortem samples.

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3.2. Paper III

Determination of "new psychoactive substances" in postmortem matrices using microwave derivatization and gas chromatography–mass spectrometry

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Determination of "new psychoactive substances" in postmortem matrices using microwave derivatization and gas chromatography–mass spectrometry

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ABSTRACT

Despite worldwide efforts aiming to ban the marketing and subsequent abuse of psychoactive substances such as synthetic cathinones and phenethylamines, there has been an alarming growth of both in recent years. Different compounds similar to those already existing are continuously appearing in the market in order to circumvent the legislation.

An analytical methodology has been validated for qualitative and quantitative determinations of D-cathine (D-norpseudoehedrine), ephedrine, methcathinone, 1-(4-methoxyphenyl)-propan-2-amine (PMA), mephedrone, methedrone, 2,5-dimethoxy-4-methylamphetamine (DOM), 4-bromo-2,5-dimethoxyamphetamine (DOB), 2,5-dimethoxyphenethylamine (2C-H), 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 4-iodo-2,5-dimethoxyphenethylamine (2C-I), 2-[2,5-dimethoxy-4-(ethylthio)phenyl]ethanamine (2C-T-2), 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4) and 2-[2,5-dimethoxy-4-(propylthio)phenyl]ethanamine (2C-T-7), in low volumes of vitreous humor (100 μL), pericardial fluid (250 μL) and whole blood (250 μL), using deutered amphetamine, ephedrine and mephedrone as internal standards. The validation parameters included selectivity, linearity and limits of detection and quantification, intra- and interday precision and trueness, recovery and stability. The method included mixed-mode solid phase extraction, followed by microwave fast derivatization and analysis by gas chromatography-mass spectrometry operated in selected ion monitoring mode. The procedure was linear between 5 and 600 ng/mL, with determination coefficients higher than 0.99 for all analytes. Intra- and interday precision ranged from 0.1 to 13.6%, while accuracy variability was within 80–120% interval from the nominal concentration at all studied levels. The extraction efficiencies ranged from 76.6 to 112.8%. Stability was considered acceptable for all compounds in the studied matrices. The developed assay was applied to authentic samples of the Laboratory of Chemistry and Forensic Toxicology, Centre Branch, of the National Institute of Legal Medicine and Forensic Sciences, Portugal.

1. Introduction

The phenomenon of new psychoactive substances consumption depends both on the social context and the social representations of its consumers. Even though the fact that same substances are scattered throughout the world its abuse depends on local legal control. Similar to what happened in other countries, in Portugal, this phenomenon only received proper attention after having reached alarming proportions. Between 2007 and 2013, there was an exponential increase of the consumption of "new psychoactive substances" (NPS) or "designer drugs" suffered an exponential increase especially amongst the younger age groups, coincident with the opening of the so called *smartshops* where this type of products were freely sold camouflaged under the most varied forms (incense, pills, powders, bath salts, decorative snow, plant feeder) and they are sometimes labelled "not for human use", "not for sale to minors", "keep out of the children's reach"

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or "not tested for hazards or toxicity" [1–7]. As an attempt to control this situation, in April of 2013, a list of 139 psychoactive substances was published prohibiting the production, importation, exportation, advertising, distribution, sale, possession and availability of those drugs. Currently, that list includes a total number of 159 NPS that comprise phenethylamines, cathinones, piperazines, cocaine derivatives, plants and respective active compounds and synthetic cannabinoids. This legislation also included the closure of the *smartshops* [8]. The information reported by the European Monitoring Centre on Drugs and Drug Addiction (EMCDDA), highlights the expansion of NPS [9]. During 2014 a total of 101 new substances were reported in Europe some of which included 31 cathinones and 9 phenethylamines [10]. The synthetic stimulants are difficult to regulate due to growing availability of different formulations constantly emerging. Their similarity in chemical structures within the groups is an attempt to circumvent the existing drug legislation [3,11,12]. The high potency of some of those substances hinders its detection, since they exist in blood at low concentrations.

One of the greater and most important groups of psychostimulants drugs are the phenethylamine derivatives. These newer alternative drugs have significant differences from traditional amphetamines. Despite the similarity in chemical structure among many of these synthetic derivatives, small changes can have a great impact in potency, hallucinogenic effects and in human undesirable symptoms [13–17].

Cathinone derivatives are synthesized by adding different substituents at distinct positions of the cathinone molecule. These classes of substances can be classified into 4 groups: cathinones, phenethylamines, piperazines and tryptamines [12]. They induce sympathomimetic effects and stimulation of the central nervous system, they easily cross the blood-brain barrier and they are mainly concentrated in the kidneys, lungs, brain and cerebrospinal fluid. Despite most of these NPS are potent inhibitors of noradrenaline reuptake transporter, there are significant differences in their inhibition profiles regarding dopamine and serotonin reuptake transporters as well as their ability to release monoamines [18]. The most common adverse clinical symptoms in synthetic cathinone users are related to cardiac, neurological, psychological and gastrointestinal complications that require medical assistance [1,19,20]. The abuse of this NPS was first reported in Europe and United Sates in 2009 [21].

This recent reality became a real challenge for the forensic toxicologist in the development of analytical procedures for the determination of these substances in several biological matrices. Indeed, in recent years some analytical methods have been reported for the determination of synthetic cathinones and phenethylamines in human biological specimens, namely blood [2,22–26], plasma [23–25,27–29], serum [14,24], urine [2,14,17,24,28–36] and hair [1,14,22,37], involving both gas [1,14,17,22,24,25,27,30,32,33,37] and liquid chromatography [1,2,14,17,22–26,29,31,34–36] coupled to mass spectrometry and also liquid chromatography with UV [28]. Sample preparation were performed mainly by means of liquid–liquid extraction [24–26,29,30,32,33] or solid-phase extraction [17,23–25,27,28,31,34–36].

It is relevant to highlight the importance of studying this type of drugs in alternative specimens to blood and urine in order to overcome situations where their collection is unavailable (e.g. exsanguination following severe traumatic injuries, advanced putrefaction, carbonization and embalming). In these cases, the vitreous humor and pericardial fluid matrices could be useful to provide valuable information about the intake of certain drugs. The advantages and disadvantages of the use of these matrices were previously described by the authors [38].

Despite the numerous well-known advantages that GC–MS technique offers in the field of forensic and clinical toxicology, the derivatization step is often required to improve the detectability and the stability of the compounds [39]. Derivatization technique by microwaves used for the determination of drugs of abuse by GC–MS has been used for some authors which indicates its worth [40–45]. However, its adoption has not yet been established between the majority of the scientific community [45]. In this work there was a presentation of a simple and fast microwave derivatization procedure that spent 90 s to complete derivatization of the compounds. This score represents a major improvement when compared to the 80 min usually spent in the classical methodologies, and even with the time spent on most recent works (about 5 min) of drugs of abuse carried out by microwave-assisted derivatization procedures such as mephedrone, butylone and methylone in urine [32], amphetamines and methamphetamines in urine [40], opiates and cannabinoids [42], amphetamines in hair [44] and gamma-hydroxybutyric acid (GHB) in human plasma and urine [46].

The aim of this work was the development of an analytical methodology for the accurate quantification of synthetic cathinones and phenethylamines in low volumes of vitreous humor, pericardial fluid and whole blood, which includes a fast sample derivatization step able to dramatically reduce the time consumed for the analysis. Also, in this particular study, we have chosen an instrumental methodology accessible in most laboratories: single quadrupole MS.

To our knowledge, this is the first developed study that allows simultaneous determination of these analytes in postmortem samples.

2. Material and methods

2.1. Standards and reagents

Analytical reference materials were used for validation study and quantitative analysis. D-cathine (D-norpseudoephedrine), ephedrine, D,l-methcathinone, 1-(4-methoxyphenyl)-propan-2-amine (PMA), mephedrone (4-methylmethcatinone), methedrone (4-methoxy-*n*-methylcathinone), 4-bromo-2,5-dimethoxyamphetamine (DOB), 2,5-dimethoxyphenethylamine (2C-H), 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 4-iodo-2,5-dimethoxyphenethylamine (2C-I), 2-[2,5-dimethoxy-4-(ethylthio) phenyl]ethanamine (2C-T-2), 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4) and 2-[2,5-dimethoxy-4-(propylthio) phenyl]ethanamine (2C-T-7) were obtained from Lipomed AG (Arlesheim, Switzerland) in solution at a concentration of 1 mg/mL. The selection of the substances was carried out by taking into account both the substances with higher ranks of consumption in Portugal and requests that arrived at the laboratory for the identification of these compounds in forensic context. The deuterated internal standards (IS) were purchased from Cerilliant (Round Rock, TX, USA) in solution at a concentration of 1 mg/mL (amphetamine-d6 and ephedrine-d3) and 100 μg/mL (mephedrone-d3). Water, methanol, 2-propanol, dichloromethane and *n*-hexane were of ACS grade and hydrochloric acid, ammonium hydroxide and potassium dihydrogen phosphate were of analytical grade. All solvents and reagents were acquired from Merck Co (Darmstadt, Germany). The acylation reaction was carried out by the derivatization reagent *n*-methyl-bis-(trifluoroacetamide) (MBTFA), which was purchased from Macherey–Nagel GmbH & Co (Düren, Germany).

Oasis® MCX (mL, 60 mg) extraction cartridges were obtained from Waters (Milford, MA, USA).

For calibrators, working standard solutions of all substances were prepared by dilution of the stock solutions with methanol to the appropriate concentrations (50, 5 and 0.5 μg/mL). Additional work solutions, at the same concentrations, were prepared to be

used in the quality control samples (QC). A working solution of the IS at 5 μg/mL was also prepared in methanol. All these solutions were protected from light and stored at a temperature between 2 and 8 °C.

Potassium dihydrogen phosphate solution was prepared by dissolving 13.61 g of potassium dihydrogen phosphate in deionized water, obtaining a final volume of 1000 mL of a 0.1 M buffer solution.

Daily, were prepared the washing and elution solvents, diclorometane:methanol (70:30, v/v) and dichloromethane:2-propanol:ammonium hydroxide (78:20:2, v/v/v), respectively.

2.2. Gas chromatography–mass-spectrometry conditions

Gas chromatography–mass-spectrometry (GC–MS) analysis was performed on a HP 6890 system (Hewlett-Packard, Waldbronn, Germany) coupled to a 5973 mass selective detector (Hewlett-Packard, Waldbronn, Germany). A capillary column (30 m × 0.32 mm I.D., 0.25 mm film thickness) with 5% phenylmethylsiloxane (HP-5 MS, Agilent J&W Scientific, Folsom, CA, USA), was used.

The injector was operated in split injection mode (split ratio of 1:6) with a constant flow rate (1.2 mL/min) of highly purified helium, which was used as carrier gas. The temperatures of the injection port and detector were set at 250 and 280 °C, respectively. The initial oven temperature was 90 °C (held for 2 min), which was increased by 20 °C/min–300 °C and held for 3 min. The total run time was 15.5 min. The mass spectrometer was operated in the electron ionization (EI) mode with energy of 70 eV and emission current of 300 μA. After the identification of the analytes in full-scan mode, specific ions were monitored in the selected ion monitoring (SIM) mode for confirmation and quantitation the compounds, as can be seen in Table 1. To confirm the presence of the compounds characteristic fragment ions must be extracted. The criteria for analytes identification was established according to the recommendations of the World Anti-Doping Agency [47]: the relative retention time of the substance had to be within a 1% window, or 0.2 min in absolute terms, from that of the same compound in a QC sample prepared and analysed contemporaneously. Positive peaks must include at least three diagnostic ions, and their relative intensities should not differ by more than a tolerated amount from those generated by the same compound in a QC sample prepared and analyzed contemporaneously: if the relative intensity of the ion is within a 25–50% interval of the base peak in the QC sample, a maximum relative tolerance of ±20% will be allowed for the same ion in the sample; if this intensity is less than 25% or higher than 50% in the QC sample, then absolute tolerances of ±5 and ±10%, respectively, will be allowed for the ion in the sample. The IS used to quantitate each compound were as follows: ephedrine-d3 for ephedrine, mephedrone-d3 for mephedrone and amphetamine-d6 for all other analytes (D-cathine, methcathinone, PMA, 2C-H, methedrone, DOM, DOB, 2C-B, 2C-I, 2C-T-2, 2C-T-4, 2C-T-7). Only one ion was monitored for each IS.

A representative ion chromatogram in SIM mode of a spiked pericardial fluid sample is shown in Fig. 1.

2.3. Biological samples

Blank blood (BL) samples used in the validation experiments and in the confirmation and calibration purposes were obtained from a local blood donation bank. Vitreous humor (VH), pericardial fluid (PF) and postmortem blank blood were collected during autopsies performed at the Medico-Legal Office of the National Institute of Legal Medicine and Forensic Sciences, Centre Branch, Aveiro, Portugal. These samples were drug-free, as they were screened before being used for both calibrators and control samples. All specimens were pooled and stored at −15 °C before being used.

Authentic samples were collected from subjects under psychiatric evaluation, traffic legislation and during the autopsies performed at the Forensic Services of Clinical and Pathology of the National Institute of Legal Medicine and Forensic Sciences, Centre Branch, Portugal. These samples were gathered out of presumably intoxicated with drugs of abuse individuals and to those whose death were suspicious or relates with unidentified situations e.g.: projected height falls, road accidents, labor accidents or absence of apparent cause, road-site testing of vehicle drivers.

These samples belong to deceased individuals who were autopsied and are related to drug-influenced driving. Usually, they are sent to the laboratory by a Court-of-Law, as the Portuguese legislation so requires, and toxicological analysis are normally requested for clarification of death, as part of day-to-day routine work.

2.4. Sample preparation and extraction procedure

Samples of VH (100 μL), PF (250 μL) and BL (250 μL) were prepared by the addition of 3 mL of 0.1 M phosphate buffer (pH 4.4) and 25 μL of deuterated IS solution. Then, samples were homogenized and centrifuged at 3000 rpm for 5 min. The aqueous phases were added to the mixed-mode extraction cartridges, previously conditioned with 2 mL methanol and 2 mL of deionised water. After the samples had passed through, the cartridges were washed sequentially with 2 mL of each deionised water, hydrochloric acid 0.1 M, a mixture of diclorometane:methanol (70:30, v/v) and finally with 3 mL of *n*-hexane. After drying under full vacuum the compounds were eluted with a 3 mL of a mixture of dichloromethane:2-propanol:ammonium hydroxide (78:20:2, v/v/v). Eluates were dried under a nitrogen stream at 40 °C after the addition of 30 μL of MBTFA to prevent loss of the analytes of interest during evaporation. Finally, 60 μL of MBTFA were added to the dried extracts and mixed by vortexing for approximately 20 s. The mixtures were derivatized in a microwave reactor at 900 W during 90 s and, after cooling down to room temperature, the glass vials were opened and the solutions were transferred to the GC autosampler vials to be injected a 2 μL aliquot into the GC–MS system.

3. Results and discussion

3.1. Derivatization procedure

For derivatization procedure, a domestic digital microwave oven (Candy CMG 25D CS) with a nominal power of 900 W, was used in this study. The stability of the microwave was monitored from different measurements over a period of 1 month, by heating, in a glass beaker, 200 mL of distilled water for 90 s at 100% nominal power. The effective absorbed power calculated was 558 ± 15 W [42,43,45,48–50].

To evaluate the potential of the heating reaction when using microwave energy, comparison was made with the conventional reaction heating block at 80 °C during 30 min. For this purpose 2 calibration curves were made by spiking 9 blank samples of each matrix (VH, PF and BL) with all the analytes at 9 concentration levels (5, 10, 15, 20, 50, 200, 400, 500, 600 ng/mL) and 2 sets of 3 QC samples spiked at 100 ng/mL were prepared. All the samples were extracted and analysed according to the aforementioned method. For derivatization with MBTFA one set of samples (calibration curve and 3 QC samples at 100 ng/mL) was heated for 90 s in the microwave oven, while the other set of samples (calibration curve and 3 QC samples at 100 ng/mL) was treated by conventional heating for 30 min at 80 °C. Similar results were obtained for both sets of experiments: calibration curves presented

Table 1
Retention times (RT) and ions monitored for each substance.

Substances	RT (min)	Quantitation ion (*m*/*z*)	Qualification ion 1 (*m*/*z*)	Qualification ion 2 (*m*/*z*)
Amphetamine-d6	5.59	144	–	–
D-Cathine	5.85	230	140	203
DOB	9.44	256	229	231
DOM	8.39	305	165	192
Ephedrine	6.32	154	110	244
Ephedrine-d3	6.31	247	–	–
Mephedrone	7.39	154	119	110
Mephedrone-d3	7.38	157	–	–
Methcathinone	6.69	154	105	110
Methedrone	8.33	135	154	110
PMA	7.22	121	148	261
2C-B	9.56	242	229	355
2C—H	8.17	277	164	121
2C-I	10.05	290	403	277
2C-T-2	10.12	337	211	224
2-C-T-4	10.13	351	225	183
2-C-T-7	10.47	351	225	238

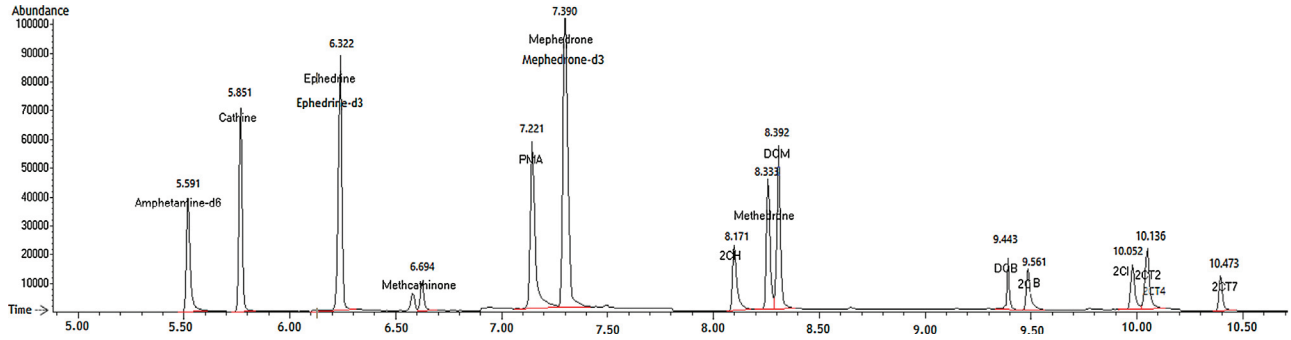

Fig. 1. Total ion chromatogram of a spiked pericardial fluid sample (100 ng/mL).

correlation coefficients higher than 0.99 for all analytes and the P-values obtained were between 0.1764 and 0.4464, which means that there were no significant differences between both groups of QC samples after t-Test application with a significance level of 0.05. Both derivatization procedures were considered comparable. Therefore, best results were achieved with the microwave heating procedure. Subsequently, in order to verify the uniformity of the microwave irradiation during thermally-assisted chemical reactions during 90 s, several experiments were performed using QC samples at three concentration levels (5, 100 and 600 ng/mL), each one in sets of 2, 4, 8, 12, 16, 20 and 24 glass tubes. We found that the relative areas of the derivatives were constant within each level of concentration and not influenced by the number of the glass tubes. Therefore, these results indicated that 24 glass tubes can be used for simultaneous microwave-assisted heating reactions, which symbolizes a maximum efficiency of derivatization without decomposition the products obtained. This technique has offered a considerable reduction of the entire analysis time.

3.2. Method validation

The described analytical procedure was performed according to accepted international guidelines on bioanalytical method validation [51]. Calibrators and QC samples were prepared by fortifying blank samples of VH, PF and BL. Selectivity, linear range, carry-over, limits of detection (LOD) and quantitation (LLOQ), precision (intra-day and intermediate) and bias, extraction efficiency and stability (autosampler, bench-top and short-term freeze/thaw) were studied.

3.2.1. Selectivity

Selectivity was studied by analyzing 10 pools from different sources of blank samples of each matrix (VH, PF and BL). They were investigated for interferences at the retention times and monitored ions for each significant analyte and respective IS. In addition, the ability to identify the analytes of interest in the presence of 100 other substances routinely analysed in our laboratory (drugs of abuse, medical substances and pesticides) was also checked. Two sets of samples (n = 10) from each pool, spiked with the same concentration (100 ng/mL) of all the compounds were prepared into 10 mL glass tubes. From each matrix a group of 10 of these samples were further spiked with 5 ng/mL of all analytes of interest (D-cathine, methcathinone, ephedrine, PMA, mephedrone, 2C—H, methedrone, DOM, DOB, 2C-B, 2C-I, 2C-T-2, 2C-T-4, 2C-T-7). Ten positive and ten negative samples of each matrix were obtained, and they were submitted to the above-mentioned methodologies. All analytes were successfully identified in all the spiked matrices. Further, by the analysis of the negative blank pools no matrices interferences were observed at the retention times and at *m*/*z* values of the monitored ions. This suggests that neither endogenous matrices constituents nor any of the compounds added do interfere with the analysis of the studied analytes. Accordingly to these results the described procedures were considered selective for the determination of the selected substances in all matrices studied since, interferences which may obstruct the capability to detect, identify, or quantitate the compounds of interest were not present.

3.2.2. Linearity

The calibration curves ($n = 6$) were constructed by linear regression using the peak area ratio of each analyte and their analogous IS, plotted against the corresponding theoretical concentration. The criteria for acceptance included coefficients of determination (r^2

Table 2
Linearity results for all compounds studied in BL, PF and VH.

Analytes	Matrices	Linear Range (ng/mL)	Linearity			LOD/LLOQ (ng/mL)
			Slope[a]	Intercept[a]	r^{2} [a]	
D-CATHINE	BL	5–600	5.4E-03 ± 2.3E-03	2.0E-03 ± 7.0E-03	0.9980 ± 6.4E-04	5
	PF	5–600	9.0E-04 ± 0.0E+00	−6.0E-04 ± 2.8E-04	0.9962 ± 3.5E-04	5
	VH	5–600	3.9E-01 ± 2.5E-01	2.3E-01 ± 2.1E-02	0.9996 ± 3.5E-04	5
DOB	BL	5–600	6.2E-04 ± 2.1E-05	−3.8E-03 ± 1.1E-03	0.9995 ± 7.1E-05	5
	PF	5–600	8.0E-04 ± 0.0E+00	−2.3E-03 ± 2.8E-04	0.9955 ± 2.8E-03	5
	VH	5–600	8.0E-04 ± 1.4E-04	−3.9E-03 ± 7.1E-05	0.9998 ± 1.4E-04	5
DOM	BL	5–600	7.0E-04 ± 1.4E-04	−2.4E-03 ± 1.7E-03	0.9982 ± 2.3E-03	5
	PF	5–600	1.0E-03 ± 0.0E+00	−4.2E-03 ± 1.3E-03	0.9976 ± 2.8E-03	5
	VH	5–600	5.5E-04 ± 7.1E-05	−1.8E-03 ± 7.8E-04	0.9983 ± 2.3E-03	5
Ephedrine	BL	5–600	3.1E-02 ± 1.6E-02	5.8E-02 ± 7.9E-02	0.9989 ± 2.8E-04	5
	PF	5–600	4.8E-02 ± 4.2E-04	−1.6E-01 ± 2.0E-02	0.9998 ± 7.1E-05	5
	VH	5–600	2.2E-02 ± 2.9E-02	5.3E-02 ± 7.3E-02	0.9993 ± 8.5E-04	5
Mephedrone	BL	5–600	2.1E-03 ± 0.0E+00	5.4E-03 ± 8.5E-04	0.9997 ± 2.8E-04	5
	PF	5–600	2.2E-03 ± 0.0E+00	−5.5E-03 ± 2.1E-03	0.9995 ± 3.5E-04	5
	VH	5–600	2.1E-03 ± 0.0E+00	6.5E-03 ± 1.1E-03	0.9998 ± 1.4E-04	5
Methcathinone	BL	5–600	6.6E-03 ± 3.3E-03	−8.0E-03 ± 2.1E-02	0.9956 ± 4.9E-03	5
	PF	5–600	2.5E-03 ± 0.0E+00	−1.4E-02 ± 8.5E-04	0.9999 ± 7.1E-05	5
	VH	5–600	4.2E-03 ± 2.1E-04	−1.9E-02 ± 6.1E-03	0.9960 ± 4.2E-03	5
Methedrone	BL	5–600	2.5E-03 ± 7.1E-05	−6.4E-03 ± 6.3E-03	0.9994 ± 2.8E-04	5
	PF	5–600	8.0E-04 ± 0.0E+00	−3.3E-03 ± 2.5E-03	0.9989 ± 1.4E-04	5
	VH	5–600	2.2E-03 ± 0.0E+00	−6.5E-04 ± 3.5E-03	0.9998 ± 2.1E-04	5
PMA	BL	5–600	3.0E-03 ± 7.1E-05	−8.4E-03 ± 9.2E-04	0.9992 ± 7.8E-04	5
	PF	5–600	4.0E-03 ± 0.0E+00	−3.6E-03 ± 3.5E-03	0.9996 ± 2.8E-04	5
	VH	5–600	1.6E-03 ± 1.9E-03	−5.0E-03 ± 5.7E-03	0.9998 ± 7.1E-05	5
2C-B	BL	5–600	6.5E-04 ± 7.1E-05	−4.0E-04 ± 2.7E-03	0.9963 ± 4.2E-03	5
	PF	5–600	7.5E-04 ± 7.1E-05	−1.7E-03 ± 2.8E-04	0.9998 ± 7.1E-05	5
	VH	5–600	6.5E-04 ± 7.1E-05	−1.4E-03 ± 2.3E-03	0.9997 ± 1.4E-04	5
2C-H	BL	5–600	8.0E-04 ± 0.0E+00	−3.5E-04 ± 3.0E-03	0.9973 ± 2.8E-04	5
	PF	5–600	1.0E-03 ± 0.0E+00	−3.3E-03 ± 7.8E-04	0.9967 ± 4.5E-03	5
	VH	5–600	8.0E-04 ± 0.0E+00	−6.5E-04 ± 1.9E-03	0.9999 ± 0.0E+00	5
2C-I	BL	5–600	8.5E-04 ± 7.1E-05	−4.5E-03 ± 1.2E-03	0.9993 ± 1.4E-04	5
	PF	5–600	8.0E-04 ± 1.4E-04	−2.9E-03 ± 7.1E-05	0.9985 ± 1.5E-03	5
	VH	5–600	7.5E-04 ± 7.1E-05	−2.6E-03 ± 2.8E-04	0.9998 ± 2.1E-04	5
2C-T-2	BL	5–600	7.0E-04 ± 7.1E-04	−4.5E-04 ± 1.1E-03	0.9954 ± 3.0E-03	5
	PF	5–600	1.2E-03 ± 2.8E-04	2.6E-03 ± 6.6E-03	0.9998 ± 1.4E-04	5
	VH	5–600	8.0E-04 ± 2.8E-04	−3.6E-03 ± 2.1E-04	0.9998 ± 1.4E-04	5
2C-T-4	BL	5–600	1.2E-03 ± 2.1E-04	−3.0E-03 ± 1.5E-03	0.9978 ± 6.4E-04	5
	PF	5–600	1.3E-03 ± 0.0E+00	−3.6E-03 ± 1.1E-03	0.9991 ± 9.2E-04	5
	VH	5–600	1.2E-03 ± 0.0E+00	−3.1E-03 ± 4.9E-04	0.9998 ± 1.4E-04	5
2C-T-7	BL	5–600	1.1E-03 ± 4.2E-04	−2.8E-03 ± 1.5E-03	0.9971 ± 2.8E-03	5
	PF	5–600	4.8E-03 ± 4.6E-03	−1.3E-03 ± 4.0E-03	0.9993 ± 4.2E-04	5
	VH	5–600	1.4E-03 ± 5.7E-04	6.0E-04 ± 3.8E-03	0.9999 ± 7.1E-05	5

[a] Mean values ± standard deviation (n = 6).

values) ≥0.99 and the calibrators quantitated within ±20%. The linear range for each substance was from 5 to 600 ng/mL (5, 10, 15, 20, 50, 100, 200, 300, 400, 500, 600 ng/mL) in the 3 biological matrices studied (VH, PF, BL). In conjunction with each calibration curve, a blank sample with IS added, and 4 different concentration levels (5, 20, 100 and 500 ng/mL) of QC samples ($n = 3$) were also analysed. All acceptance criteria have been accomplished for calibration data (Table 2). The possibility of carryover was investigated by injecting extracted blank samples of each matrix, promptly after analysis of the highest calibrator of each calibration curve and check for the presence of eventual ion signals at the retention times of each compound under investigation. Carryover was not observed in all matrices analysed for each analyte. For the LOD and LLOQ determinations blank samples of VH, PF and BL were fortified with decreasing concentrations of the substances. The LOD was determined as the lowest concentration that resulted in a signal-to-noise ratio (S/N) of at least 3/1.The LLOQ was evaluated as the minimum concentration of the studied analytes that could be measured with adequate precision (Coefficient of variation, CV < 20%) and bias (±20%). The LLOQ and LOD were both determined at 5 ng/mL for all substances and they were evaluated in 6 different days (n = 30). These values were obtained with lower sample volumes (100 μL of VH and 250 μL of PF and BL) than those found in previous studies in plasma and blood [23,26,28,29].

3.2.3. Precision and accuracy

Precision was expressed in terms of coefficient of variation (CV, %) and calculated by the one-way analysis of variation (ANOVA). The intra-day precision and bias were determined by analysing batches of 5 QC samples at 4 different concentration levels (5, 20, 100 and 500 ng/mL) in each matrix (VH, PF and BL) at the same day. The intermediate precision and bias were evaluated by the analysis of 4 QC samples (5, 20, 100 and 500 ng/mL) in each matrix over 6 consecutive days (n = 24). The bias was calculated for each concentration group of QC samples as the [(mean of measured concentrations − theoretical concentration)/theoretical concentration x 100]. Acceptable values were established for CV < 20% and bias variability of ±20%. The obtained values for intra-day precision (CV < 13.6%) and bias (0.0–19.4%), as well as to the intermediate precision (CV < 17.1%) and bias (0.0–20.0%) were acceptable at all concentrations in the studied matrices. These results are summarized in Tables 3 and 4.

3.2.4. Recovery

The extraction efficiency was measured by comparing 2 sets of 6 different QC samples at each concentration level (20, 100 and 500 ng/mL) in which the IS was added after the extraction. In the set 1 blank samples were fortified with all analytes before extraction, while set 2 the analytes were added to the QC samples after the

Table 3
Intra-day precision (n = 5).

Substances	Biological Specimens	Spiked Concentration (ng/mL)											
		5			20			100			500		
		Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)
D-Cathine	BL	5.4	0.3	8.6	21.7	0.4	8.6	94.2	3.7	−5.8	497.0	9.6	−0.6
	PF	5.2	5.0	4.0	20.2	3.5	0.9	101.0	3.4	1.0	501.2	2.3	0.2
	VH	5.7	3.7	13.0	19.7	4.8	−1.5	96.9	3.7	−3.1	490.6	3.3	−1.9
DOB	BL	6.0	0.9	19.4	18.6	3.7	−6.8	88.9	2.0	−11.1	509.5	2.8	1.9
	PF	5.7	4.3	14.0	19.4	5.3	−2.9	94.5	4.9	−5.5	494.5	2.6	−1.1
	VH	5.0	12.7	0.2	21.2	2.0	5.9	103.3	6.2	3.3	493.8	4.6	−1.2
DOM	BL	5.9	3.8	18.6	18.6	9.4	−7.2	98.8	0.6	−1.2	502.7	1.1	0.5
	PF	5.7	4.3	14.0	19.4	5.3	−2.9	94.5	4.9	−5.5	494.5	2.6	−1.1
	VH	5.7	4.2	13.8	19.7	8.4	−1.6	100.9	11.3	0.9	491.1	2.4	−1.8
Ephedrine	BL	5.0	12.5	0.0	19.6	7.0	−2.2	103.3	3.8	3.3	514.7	3.2	2.9
	PF	5.4	4.5	8.0	20.0	0.4	−0.1	97.3	8.6	−2.7	497.2	2.0	−0.6
	VH	4.8	5.0	−3.8	20.9	4.5	4.3	101.0	5.0	1.0	491.4	2.1	−1.7
Mephedrone	BL	5.0	7.5	−0.6	18.2	7.3	−9.1	97.1	3.9	−2.9	479.9	6.0	−4.0
	PF	5.0	5.3	−0.4	22.4	5.3	11.9	93.2	4.2	−6.8	486.1	4.2	−2.8
	VH	4.6	5.9	−8.6	19.2	2.6	−4.0	96.6	3.9	−3.4	495.8	0.8	−0.8
Methcathinone	BL	5.6	0.2	12.4	19.3	8.4	−3.6	91.5	4.8	−8.6	425.6	2.7	−14.9
	PF	5.9	0.7	17.4	21.7	4.8	8.4	98.6	6.4	−1.4	497.0	1.5	−0.6
	VH	4.7	2.0	−6.8	20.3	4.4	1.6	99.0	7.5	−1.0	491.9	2.0	−1.6
Methedrone	BL	4.8	4.0	−4.2	19.7	9.8	−1.5	84.4	8.9	−15.6	502.7	1.1	0.5
	PF	5.0	11.9	−0.6	20.8	8.7	4.1	107.4	1.5	7.4	483.0	0.5	−3.4
	VH	5.1	6.0	2.4	20.6	4.6	3.1	102.0	4.5	2.0	493.7	1.5	−1.3
PMA	BL	5.6	8.5	11.6	18.8	10.2	−6.1	91.8	5.7	−8.2	500.3	7.9	0.1
	PF	4.8	9.7	−4.2	20.1	2.1	0.5	102.4	4.4	2.4	487.7	4.4	−2.5
	VH	5.4	6.9	7.8	20.4	0.5	2.0	104.1	5.8	4.1	476.1	5.8	−4.8
2C-B	BL	5.8	0.7	16.0	19.8	6.2	−1.0	95.7	5.7	−4.3	549.1	4.2	9.8
	PF	5.1	4.8	1.2	19.0	3.5	−5.2	98.5	4.6	−1.5	481.4	5.4	−3.7
	VH	5.8	2.0	16.2	19.8	0.9	−1.2	104.7	3.4	4.7	492.0	4.6	−1.6
2C-H	BL	5.7	10.7	14.2	18.9	6.4	−5.3	92.5	11.1	−7.5	485.7	1.0	−2.9
	PF	5.4	8.1	7.4	96.3	5.6	−6.6	96.3	5.6	−3.7	492.8	3.8	−1.4
	VH	4.8	1.3	−3.6	21.2	3.3	5.8	102.2	3.6	2.2	493.4	1.3	−1.3
2C-I	BL	5.1	3.6	2.4	20.6	3.5	3.1	91.7	7.6	−8.3	527.4	10.4	5.5
	PF	5.7	5.7	13.0	19.8	5.6	−1.2	93.8	3.9	−6.2	454.8	8.7	−9.0
	VH	4.9	4.3	−2.8	20.1	4.3	0.4	100.6	6.1	0.6	490.6	0.9	−1.9
2C-T-2	BL	5.3	7.5	6.0	21.3	5.7	6.5	105.8	1.0	5.8	459.3	6.4	−8.1
	PF	4.8	7.5	−3.8	20.9	3.3	4.7	106.0	2.9	6.0	489.3	1.6	−2.1
	VH	5.0	2.5	−0.6	20.7	1.2	3.7	103.1	0.4	3.1	489.9	1.1	−2.0
2C-T-4	BL	4.8	13.6	−4.4	19.9	12.0	−0.6	102.3	6.4	2.3	517.3	4.6	3.5
	PF	5.5	5.8	10.4	19.6	10.6	−2.0	102.7	3.8	2.7	469.6	0.6	−6.1
	VH	5.2	8.5	3.8	19.5	0.2	−2.6	104.7	3.6	4.7	492.3	2.6	−1.5
2C-T-7	BL	5.0	3.1	0.8	21.0	7.0	4.8	95.6	2.2	−4.4	505.1	6.7	1.0
	PF	4.8	0.2	−4.0	20.3	1.4	1.5	98.2	9.5	−1.8	488.3	2.0	−2.3
	VH	5.0	9.7	−0.2	19.6	6.6	−1.9	93.0	4.9	−7.0	505.3	1.5	1.1

extraction procedure (100% recovery), the mean peak areas ratios obtained were compared as follows: Set1/Set2 × 100%. Extraction efficiencies were between 76.6 ± 3.3 (2C-T-4 at 20 ng/mL) and 112.8 ± 5.8 (2C-H at 500 ng/mL) as shown in Table 5.

3.2.5. Stability

The stability was examined in 2 QC samples fortified with different concentrations (5 and 500 ng/mL) in all matrices as follows: stability of processed samples in the autosampler was investigated by reinjection the extracts under the conditions of GC–MS analysis during 48 h and calculating each concentrations against the original calibration curve; bench-top stability was evaluated by leaving the spiked QC samples at room temperature for 3 h, after that they were extracted and compared with freshly spiked samples at the same concentrations; short-term freeze/thaw cycles (n = 3) were evaluated with spiked QC samples frozen at −15 °C and completely thawed after periods of 1, 3 and 7 days, samples were analysed after the last storage period. Comparisons were made against freshly spiked QC. All the samples were prepared and processed by the aforementioned procedure and the criteria for stability acceptance included concentrations of the QC samples within ± 20% of the freshly spiked QC samples. In the post-preparative study, the analytes were found stable in the derivatized extracts left in the autosampler tray for 48 h. The experiments also revealed that all compounds exhibit stability in each matrix at room temperature for 3 h, and after the 3 freeze/thawed cycles. No significant deterioration was observed for any of the substances studied during the evaluation of the stability parameters (Table 6).

The results obtained for all validated parameters were in accordance with the acceptance criteria.

3.3. Method application

In order to evaluate the applicability of the method, the presented methodology has been applied to 1093 blood samples, 187 vitreous humor samples and 85 pericardial fluid samples, which were collected during autopsies from suspected drug abusers and high-risk individuals, between 2011 and 2015. These samples were analysed by the developed procedure after obtaining positive results by immunoassay screening for amphetamines and methamphetamines.

Table 4
Intermediate precision and trueness data (n = 24).

Substances	Biological Specimens	Spiked Concentration (ng/mL)											
		5			20			100			500		
		Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)
D-Cathine	BL	5.2	7.2	4.0	20.2	10.4	1.0	94.3	0.3	−5.7	497.3	0.1	−0.5
	PF	5.2	1.7	4.0	19.7	3.5	−1.5	100.2	1.2	0.2	500.1	0.3	0.0
	VH	5.3	10.7	6.0	20.6	6.5	3.0	96.0	1.3	−4.0	493.8	0.9	−1.2
DOB	BL	5.5	12.3	10.0	19.2	4.0	−4.0	90.3	2.3	−9.7	504.0	1.5	0.8
	PF	5.8	3.3	16.0	19.9	3.1	−0.5	96.8	3.5	−3.2	491.1	1.0	−1.8
	VH	5.5	12.5	10.0	20.6	3.7	3.0	100.1	4.5	0.1	483.7	3.0	−3.3
DOM	BL	6.0	1.0	20.0	18.9	2.7	−5.5	92.4	9.8	−7.6	506.1	1.0	1.2
	PF	5.8	3.3	16.0	19.9	3.1	−0.5	96.8	3.5	−3.2	491.1	1.0	−1.8
	VH	5.8	2.9	16.0	19.1	4.1	−4.5	99.8	1.5	−0.2	496.9	1.7	−0.6
Ephedrine	BL	4.8	6.7	−4.0	19.6	5.7	−2.0	103.3	3.1	3.3	514.7	2.7	2.9
	PF	5.1	8.7	2.0	20.4	3.2	2.0	99.5	3.2	−0.5	494.9	0.7	−1.0
	VH	4.9	2.9	−1.8	20.5	2.4	2.5	102.5	2.0	2.5	496.7	1.5	−0.7
Mephedrone	BL	4.9	1.5	−2.0	18.5	2.4	−7.5	97.4	2.2	−2.6	480.2	0.1	−4.0
	PF	5.1	3.8	2.0	21.9	2.9	9.5	97.3	6.0	−2.7	489.3	0.9	−2.1
	VH	4.7	4.0	−6.0	19.2	0.2	−4.0	97.8	8.5	−2.2	490.7	1.5	−1.9
Methcathinone	BL	5.5	4.1	9.2	19.8	3.9	−0.8	101.2	13.6	1.2	467.9	12.8	−6.4
	PF	5.6	6.0	12.0	21.4	1.6	6.8	98.4	0.4	−1.6	496.7	0.1	−0.7
	VH	5.1	13.2	2.8	19.8	3.7	−1.0	95.2	5.6	−4.8	458.7	10.2	−8.3
Methedrone	BL	4.8	11.6	−4.0	20.4	4.7	2.0	87.1	4.3	−12.9	489.7	2.6	−2.1
	PF	4.9	1.2	−2.0	20.9	0.4	4.5	105.2	2.9	5.2	492.7	2.8	−1.5
	VH	4.9	6.3	−2.0	19.6	7.2	−2.0	101.1	1.3	1.1	496.9	1.5	−0.6
PMA	BL	5.4	4.3	8.0	19.3	4.0	−3.5	93.2	9.7	−6.8	510.8	2.9	2.2
	PF	4.7	1.7	−6.0	19.5	4.3	−2.5	101.9	0.7	1.9	492.6	1.4	−1.5
	VH	5.6	4.5	12.0	20.2	1.4	1.0	103.7	2.5	3.7	488.4	3.6	−2.3
2C-B	BL	5.8	0.7	16.0	19.8	6.2	−1.0	95.7	5.7	−4.3	549.1	4.2	9.8
	PF	5.2	4.4	4.0	19.2	1.7	−4.0	99.2	0.9	−0.8	482.9	0.4	−3.4
	VH	5.5	7.5	10.0	19.9	0.7	−0.5	103.7	1.3	3.7	487.9	1.2	−2.4
2C-H	BL	5.5	4.6	10.0	19.3	2.5	−3.5	95.2	4.1	−4.8	524.5	10.5	4.9
	PF	5.4	0.6	8.0	19.7	7.4	−1.5	100.0	5.2	0.0	494.3	0.4	−1.1
	VH	5.2	10.8	4.0	20.6	4.1	3.0	99.6	3.7	−0.4	496.2	0.8	−0.8
2C-I	BL	5.6	12.8	12.0	20.2	3.1	1.0	87.4	7.0	−12.6	514.7	3.5	2.9
	PF	5.8	2.5	16.0	19.5	2.2	−2.5	91.7	3.2	−8.3	471.1	4.9	−5.8
	VH	5.2	8.1	4.0	20.1	0.1	0.5	100.7	0.1	0.7	496.3	1.6	−0.7
2C-T-2	BL	5.0	8.6	0.0	21.0	2.0	5.0	102.5	4.6	2.5	484.1	7.3	−3.2
	PF	5.0	4.4	0.0	20.4	3.5	2.0	101.5	6.2	1.5	483.3	1.8	−3.3
	VH	5.7	17.1	14.0	20.8	0.1	4.0	101.5	2.3	1.5	495.7	1.7	−0.9
2C-T-4	BL	5.3	13.6	6.0	19.3	4.3	−3.5	98.8	5.0	−1.2	516.8	0.1	3.4
	PF	5.6	2.9	12.0	20.4	5.6	2.0	99.4	4.7	−0.6	484.4	4.3	−3.1
	VH	5.1	1.4	2.0	20.1	4.5	0.5	101.3	4.8	1.3	495.0	0.8	−1.0
2C-T-7	BL	5.2	5.4	4.0	20.0	7.1	0.0	98.0	3.4	−2.0	502.2	0.8	0.4
	PF	5.2	11.3	4.0	20.4	0.6	2.0	100.2	2.8	0.2	490.5	0.7	−1.9
	VH	4.9	1.5	−2.0	19.8	1.6	−1.0	98.5	7.9	−1.5	501.8	1.0	0.4

Table 5
Extraction efficiency.

Analytes	Extraction efficiency (mean values ± standard deviation) (n = 6)		
	20	100	500
	(ng/mL)		
D-Cathine	106.2 ± 1.8	103.9 ± 1.1	106.4 ± 4.6
DOB	101.3 ± 0.9	99.2 ± 3.0	78.4 ± 4.7
DOM	110.6 ± 3.4	92.1 ± 0.3	84.0 ± 5.2
Ephedrine	108.7 ± 2.8	106.9 ± 0.0	93.1 ± 0.1
Mephedrone	82.9 ± 6.0	80.7 ± 3.6	90.9 ± 0.8
Methcatinone	97.5 ± 2.0	83.1 ± 0.7	75.7 ± 5.3
Methedrone	93.9 ± 24.3	98.5 ± 2.3	81.2 ± 5.1
PMA	110.3 ± 8.4	102.4 ± 3.7	88.2 ± 0.6
2C-B	102.5 ± 13.2	96.8 ± 0.2	105.0 ± 4.8
2C—H	109.0 ± 8.6	96.6 ± 6.7	112.8 ± 5.8
2C-I	111.9 ± 5.0	85.4 ± 0.9	105.1 ± 6.6
2C-T-2	81.0 ± 2.0	87.6 ± 2.0	86.0 ± 4.6
2C-T-4	76.6 ± 3.3	88.6 ± 0.1	79.0 ± 1.5
2C-T-7	96.5 ± 3.2	82.1 ± 1.8	85.2 ± 0.6

Table 6
Stability data (%difference) after storage at room temperature 3 h and after 3 freeze-thaw cycles.

Compounds	Matrices	Bench-top 3 h (Room temp)		Freeze-thaw (−15 °C) 7 days (3 cycles)	
		5	500	5	500
		(ng/mL)		(ng/mL)	
D-Cathine	BL	−4.2	0.6	−9.3	6.4
	PF	0.8	−0.8	0.1	−6.7
	VH	−6.2	0.7	4.6	−0.9
DOB	BL	−2.8	−3.6	6.8	8.9
	PF	−3.5	1.9	−1.2	−4.4
	VH	9.8	−2.0	−5.4	−3.1
DOM	BL	−2.2	−2.3	7.2	−2.0
	PF	5.3	2.3	−2.7	−2.2
	VH	−2.0	−1.8	−2.9	−1.1
Ephedrine	BL	2.0	−3.8	4.3	−3.7
	PF	−11.1	3.5	−1.9	6.2
	VH	2.1	1.1	−1.7	−8.7
Mephedrone	BL	−2.5	−1.9	−17.0	−0.2
	PF	−1.6	−1.2	−17.3	4.5
	VH	2.8	−1.0	−12.7	1.3
Methcatinone	BL	−0.4	−14.3	−9.8	7.6
	PF	−7.0	−5.9	−8.4	2.6
	VH	−7.8	−6.7	−1.0	−3.8
Methedrone	BL	2.3	−1.5	6.1	−9.8
	PF	−3.4	1.4	−2.1	−15.2
	VH	−4.3	0.7	−4.9	−1.9
PMA	BL	−15.8	−1.5	3.8	−9.9
	PF	−9.3	4.7	−4.0	−9.0
	VH	−5.2	1.0	−0.9	−0.3
2C-H	BL	−5.4	1.8	−4.1	−7.5
	PF	2.4	−1.5	3.3	−1.1
	VH	−9.6	0.6	−5.1	−2.5
2C-B	BL	−10.3	−12.1	−3.0	3.7
	PF	−7.1	−1.1	4.4	−2.9
	VH	−5.3	−0.8	0.7	−0.9
2C-I	BL	−12.1	−10.7	−5.4	0.0
	PF	−0.9	−11.6	2.2	−6.8
	VH	−5.8	1.2	0.0	0.1
2C-T-2	BL	−5.7	5.2	−4.2	−4.1
	PF	4.0	−1.1	0.3	−3.3
	VH	−12.3	1.2	−1.6	−1.6
2C-T-4	BL	−14.3	−6.4	−2.5	−2.9
	PF	−4.0	−3.7	−1.6	−3.8
	VH	−1.7	0.5	3.2	−3.3
2C-T-7	BL	3.2	−2.9	−2.7	4.8
	PF	8.3	−2.8	−1.5	−0.2
	VH	−1.8	−0.7	0.9	5.9

The cases where it was possible to detect any of the substances covered by the developed procedure, were associated with sudden death by undetermined cause, traffic accidents, and falls by projection from a high place.

In the majority of the analysed positive cases there was a confirmation on the presence of ephedrine in blood, pericardial fluid and vitreous humor. This was very likely related either with medical administration (existing in the formulations of nasal decongestants and in the cold medications) or with the intake of nutritional supplements (containing plant material or extracts of *Ephedra* species), given the fact that the calculated concentrations were within the range of concentrations considered therapeutic for this substance (0.02–2 μg/mL of blood) [26].

In one single case, the presence of cathine (probably resultant from the metabolism of pseudoephedrine, a substance used in medications for the treatment of allergies and colds) was confirmed.

In two of the analysed cases there was a confirmation of the presence of both ephedrine and methcathinone in blood. Methcathinone has not been marketed for therapeutic purposes and may result from the chemical oxidation of ephedrine. In addition, medications containing pseudoephedrine and ephedrine do not metabolize in methcathinone after ingestion. Consequently, the presence of these two compounds associated, suggests the intake of methcathinone [30].

Finally, in only one blood case was possible to simultaneous confirm the presence of ephedrine, methcathinone and mephedrone. The presence of the mephedrone in the blood sample is suitable to infer that there was consumption of NPS.

This small number of positive cases, confirmed by the developed procedure, can be explained with the absence of pathologists' requests caused by their unawareness of this recent reality and with the fact that a vast majority of cases of poisoning have not resulted in death (at least immediately) and, as such, was solved in the hospital (which means they have not reached the Laboratory of Chemistry and Forensic Toxicology, Centre Branch, SQTFC, of the National Institute of Legal Medicine and Forensic Sciences, INMLCF). Moreover, the samples storage conditions until its arrival to the SQTFC of the INMLCF, may interfere with the stability of the synthetic cathinones [26,36,52] as though it is a subject that can not be neglected.

4. Conclusion

We developed a sensitive and selective procedure for the simultaneous determination of 14 synthetic cathinones and phenethylamines in blood, VH and PF specimens by GC–MS.

Only 100 μL (VH) and 250 μL (PF and BL) of sample volume were required to achieve a 5 ng/mL LOQ for all analytes studied. Moreover, the procedure is rather simple to perform with a very fast microwave derivatization step (90 s) saving laboratory time consumed. We can conclude that the validated method is adequate for qualitative and quantitative determinations of these substances in both conventional and unconventional biological matrices and it is useful to help the evaluation of the consumption of these psychoactive substances. The authors highlight that this is the first study developed that allows simultaneous determination of these analytes in postmortem samples using a fast derivatization microwave procedure for the GC–MS analysis.

Despite all the efforts that have been made in the development of procedures for the detection and identification of this type of substances in biological matrices, the unpredictability of its appearance in a routine analysis, increases the difficulty of its identification. So, this method is an important contribution for the comprehensive procedures that need to be developed for simultaneous determination of various cathinones and phenethylamines, due to the considerable number of distinct compounds currently available in the market. In addition, because this type of substances have serious adverse effects to the health, it is imperative that forensic laboratories continue to invest in the development of analytical methodologies for its determination in biological specimens.

Compliance with ethical standards

Both the previous study's subject and the performed methods and techniques respect the ethical and procedural requirements established in the juridical regime of corpses and samples using for scientific investigation purposes. Consequently, a subsequent approval was obtained by the Committee of Ethics of the Faculty of Medicine of the University of Coimbra (Code number: CE-017/2016).

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CHAPTER 4

DISCUSSION

In this chapter it is intended to present a general and comprehensive discussion of the most prominent points of this project, since "Chapter 3" is composed of the published experimental articles of this work where each one has its own discussion.

According to the "EU Drugs Markets Report 2016" there are no signs of a slowdown in the number, type or availability of NPS [33]. The picture of the NPS market still has significant gaps around prevalence of consumption and acquisition. However, despite the limitations due to unavailable data, there is now strong evidence that new substances are causing a wide range of serious harm in Europe. These include an increase in the number of serious acute poisonings, including deaths, as well as harm arising from changes in the patterns of drug injection, as injecting drug users switch to new substances [33,185,186]. Nevertheless, there is a deficiency in knowledge of acute and longer-term health damage as well as the social problems that may be associated. Likewise, there is a profound ignorance regarding driving under the influence of NPS, since it is expected that the degradation of CNS functions, caused by the consumption of these substances affects both reaction and driving capacity and, consequently, increases the risk of accidents. Information on the occurrence of crime, disorder, violence and deaths in the context of the use of NPS also remains limited. So, public and policy concern about the use of NPS has grown considerably in a short time. However, the recreational abuse of NPS increases irrespective of the present legislative framework, since law is not effective in changing consumer behaviour, despite legislative initiatives to prohibit their use. So, it would be quite helpful, to encourage and support forensic toxicology laboratories in identifying new substances and precursors in biological samples. Alongside this, mechanisms should be created that facilitate accessing and sharing of reference materials, analytical data and professional experience in this area.

As the consumption of these substances can lead to multiorgan failure and death there is an urgent need to consider these substances highly dangerous to public health, and at least the most common compounds should be included in screening procedures in clinical and forensic toxicology.

Gathering some clinical data and information reported on the *internet,* it is possible to verify, as a result of medical findings, that synthetic cathinones are endowed with numerous adverse reactions. This reality makes it hard for health professionals in the medical treatment of intoxications by these substances. The synthetic cathinones and phenethylamines together belong to the major groups of NPS reported every year to the early warning advisory systems by a large number of countries and territories throughout the world [5,6].

In relation to the *Salvia divinorum* (SD), according to the published literature, it seems to be a substance of occasional use, and there are few who repeat the experience due to the undesirable effects that it often causes. According to data reported from the National Institute on Drug Abuse (NIDA) SD use among young people during the years of 2012 and 2013 was higher than in 2015 [187], it being unclear regarding the dangers of both its short and long-term use. During the year of 2012, in Portugal (on the Island of Madeira), several

undesirable emergency episodes occurred and even deaths associated with SD consumption and with other NPS. Indeed, the requests for toxicological analyses of these cases were never submitted and as a result these deaths have not been confirmed as related to NPS. However, these cases were an alert to a new reality and to the need for toxicology laboratories to be prepared with sensitive and suitable methodologies to determine these NPS in biological samples. So, it is desirable to ensure the existence of analytical procedures in forensic and clinical toxicological laboratories for the determination of these substances in biological matrices. On the other hand, these methods must be fully validated to objectively demonstrate their applicability for the intended routine use, with reliable analytical data being a prerequisite for a correct interpretation of toxicological results in daily routine work. Only in this way will the possibility of one result being contested in court be greatly reduced, likewise unjustified legal consequences for a person or the suspicion of an erroneous therapy to a patient [188,189].

Prior to laboratory experiments a validation plan must be implemented that includes the instrumental methods and the sample preparation techniques that will be used for each specific method [188–191]. Thus, two distinct validation plans were designed (for salvinorin a and for cathinones and phenethylamines) that allowed the successful achievement of the scope of this work. To fulfil the outlined plan both methodologies included the development of the chromatographic methods by GC-MS/EI to detect and identify the studied substances, the optimization of the microwave derivatization procedure that increased the chromatographic efficiency of the synthetic cathinones and phenethylamines and the development of the solid-phase extraction techniques used for each method. Before starting the validation studies the parameters to be evaluated and the respective acceptance criteria were defined. The validation experiments were conducted in a similar manner to casework and the following parameters were assessed: selectivity (interference studies), calibration model (linearity), carryover, limits of detection (LOD) and quantitation (LLOQ), precision (intra-day and intermediate), accuracy, extraction efficiency and stability (autosampler, bench-top and short-term freeze/thaw) [191]. The main objective of the methods developed is their application in the routine of toxicological analyses, the evaluation being of prime importance in potential interferences from prescription drugs, pesticides, drugs of abuse and other toxic agents.

4.1. Gas chromatography-mass spectrometry

Gas chromatography was selected as the analytical instrumental method for the detection and quantitation of the studied substances, since they are volatile and well separated with high resolution by this technique. An HP 6890 gas chromatography–mass spectrometry (Hewlett-Packard, Waldbronn, Germany) system was used, equipped with a capillary column

(30 m × 0.32 mm I.D., 0.25 mm film thickness) with 5% phenylmethylsiloxane (HP-5MS) supplied by J&W Scientific (Folsom, CA, USA) and coupled to a 5973 mass-selective detector (Hewlett-Packard, Waldbronn, Germany). With the aim of achieving a proper efficiency in the separation of the compounds there were developed, for each method, programmed temperatures of the column. Helium was the highly purified carrier gas used with a constant flow rate (1.2 mL/min). The mass spectrometer was operated with a filament current of 300 μA at electron energy of 70 eV in the electron ionization (EI) mode. The temperatures of the injection port and detector were set at 250 and 280ºC, respectively. The scan mode (*full scan*), with a range of *m/z* 50-600, was used to measure the retention times (RT) and the corresponding mass spectrum (all masses of the operating spectrum) of each compound. This step is crucial for the correct identification of the characteristic ions of each substance. Then, in the selected ion monitoring mode (SIM mode), three selected ionic fragments with higher abundance and specificity to each compound in the corresponding RT [192] were used, aiming the increase of sensitivity and achievement of lower limits of detection. Thus, among the various advantages of the mass spectrometry is the detection of low levels of the compounds in a precise and specific manner which allows the clear identification of the compounds. Nevertheless, despite the many advantages of the GC-MS, time-consuming derivatization of the extracts are often required in order to improve the chromatographic efficiency of the compounds, which require long reaction time (up to 30min) at high temperatures. Thus, to decrease the time of the derivatization without compromising chromatographic effectiveness, a derivatization method by microwave was developed which demonstrated effectiveness in reducing that time and shortening the overall time of analysis. After GC separation of the components of a mixture, they are fragmented into ionized form to be separated based on their *m/z* ratio. Quantitation of all the compounds was done in the selected ion monitoring (SIM) mode, using one of the selected ions for each substance.

In contrast to our GC methods using only single quadrupole MS detection are the tandem mass spectrometry techniques (such as GC-MS-MS or UPLC-MS-MS) with which it is possible to achieve lower LOQs. However, such instruments are not available in our laboratory as in many other forensic laboratories in Europe and worldwide. Indeed, the lower LOQs achieved in all the substances studied (5 ng/mL) in reduced sample volumes demonstrate that this is a sensitive analytical instrumentation that is accessible in most laboratories nowadays.

4.2. Biological matrices

In recent years, unconventional samples are gaining greater importance in virtually all fields of toxicological analysis. In forensic toxicology, the requests for analysis in these matrices are increasing. On the other hand, in the vast majority of forensic toxicological laboratories that routinely analyse case samples there is a need to make extensive analysis to search many different compounds using several analytical methodologies (for e.g. drug screening using antibody-based tests, confirmation and quantitation of drugs, medicines and pesticides using

more rigorous techniques such as GC and LC mass spectrometry detection) in reduced volume of biological samples. So, to prevent these situations it is increasingly necessary to develop sensitive methods that use low volumes of conventional and non-conventional specimens to give a better response in the routine of a forensic toxicology laboratory.

Blood (BL) is the specimen of choice to assess intoxication situations in forensic toxicology, since it represents the dynamic state of drug distribution in the body, with the best correlation to the state of the person when death occurred and it also offers the possibility of correlation between concentrations and observed effects. Therefore, a BL sample that contains a drug is more likely to indicate recent usage compared to other biological sample (for e.g. urine). However, combining the accessibility and understanding of the pharmacokinetics and distribution of drugs in the body, it arouses the interest of the forensic toxicologist to use other specimens alternative or complementary to the BL. These specimens may offer several advantages in being analysed, such as cumulative information on drug use, or inability to BL collection.

The physicochemical properties of the drugs and biological fluids can be useful to predict the drugs distribution between the various compartments of the body. For the most drugs, the principal mechanism of transfer is passive diffusion, while the transfer of drugs from the circulating BL to another biological fluid involves transport across membranes that are an effective barrier against ionized, highly polar substances. Protein binding, pKa, lipid solubility and biological fluid composition largely determine the extent to which the drug is present in the body [126,128].

The VH is one of the most utilised alternative postmortem specimens. It consists essentially of water and is the matrix with the least interference, followed by PF when compared to BL. The main drawback of this matrix is the reduced volume able to be collected during an autopsy and its lack of data on drug disposition to assist in the interpretation of analytical results [150]. Nevertheless, in cases where death has involved trauma of the central organs, VH may be a useful alternative specimen as an adjunct to cardiac BL when potentially contaminated from adjacent tissues and stomach contents. Based on limited existing data which requires more studies, it seems that when a drug is detected in the BL it is also detectable in the VH [140–144,152,154–156,193–196].

The PF has not aroused great attention as an alternative specimen for drug analysis until now [140–144,152,155,156,196]. This specimen has the advantage of the larger volume that can be collected during an autopsy, without significant contamination, and has less interference in its composition than BL [126,133,150]. However, postmortem diffusion of certain drugs into PF and cardiac BL from the gastric content can occur, complicating the interpretation of concentrations obtained in these biological fluids [195]. Similarly to VH, the database is limited in the studied substances to assist the interpretation of the results obtained [140–144,152,154–156,193–196]. Despite its advantages, this specimen has been poorly studied and further investigations are needed for it to be routinely used in toxicological analysis of drugs.

Although, it seems to be consensus that there is good correlation between the femoral BL and the PF, suggesting that drug concentrations in this specimen is useful for estimation of intoxication degree [140,157].

This work combined the desirable situations referred to above: validation of two analytical methodologies in low volumes of postmortem alternative specimens (100 µL of VH and 250 µL of PF) in conjunction with whole BL (250 µL).

The extraction of the compounds of interest from the biological matrices is an important step in the development of the analytical methodology, because it will affect the overall sensitivity and selectivity of the method. In sample preparation the removal of potential interference makes the method more selective and reproducible. In general, the extraction of non-volatile compounds is performed through liquid-liquid and solid-phase extraction (LLE and SPE). Most biological samples require pre-treatment before extraction of the analytes. In most cases, dilution and buffering are sufficient and if there are solid particles, centrifugation or filtering may be needed. However, several sample cleanup procedures have been used, namely through protein precipitation, by addition of an organic solvent (e.g. methanol or cold acetonitrile), strong salt solution (zinc chloride) or a strong acid solution (perchloric, formic or trichloroacetic acid), or through deconjugation of glucoronides by hydrolysis with strong acids and bases, or enzymes (with β-glucuronidase) [197].

Usually, before sample application to the SPE column sample cleanup is required as high concentrations of macromolecules (e.g. proteins) or material in suspension can be present and result in the clogging of the pores of the solid adsorbent of the SPE column during the extraction phase. To avoid similar situations, in these two procedures all the specimens were diluted with 0.1M phosphate buffer (pH 4.4) in order to reduce the viscosity and also to control its pH. Then, the diluted samples were centrifuged to promote the sedimentation of the solids, and thus reduce interference and the risk of clogging the cartridges adsorbents. Later, these samples were subjected to the respective SPE procedure to remove interfering compounds and to concentrate the substances of interest. Both techniques produced clean extracts with good recoveries and reproducible results.

4.3. Determination of salvinorin a in blood, plasma, vitreous humor and pericardial fluid

A sensitive analytical procedure was developed and fully validated for the determination of SA in BL, plasma, VH and PF. The initial oven temperature was set as 70◦C held for 3 min, which was increased by 30◦C/min to 300◦C and held for 6 min. A volume of 2µL was used in the the splitless injection mode. The ions were monitored at m/z 318, 359, 404 and 432 (quantitation ion) for SA, and only one ion was monitored at m/z 231 for the internal standard (ethion). The established chromatographic conditions allowed good resolution between the SA and the ethion (IS) and a suitable analytical run time (16.67 min). The

application of the existing criteria at the SQTF-C, according to WADA's recommendations [192], allowed an unequivocal identification of the compounds.

It is important to make some considerations about the choice of the internal standard of this method, since it wasn't an easy choice to make, because it is not usual to use a pesticide as internal standard for this kind of compound. Concerning this issue, indeed, the best choice for the IS would be a deuterated analogue of SA, but unfortunately it was not commercially available when this study was developed. For this reason, an attempt was first made to use a structurally related compound, namely 16-dehydropregnenolone acetate (16-DPA), a steroid precursor normally used in chemical synthesis. However, the fragmentation pattern of this compound by mass spectrometry electron ionization was extremely poor, and its chromatographic performance was not adequate. Secondly, the possibility of using a medicinal compound was also considered, but the probability of appearance in authentic samples positive for SA would be quite high. Another attempt that apparently would be a good choice was with deuterated analogues of other drugs but extracts would have to be derivatized prior to GC-MS analysis, which would introduce further chromatographic interferences and impart an unnecessary increase on the cost of the toxicological analysis. For these reasons, an organophosphorus pesticide not commercially available in our country was used, since its chromatographic behavior was adequate, presented a good fragmentation pattern, and the probability of appearance in authentic samples simultaneously with SA is extremely low. Furthermore, the compound originates a perfectly shaped chromatographic peak without derivatization, and did not interfere with the extraction and analysis of the compound under study, as was shown on the obtained parameters during method validation (namely concerning precision and accuracy).

The extractions of the analytes from within the matrices were simple and rapid and were performed using solid reversed-phase extraction cartridges, Oasis® HLB (3 mL, 60 mg) due to the non-polar nature of SA. With this technique high reproducibility and extractive efficiency was achieved and during the drying process of the columns adsorbent there was no loss of the substances of interest. After the optimization of analyte extraction, the method had been fully validated. Concerning the method´s selectivity it has been shown that the matrices constituents do not interfere significantly with the SA, as they were not detected in the 10 analyzed blank samples (negative samples) of different origins, while in the 10 spiked samples (positive samples) the laboratory´s criteria for identification of the compounds [192] was fulfilled, both in terms of ion ratios and relative retention time. Also, no interferences were observed at the RT and selected ions of the compounds of interest, from the analysis of the additional substances most commonly encountered in routine casework. The method´s LOD and LOQ were both determined at 5 ng/mL, fulfilling the criteria for compound identification [192]. These values are comparable or lower to those described in previous published works in plasma and BL, taking into account those which were obtained using lesser sample volumes [198–200]. Regarding the values obtained in VH and PF, there are no published studies for the

determination of SA in these matrices with which to compare. It seems however that reduced volumes of sample are very important in those situations where the sample available is scarce. Extraction efficiencies values higher than 88% (except the value 80% for VH at the level 100 ng/mL) were obtained for all matrices at the three concentration levels, which means that SA losses during SPE is minimal in its contribution to the low limits achieved. The calculated coefficient of variation for intra-day and intermediate precision were below 12% presenting a mean relative error within a ±9% interval, for all matrices at all concentration levels. These are good results, even though a non-deuterated internal standard was used. To simulate the conditions that a sample is subjected to during the processing of toxicological analysis, the stability of the SA in the autosampler, during bench-top and freeze/thaw cycles was studied. Stability of processed samples in the autosampler was guaranteed for 24 h. The study also revealed that SA is stable in each matrix for 3 h at room temperature and for the three freeze/thaw cycles during 7 days the acceptance criteria were fulfilled (concentrations of the spiked samples within ± 20% of the freshly spiked samples).

Blood is not the best sample of choice for SA determinations in clinical toxicology, because patients under the effect of this drug do not present physical conditions for BL collection without putting themselves and health professionals at risk, due to the invasive collection procedure involving needles. Otherwise, in forensic toxicology whole BL could be a very useful specimen to confirm the consumption of SD if death occurs in a short period after exposure, in order to be able to detect the active metabolite SA.

VH can be useful in forensic toxicology for SA analysis as a complementary sample to BL and/or urine when considering intake of the studied substance, even though this is the first study of SA determination in VH. However, further studies should be done to evaluate the correlation of the VH concentration of SA to BL, since VH and BL concentrations do not correlate for all substances. So, the limitations of VH for the purpose of forensic toxicology largely concern quantitative interpretation [126,133]. Similarly to the VH, further studies should be done to evaluate the correlation of the PF concentration of SA to BL, since this is the first study in this matrix. On the other hand, the method has not yet been applied to positive cases, as no positive routine samples were available at the time in the SQTF-C. Although, despite the impossibility to assess positive results in authentic biological samples with this method, positive results were obtained with SA determinations in non-biological products sold at *smartshops* using the developed methodology.

4.4. Determination of synthetic cathinones and phenethylamines in blood, vitreous humor and pericardial fluid

A simple and sensitive analytical methodology was developed and validated for the confirmation and quantitation of d-cathine (d-norpseudoehedrine), ephedrine, methcathinone, 1-(4-methoxyphenyl)-propan-2-amine (PMA), mephedrone, methedrone, 2,5-Dimethoxy-4-methylamphetamine (DOM), 4-Bromo-2,5-dimethoxyamphetamine (DOB), 2,5-

Dimethoxyphenethylamine (2C-H), 4-Bromo-2,5-dimethoxyphenethylamine (2C-B), 4-Iodo-2,5-dimethoxyphenethylamine (2C-I), 2-[2,5-Dimethoxy-4-(ethylthio)phenyl]ethanamine (2C-T-2), 2,5-Dimethoxy-4-isopropylthiophenethylamine (2C-T-4) and 2-[2,5-Dimethoxy-4-(propylthio)phenyl]ethanamine (2C-T-7) in BL, VH and PF. It was decided to test the conditions laid down in the accredited experimental procedure validated in the SQTF-C for the extraction of amphetamines in whole BL due to the structural similarity between the studied compounds. So, the extracts were cleaned up by means of mixed-mode solid-phase extraction with Oasis MCX® cartridges and derivatized with N-Methyl-bis(trifluoroacetamide) (MBTFA) prior to GC-MS analysis. When GC-MS is utilized for the analysis of primary and secondary amines it is recommended to use the MBTFA reagent to obtain the trifluoroacetyl derivatives and the addition of this reagent, prior to the evaporation step, is also recommended to avoid losses of volatile low molecular compounds [201].

The derivatization procedure was extensively optimized using a domestic digital microwave oven with a nominal power of 900 W. The initial experiments focused on establishing optimum microwave conditions for the acetylated derivatives. When using microwave derivatization, reaction solvent, microwave power and reaction time are important parameters to study. For this purpose, these parameters have been evaluated on the basis of studies carried out with other drugs of abuse and different derivatization reagents [202–206]. The stability of the microwave was monitored from repeated measurements over a period of 1 month, by heating, in a glass beaker, 200 mL of distilled water for 90 s at 100% nominal power. The effective absorbed power calculated was 558±15W [202–204,206,207]. To investigate the yield of the derivatization reaction when using microwave energy, comparison was made with conventional heating in a heating block at 80 ºC during 30 min. Therefore 2 calibration curves were made by spiking 9 blank samples of each matrix (VH, PF and BL) with the studied substances at 9 different concentration levels (5, 10, 15, 20, 50, 200, 400, 500, 600 ng/mL) and 2 sets of 3 quality control (QC) samples spiked at 100 ng/mL were prepared. All the samples were extracted and analysed according to developed method. For derivatization with MBTFA the first set of extracts (calibration curve and 3 QC samples at 100 ng/mL) was heated for 90 s in the microwave oven, while the second was by conventional heating for 30 min at 80ºC. Similar results were obtained for both sets of experiments, i.e., calibration curves presented correlation coefficients higher than 0.99 for all analytes and the P-values obtained were between 0.1764 and 0.4464, which means that there were no significant differences between both groups of QC samples after t-Test application with a significance level of 0.05. Also verified was the uniformity of the microwave irradiation during thermally-assisted chemical reactions during 90s, using QC samples at three different concentration levels (5, 100 and 600 ng/mL), each one in sets of 2, 4, 8, 12, 16, 20 and 24 glass tubes. The relative areas of the derivatives were found constant within each level of concentration and not influenced by the number of the glass tubes. Therefore, these results indicated that 24 glass tubes can be used for simultaneous microwave-assisted heating

reactions, which symbolizes a maximum efficiency of derivatization without decomposition of the products obtained. This technique has offered a considerable reduction of the derivatization procedure from 30min to 90s.

The developed analytical methodologies have been fully validated. Concerning selectivity, 10 pools from different origins of blank samples of each matrix were tested for interferences at the retention times, ions were monitored and the ability to identify the compounds of interest in the presence of 100 other substances routinely analysed in the SQTF-C was also investigated. All compounds were successfully identified in all the spiked samples. In addition, no interfering signals, due to the endogenous constituents of the matrices or the substances added, at the retention times and at the *m/z* values of the monitored ions, were observed in the negative blank pools. The methods were considered selective for the determination of the selected substances in all matrices studied. The method´s limits (LOD and LLOQ) were determined at 5 ng/mL for all analytes and were comparable to those published in previous studies in plasma and BL [101,102,112,113], considering that they were obtained with lower sample volumes. The results obtained showed a high, reproducible, and fully concentration-independent recovery for all the compounds in all three analysed matrices. Concerning the method´s extraction efficiencies, values higher than 76% were obtained for all matrices at the three concentration levels, which means that analytes loss during SPE contributed only minimally to the low limits achieved. Acceptable values were established for CV < 20% and relative error variability of ±20%. The calculated coefficient of variation for intra-day and intermediate precision were below 17% presenting a mean relative error within a ±19% interval, for all matrices at all concentration levels. These are good results throughout the concentration range studied and the method complies with the purpose of applicability in routine analysis of the SQTF-C. It is evident that further work needs to be done in PF, VH and also in BL to evaluate the correlation of these matrices to BL in the determination of the studied substances.

To simulate the conditions that the samples are subject to during the processing of the requested analyses, the stability in two different levels of concentration (low and high) was also considered, in terms of processed samples in the autosampler, bench-top and freeze-thaw cycles (n=3) after periods of 1, 3 and 7 days and comparisons were made against freshly spiked samples. The criteria for stability acceptance included concentrations of the spiked samples within ± 20% of the freshly spiked samples. In the post-preparative study, the analytes were found stable in the derivatized extracts left in the autosampler tray for 48 h. The experiments also revealed that all compounds exhibit stability in each matrix at room temperature for 3 h, and after the 3 freeze/thawed cycles. No significant deterioration was observed for any of the substances studied during the evaluation of the stability parameters. However, some studies suggests that the samples storage conditions prior to arrival in the forensic toxicology laboratory, may interfere with the stability of the synthetic cathinones [101,103,108].

The methods were applied to routine samples with positive results for ephedrine in BL, PF and VH, cathine in BL and methcathinone and mephedrone in BL. This small number of positive cases, can be explained with the absence of pathologists' requests caused by their unawareness of this recent reality and with the fact that a vast majority of cases of poisoning have not resulted in death (at least immediately) and, as such, were solved in the hospital (which means they have not reached the SQTF-C).

CHAPTER 5

CONCLUSIONS

This work comprised the development and full validation of two analytical methodologies in conventional and unconventional human specimens for the determination of salvinorin a, synthetic cathinones, ephedrines, and phenethylamines belonging to the D and 2C series, using solid-phase extraction followed by analysis by gas chromatography-mass spectrometry with prior microwave derivatization.

The main achievements and conclusions obtained through the work subjacent to the present thesis are the following:

- A simple and sensitive GC-MS method was developed and fully validated for the determination of Salvinorin a in biological specimens. Using samples amounts of 100 µL of vitreous humor and 250 µL of plasma, whole blood and pericardial fluid low limits of detection and quantitation (5 ng/mL) were achieved, which appears to be extremely useful in those situations where there is a small amount of sample available for toxicological analysis, as frequently occurs in forensic toxicology laboratories. Moreover, the analytical methodology showed to be selective, linear in the studied range (5-100 ng/mL), precise (<12%) and accurate (below ±9%), even using a non-deuterated internal standard, and the cleanup efficiency using reversed-phase solid extraction ranged from 79.6% and 100.6% in the studied concentrations (10, 25 and 100 ng/mL) for all matrices.

- An analytical procedure for the determination of d-cathine (d-norpseudoehedrine), ephedrine, methcathinone, 1-(4-methoxyphenyl)-propan-2-amine (PMA), mephedrone, methedrone, 2,5-Dimethoxy-4-methylamphetamine (DOM), 4-Bromo-2,5-dimethoxyamphetamine (DOB), 2,5-Dimethoxyphenethylamine (2C-H), 4-Bromo-2,5-dimethoxyphenethylamine (2C-B), 4-Iodo-2,5-dimethoxyphenethylamine (2C-I), 2-[2,5-Dimethoxy-4-(ethylthio)phenyl]ethanamine (2C-T-2), 2,5-Dimethoxy-4-isopropylthiophenethylamine (2C-T-4) and 2-[2,5-Dimethoxy-4-(propylthio)phenyl]ethanamine (2C-T-7), in several biological specimens using mixed-mode solid phase extraction followed by a fast microwave-assisted derivatization and analysis by GC-MS, has been developed and fully validated. Low sample volumes were used, namely 100 µL for vitreous humor and 250 µL for pericardial fluid and whole blood. This method was linear within the studied ranges with determination coefficients higher than 0.99 for all analytes. Intra- and interday precision and accuracy were according the studied criteria. Extraction efficiencies ranged from 76.6 to 112.8%. In addition, low limits of detection and quantitation (5 ng/mL) were obtained for all analytes in the studied matrices.

- The proposed procedure is rather simple to perform and includes a very fast microwave-assisted derivatization step, offering an effective reduction in the total time of the analysis when compared to classical derivatization procedures.

- According to the low volumes of samples used, and the low limits achieved for all substances using a single quadruple mass spectrometer, which is available in most laboratories, we can conclude that the validated methodologies are sensitive and specific for the analysis of the studied compounds in conventional and unconventional specimens.

- These are the first studies for such substances analysed in vitreous humor and pericardial fluid. These specimens can contribute by providing more information about their distribution in the human body after massive ingestion. On the other hand, they offer an alternative in situations where the blood sample is not available for analysis.
  However, more experiments are necessary to establish correlations with blood concentrations in order to contribute to a better understanding of the action of these substances in the human body, when there is suspicion of their intake.

- In Portugal, the real knowledge of the intoxications with this kind of substances is still scarce, so it is important to invest in the development of methods that allow their detection and quantification in biological samples.

- Finally, it would be wise to establish protocols of action between Hospital Services in direct contact with these situations, and the laboratories of Forensic Toxicology of the INMLCF I.P as a competent entity to make this kind of toxicological analysis, in cases with suspicion of intoxication with these types of substances. Thus, there would be an awareness of how these substances are influencing the public health in Portugal.

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Annexes

Scientific presentations

The results obtained in this study were presented in several scientific meetings as poster and oral communications.

Oral scientific communications:

FATAL INTOXICATION WITH METHOXETAMINE: AN UNPRECEDENTED CASE IN PORTUGAL
Cláudia Margalho, Alice Castanheira, Fernando Castanheira, João Franco
XVI Latin-American Congress on Chromatography and 9th National Meeting on Chromatography
Lisboa, Portugal, 5–9 Janeiro de 2016

"NOVAS DROGAS": UM CASO INÉDITO EM PORTUGAL
C. Margalho
II Conferência do Instituto Nacional de Medicina Legal e Ciências Forenses
Coimbra, Portugal, 29-30 Outubro 2015

DETERMINAÇÃO DE "NOVAS SUBSTÂNCIAS PSICOATIVAS" EM MATRIZES BIOLÓGICAS: APLICAÇÃO A CASOS FATAIS RELACIONADOS COM O SEU CONSUMO
C Margalho, A Castanheira, J Franco, F Corte Real, M López-Rivadulla, E Gallardo
I Conferência Nacional de Medicina Legal e Ciências Forenses
Coimbra Portugal, 30-31 Outubro 2014

DESENVOLVIMENTO DE UMA METODOLOGIA ANALÍTICA PARA A DETERMINAÇÃO DE NOVAS SUBSTÂNCIAS PSICOATIVAS EM AMOSTRAS BIOLÓGICAS
Cláudia Margalho
8º Encontro Nacional de Cromatografia
Covilhã, Portugal, 2-4 de Dezembro de 2013

A IMPORTÂNCIA DAS AMOSTRAS ALTERNATIVAS *POST MORTEM* EM TOXICOLOGIA FORENSE: DETERMINAÇÃO DE CATINONAS SINTÉTICAS
Cláudia Margalho
12º Congresso Nacional de Medicina Legal e Ciências Forenses
Porto, Portugal, 7-9 de Novembro de 2013

ANÁLISE DE DROGAS ILÍCITAS E LÍCITAS

Cláudia Margalho

II Jornadas Internacionais de Ciências Forenses e Criminais, "Perspetivas Forenses sobre Segurança-Documentos, Substâncias Ilícitas e Terrorismo".

Instituto Superior de Ciências da Saúde Egas Moniz, 15 de Junho de 2012

AS NOVAS SUBSTÂNCIAS PSICOATIVAS E A TOXICOLOGIA FORENSE

Cláudia Margalho

ENEBT´13 – I Encontro Nacional de Estudantes de Biotecnologia

Covilhã, Portugal, 24-26 Maio de 2013

DROGAS LÍCITAS: DESENVOLVIMENTO E VALIDAÇÃO DE UMA METODOLOGIA ANALÍTICA PARA A DETERMINAÇÃO DE SALVINORINA A EM AMOSTRAS BIOLÓGICAS

Cláudia Margalho, Eugenia Gallardo, Alice Castanheira, João Franco, Duarte Nuno Vieira, Francisco Corte Real

11º Congresso Nacional de Medicina Legal e Ciências Forenses

Évora, Portugal, 9-10 de Novembro de 2012

Poster presentations:

SIMULTANEOUS DETERMINATION OF CATHINONE DERIVATIVES AND OTHER DESIGNER DRUGS IN POST-MORTEM ALTERNATIVE SAMPLES

Cláudia Margalho, Manuel López-Rivadulla, Eugenia Gallardo, Francisco Corte Real

IALM 2016 Intersocietal Symposium

Veneza, Itália, 21-24 Junho de 2016

"NEW DRUGS", LEGISLATION AND INTERNET: A "GAME" WITHOUT RULES

Cláudia Margalho, Alice Castanheira, Fernando Castanheira, João Franco, Francisco Corte Real

XVI Latin-American Congresso on Chromatography and 9th National Meeting on Chromatography

Lisboa, Portugal, 5–9 Janeiro de 2016

O QUE "ESCONDEM" AS NOITES ACADÉMICAS…

C. Margalho, A. Castanheira, F. Castanheira, J. Franco, F. Corte Real

II Conferência do Instituto Nacional de Medicina Legal e Ciências Forenses

Coimbra, Portugal, 29-30 Outubro 2015

DETERMINATION OF COCAINE ANALYTES IN BIOLOGICAL SPECIMENS BETWEEN 2010 AND 2014

Cláudia Margalho, Catarina Pinto, Alice Castanheira, João Franco

53rd TIAFT meeting 2015

Florença, Itália, 30 Agosto – 4 Setembro

CONSUMO SIMULTÁNEO DE DIFERENTES DROGAS DE ABUSO Y SU DETERMINACIÓN EN DISTINTAS MATRICES BIOLÓGICAS: PRESENTACIÓN DE CASOS REALES

Margalho C, Castanheira A, Pinto C, Gallardo E, Franco J

XXI Congreso Español de Toxicología y V Iberoamericano

León, Espanha, 17-19 Junho 2015

PROCEDIMIENTO ANALÍTICO PARA LA DETERMINACIÓN DE COCAÍNA Y SUS METABOLITOS EN DISTINTAS MATRICES BIOLÓGICAS: APLICACIÓN A MUESTRAS DE RUTINA

Margalho C, Castanheira A, Franco J, Corte Real F, Gallardo E, López-Rivadulla M

XXI Congreso Español de Toxicología y V Iberoamericano

León, Espanha, 17-19 Junho 2015

DETERMINAÇÃO DE "NOVAS SUBSTÂNCIAS PSICOATIVAS EM MATRIZES BIOLÓGICAS: APLICAÇÃO A CASOS FATAIS RELACIONADOS COM O SEU CONSUMO

C Margalho, A Castanheira, J Franco, F Corte Real, M López-Rivadulla, E Gallardo

I Conferência Nacional de Medicina Legal e Ciências Forenses

Coimbra Portugal, 30-31 Outubro 2014

MUERTES QUE IMPLICAN EL CONSUMO DE NUEVAS SUSTANCIAS PSICOATIVAS

Margalho C, Castanheira A, Corte Real F, López-Rivadulla M, Gallardo E

II Jornadas de Formación en Toxicología

Valência, Espanha, 24 Junho 2014

VALIDATION OF AN ANALYTICAL PROCEDURE FOR QUANTITATION OF SYNTHETIC CATHINONES AND PHENETHYLAMINES IN DIFFERENT BIOLOGICAL SPECIMENS USING MICROWAVE FAST DERIVATISATION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Cláudia Margalho, Eugenia Gallardo, Ana Ferreira, Alice Castanheira, Mário Dias, Duarte Nuno Vieira, Manuel López-Rivadulla, Francisco Corte Real

51ST Annual Meeting of the International Association of Forensic Toxicologists (TIAFT)

Funchal, Madeira, Portugal, 2-6 Setembro de 2013

ANALYSIS OF OPIOIDS IN CONVENCIONAL AND UNCONVENTIONAL BIOLOGICAL SPECIMENS USING MICROWAVE DERIVATIZATION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Cláudia Margalho, Eugenia Gallardo, Alice Castanheira, Fernando Castanheira, Mário Dias, Duarte Nuno Vieira, Manuel López-Rivadulla, Francisco Corte Real
51ST Annual Meeting of the International Association of Forensic Toxicologists (TIAFT)
Funchal, Madeira, Portugal, 2-6 Setembro de 2013

A VALIDATED METHOD FOR QUANTITATION OF COCAINE AND ITS METABOLITES IN BIOLOGICAL SPECIMENS USING MICROWAVE DERIVATIZATION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Cláudia Margalho, Eugenia Gallardo, Alice Castanheira, Mário Dias, Duarte Nuno Vieira, Manuel López-Rivadulla, Francisco Corte Real
51ST Annual Meeting of the International Association of Forensic Toxicologists (TIAFT)
Funchal, Madeira, Portugal, 2-6 Setembro de 2013

DETERMINACIÓN SIMULTÁNEA DE CATINONAS SINTÉTICAS Y FENILETILAMINAS EN DISTINTOS ESPECÍMENES BIOLÓGICOS POR CROMATOGRAFÍA DE GASES-ESPECTROMETRÍA DE MASAS

Margalho C, Gallardo E, Castanheira A, Ferreira A, Castanheira F, Vieira D N, López-Rivadulla M, Corte Real F
XX Congreso Español de Toxicologia y IV Iberoamericano (aetox)
Salamanca, Espanha, 26-28 Junho de 2013

VALIDACIÓN DE UNA METODOLOGÍA ANALÍTICA PARA LA DETECCIÓN Y CUANTIFICACIÓN DE SALVINORINA A EN DISTINTAS MATRICES BIOLÓGICAS

Margalho C, Gallardo E, Castanheira A, Vieira D N, López-Rivadulla M, Corte Real F
XX Congreso Español de Toxicologia y IV Iberoamericano (aetox)
Salamanca, Espanha, 26-28 Junho de 2013

DEVELOPMENT AND VALIDATION OF AN ANALYTICAL PROCEDURE FOR DETECTION AND QUANTITATION OF SALVINORIN A IN BIOLOGICAL SPECIMENS USING SOLID PHASE EXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Cláudia Margalho, Eugenia Gallardo, Alice Castanheira, João Franco, Duarte Nuno Vieira, Manuel López-Rivadulla, Francisco Corte Real
22nd Congress of the International Academy of Legal Medicine IALM 2012
Istanbul, TurKiye, 5-8 Julho de 2012

Research project proposal:

"Determinação de novas drogas psicotrópicas em amostras biológicas":

Collaboration agreement between Instituto Nacional de Medicina Legal e Ciências Forenses, I.P. (INMLCF, I.P.) and Centro Hospitalar da Universidade de Coimbra E.P.E (CHUC, E.P.E.) for scientific research to pursuit new psychoactive substances, 04 Março de 2013.