UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Determination of new psychoactive substances in forensic samples by gas chromatography coupled to mass spectrometry

(Versão final após defesa)

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Resumo Alargado

O termo “novas” para as novas substâncias psicoativas remete não só para a síntese de novos compostos como também para a utilização pela primeira vez de forma inapropriada de alguns produtos já existentes. Geralmente, a síntese e produção destas novas substâncias tende a mimetizar os efeitos de algumas substâncias clássicas, como são os casos da cocaína e anfetaminas, que já se encontram controladas pela legislação e estão listadas como drogas controladas pelo *United Nations Office on Drugs and Crime* e pelo Observatório Europeu da Droga e da Toxicodependência.

Dentro das novas substâncias psicoativas fazem parte as catinonas sintéticas e as feniletilaminas que juntas são consideradas um dos maiores grupos monitorizados pelo Observatório Europeu da Droga e da Toxicodependência. Em 2015, as catinonas foram as substâncias mais apreendidas e atualmente foram já detetadas 118 substâncias deste grupo, sendo que 14 apareceram pela primeira vez no ano de 2016.

De forma a permitir um maior controlo no aparecimento destas substâncias, o Observatório Europeu da Droga e da Toxicodependência, juntamente com a Europol, criaram um sistema de alerta rápido em que consiste numa partilha de informação entre todos os 28 estados membros da União Europeia, Turquia e Noruega de forma estabelecer um procedimento de risco, permitindo à União Europeia responder rapidamente a possíveis ameaças. No entanto, estão constantemente a surgir novos compostos, derivados dos que foram previamente sintetizados, o que torna o controlo e o planeamento de mecanismos de ação e resposta rápida algo complicado.

Desta forma, a criação por parte dos laboratórios de toxicologia forense de metodologias para a deteção e quantificação deste tipo de compostos em amostras biológicas possui grande importância e é de grande interesse público e científico, principalmente em amostras de sangue.

Apesar do método instrumental utilizado possuir grande importância para a obtenção de uma maior sensibilidade na deteção de compostos, também a preparação da amostra tem um enorme papel no que diz respeito a esse aspeto. A extração em fase sólida apresenta algumas vantagens relativamente a outras técnicas de extração, nomeadamente à diminuição de formação de emulsões (quando comparado com a extração líquido-líquido), maior eficiência no processo de extração e na possibilidade de extrair várias amostras ao mesmo tempo. Quanto ao processo de derivatização, atualmente, alguns investigadores já adotaram o método de derivatização rápida utilizando um micro-ondas, permitindo ao toxicologista economizar tempo neste passo de preparação da amostra.

O objetivo desta dissertação foi desenvolver e validar uma metodologia analítica para a deteção e quantificação de oito catinonas sintéticas (catinona, flefedrone, bufedrona, α-PVP, metilona, etilona, pentilona e MDPV) e de três feniletilaminas (4-MTA, 2C-P e dragonFLY), usando a extração em fase sólida e um método de derivatização rápida por micro-ondas para

posterior análise das substâncias por cromatografia de gases acoplada à espetrometria de massa. Foi usado o método cromatográfico já implementado na rotina do Serviço de Química e Toxicologia Forenses da Delegação do Centro para análise de anfetaminas, metanfetaminas e de um grupo de catinonas e fenetilaminas. Pretendeu-se com este trabalho acrescentar estas novas substâncias ao método já implementado na rotina do Laboratório de Química e Toxicologia Forenses da Delegação do Centro do Instituto Nacional de Medicina Legal e Ciências Forenses. Os padrões internos utilizados foram anfetamina-d6, metanfetamina-d9, MBDBD-d5, MDMA-d5 e MDEA-d5 e foi utilizado o sangue como matriz biológica.

O método desenvolvido foi validado de acordo com as normas do *Scientific Working Group for Forensic Toxicology* e foram avaliados os seguintes parâmetros: seletividade, linearidade, limites de deteção e quantificação, precisão, exatidão, estibilidade, fator de diluição e recuperação.

O método mostrou ser linear entre 5-500 ng/mL para a catinona, bufedrona, 4-MTA, metilona, 2C-P e dragonFLY, 10-500 ng/mL para a flefedrona, etilona, pentilona e MDPV e 40-500 ng/mL para o α-PVP, com coeficientes de determinação superiores a 0,99. Os limites de deteção e quantificação foram de 5 ng/mL (catinona, bufedrona, 4-MTA, metilona, 2C-P e dragonFLY), 10 ng/mL (flefedrona, etilona, pentilona e MDPV) e 40 ng/mL (α-PVP). A precisão intradia revelou valores de coeficiente de variação inferiores a 8,1% e, relativamente à precisão intermedia, obtiveram-se valores de coeficiente de variação, ao longo de 5 dias, inferiores a 8.6%. Quanto à estabilidade, esta foi estudada em amostras processadas no carrossel do equipamento cromatográfico, à temperatura ambiente durante 24 horas (estabilidade de bancada) e após 3 ciclos de congelamento/descongelamento. Todas as substâncias mostraram ser estáveis no carrossel até 72 horas e até 24 horas deixadas na bancada de trabalho à temperatura ambiente. Relativamente aos ciclos de congelamento/descongelamento foi avaliada a estabilidade ao fim de 7, 20 e 28 dias, onde todas as substâncias demonstraram ser estáveis até ao 28º dia excetuando o α-PVP e o 2C-P que apresentaram instabilidade ao 7ºdia e o dragonFLY e a metilona que foram consideradas instáveis a partir do 20º e 28º dia, respetivamente. Além disso, foram realizados estudos para a eficiência de extração onde foram obtidas recuperações entre 70 e 116%.

É importante salientar que esta é a primeira metodologia descrita sobre a utilização do sangue como matriz biológica para a deteção e quantificação deste grupo de compostos estudados utilizando um procedimento de extração em fase sólida simples associado ao processo de derivatização rápida dos compostos com o reagente *N*-metil-bis(trifluoroacetamida) por micro-ondas.

Palavras-chave

Novas substâncias psicoativas, catinonas sintéticas, feniletilaminas, derivatização rápida por micro-ondas, cromatografia de gases acoplada à espetrometria de massa.

Abstract

The term “new” for new psychoactive substances does not only refers to the newly synthesized compounds, but also to the ones used in an inappropriate way. These substances are produced to mimic the effects of the already controlled substances, like cocaine and amphetamine and despite the efforts to reduce their appearance, with the easy access to information and purchase, their presence in the streets had an alarming increase.

For this work, it was developed and validated an analytical methodology to determine a group of eight synthetic cathinones (cathinone, flephedrone, buphedrone, α-PVP, methylone, ethylone, pentylone and MDPV) and three phenethylamines (4-MTA, 2C-P and dragonFLY). It was used blood as a biological matrix and deutered internal standards (amphetamine-d6, methamphetamine-d9, 3,4-methylenedioxymethamphetamine-d5, 3,4-Methylenedioxy-N-ethylamphetamine-d5 and N-Methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine-d5). The preparation of the sample consisted in a mixed-mode solid phase extraction followed by a fast derivatization process utilizing a microwave and the N-Methyl-bis(trifluoroacetamide) as the derivatization reagent, and the extracts were then analysed by gas chromatography-mass spectrometry operating in electron ionization mode analysis. The procedure was validated following the Scientific Working Group of Forensic Toxicology guidelines and the parameters studied were selectivity, linearity, limits of detection and quantification, intra- and interday precision and trueness, extraction efficiency and stability. The methodology presented linearity between 5-500 ng/mL for cathinone, buphedrone, 4-MTA, methylone, 2C-P and dragonFLY, 10-500 ng/mL for flephedrone, ethylone, pentylone and MDPV and 40-500 ng/mL for α-PVP, with determination coefficients above 0.99 for all substances. The limits of detection and quantification ranged between 5 ng/mL and 40 ng/mL, depending on the substance. For intra-day and interday precision the values of coefficient of variations varied between 1.1 and 8.6% and extraction efficiencies ranged from 70.3 to 116.6%. The method was subsequently applied to more than one hundred authentic samples of the Laboratory of Chemistry and Forensic Toxicology, Centre Branch, of the National Institute of Legal Medicine and Forensic Sciences, Portugal.

Keywords

New psychoactive substances, synthetic cathinones, phenethylamines, microwave fast derivatization, gas chromatography coupled to mass spectrometry.

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Abbreviations

25B-NBOMe	2-(4-Bromo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine
25C-NBOMe	2-(4-Chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine
25D-NBOMe	2-(2,5-Dimethoxy-4-methylphenyl)-N-(2-methoxybenzyl)ethanamine
25E-NBOMe	2-(2,5-Dimethoxy-4-ethylphenyl)-N-(2-methoxybenzyl)ethanamine
25H-NBOMe	2-(2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine
25I-NBOMe	2-(4-Iodo-2,5-dimethoxyphenyl)-N-((2 methoxyphenyl)methyl)ethanamine
25T4-NBOMe	2,5-Dimethoxy-4-isopropylthiophenethylamine-N-(2-methoxybenzyl)ethanamine
2C-B	4-bromo-2,5-dimethoxy-ß-phenethylamine
2C-B-FLY	2-(4-Bromo-2,3,6,7-tetrahydrofuro(2,3-f)(1)benzofuran-8-yl)ethanamine
2C-C	2,5-Dimethoxy-4-chlorophenethylamine
2C-D	2,5-Dimethoxy-4-methylphenethylamine
2C-E	2,5-Dimethoxy-4-ethylphenethylamine
2C-H	2,5-Dimethoxyphenethylamine
2C-I	2,5-Dimethoxy-4-iodophenethylamine
2C-N	2,5-Dimethoxy-4-nitrophenethylamine
2C-P	2,5-Dimethoxy-4-propylphenethylamine
2C-T-2	2,5-Dimethoxy-4-(ethylthio)phenethylamine
2C-T-4	2,5-Dimethoxy-4-isopropylthiophenethylamine
2C-T-7	2,5-Dimethoxy-4-propylthiophenethylamine
3C-P	Alpha-Methyl-4-propoxy-3,5-dimethoxyphenethylamine
5-APDB	5-(2-Aminopropyl)-2,3-dihydrobenzofuran
5-HT	Serotonin
5-HT2	Serotonin receptor subtype 2
6-APB	6-(2-Aminopropyl)benzofuran
ACN	Acetonitrile
ATS	Amphetamine Type-Substances
BDB	Benzodioxolylbutanamine
BMC	Bromomethcathinone
BMDP	3,4-Methylenedioxy-N-benzylcathinone
CMC	Chloromethcathinone
CNS	Central Nervous System
COMT	Cathecol O-Methyl Transferase
CYP	Cytochrome P450
DA	Dopamine
DAT	Dopamine transporter
DEA	Drug Enforcement Administration
DLLME	Dispersive Liquid-Liquid Microextraction
DMC	Dimethylcathinone
DMMC	Dimethylmethcathinone
DOB	2,5-dimethoxy-4-bromoamphetamine
DOC	2,5-dimethoxy-4-chloroamphetamine
DOET	Dimethoxy-4-ethylamphetamine
DOI	2,5-dimethoxy-4-iodoamphetamine
DOM	2,5-dimethoxy-4-methylamphetamine
EI	Electron Impact

EMC	Ethylmethcathinone
EMCDDA	European Monitoring Centro for Drugs and Drug Addiction
ESI	Electrospray Ionization
EU	European Union
EWS	European Warning System
FMC	Fluoromethcathinone
GC	Gas Chromatography
GI	Gastrointestinal
HCl	Hydrochloric acid
HESI	Heated Electrospray Ionization
HFBA	Heptafluorobutyric Acid
HHMC	3,4-Dihydroxymethcathinone
HMMC	4-Hydroxy-3-methoxymethcathinone
HRMS	High Resolution Mass Spectrometry
INMLCF	National Institute of Legal Medicine and Forensic Sciences
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
LSD	Lysergic acid Diethylamide
MAO	Monoamine oxidase
MBC	Methylbenzylcathinone
MBTFA	N-Methyl-bis(trifluoroacetamide)
MDMA	3,4-methylenedioxy-N-methylamphetamine
MEC	Methylethcathinone
MEKC	Micellar Electrokinetic Chromatography
MeOH	Methanol
MEPS	Microextraction by Packed Sorbent
MDC	3,4-Methylenedioxycathinone
MDPBP	3,4-Methylenedioxy-α-pyrrolidinobutyrophenone
MDPPP	3,4-Methylenedioxy-α-pyrrolidinopropiophenone
MDPV	3,4-Methylenedioxypyrovalerone
MMC	Methylmethcathinone
MPBP	4-Methyl-α-pyrrolidinobutiophenone
MPHP	4-Methyl-α-pyrrolidinohexiophenone
MS	Mass spectrometry
MTA	Methylthioamphetamine
MTTA	Mephtetramine
NaOH	Sodium Hydroxide
NE	Norephedrine
NEB	N-Ethylbuphedrone
NET	Norephedrine transporter
NPS	New Psychoactive Substances
PBP	Pyrrolidinobutiophenone
PFPA	Pentafluoropropionic Anhydride
PiKHAL	Phenethylamines I Have Known And Loved
PMA	para-Methoxyamphetamine
PPA	Phenylpropanolamine
PPP	Pyrrolidinopropiophenone

PV8	α-Pyrrolidinopentiophenone
PVP	Pyrrolidinopentiophenone
PVT	Pyrrolidinopentiothiophenone
QTOF	Quadrupole Time of Flight
RP	Reverse phase
SALLE	Salting-out-assisted Liquid-Liquid Extraction
SAR	Structure-activity relationship
SERT	Serotonin transporter
SPE	Solid-Phase Extraction
SQTFC	Laboratory of Chemistry and Forensic Toxicology - Centre Branch
TBME	t-butyl methyl ether
TFAA	Trifluoroacetic Anhydride
UHPLC	Ultra-High Performance Liquid Chromatography
UNODC	United Nations Office on Drug and Crime
UPLC	Ultra Performance Liquid Chromatography

Justification and Objectives

Synthetic cathinones and phenethylamines represent the second largest group of new psychoactive substances monitored by the EMCDDA. The consumption and synthesis of these compounds have become a problem of public health and safety. Chewing khat leaves for their psychostimulant effects has been a social and cultural habit among Saudi Arabian and East African communities for several centuries. Cathinone is the main psychoactive compound found in those leaves and it has been widely used since is a β-keto analogue of amphetamine, thus detain similar main effects, such euphoria and increased energy. Due to that similarity, there have been developed synthetic cathinones and introduced in the recreational drug market as 'bath salts', trying to circumvent the law selling legal alternatives to illicit drugs such 'ecstasy' and cocaine.

Therefore, the development of a method to detect and quantify synthetic cathinones and phenethylamines in blood samples has a great importance in the forensic toxicological world for cases of drug intoxication and/or overdose, since authorities can request laboratories to run analysis to verify the presence of these substances. If the detection tests come positive, identification and quantification analysis can also be solicitated. Additionally, it is important that these methods are reliable, easy to perform, time-saving and economic.

The objective for this work was the development, optimization and validation of an analytical methodology to determine the presence of synthetic cathinones (cathinone, flephedrone, buphedrone, α-PVP, methylone, ethylone, pentylone and MDPV) and phenethylamines (4-MTA, 2C-P and dragonFLY) in whole blood samples by mixed-mode solid phase extraction followed by a fast microwave derivatization step with *N*-methyl bis(trifluoroacetamide) (MBTFA) and gas-chromatography analysis. Other objective for this work was the applicability of this methodology to real samples.

These compounds were chosen to complement the already implemented method of the Service of Forensic Chemistry and Toxicology of the Centre Branch (SQTFC) of National Institute of Legal Medicine and Forensic Sciences, I.P. (INMLCF, I.P.). The method developed utilizes the more commonly used chromatographic analysis instrument in forensic toxicology laboratories for the determination of unknown compounds.

The present dissertation is divided into two chapters:

Chapter I corresponds to the introduction of the theme, where are approached physical, chemical and toxicological aspects of these two class of substances. Also, some published case reports are highlighted as a critical review is made on some cases.

Chapter II describes the entire experimental and instrumental part of the present dissertation and corresponds to the submitted article entitled "Determination of "new psychoactive substances" in whole blood using microwave derivatization and GC/MS".

This article describes a method for the determination of the above-mentioned synthetic cathinones and phenethylamines in whole blood using solid phase extraction and gas-chromatography followed by a fast microwave derivatization step.

Chapter 1: Introduction

New Psychoactive Substances

Recently there has been a rapid and constant growth of the synthesis and of New Psychoactive Substances (NPS), despite the efforts created to eradicate them. That became a problem of public health and safety, at global level, mainly due to its rapid propagation and high chemical composition variety, making the monitoring for its appearance a complex task, without a timely appropriate action on the legal status of NPS. The problem exacerbated with the opening of the so-called *smartshops* and the development of the internet drug market, where these substances are sold under the name of classic drugs or as "bath salts", "legal highs", "incense" or "plant food" and are many times labelled "not for human consumption" or "not tested for hazards or toxicity" to circumvent drug legislation. Therefore, understanding this phenomenon and act on it has become a major concern by both governments and international groups responsible for regulating and monitoring the drug phenomenon in order to reduce the demand and supply, namely the United Nations Office on Drugs and Crime (UNODC) and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (1,2,11,12,3–10).

NPS are a vast group of substances that are produced to mimic the effects of common illicit drugs and are not controlled by international law. According to the Intervention on the Addictive Behaviours and Dependencies Services, NPS are a pure or made in preparation new narcotic or psychotropic that is not controlled by the 1961 Single Convention of the United Nations about the narcotic, neither by the 1971 Convention of the United Nations about the psychotropic substances: despite that, they can be a much bigger threat compared with the substances listed on those conventions. The term "new" refers not only to the newly invented and synthesized substances, but also to the ones available on the market used in an inappropriate way. Despite the similarity with controlled psychoactive molecules, they are sufficiently different to bypass the law, as they are not cited in the regulated substances lists. It is been verified that at the same time that the NPS are being controlled, some variants are being created (1,2,4,6,13).

Initially, the appearance of NPS was relatively rare: designers fentanyls appeared for the first time in the 1980s alongside with some ring-substituted phenethylamines and in the 1990s a variety of *ecstasy*-type substances and tryptamines also arise. With the development of the *internet*, the NPS emergence and development had an enormous impact on the drug market, as it provides a source of information for these substances (chemical composition, psychoactive effects), not only for the consumers but also to the producers and suppliers.

Most of the NPS appeared as experimental substances for medical researches, substances derivatives or previously approved substances for human consumption (e.g. amfepramone). Currently, young population are the main consumers for these drugs (14), but addicts and marginalized individuals can also be indicated as abusers.

As they are considered to be a vast and heterogeneous group, NPS can be distinguished in different categories based on their psychopharmacological activity: psychostimulants, synthetic cannabimimetics and hallucinogens. However, the majority of the active components place NPS in one of four chemical classes: phenethylamines, tryptamines, piperazines and cathinones (1,4,6,12,14–18).

The last published report by EMCDDA (14) refers that NPS continue to represent a huge threat to public health. The issues associated to the consumption of these substances are related to seizures, psychoses, hallucinations, paranoia, hyperthermia and when consumed in combination with other substances, like opioids, it can lead to death (19). During 2016, 66 NPS had been detected for the first time in Europe and by the end of the year, more than 620 NPS were being monitored in EU Members States, Norway and Turkey by the Early Warning System (EU EWS), 70% of those appearing in the last five years (Figure 1). In 2015, cathinones were the most apprehended substances: with a total of 80 000 seizures, these drugs represent 33% of the total (Figure 2). In general, synthetic cathinones are the second most monitored group of substances by the EMCDDA, with 118 compounds detected in total, which 14 were noticed for the first time in 2016 (14,19).

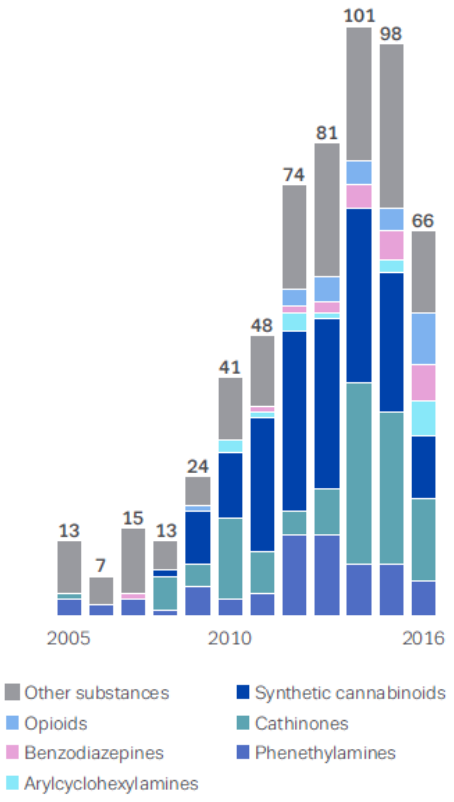

Figure 1 - Substances monitored from 2005 to 2016 according to EMCDDA report of 2017 (14).

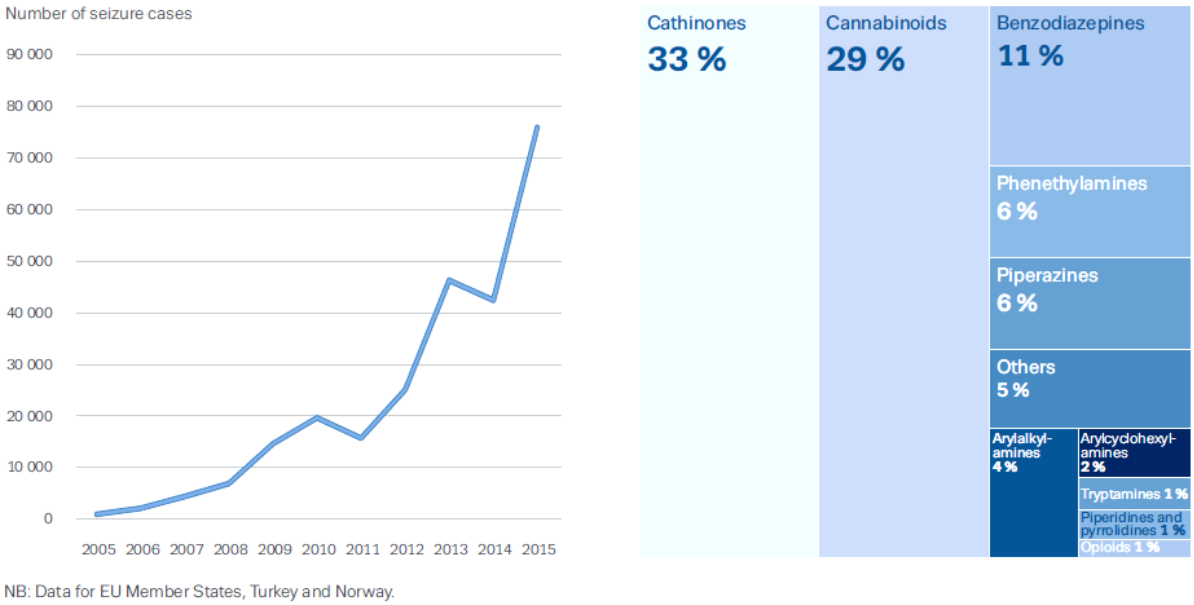

Figure 2 - Substances seized in 2015 according to EMCDDA report of 2017 (14).

Since the early 1990s, many designer drugs were regularly discovered in the European Union. They were often psychotropic substances and their appearance raised questions about possible health risks and problems related to international law enforcement. Along with Europol, EMCDDA created the EU Early Warning System, which consists in all 28 EU member states, Turkey and Norway, to share information and establishing a risk-assessment procedure and mechanism for eventual drug control, allowing the EU to respond rapidly to emerging threats (20,21).

Later in this dissertation two families of NPS (phenethylamines and synthetic cathinones) will be approached, as they were studied for the purpose of the present investigation.

Phenethylamines

Inside NPS sub-categories, phenethylamines are the substances that provide the base structure for several other classes of NPS (Figure 3). As a drug of abuse, phenethylamines had their appearance related with the introduction of *ecstasy* (more known as 3,4-methylenedioxy-*N*-methylamphetamine hydrochloride or MDMA) in the late eighties. Its inclusion in the anti-drug legislation changed the problem and MDMA and amphetamine were used to make several modifications, primarily at the aromatic system (less frequently at the alkyl chain and amine function), to circumvent that legislation (22,23).

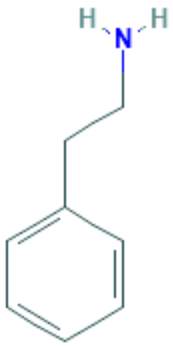

Figure 3 - Phenethylamine structure (24).

Phenethylamines are a broad and diverse category of NPS which includes stimulants, psychedelics, entactogens, anorectics, bronchodilators and anti-depressants. However, it is possible to divide them in three main groups: dimethoxyphenylethanamines, dimethoxyphenylpropanamines and beta-keto compounds (23,25).

From the dimethoxyphenylethanamines group, the so-called 2Cs are the most popular (Figure 4). They contain a phenethylamine backbone with two methoxy groups on the aromatic ring, in positions 2 and 5, and different substituents at position 4. The name 2C derive from the two carbon atoms between the amine and the benzene ring. Most of the currently known 2Cs were synthesized for the first time by Alexander Shulgin, an american pharmacologist, in the eighties and published in his book PiHKAL (*"Phenethylamines I Have Known And Loved"*). The first 2C-serie synthesized was 4-bromo-2,5-dimethoxy-ß-phenethylamine (2C-B) in mid-1980s and was sold as an aphrodisiac in Germany and Netherlands, under the brand names of Nexus, Erox and Performax. It popularity increased rapidly but was only available for a short period due to Dutch authorities that schedule 2C-B provisionally in the list of psychotropic and narcotic substances. Despite the incorrect registration of this drug in the respective law, 2C-B did not re-appear in shops and was succeed by other two 2C-series compounds: 2C-T-2 and 2C-T-7. According to Analogue Statute of the Controlled Substances Act, besides the schedule substances, it is also illegal to traffic any analogues of the already schedule phenethylamines, which includes currently available and previously synthesized drugs (11,23,25–27).

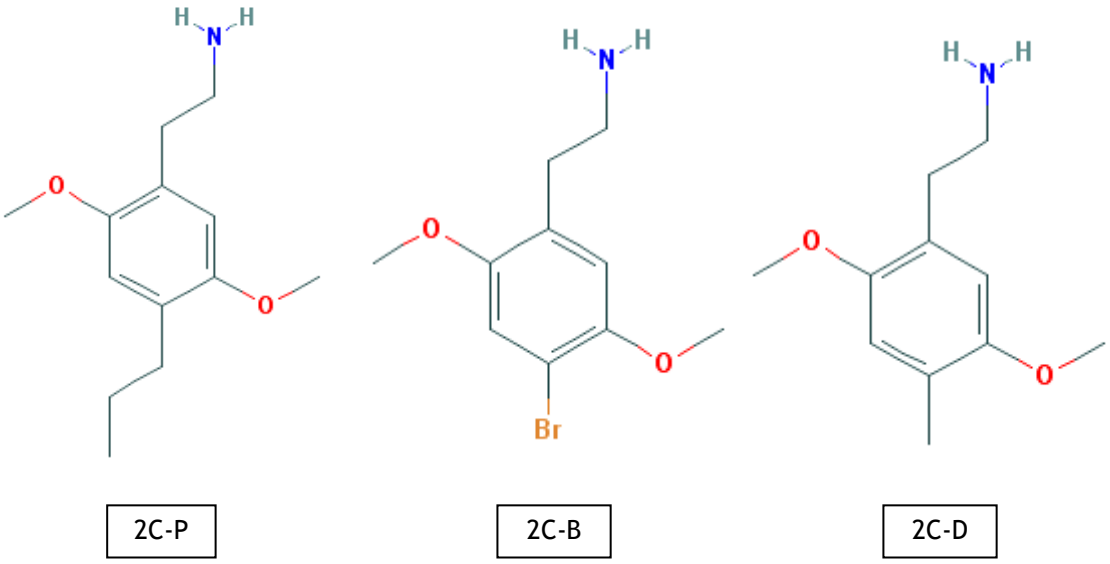

Figure 4 - Variants of the 2C family (28–30).

The hallucinogenic 2Cs are substances that have shown affinity for the serotonin (5-HT_2) and alpha-adrenergic receptors, however, depending on the receptor subtypes, they can exhibit either agonistic or antagonistic properties, being extremely active even at very small doses. Studies for the structure-activity relationship (SAR) of 2Cs have demonstrated that the primary amine separated from the phenyl ring by two carbon atoms, the presence of methoxy groups at positions 2 and 5 at the aromatic ring and a hydrophobic substituent at position 4 (alkyl, alkylthio, halogen, etc.) are responsible for the hallucinogenic effects on these compounds. So new hallucinogenic 2Cs can be obtained with the insertion of different substituents at positions 2, 4 or 5 in the phenyl ring and designer substitutions in these substances structure can enhance their hallucinogenic activity, such homologation reactions in the 4-substituent (8,11,25,27).

Generally, and depending on the 2C, these substances are available in liquid form, powder, capsule or tablet and the consumption is made by oral ingestion or by insufflation, being the last one the way of consumption that produces more intense and rapid effects: oral ingestion has an onset action of 1 to 3 hours with a duration of 5 to 7 hours, but insufflation has a start action in 5 to 15 minutes and a duration of 2 to 4 hours. Both ways of administration leave the individual with hangover symptoms and with lasting effects that can continue from 1 to 7 days. Also, these substances are often sold in tablets as *Ecstasy* in several different doses (8,27,31).

The metabolism of 2Cs occurs by *O*-demethylation in the positions 2 and 5 of the aromatic ring: deamination followed by oxidation to the corresponding acid at position 2 or reduction to the corresponding alcohol at position 5. The process of deamination of 2C substances involves mainly the enzymes monoamine oxidase (MAO)-A and MAO-B. These are enzymes present in the outer mitochondrial membrane and could be found, not only in neural and glia

cells, but also in liver. They have the ability to catalyse the oxidation of primary, secondary and tertiary amines into their corresponding protonated imines and posterior non-enzymatic hydrolysis of the imines products to the corresponding aldehyde. The substrates of MAO are dopamine and noradrenaline, neurotransmitters that show structural similarity with 2Cs substances. Besides that, and due to the involvement of these enzymes, there are possible drug-drug interactions with the inhibitors of MAO, increasing the concentration and further toxicity of 2Cs. However, cytochrome P450 (CYP) enzymes are also involved in the metabolism of these class of compounds. They are located in membranes, essentially in the endoplasmic reticulum, that can be found generally in the liver. The action of these enzymes consists to catalyse the deamination via oxidation of the α-carbon atom subsequent to the nitrogen. CYP2D6 has shown to have some involvement in the metabolism of some variants of 2Cs (2C-D, 2C-E, 2C-T-2 and 2C-T-7), but in small extent (8,11,27,32). As for the excretion of the 2C agents, there is not yet enough information. However, some investigations have been made on this topic involving animal tests and showing that the primary excretion route for some species of 2Cs is the urinary (33).

The effects caused by 2C substances toxicity are a combination of hallucinogenic and stimulating effects. Depending on the dose, patients with 2C intoxication can exhibit sympathomimetic syndrome, serotonin syndrome, hallucinations, or a combination of any of these last three - euphoria, empathy, hallucinations, agitation, nausea, vomiting, seizures, respiratory depression, hypertension and tachycardia. The dose for the 2C substances ranges between 10 to 30 mg, depending on the variant of this family of drugs, however and according to DEA (Drug Enforcement Administration) (31), the 2C-B, first synthesized 2C, it is active between 0.1-0.2 mg/kg lasting for 6 to 8 hours. According to consumers, the abuse of these substances promotes enhanced visual, tactile, auditory and olfactory senses. At lower doses, 2C drugs usually exhibit stimulating effects and increased visual, auditory and tactile sensation; at moderate doses, it may produce hallucinations; and, at higher doses, the consumers may experience displeasing hallucinations and sympathomimetic syndrome leading to tachycardia, hypertension and hyperthermia. Besides that, the consumption of these compounds can lead to excited delirium due to the increased dopamine release, along with the inability of the brain to efflux that dopamine and consequently overstimulation of the postsynaptic receptors. This event can lead the consumer to death since is characterized to follow a sequence of symptoms starting with delirium and agitation, moving violence, hyperactivity, hyperthermia and, at last, resulting often in a sudden and unexpected cardiopulmonary arrest. The treatment for the intoxication with 2C series is only symptom-based supportive care, since there are not any antidotes available yet. Individuals intoxicated should rest in a calm and quiet environment until they are stabilized and receive supportive care, fluid resuscitation, sedation and cooling measures, always under continuous cardiac monitoring and baseline electrocardiogram. For patients with excited delirium, it is common for them to exhibit autonomic hyperactivity due to abnormal dopamine processing. In that case, the main goal is to obtain a rapid sedation of the individual and attenuation of the

symptoms using catecholamines. Also, benzodiazepines, neuroleptics or a combination of both are commonly used to treat this symptom, as well as sympathomimetic signs of hypertension, tachycardia and hyperthermia. At last, a gastrointestinal (GI) decontamination must be indicated, depending on the route of administration and time past since the ingestion (8,27,31).

Inside the dimethoxyphenylpropanamines, the main group of substances are the so-called DOxx (Figure 5) and, as well as 2C series, most of the substances in this group were described by Shulgin on his book (11). Also named as 2,5-dimethoxy-4-amphetamines, the DOxx, structurally, are similar to 2Cs, with two methoxy groups in positions 2 and 5 of the phenyl ring and a hydrophobic substituent at position 4, particularly a halogen. However, instead of a phenethylamine backbone, the DO family presents an amphetamine backbone structure (34-36).

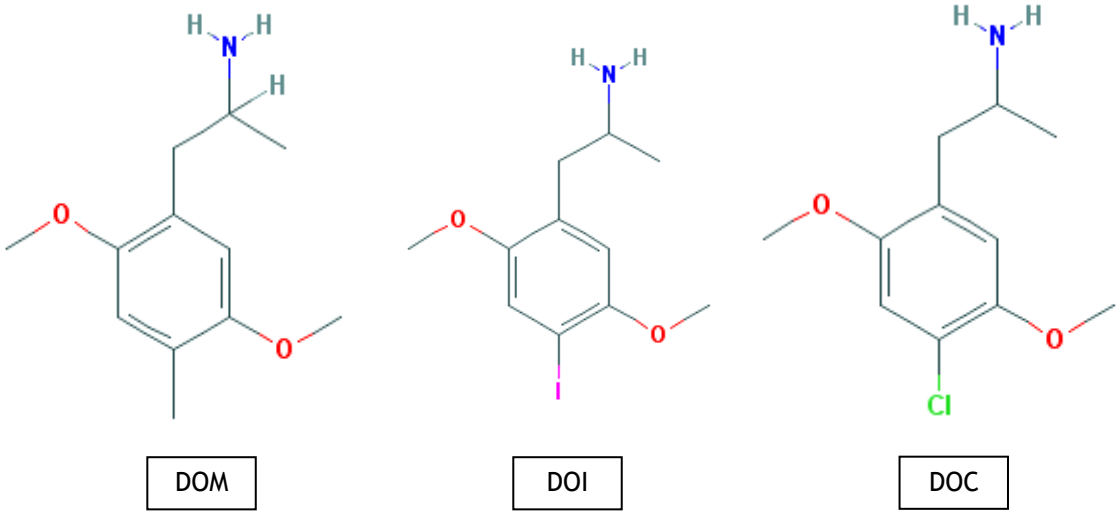

Figure 5 - Variants of the DO family (37–39).

The DOxx have shown affinity for the subtype 2 of the serotonin receptor (5-HT_2) exhibiting agonistic properties. Studies on the SAR of these substances have revealed that hallucinogen-like activity was also similar to the one demonstrated by 2C family, which is attributed to the primary amine function separated from the phenyl ring by two carbons, the presence of methoxy groups at positions 2 and 5 and the lipophilic 4-substituent, in this case, specially, a halogen. However, differently of 2Cs, DOxx possesses a methyl group at the α-carbon that is responsible for the enhancement of the hallucinogenic properties of these substances, increasing *in vivo* potency and duration of action compared to dimethoxyphenethylamines (e.g. 2,5-dimethoxy-4-bromoamphetamine (DOB) possesses similar chemical characteristics with 2C-B, but produces lasting effects) (40–42). These types of substances were many times combined with other drugs, so their sale in *smartshops* was made alone or mixing them with another designer drugs in the form of powders, tablets, liquids or blotters. Due to the major number of substances comprehended in this family, the dose range varies according to each DOxx analogue, but the common drug abusers dose varies, generally, between 1 to 10 mg.

According to some studies, substances belonging to DO family can be orally or intravenous administrated, and the time and duration of action vary according to the route of administration. Some studies revealed that, when administrated orally, these substances reached a plasma concentration value 1 hour after their consumption. Relatively to subcutaneous application, a plasma concentration was also achieved 1 hour post application. However, by subcutaneous consumption, the plasma concentration was much higher compared to the one reached when consumed orally (11,34–36,42–45). Nevertheless, the data on its toxicokinetics are still scarce.

Nowadays, DOxx are scheduled under the Convention on Psychotropic Substances, whereby they are not common to find in toxicological reports and cases latterly, so the metabolism of these compounds was more extensively studied in animals. The metabolic pathway for the dimethoxyphenylpropanamines vary according to the different analogues of these class of substances: chloro (2,5-dimethoxy-4-chloroamphetamine-DOC) and iodo (2,5-dimethoxy-4-iodoamphetamine-DOI) subtypes suffers *O*-demethylation reaction of the methoxy groups at positions 2 and 5 of the phenyl ring, the methyl variant (2,5-dimethoxy-4-methylamphetamine-DOM) undergoes oxidative deamination and aliphatic hydroxylation and the bromo (DOB) analogues are metabolized by a combination of the above-mentioned reactions (46). The enzymes involved in the metabolic processes are still not well described, but according to studies made for the identification of CYP isoenzymes involved in the metabolic process of DO substances, it has shown that the only responsible for the *O*-demethylation and hydroxylation, the main metabolic steps for these substances, is the isoenzyme CYP2D6. However, due to a competitive inhibition exhibit by the DOxx and substances derived, the metabolites formed appeared in very small amounts (41). As the metabolic pathway for these substances is somewhat incomplete, there is not too much information relatively the excretion. Nevertheless, the main metabolites for some variants of the DO family appear by the urinary route (34–36,47).

The effects associated to the consumption of these class of substances are highly dose-dependent and due to the lack of cases reported, there is not much information on the dose responsible for the effects caused in consumers. Nevertheless, generally, the DO compounds are capable of producing long-lasting hallucinogenic effects compared to 2Cs, nausea, tachycardia, central nervous system (CNS) effects, euphoria, enhanced visual, auditory, olfactory and physical sensations similar to LSD, agitation and vasospasms. Also sense of well-being, emotional awakening, blurred vision, dehydration, vomiting, headache, muscle tension and dilated pupils are some other effects experienced by consumers of these type of substances. For compounds such as the bromo analogue (DOB), the consumption of dosages ranging the 2.8 mg can produce adverse effects such as cramps with muscular pain and flashes of hallucination. Higher doses (over 3.5 mg) can lead to an overdose situation with memory loss, irrational and even violent behaviour. Despite the drug effects of this variant last for a long time (8-24 hours), depending on the person condition and administrated dosage, it takes a long time for onset action (over 3 hours) which lead the user to take a

second dose, because the first has not produce yet any effect, generally causing an intoxication. In case of acute intoxication, the individuals experience convulsion, arterial vascular spasms, vomit, diarrhea and optical hallucinations (40,42,44,48,49).
The third group of phenethylamines are the beta-ketones compounds and, within these, the synthetic cathinones are the main substances belonging to this class.

Synthetic Cathinones

Synthetic cathinones are derivatives from a natural occurring alkaloid called cathinone (Figure 6), found in the leaves and twigs of *Catha edulis* (or commonly named "*Khat*"), a plant originally from the northeastern Africa and the Arabian Peninsula. The act of chewing *khat* for its amphetamine-like effects it is a centenary practice that is still common today in some Middle Eastern and East African countries. The plant became known in Europe thanks to Swedish botanist Peter Forssakal in the late eighteenth century, who catalogued it. Years later, in the nineteenth century, with the advance of chemistry, it was possible to isolate the active compounds from extracts of the plant in order to identify them and study their active principles (7,50–52).

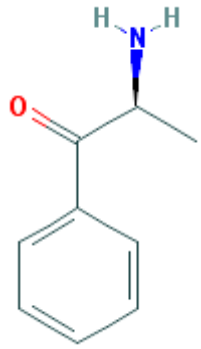

Figure 6 - Cathinone structure (53).

The introduction of most of synthetic cathinones in the drug market and, consequently, their recreational use, started in the mid-2000s. However, the first cathinone derivative appeared in 1928 with the synthesis of a methylated analogue of cathinone named methcathinone (ephedrone). Initially, these substances were developed for therapeutic purposes: methcathinone (ephedrone), was commercialized in the USSR as an antidepressant between 1930 and 1940 and later, a pharmaceutical company utilized it as a potential CNS stimulant; pyrovalerone was also tested as a stimulant in France and USA, for patients with chronic fatigue; diethylpropion (amfepramone) was marketed as an appetite suppressant (12,16,50,52). Due to its similarity of effects with some classic and controlled drugs, synthetic cathinones are currently available in *smartshops* and online, being sold under several names (*Bliss, Blue Silk, Cloud Nine, Ivory Wave, Meow Meow*) as "bath salts", "research chemicals", "plant food", and labelled "not for human consumption" or "not tested for hazards and toxicity" in order to circumvent the law (Figure 7) (54).

Figure 7 - Illustrative image of packages of 'bath salts' (55).

Phenethylamines are structurally related to amphetamines. This led to describe cathinone as a natural amphetamine consisting in an aromatic ring-substituted group linked to an amino function, differing only by a beta keto functional group at the beta carbon, which increases polarity and decreases its ability to penetrate CNS through the blood-brain barrier (BBB), resulting in less intense effects. This ketone group allows synthetic cathinones to exist in two stereoisomeric forms, that may vary their potency, or to be in racemic mixtures. Due to its structural similarity, in general cathinones derivatives produces amphetamine-like effects acting as release stimulators of norephedrine (NE), dopamine (DA) and serotonin (5-HT) to the synaptic cleft and inhibitors of monoamines reuptake by blocking the neurotransmitters transporters (NET-norephedrine transporter; DAT-dopamine transporter; SERT-serotonin transporter), but their action can vary from substance to substance: methylone, like amphetamine and MDMA, act as a substrate for a nonspecific monoamine transporter, increasing the release of neurotransmitters; however, similar to cocaine, MDPV inhibits the reuptake of monoamine at the SERT, NET and DAT. So, since cathinones derivatives are many times used as substitutes of classic and controlled drugs, for their similar effects, they can divided into three groups according to their action mechanism on the CNS: cocaine-MDMA mixed type, that includes substances methylone and ethylone, act as nonspecific monoamine reuptake inhibitors, blocking mainly the DAT; methamphetamine-like type substances, involves cathinone and flephedrone, promotes DA release and inhibit the reuptake of DA and NE; pyrovalerone type compounds, such MDPV, plays as a selective inhibitors for monoamines reuptake. These actions create an excess of the neurotransmitters within the synaptic cleft leading to both acute and long-term deficiencies in the NE, DA, 5-HT systems (7,12,16,17,50,52,56,57).

Synthetic cathinones are presented most of the times as a white or brown amorphous or crystalline powder or in liquid formulation, but it is also common to see them in tablet and capsule forms. They can be absorbed by multiple routes and by many ways including nasal insufflation, oral ingestion, intramuscular, intravenous, rectal insertion, gingival application, smoking, inhalation, "bombing" and "keying". These last two are common terms utilized for the consumption of these type of substances that means wrapping the powder in a cigarette

paper and swallow it ("bombing") and dip a key into the powder and then insufflate it ("keying") (7,12,16,50,51,54,57,58). Despite all these forms of consumption, the most common and more utilized ways are the nasal insufflation and oral ingestion of cathinones, being this last one rapidly absorbed with the maximum peak being reached after 1.5 hours and with lasting effects from 1 to 6 hours. As the group of synthetic cathinones includes several substances, an accurate onset of action it is difficult to precise, since it depends on the substance itself and the dose administered (once the purity of the consumed drug is not known, it becomes difficult to precise the dose) (7,17,57).

The use of synthetic cathinones leads to psychostimulant-like effects on the mind such euphoria, alertness, psychosis, agitation and confusion. Physical symptoms include hypertension, tachycardia, hyperthermia, seizures and delusions. Reports of death are mentioned in cases of individuals that highly abuse the consumption of these substances (1,50,51,54,56,57). These symptoms are much similar to those demonstrated by the consumption of classic substances such cocaine and amphetamines. However, due to their lower liposolubility, higher doses of cathinones are commonly self-administered in order to exhibit the same effects. Also, it is strongly desired by the consumers of these substances second doses (7,54,56,57,59).

The metabolic pathways of cathinone derivatives depend on their chemical structures, specially the phase I metabolism. Thus, according to the variants of synthetic cathinones, they can be metabolized by reduction of the ß-ketone moiety, *N*-dealkylation, demethylenation followed by *O*-methylation for 3,4-methylenedioxyphenyl cathinones, hydroxylation followed by dehydrogenation and ring opening for the *N*-pyrrolidine cathinones and metabolism of benzene ring substituents.

Reduction of the ß-ketone moiety to form hydroxyl metabolites represents one of the major metabolic pathways for synthetic cathinones, since the ß-ketone is a characteristic structure for this class of substances. *N*-dealkylation reaction is mediated by CYP2D6, which is the major metabolic pathway for N-alkyl cathinones and is also the reaction responsible for the opening of pyrrolidine ring in pyrrolidine derivatives. The process of demethylenation followed by *O*-methylation for 3,4-methylenedioxyphenyl cathinones consists in the demethylenation of the 3,4-methylenedioxy moiety to dihydroxy metabolite which is catalysed by CYP2D6 and CYP2C19. The *O*-methylation of dihydroxy metabolite is mediated by cathecol *O*-methyl transferase (COMT). Pyrrolidine derivatives commonly suffer a reaction of hydroxylation at the *N*-pyrrolidine moiety. The hydroxyl metabolite generated is later metabolized through dehydrogenation and, finally, the pyrrolidine ring opens to create an aliphatic aldehyde metabolite. At least, the metabolism of benzene ring substituents is observed in some derivatives. For 4-methoxyphenyl cathinones, the reaction of *O*-demethylation produces 4-hydroxyl-phenyl metabolites and, in case of 4-methylphenyl cathinones, the reaction that often occurs is the hydroxylation of the 4-methyl moiety of the phenyl ring, which generates a hydroxyl-methyl metabolite (1,16,52,59).

Although limited information is available relatively to the phase II metabolism of synthetic cathinones, glucuronidation and sulfation pathways are described for hydroxyl metabolites and even for unchanged cathinone derivatives (1,52). The excretion of these substances occurs mainly through the urine but in also through the biliary system (7,52,59).

The treatment associated to consumers of these class of substances is purely supportive. Primarily, it should be focused on reducing agitation, psychosis, hypertension, tachycardia, hyperthermia and seizures by sedating the patient with large doses of benzodiazepines. Also, internal and external cooling for hyperthermia and, in cases of significant toxicity, neuromuscular blockade and intubation may be necessary (7,51).

Detection of NPS in biological samples

The detection and quantitation of NPS in biological specimens is of overwhelming relevance for the identification and documentation of its use and abuse. For this reason, researchers have made numerous efforts to create databases in order to have more information for each substance. Given the increased consumption of NPS in the recent years, great efforts have been made to identify and quantify these compounds. The fact that these substances are constantly emerging poses a challenge for a rigorous determination by forensic toxicology laboratories. For instance, conventional screening tests are very fallible since they are based on immunoassays which are not specific (7,51,59,60). Nowadays, the use of highly specific and sensitive analytical techniques is mandatory to determine these substances and metabolites, and a careful selection of the biological sample to be analysed is important as well.

Blood/plasma/serum and urine are the biological matrices more applied at the research of drugs of abuse due the advantages of these samples. Pasin *et al*. applied a salting-out-assisted LLE for 100 µL of blood to detect and quantify 37 new designer drugs, where 23 were cathinones and 8 were phenethylamines by UPLC-TOF-MS. The development of this analytical method allowed them to achieve recoveries ranging from 71 to 100% and limits of detection and quantification 0.007-0.07 µg/mL and 0.05-0.1 µg/mL, respectively (61). Also, Lehmann *et al*. developed and validated a method utilizing small volumes of serum (150 µL) and a fully automated in-line SPE which offers the entire process of conditioning, sample introduction, washing and elution during defined flow rates to detect 74 NPS. This SPE technique allows the utilization of small volumes of solvent making it economic and eco-friendly. However, the guidelines were fully achieved only for 62 of these compounds with limits of detection between 0.2 and 4 µg/L and limits of quantification of 5 µg/L. The analytes extraction resulted in recoveries of over 50% for 69 substances. Besides that, Lehmann *et al*. tested their validated method in the detection of further 21 NPS, totalling 95 substances and obtained a LOD between 0.0004-0.0016 µg/mL (62). Odoardi *et al*. also developed a method for the screening in whole blood of several NPS, pertaining to different classes, but in their case,

they apply a DLLME for the detection of 78 analytes. This technique for sample preparation could be an alternative to classical liquid-liquid and solid-phase extraction procedures due to its quickness it use only microliters amounts of organic solvents, being considerate an economic method and allows to extract simultaneously a large number of substances from different chemical classes (63). In case of Ambach *et al.*, they published a method for the detection and quantification of 56 NPS in both urine and whole blood by a LC-MS/MS. Despite the values achieved and the validation of the method according to the guidelines of the German Society of Toxicological and Forensic Chemistry, they presented a methodology with a vast number of NPS with eight different substances classes. However, quantification was only possible for 45 compounds in whole blood and 44 in urine, whereas the remaining can be evaluated semi quantitatively, that should be sufficiently for most clinical and forensic questions as there are no legal limits regarding the concentrations of these NPS in these biological matrices (64).

However, there are others that can also be utilized in this process and their study and application have been growing, like oral fluid/saliva and hair. This last one is a valuable tool that can provide information about the current diffusion of NPS among the population and the social characteristics of these drug's users. Salomone *et al.* developed a method to determine 31 substances by using a UHPLC-MS/MS system. Regardless the good recoveries obtained for a large number of compounds, the sample preparation for this analysis shows a time-consuming process with the incubation in methanol at 55°C for 15 h. Nevertheless, the utilization of a UHPL-MS/MS for the analysis with a total run time of 5.5 min reduce the time required compared to other instrumentation equipment (65). Likewise, Strano-Rossi *et al.*, utilized a UHPLC-MS/MS to screening 50 NPS. The sample preparation consisted in an incubation overnight under sonication but with different conditions depending on the classes of the analytes to be extracted (66).

The utilization of oral fluid for drug testing has the benefits of being non-invasive and offering facile sampling. Rocchi *et al.* developed and validated a methodology using 90 µL of this matrix for simultaneous identification and quantification of 31 NPS based on a MEPS-UHPLC-MS/MS. Besides the low limits of detection and quantification obtained (0.005-0.850 ng/mL and 0.015-2.600 ng/mL, respectively), the reduced matrix effects found at different concentrations for all analytes were provided for the MEPS clean-up showing an effective removal of interfering compounds, despite the non-quantitative recoveries for some analytes (67). Mercolini *et al.* also developed and validated a method using not only oral fluid, but also blood and urine, to determine the main cathinones analogues (methylone, ethylone, butylone, 4-MMC, 4-MEC and MDPV) by VAMS-LC-MS/MS. This procedure is a miniaturised sampling technique that allows to reduce the sampling volume and overcome some limitations related to biological fluids (68).

Less explored are the matrixes like vitreous humour, meconium, pericardial liquid, etc. Despite, their application, these matrixes (also-called unconventional or alternative biological matrixes), have some advantages over blood (eg. more resistance to the phenomena of

putrefaction) and urine (eg. infringe the privacy of the individual with the supervised urine collection). Margalho *et al*. validated an analytical methodology for the simultaneous qualitative and quantitative determinations of 14 synthetic cathinones and phenethylamines using both conventional (blood) and unconventional (pericardial fluid and vitreous humour) biological matrices. Despite the low limits achieved (LOD and LOQ of 5 ng/mL), the highlight of this study were the sample preparation that consisted in utilizing a fast derivatization microwave procedure (90 s) for the GC-MS analysis, allowing to save laboratory time consumed (69). Also, Gerace *et al*. presented a case report where they analysed several matrices in order to evaluate mephedrone as cause of dead of a 25-year-old man. They collect samples of blood, urine and gastric contents and submitted them to toxicological analysis. Also, brain, bile, lung and hair samples were used as alternative and additional matrices for their analysis. The detection of this substance was performed by a validated GC-MS/EI method. However, mephedrone only were detected in blood, urine and gastric contents and the calculated LOD and LOQ were, respectively, 0.006 µg/mL and 0.02 µg/mL for both blood and urine matrices (70).

Amniotic fluid is considered an unconventional matrix of invasive and difficult collection. However, it can give a specific and direct measure of fetal exposure, indicates actual drug concentrations that have passed the placental barrier, rather than maternal blood and Burrai *et al*. presented a methodology utilizing this matrix that identify and quantify 13 illicit phenethylamines by a SPE using a hydrophilic-lipophilic balance cartridges and LC-MS/MS, obtaining limits of detection and quantification between 0.003-0.006 µg/mL and 0.009-0.02 µg/mL, respectively, and recoveries ranging between 72 and 96% (71).

Table 1-5 resume the main sample procedures and analytical instrumentation used in order to determine the studied compounds in biological human matrices. Literature search was performed using the PubMed database, and the search strings were “new psychoactive substances”, “cathinones”, “synthetic cathinones” and “phenethylamines” in the different types of human biological specimens.

Table 1 - Bioanalytical procedure for determination of synthetic cathinones and phenethylamines in blood, serum and plasma (2014 to present).

Analytes	Sample Amount	Sample Preparation	Stationary and mobile phase (when applicable)	Detection mode	LOD; LOQ	Recovery	References
2C-H, 2C-I, 2C-B, 2C-T-2, 2C-T-7, 2C-D, 2C-E, 2C-P, 2C-N, 2C-B-FLY, 25H-NBOMe, 25C-NBOMe, 25B-NBOMe, 25I-NBOMe, 25E-NBOMe (plus LSD, fentanyl and their metabolites)	1 mL blood	LLE (with saturated aqueous sodium sulfate solution, diethyl ether-ethyl acetate mixture (1:1), sodium hydroxide)	Stationary phase: ThermoFisher Accucore Phenyl Hexyl LC column (100 mm x 2.1 mm, 2.6 µm) Mobile phase A: 2 mM aqueous ammonium formate at pH 3.4 containing 0.1% formic acid; Mobile phase B: MeOH/ACN (50:50, v/v) containing 0.1% formic acid	LC-HRMS-ESI	LOD: 0.0001 µg/mL; LOQ: 0.00025 µg/mL	-	(72)
25B-NBOMe and 4-CMC	0.2 mL blood	LLE (with ethyl acetate)	Stationary phase: C18 column (100 mm x 4.6 mm, 2.7 µm) Mobile phase: 10 mM ammonium formate with 0.05% v/v of formic acid in water/ACN with 0.05% v/v of formic acid (9:1 v/v)	LC-MS-ESI	LOD: 0.0000053 – 0.0000013 µg/mL; LOQ: 0.0000159 – 0.0000049 µg/mL	84.7-102% (25B-NBOMe) and 91.1-98.5% (4-CMC)	(73)

1-Naphyrone, 25D-NBOMe, 25H-NBOMe, 2C-E, 2C-N, 2-Fluoromethcathinone, 3,4-DMMC, 3-MMC, 4-Fluoromethcathinone, 4-Methylethcathinone, Buphedrone, Butylone, Dimethylcathinone, Ethcathinone, Ethylone, MDPV, Mephedrone, Methcathinone, Methedrone, Methylone, Naphyrone, Pentedrone, Pentylone, (plus amphetamines, 28 synthetic cannabinoids, 3 indanes, 2 piperazines, 2 tryptamines, 2 phencyclidines, methoxetamine, ketamine and its metabolite)	0.2 mL blood	Precipitation with ACN	Stationary phase: C18 column Mobile phase A: 5 mM aqueous formic acida; Mobile phase B: 5 mM CAN	LC-MS/MS-ESI	LOD: 0.00005-0.0003 µg/mL ; LOQ: 0.0001-0.0005 µg/mL	72-110%	(74)
Cathinone, Methcathinone, 4-MEC, 3,4-DMMC, Mephedrone, Methedrone, Buphedrone, Pentedrone, Flephedrone, Benzedrone, Naphyrone, Methylone, Ethylone, Butylone, Pentylone, Bupropion, Pyrovalerone, α-PVP, MDPV, PPP, MDPPP, MPBP, MDPBP, 2C-B, 2C-E, 2C-H, 2C-I, 25B-NBOMe, 25C-NBOMe, 25H-NBOMe, 25I-NBOMe (plus piperazines)	0.1 mL whole blood	SALLE (with ACN)	Stationary phase: C18 column (150 mm x 2.1 mm, 1.8 µm) Mobile phase A: 5 mM ammonium formate; Mobile phase B: ACN containing 0,1% (v/v) formic acid	UPLC-QTOF-MS-ESI	LOD: 0.007-0.07 µg/mL; LOQ: 0.05-0.1 µg/mL	71-100%	(61)

Methcathinone, Methylone, DMC, 4-FMC, Ethylcathinone, Ethylone, Methedrone, Buphedrone, Butylone, 4-MEC, Pentedrone, Pentylone, 3,4-DMMC, MDPV, 1-Naphyrone, Naphyrone (plus synthetic cannabinoids)	0.25 mL plasma	Precipitation with ACN-MeOH	Stationary phase: BetaBasic18 column (150 mm x 2.1 mm) Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in ACN	LC-HRMS-ESI	LOD: 0.000005-0.00008 µg/mL; LOQ: 0.03-0.4 ng/mL	>75%	(75)
2C-B, 2C-D, 2C-E, 2C-H, 2C-I, 2C-P, 2C-T-2, 2C-T-4, 2C-T-7, 4-MEC, 4-MTA, Butylone, Cathinone, DOB, DOET, DOM, Ethcathinone, Ethylamphetamine, Ethylone, MDPPP, MDPV, Mephedrone, Methcathinone, Methedrone, Methylone, Naphyrone, Norephedrine, Pyrovalerone (plus amphetamines, piperazines and indanes)	0.5 mL whole blood	SPE - Bond Elut Certify cartridges	Stationary phase: Synergi Polar-RP column (100 mm x 2.0 mm, 2.5 µm) Mobile phase A: 0.1% formic acid in 10 mM aqueous solution of ammonium formate; Mobile phase B: 0.1% formic acid in MeOH	LC-MS/MS-ESI	-; LOQ: 0.001-0.01 µg/mL	-	(64)

25B-NBOMe, 25H-NBOMe, 2C-B, 2C-B-FLY, 2C-D, 2C-E, 2C-H, 2C-N, 2C-P, 2C-T-7, 2-FMC, BDB, Bromo-DragonFLY, Butylone, Cathine, Cathinone, DMC, DOB, DOET, DOM, Eutylone, Flephedrone, MDPBP, MDPPP, MDPV, Mephedrone, Methedrone, Methylone, MPBP, Naphyrone, NEB, Pentylone, (plus amphetamines, aminoindanes, phencyclidine-type substances, ketamine, tryptamines, piperazines and other substances)	0.15 mL serum	Automated-SPE with sample processor PAL HTS - 10-APCXP cartridges	Stationary phase: Kinetex Biphenyl Core-Shell column (100 mm x 2.1 mm, 2.6 µm) Mobile phase A: 2 mM ammonium formate in water with 0.1% formic acid; Mobile phase B: 2 mM ammonium formate in ACN with 0.1% formic acid	LC-MS/MS-ESI	LOD: 0.0002-0.004 µg/mL; LOQ: 0.005 µg/mL	>50% for 69 analytes and <50% for MDAI, NMT, MDAT, 6-TMA and methylphenidate	(62)
Cathine, DOB, DOM, Ephedrine, Mephedrone, Methcathinone, Methedrone, PMA, 2C-B, 2C-H, 2C-I, 2C-T-2, 2C-T-4 and 2C-T-7	0.25 mL whole blood	SPE – Oasis MCX cartridges MBTFA + Microwave derivatization	Stationary phase: HP-5 MS column (30 m x 0.32 mm x 0.25 mm) with 5% phenylmethylsiloxane Mobile phase: Helium	GC-MS-EI	LOD: 0.005 µg/mL; LOQ: 0.005 µg/mL	76.6-112.8%	(69)

α-PVP, 3,4-DMMC, 4-MEC, bk-2C-B, Buphedrone, Butylone, Ethylone, Cathinone, Clephedrone, Ethcathinone, Ethylphenidate, Flephedrone, MDPV, Methylmethcathinone, Methedrone, Methylone, MTA, MTTA, Naphyrone, Norephedrine, Norpseudoephedrine, Pentedrone, Pentylone (plus amphetamines, synthetic cannabinoids and other stimLuants	0.5 mL blood	DLLME (with chloroform and MeOH)	Stationary phase: C18 column (100 mm x 2.1 mm, 2.6 µm) Mobile phase A: 5 mM ammonium formate containing 0.1% formic acid; Mobile phase B: MeOH with 0.1% formic acid	UHPLC-MS/MS-ESI	LOD: 0.0002-0.002 µg/mL; -	5-110%	(63)
Methcathinone, 3-FMC, 4-FMC, Methylone, Ethcathinone, Ethylone, Methedrone, Buphedrone, Butylone, Mephedrone, Eutylone, 4-MEC, MDPBP, Pentedrone, Pentylone, 3,4-DMMC, α-PVP, 4-EMC, MPBP, MDPV, Pyrovalerone and Naphyrone.	2 mL blood	SPE – PolyChrom Clin II columns	Stationary phase: C18 column (100 mm x 2.1 mm, 2.7 µm) Mobile phase A: 0.1% formic acid in deionized water; Mobile phase B: 0.1% formic acid in ACN	LC-QTOF-MS-ESI	LOD: 0.001-0.005 µg/mL; LOQ: 0.001-0.005 µg/mL	81-93%	(76)
Methylone, ethylone, butylone, mephedrone, 4-MEC and MDPV	0.09 mL plasma	Precipitation with MeOH	Stationary phase: C18 column (50 mm x 2.1 mm, 3.5 µm) Mobile phase A: 0.1% formic acid in ACN; Mobile phase B: 0.1% formic acid in water	LC-MS/MS-ESI	LOD: 0.003 µg/mL; LOQ: 0.01 µg/mL	75.1-88.5%	(68)

Methylone, HMMC, MDC and HHMC	0.1 mL plasma	SPE – SOLA SCX cartridges	Stationary phase: Synergi Polar-RP column (100 mm x 2 mm, 2.5 µm) Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in ACN	LC-MS/MS-ESI	LOD: 0.00025-0.01 µg/mL; LOQ: 0.0005-0.01 µg/mL	49.2-81.7%	(77)
2C-B, 2C-D, 2C-E, 2C-H, 2C-I, 2C-P, 2C-T-2, 2C-T-4, 2C-T-7, 3-FMC, 4-MEC, 4-MTA, Butylone, Cathinone, DOB, DOET, DOM, Ethcahinone, Ethylamphetamine, Ethylone, Flephedrone, MDPPP, MDPV, Mephedrone, Methcathinone, Methedrone, Methylone, Naphyrone, Norephedrine, Norpseudoephedrine, Pentylone, Pseudoephedrine, Pyrolvalerone (plus amphetamines, piperazines and tryptamines)	0.01 mL blood	Precipitation with MeOH	Stationary phase: Synergi Polar-RP column (100 mm x 2 mm, 2.5 µm) Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in ACN	LC-MS/MS-ESI	LOD: 0.001-0.01 µg/mL; LOQ: 0.0025-0.01 µg/mL	-	(78)

25B-NBOMe, 25C-NBOMe, 25H-NBOMe, 25I-NBOMe, 2C-B, 2C-C, 2C-D, 2C-E, 2C-H, 2C-I, 2C-N, 2C-T-2, 2C-T-7, 2-MMC, 3,4-DMMC, 3C-P, 3-FMC, 3-MMC, 4-BMC, 4-CMC, 4-EMC, 4-FMC, 4-MBC, 4-MEC, 4-MTA, α-PBP, α-PPP, α-PVP, BDB, BMDP, Buphedrone, Bupropion, Butylone, Dibutylone, Diethylpropion, Dimethylone, DOET, DOI, DOM, Ephedrone, Ethcathinone, Ethylone, Eutylone, MDPBP, MDPPP, MDPV, Mephedrone, Metamfepramone, Methedrone, Methylone, MPBP, Naphyrone, NEB, Pentedrone, PPA (plus amphetamines, tryptamines, piperazines, piperidines, synthetic cannabinoids, arylalkylamines, arylcyclohexylamines, aminoindanes and other drugs)	0.2 mL blood	Precipitation with ACN	Stationary phase: C18 column (50 mm x 2.1 mm, 1.8 µm) Mobile phase A: 0.1% formic acid in ACN (v/v) and 0.1% formic acid in water (v/v)	LC-MS/MS-ESI	LOD: 0.1-3.09 ng/mL; -	1.8-133%	(79)
Mephedrone	2 mL blood	Extraction with TBME TFAA derivatization	Stationary phase: HP-5 (17 m x 0.2 mm, 0.33 µm) Mobile phase: Helium	GC-MS-EI	LOD: 0.006 mg/L; LOQ: 0.02 mg/L	-	(70)

Table 2 - Bioanalytical procedures for determination of synthetic cathinones and phenethylamines in urine (2014 to present).

Analytes	Sample Amount	Sample Preparation	Stationary and mobile phase (when applicable)	Detection mode	LOD; LOQ	Recovery	References
Cathinone, Methcathinone, Methylone, Methedrone, 2C-H, Butylone, Ethylamphetamine, Mephedrone, BDB, 4-MEC, 4-MTA, 2C-B, MDPV, DOM, DOB, 2C-I, 2C-T-2, DOET, 2C-T-4, 2C-T-7 (plus amphetamines, Ketamine, Ritalinic acid, piperazines and piperidines)	200 µL	SALLE (with ACN)	Stationary phase: C18 column (4.0 mm x 2.0 mm) Mobile phase A: 5 mM ammonium buffer adjusted to pH 4 with formic acid; Mobile phase B: pure ACN with 0.1% formic acid	UPLC-QTOF-MS-ESI	LOD: 1-9 µg/L; LOQ: 1-21 µg/L	23.0-62.1%	(80)
2C-B, 2C-C, 2C-D, 2C-E, 2C-H, 2C-I, 2C-T-2, 2C-T-4, 2C-T-7, 4-MTA, DOB, DOC, DOET, DOI and DOM	2 mL	SPE - PolyChrom Clin II columns	Stationary phase: C18 column (100 mm x 2.1 mm, 1.8 µm) Mobile phase A: 50 mM ammonium acetate in deionized water/MeOH (95:5); Mobile phase B: 50 mM ammonium acetate in a mixture of ACN/deionized water (90:10)	LC-MS/MS-ESI	LOD: 0.5 ng/mL (except 2C-B: 1 ng/mL); LOQ: 0.5 ng/mL (except 2C-B: 1 ng/mL)	64-93%	(81)
2C-B, 2C-D, 2C-E, 2C-H, 2C-I, 2C-P, 2C-T-2, 2C-T-4, 2C-T-7, 4-MEC, 4-MTA, Butylone, Cathinone, DOB, DOET, DOM, Ethcathinone, Ethylamphetamine, Ethylone, MDPPP, MDPV, Mephedrone, Methcathinone, Methedrone, Methylone, Naphyrone, Norephedrine, Pyrovalerone (plus amphetamines, piperazines and indanes)	250 µL	LLE (with ethyl acetate)	Stationary phase: Synergi Polar-RP column (100 mm x 2.0 mm, 2.5 µm) Mobile phase A: 0.1% formic acid in 10 mM aqueous solution of ammonium formate; Mobile phase B: 0.1% formic acid in MeOH	LC-MS/MS-ESI	-; LOQ: 1-10 ng/mL	-	(64)

Methcathinone, 3-FMC, 4-FMC, Methylone, Ethcathinone, Ethylone, Methedrone, Buphedrone, Butylone, Mephedrone, Eutylone, 4-MEC, MDPBP, Pentedrone, Pentylone, 3,4-DMMC, α-PVP, 4-EMC, MPBP, MDPV, Pyrovalerone and Naphyrone.	1 mL	SPE - PolyChrom Clin II columns	Stationary phase: C18 column (100 mm x 2.1 mm, 2.7 µm) Mobile phase A: 0.1% formic acid in deionized water; Mobile phase B: 0.1% formic acid in ACN	LC-QTOF-MS-ESI	LOD: 0.25-5 ng/mL; LOQ: 0.25-5 ng/mL	-	(76)
Methylone, ethylone, butylone, mephedrone, 4-methylethcathinone and MDPV	90 µL	Precipitation with MeOH	Stationary phase: C18 column (50 mm x 2.1 mm, 3.5 µm) Mobile phase A: 0.1% formic acid in ACN; Mobile phase B: 0.1% formic acid in water	LC-MS/MS-ESI	LOD: 3 ng/mL; LOQ: 10 ng/mL	75.1-88.5%	(68)
4-MTA, Bromo-DragonFLY, DOB, DOET, DOM, Ethylamphetamine, 2C-B, 2C-H, 2C-I, 2C-T-2, 2C-T-4, 2C-T-7, Mescaline, Ethylone, Mephedrone, Methcathinone, Methedrone, Methylone, MDPV (plus amphetamines, opiates, cocaine and metabolites, ketamine and metabolites/analogues, benzodiazepines and metabolites, cannabinoids, piperazines, tryptamines, mitragynine, salvinorin A, and other conventional drugs of abuse)	1 mL	SPE – Oasis MCX cartridges	Stationary phase: C18 column (100 mm x 3.0 mm, 1.8 µm) Mobile phase A: 5 mM ammonium formate, 0.1% formic acid in water; Mobile phase B: 100% MeOH.	LC-MS/MS-ESI	LOD: 1-250 ng/mL; -	-	(82)
5-APDB, 6-APB, 3,4-DMC, 4-FMC, 4-MEC, 4-Methoxy-α-PVP, 4-Methoxymethcathinone, 4-MPBP, α-	100 µL	SPE - SOLA SCX cartridges	Stationary phase: C18 column (100 mm x 2.1 mm, 2.6 µm) Mobile phase A:	LC-HRMS-HESI	LOD: 1-5 µg/L; LOQ: 2.5-5 µg/L	-	(83)

Ethylaminopentiophenone, α-PBP, α-PPP, α-PVP, α-PVT, Benzedrone, Buphedrone, Butylone, Cathinone, Diethylcathinone, Ethylcathinone, Ethylone, MDPBP, MDPPP, MDPV, Mephedrone, Methcathinone, Methylone, MPHP, Naphyrone, Pentedrone, Pentylone, PV8, Pyrovalerone, 4-MEC metabolite, Normaphedrone, Buphedrone ephedrine (plus amphetamines, antidepressants and piperazines)			0.1% formic acid in water; Mobile phase B: 0.1% formic acid in ACN				
Mephedrone	2 mL	Extraction with TBME TFFA derivatization	Stationary phase: HP-5 (17 m x 0.2 mm, 0.33 µm) Mobile phase: Helium	GC-MS-EI	LOD: 0.006 mg/L; LOQ: 0.02 mg/L	-	(70)
Cathinone, Buphedrone, Mephedrone, 4-Methylethcathinone, Methedrone, 3-FMC, Flephedrone, Ethylone, Methylone, Ephedrone, MDPV and Naphyrone	10 mL	SPE – Supel-Select SCX cartridges	-	MEKC-ESI-MS/MS	LOD: 10-67 ng/mL; LOQ: 13-106 ng/mL	25.5-137.0%	(84)
Mephedrone, methylone, butylone, ethylone, pentylone and MDPV	2 mL	SPE – SPEC® DAU Derivatization with HFBA	Stationary phase: HP-5MS (30 m x 0.25 mm, 0.25 µm) Mobile phase: Helium	GC-MS-EI	LOD: 5 ng/mL (except MDPV: 20ng/mL);	82.34-104.46%	(85)

Table 3 - Bioanalytical procedures for determination of synthetic cathinones and phenethylamines in hair (2014 to present).

Analytes	Sample Amount	Sample Preparation	Stationary and mobile phase (when applicable)	Detection mode	LOD; LOQ	Recovery	References
Butylone, Ethylone, Flephedrone, Mephedrone, Methylone, Methedrone, 4-MEC, MDPV, Pyrovalerone (plus amphetamines)	10 mg	SPE – CSDAU206	Stationary phase: C18 column (50 mm x 2 mm, 5 µm) Mobile phase A: deionized water containing 0.1 % formic acid; Mobile phase B: ACN containing 0.1% formic acid	LC-MS/MS	LOD: 0.05 ng/mg; LOQ: 0.1 ng/mg	95% (±2%)	(86)
Methylone, Ethcathinone, Buphedrone, Amfepramone, Butylone, Mephedrone, 6-APB, 4-MEC, Pentedrone, α-PVP, MDPV, 2C-B, 25H-NBOMe, 2C-P, 25C-NBOMe, 25B-NBOMe, 25I-NBOMe (plus other stimulant, psychedelic, and dissociative designer drugs)	25 mg	MeOH, 55 °C (15 hours)	Stationary phase: C18 column (100 mm x 2.1 mm, 1.8 µm) Mobile phase variation from 95:5 to 45:55 (A:B, v/v) – Solvent A: water/formic acid 5 mM; Solvent B: ACN/MeOH 80:20 plus formic acid 5 mM	UHPLC-MS/MS-ESI	LOD: 0.9-17 pg/mg; LOQ: 1.8-35 pg/mg	79-115%	(65)
4-MEC and MDPV	20 mg	LLE (with a mixture of hexane:ethyl acetate (1:1) and carbonate buffer)	Stationary phase: Hypersil GOLD PFP column (100 mm x 2.1 mm, 1.9 µm) Mobile phase: gradient of ACN and formate buffer (2 mmol/L formate in formic acid 0.1%) starting from 20% of ACN to 90%	LC-MS/Ms-ESI	LOD: 0.5 pg/mg; LOQ: 1 pg/mg	66% (4-MEC) and 87% (MDPV)	(87)
3,4-DMMC, 4-MEC,	30 mg	Incubation under sonication	Stationary phase: C18	UHPLC-ESI-	LOD: 2-20	2-90%	(66)

Buphedrone, Butylone, Cathine, Cathinone, Ethcathinone, Ethylone, Flephedrone, MDPV, Mephedrone, Methedrone, Methylone, MTA, Naphyrone, Pentedrone, Pentylone (plus amphetamines, other stimulants and synthetic cannabinoids)		overnight at 45°C with MeOH (Synthetic cannabinoids) or with formic acid 0.1% (Cathinones, ketamine, piperazines, stimulants and ATS)	column (100 mm x 2.1 mm, 2.6 µm) Mobile phase A: 5 mM ammonium formate containing 0.1% formic acid; Mobile phase B: MeOH/ACN 1:1 with 0.1% formic acid	MS/MS	pg/mg; LOQ: 5-20 pg/mg		
DOM, DOET, DOB, 2C-B, 2C-I, 2C-T-2, 2C-T-7 (plus amphetamines)	100 mg	Incubation with 1% HCl in MeOH at 45°C (24 hours)	Stationary phase: Kinetex PFP column (75 mm x 2.1 mm, 2.6 µm) Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in ACN	LC-MS/MS-ESI	LOD: 0.03-0.07 ng/mg; LOQ: 0.09-0.20 ng/mg	77-103%	(88)

Table 4 - Bioanalytical procedures for determination of synthetic cathinones and phenethylamines in oral fluid (2014 to present).

Analytes	Sample Amount	Sample Preparation	Stationary and mobile phase (when applicable)	Detection mode	LOD; LOQ	Recovery	References
Cathine, Cathinone, Methcathinone (plus ephedrine)	0,5 mL	LLE (with ethyl acetate); Derivatization with HFBA	Stationary phase: VF-5MS (30 m x 0.25 mm) Mobile phase: Helium	GC-MS-EI	LOD: 10 ng/mL; LOQ: 20 ng/mL	-	(89)
Methylone, Dimethylcathinone, Buphedrone, Methedrone, Butylone, Ethcathinone, Mephedrone, 4-MEC, Methoxetamine, α-PVP, 2C-B, 3,4-MDPV (plus piperazines and synthetic cannabinoids)	90 µL	MEPS – C18	Stationary phase: C18 column (100 mm x 2.1 mm, 1.7 µm) Mobile phase A: water with 5 mM formic acid; Mobile phase B: MeOH/ACN (80:20, v/v) containing 5 mM formic acid	UHPLC-MS/MS-ESI	LOD: 0.005-0.850 ng/mL; LOQ: 0.015-2.600 ng/mL	31-96%	(67)
Cathinone, Methcathinone, Mephedrone (plus amphetamines)	0.5 mL	LLE (with NaOH and ethyl acetate); Derivatization with HFBA, PFPA, or TFAA	Stationary phase: HP-5MS (30 m x 0.25 mm, 0.25 µm) Mobile phase: Helium	GC-MS-EI	n.a; LOQ: 2.5-10 ng/mL	-	(90)
MDPV	500 µL	LLME (with chloroform)	-	Ion Mobility Spectrometry	LOD: 4.4 ng/mL; LOQ: 14.5 ng/mL	97% (±6%)	(91)
Methylone, ethylone, butylone, mephedrone, 4-MEC and MDPV	90 µL	Precipitation with MeOH	Stationary phase: C18 reversed-phase column (50 mm x 2.1 mm, 3.5 µm) Mobile phase A: 0.1% formic acid	LC-MS/MS-ESI	LOD: 3 ng/mL; LOQ: 10 ng/mL	75.1-88.5%	(68)

			in ACN; Mobile phase B: 0.1% formic acid in water				
Methylone, Methedrone, FMC, Mephedrone, MDPV (plus piperazines and amphetamines)	0,5 mL	SPE – Strata X cartridges	Stationary phase: T3 reversed-phase column (50 mm x 2.1 mm, 3 µm) Mobile phase A: formic acid .1%; Mobile phase B: ACN	LC-MS/MS-ESI	LOD: 0.025-0.1 ng/mL; LOQ: 0.2-0.5 ng/mL	87.9-134.3%	(92)
Cathinone, ephedrone, methylone, flephedrone, methedrone, DOB, 2C-T-2, DOET, 2C-T-7, naphyrone, 25C-NBOMe, 25B-NBOMe, 25T4-NBOMe, 4-MEC, butylone, pentedrone, 4-MTA, PVP, 2C-B, MDPV (plus piperazines and amphetamines)	100 µL	Precipitation with ACN	Stationary phase: F5 column (50 mm x 3 mm, 2.6 µm) Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in acetonitrile	UHPLC-MS/MS-ESI	LOD: 1 ng/mL; LOQ: 2.5 ng/mL	-	(93)

n.a: not available

Table 5 - Bioanalytical procedures for determination of synthetic cathinones and phenethylamines in other biological matrices (2014 to present).

Analytes	Sample Amount	Sample Preparation	Stationary and mobile phase (when applicable)	Detection mode	LOD; LOQ	Recovery	References
DOB, DOM, Mephedrone, Methcathinone, Methedrone, PMA, 2C-B, 2C-H, 2C-I, 2C-T-2, 2C-T-4, 2C-T-7	100 µL vitreous humor 250 µL pericardial fluid	SPE – Oasis MCX cartridges MBTFA + Microwave derivatization	Stationary phase: HP-5 MS column (30 m x 0.32 mm x 0.25 mm) with 5% phenylmethylsiloxane Mobile phase: Helium	GC-MS-EI	LOD: 5 ng/mL; LOQ: 5 ng/mL	76.6-112.8%	(69)
2C-B, 2C-I, 2C-T-2, 2C-T-7, DOB, DOET, DOM, PMA (plus amphetamines)	0,5 mL amniotic fluid	SPE - Supel™-Select HLB columns	Stationary phase: C18 column (50 mm x 2.1 mm, 2.6 µm) Mobile phase: Solvent A – 0.1% formic acid in water; Solvent B – 0.1% formic acid in ACN	LC-MS/MS-ESI	LOD: 3-6 ng/mL; LOQ: 9-20 ng/mL	72.3-96.1%	(71)

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Chapter 2: Experimental

Introduction

The last report published by the *European Monitoring Centre for Drugs and Drug Addiction* (EMCDDA) (2017) referred that new psychoactive substances (NPS) continue to represent an enormous threat to public health, mostly among the young population. The main issues associated with the investigation of these kind of substances are related to the difficulty of their detection, identification and quantification in a variety of biological specimens. By the end of 2016, 66 NPS had been detected for the first time in Europe and 620 more were being monitored, from which around 70% had appeared in the previous five years. Together, the phenethylamines and the synthetic cathinones represent the second largest monitored group of NPS by the EMCDDA and were the more apprehended psychoactive substances in 2015 (1,2).

Phenethylamines are a broad and diverse class of substances that includes psychedelics, stimulants, anorectics, bronchodilators and anti-depressants. Despite their similarity in chemical structure with the traditional amphetamines, slight changes can lead to variations in their hallucinogenic effects, potency and consumer's symptoms (3–5). One possible way to divide them is in three different main classes: dimethoxyphenylpropanamines, dimethoxyphenylethanamines and beta-keto compounds (4–10).

Cathinones derivatives are related to the phenethylamine family, differing only in a keto group at the beta carbon. Most of them are ring-substituted derivatives of the natural compound (cathinone) found in the leaves of *Catha edulis* plant (*khat*) (5,11,12). Since the mid-2000s, several cathinone derivatives with different substituents appeared in the European drug market with the intent to mimic the effects of the already controlled substances (such as cocaine and amphetamines). Despite the higher polarity caused by the keto group, which decreases their ability to cross the blood-brain barrier, they are able to reproduce the same effects on the Central Nervous System. Generally, phenethylamines induce the release of monoamines and inhibit the reuptake of neurotransmitters, blocking their transporters (5,7,13). All cathinones, including synthetic derivatives, inhibit monoamine transporters, stimulate the release of norephedrine, serotonin and dopamine and inhibit their reuptake. However, their selectivity for norephedrine (NET), serotonin (SERT) and dopamine (DAP) transporters varies from substance to substance (7,11,12,14,15). Clinically, the most common effects associated to the consumption of these two classes of substances includes psychopathological, neurological and cardiovascular symptoms: hallucinations, euphoria, seizures, palpitations, hypertension, psychomotor agitation, delusions, psychosis, headaches and chest pain (2,5,7,11–22).

In recent years, some analytical methods have been developed to determine phenethylamines and synthetic cathinones in several biological human matrices, namely blood, plasma, serum (23–41), urine (4,18,28,32,33,36,39,42–49), hair (50–55), oral fluid (33,56–61) and other biological or alternative matrices (38,62). All the previously were developed involving liquid (4,23,24,26–35,37,39–41,45–47,50–55,57,58,61–64) or gas (18,25,36,38,42,43,49,56,59) chromatography and the sample preparation was performed mostly by liquid-liquid extraction (24,28,30,37,42,52,56,59,63), protein precipitation (23,25–27,33,35,39,41,57) or solid-phase extraction (4,18,28,29,32,34,38,40,43–50,61,62).

The GC-MS technique presents several advantages but a derivatization step is important and often required for this analysis, in order to improve the detectability and stability of the substances (65). The utilization of a microwave technique rather than the conventional heating block has been used by some authors in different matrices (38,64,66–70), but has not yet been established among the majority of the scientific community (71).

The aim for this work was the development of a methodology able to detect, confirm and quantify phenethylamines and synthetic cathinones in blood samples, with a fast derivatization process that results in a considerably decrease of the time needed for the analysis. For instrumental analysis, we have chosen the gas chromatography with a single quadrupole mass spectrometer, since it is the chromatographic equipment more commonly available in most of the laboratories. On the other hand, this instrumental system was found adequate for the determination of the studied compounds in biological samples.

Material and Methods

Reagents and materials

Analytical reference materials were used for the validation study and routine analysis: l-cathinone (l-norephedrone), methylone (2-methylamino-1-(3,4-methylenedioxyphenyl)propan-1-one) and bromo-dragonFLY (1-(4-bromofuro[2,3-f][1]benzofuran-8-yl)propan-2-amine) were obtained from Cerilliant (Round Rock, TX, USA) in solution at a concentration of 1 mg/mL; 2-(2,5-dimethoxy-4-propylphenyl)ethanamine (2C-P), 4-methylthioamphetamine (4-MTA), buphedrone (2-(methylamino)-1-phenylbutan-1-one), ethylone (2-ethylamino-1-(3,4-methylenedioxyphenyl)propan-1-one), flephedrone (4-fluoromethcathinone), 3,4-methylendioxypyrovalerone (MDPV) and alpha-pyrrolidinovalerophenone (alpha-PVP) were purchased from Lipomed AG (Arlesheim, Switzerland) in solution at a concentration of 1 mg/mL; and pentylone (2-(Methylamino)-1-(3,4-methylenedioxyphenyl)pentane-1-one was obtained from LGC (Teddington, UK) in solution at a concentration of 1 mg/mL. The deuterated internal standards (IS) used (amphetamine-d6, methamphetamine-d9, MDMA-d5, MDEA-d5 and MBDBD-d5) were acquired from Cerilliant (Round Rock, TX, USA) in methanolic solution at 1 mg/mL. All reagents and solvents were obtained from Merck Co (Darmstadt, Germany). Water, methanol, dichloromethane, 2-propanol and n-hexane were of ACS grade

and ammonium hydroxide, hydrochloric acid and potassium dihydrogen phosphate were of analytical grade. The derivatization reagent used for the acylation reaction was n-methyl-bis(trifluoroacetamide) (MBTFA), which was purchased from Macherey –Nagel GmbH & Co (Düren, Germany).

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

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

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

Instrumentation

Gas chromatography-mass spectrometry analysis (GC-MS) were carried out on a HP 7890B system coupled to a 5977A mass selective detector (Agilent Technologies, California, USA) with a capillary column (30 m x 0,25 mm; 0,25mm film thickness) with 5% phenyl-methylsiloxane (HP-5MS).

The injection of the extracts was made in *splitless* mode with a constant flow rate of 1 mL/min with highly purified helium as carrier gas. The initial temperature of the oven was 90 ◦C (held for 2 min), with an increasing of 20 ◦C/min until reach a final temperature of 300 ◦C and held for 3 min - a total run time of 15.5 min. The mass spectrometer worked in electron ionization (EI) mode with energy of 70 eV and emission current of 300 µA. The identification of the analytes was performed in full-scan mode and subsequently specific ions were selected to confirm and quantify the substances using the selected ion monitoring (SIM) mode (Table 6). To confirm the presence of the substances, characteristic fragment ions must be extracted. The criteria for the identification of the analytes was established according to the World Anti-Doping Agency recommendations (72): the relative retention time of each substance must be within a 1% window or 0.2 min in absolute terms (for the same substance in a QC sample prepared and analysed contemporaneously). Positive peaks have to include at least three ions and their relative intensities should not differ by more than a tolerated amount comparing with those generated by the same compound in a QC sample prepared and analysed contemporaneously: if the relative intensity of the ions is between a range of 25-50% 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 lower 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.

Table 6 - 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	6.25	144	-	-
Methamphetamine-d9	6.98	161	-	-
Cathinone	7.09	105	77	140
Flephedrone	7.12	123	154	110
Buphedrone	7.65	168	105	110
4-MTA	8.90	137	164	277
MDMA-d5	9.07	158	-	-
Alpha-PVP	9.24	126	127	77
MDEA-d5	9.31	173	-	-
MBDB-d5	9.43	172	-	-
Methylone	9.45	149	121	154
2C-P	9.67	193	206	177
Ethylone	9.85	149	168	140
Pentylone	10.10	149	140	182
MDPV	11.16	126	127	149
DragonFLY	11.29	249	276	389

Sample Preparation

Samples of blood (500 µL) were prepared by the addition of 4 mL of 0.1 M phosphate buffer (pH 4.4) and 25 µL of IS solution. Posteriorly, samples were homogenized and centrifuged at 3000 rpm for 10 min.

Solid Phase Extraction

The aqueous phases obtained were added to the extraction cartridges, previously conditioned with 2 mL of methanol and 2 mL of deionized water. After passing the samples through the cartridges, these were washed, sequentially, with 2 mL each of deionized water, 0.1 M hydrochloric acid, a mixture of dichloromethane:methanol (70:30, v/v), and, at least with 3 mL of *n*-hexane. After drying the cartridges under full vacuum, the compounds were eluted with 3 mL of dichloromethane:2-propanol:ammonium hydroxide (78:20:2, v/v/v). Then, the eluates were collected, 30 µL of MBFTA was added and the extracts were dried under nitrogen stream at 40 ◦C. Finally, 60 µL of MBTFA was added to the dried extracts, vortexed for approximately 20 s and derivatized in a microwave oven during 90 s at maximum potency

(900W). After cooling down the tubes at room temperature, the extracts were transferred to vials and placed in the GC autosampler to inject a 2 µL aliquot into the GC-MS system.

Calibrators, quality controls and internal standards

Working standard solutions with all substances were prepared to obtain the calibrators by dilution of the stock solutions with methanol to the proper concentrations (0.5, 5 and 50 µg/mL). Additional working solutions for quality control samples (QC) were prepared at the same concentrations. The working solution of IS was also prepared with methanol at a concentration of 5 µg/mL. All these solutions were stored at a temperature between 2 and 8 ◦C and protected from light.

Derivatization procedure

A domestic microwave oven (Candy CMG 25D CW) with a potency of 900W was used for the derivatization procedure.

According to previous studies, and comparing with the conventional reaction heating block at 80◦C during 30 min, this method was already evaluated to prove the stability of the equipment, the potential of the heating reaction when using a microwave energy and in order to verify the uniformity of microwave irradiation during 90 s of thermally-assisted chemical reactions, proving that this technique is suitable for the validation of methods, offering a considerable reduction of time for the entire analysis (38).

Sample collection

Blank blood samples used in the confirmation, calibration and all the validation experiments were obtained from a local blood donation bank. These samples were stored at -15◦C and were previously screened to verify that they were drug-free, before being used both as calibrators and control samples.

Concerning authentic samples, routine samples , either from traffic legislation or autopsies performed at the Forensic Services of Clinical and Pathology of the National Institute of Legal Medicine and Forensic Sciences, Centre Branch, Portugal, from suspected intoxication of individuals with drugs of abuse or from cases with unidentified or strange situations for their deaths (e.g. road accidents, road-site testing of vehicle drivers, labour accidents, absence of apparent cause) were used.

Generally, these samples are sent to the laboratory for toxicological analysis because they are requested by a court-of-law, according to the Portuguese legislation, for clarification of death, as part of routine work.

Validation Parameters

The described and applied analytical procedure followed the accepted international guidelines of the Scientific Working Group for Forensic Toxicology (SWGTOX) on method validation (73). The selection of the substances was made according to the number of research solicitations to the toxicology service. According to EMCDDA, these two classes of compounds represent one of the largest groups of substances monitored by this agency, being commonly used for the adulteration of some classic drugs.

The fortification of blank samples of blood was used for the preparation of calibrators and QC samples. Parameters as selectivity, linear range, limits of detection (LOD) and quantitation (LLOQ), precision (intermediate and intra-day), bias, extraction efficiency, dilution effect and stability (bench-top, autosampler and short-term freeze-thaw) were studied.

Selectivity was studied to verify the presence of interferences at the retention time of monitored ions for each analysed substance and respective IS. It was also studied the method's ability to identify the analytes of interest in the presence of other routinely analysed substances at the laboratory (drugs of abuse, pesticides and medical substances). The study of this parameter was performed by the analysis of 8 different pools from different sources of blood. Three groups of samples were prepared: two positives groups (n=8, each) with spiked samples at 10 ng/mL and 40 ng/mL each, with the mixture of all the analytes of interest (cathinone, flephedrone, buphedrone, 4-MTA, alpha-PVP, methylone, 2C-P, ethylone, pentylone, MDPV and dragonFLY); the third set of samples was the negative group. One tube of each concentration of the positive sets (10 and 40 ng/mL), was contaminated with a mixture of other substances that are routinely analysed in the laboratory at 100 ng/mL. Therefore, 8 positive samples for each concentration, and 8 negative were submitted for the above mentioned methodologies.

The calibration curves (n=5/6) were constructed utilizing linear regressions with the ratio between the analyte peak area and the respective IS peak area, plotted against the corresponding theoretical concentration. The analysis of the curves follows as acceptance criteria the coefficient of correlation (r^2 values) ≥ 0,99, the inclusion of the zero at the curves intersection with the ordinates axis with a 95% confidence interval and the calibrators quantitated within ± 20%. The linear range was of 5 to 500 ng/mL (5, 10, 15, 20, 30, 40, 50, 100, 200, 300, 400 and 500 ng/mL) for cathinone, buphedrone, 4-MTA, methylone, 2C-P and dragonFLY, 10 to 500 ng/mL (10, 15, 20, 30, 40, 50, 100, 200, 300, 400 and 500 ng/mL) for flephedrone, ethylone, pentylone and MDPV and 40 to 500 ng/mL (40, 50, 100, 200, 300, 400 and 500 ng/mL) for alpha-PVP. At the same time, it was added a blank sample only with IS and QC samples (n=8), at three different concentration levels for each analyte – low (LLOQ), medium (200 ng/mL) and high (500 ng/mL) QC – to be analysed.

Carryover was also investigated by injecting a blank sample after the analysis of the highest calibrator of the curve and check for the eventual signals at the retention time and monitored ions for each compound of interest.

The LOD and LLOQ were determined by fortifying blank samples with concentrations of the calibration curve. For each substance, the LOD was determined as the concentration that results in a signal-to-noise (S/N) of at least 3 and the LLOQ as the minimum concentration measured with adequate precision (Coefficient of Variation, CV < 20%) and bias (± 20%).

The intra-day precision and bias were evaluated by the analysis of at least 5 QC samples at 5 concentration levels for compounds with LLOQ of 5 ng/mL (5, 10, 40, 200 and 500 ng/mL), 4 concentration levels for compounds with LLOQ of 10 ng/mL (10, 40, 200 and 500 ng/mL) and 3 concentrations levels for the compound with LLOQ of 40 ng/mL (40, 200 and 500 ng/mL), on the same day. Intermediate precision and bias were determined analysing, in at least 5 days, 3 QC samples for each substance –LLOQ (5, 10 or 40 ng/mL), 200 ng/mL and 500 ng/mL. For each concentration group of QC samples, the bias was calculated as the [(mean of calculated concentration – theoretical concentration)/theoretical concentration] x 100. Acceptable values of CV and bias were established (CV < 20%; Bias = ± 20%).

Extraction efficiency was evaluated by comparing two groups of QC samples with three sets of concentration each – LLOQ (5, 10 or 40 ng/mL), 200 and 500 ng/mL - for each substance in triplicate. One set of blood was spiked before the extraction procedure and the second after, before the extracts being dried under nitrogen stream (100% recovery). The extraction efficiency was calculated by (Group 1/Group 2) x 100%.

Three methods of stability were examined during this study: in the autosampler, short-term freeze/thaw and at bench-top. Autosampler stability was analysed by reinjecting all QC samples previously prepared (5, 10, 40, 200 and 500 ng/mL) and comparing them with their first injection data. This was evaluated until 72 hours in the autosampler. Short-term freeze/thaw cycles stability (n=3) was determined with spiked QC samples at 40 and 200 ng/mL, frozen and completely thawed after 7, 20 and 28 days. The analysis was made after the last storage period. For bench-top stability, two aliquots were prepared and fortified also at 40 and 200 ng/mL and left at room temperature for 24 h. Subsequently, they were extracted, analysed and compared with freshly spiked samples at the same concentration. The preparation of all samples followed the above-mentioned procedures and the acceptance criteria for the stability of these compounds included concentrations of the QC samples within ± 20% of the freshly fortified QC samples.

Results

Method validation

For the study of the selectivity of the method, all analytes were successfully identified in both groups of spiked samples and, by the analysis of the negative set of samples, no interferences were observed at the retention time and respective *m*/*z* values in the monitored ions for each substance. Therefore, and according to these results, the described

methodology was considered selective for the determination of these substances since neither the compounds added, nor the endogenous matrix constituents interfered in the analysis. According to Table 7, the method was linear, since all acceptance criteria were met. Also, no carryover was observed with this matrix for each substance.

Table 7 - Linearity results and limits of detection and quantification for all analytes studied in blood in the linear range of 5-500, 10-500 and 40-500 ng/mL.

Analytes	Linear Range (ng/mL)	Linearity			LOD/LLOQ (ng/mL)
		Slope(*)	Intercept(*)	$r^{2(*)}$	
Cathinone	5-500	1.8E-03 ± 8.8E-04	1.6E-02 ± 2.8E-02	0.998 ± 2.4E-03	5/5
Flephedrone	10-500	1.7E-03 ± 6E-04	-1.91E-03 ± 9.05E-03	0.997 ± 9.68E-04	10/10
Buphedrone	5-500	1.6E-03 ± 5.2E-04	2.1E-03 ± 1.3E-02	0.998 ± 2.9E-03	5/5
4-MTA	5-500	4.4E-03 ± 1.2E-03	-1.1E-02 ± 2.3E-03	0.999 ± 3.3E-04	5/5
PVP	40-500	1.5E-03 ± 1.1E-03	-5.8E-03 ± 3.4E-03	0.998 ± 2.1E-03	40/40
Methylone	5-500	6.6E-03 ± 8.0E-03	-1.3E-02 ± 7.4E-02	0.994 ± 8.8E-04	10/10
2C-P	5-500	3.7E-03 ± 1.7E-03	1.2E-03 ± 4.6E-03	0.995 ± 2.2E-03	5/5
Ethylone	10-500	1.1E-03 ± 7.3E-04	4.1E-03 ± 6.3E-03	0.999 ± 4.7E-04	10/10
Pentylone	10-500	3.4E-03 ± 9.4E-04	4.3E-03 ± 6.3E-03	0.999 ± 4.3E-04	10/10
MDPV	10-500	1.9E-03 ± 1.1E-03	-9.0E-03 ± 1.5E-02	0.999 ± 6.7E-04	10/10
DragonFLY	5-500	3.1E-03 ±6.2E-0.4	-8.6E-0.3 ± 2.1E-0.2	0.998 ± 9.5E-0.4	5/5

The LOD and LLOQ were of 5 ng/mL for cathinone, buphedrone, 4-MTA, methylone, 2C-P and dragonFLY, 10 ng/mL for flephedrone, ethylone, pentylone and MDPV and of 40 ng/mL for alpha-PVP. Comparing these results with other researches, in general, the limits obtained in this assay are considered to be acceptable for most of the studied substances. According to the results in Tables 8 and 9, intraday precision and bias values (CV < 7,8; Bias: -3,6 – 9,9) as well as those obtained for intermediate precision and bias (CV < 8,6; Bias: 1,9 – 7,8) were acceptable for all concentrations. Precision was analysed and expressed by the coefficient of variation (< 20%) and calculated by the one-way analysis of variation (ANOVA).

For the study of recovery and as shown in Table 10, the obtained values ranged between 70,3 ± 0,9 (dragonFLY at 5 ng/mL) and 116,6 ± 6,6 (alpha-PVP at 40 ng/mL).

Concerning stability, the analytes were found to be stable when left in the autosampler for at least 72 h. For bench-top stability, all compounds were considered to be stable at room temperature for 24h, and all substances were stable for 28 days and after 3 freeze/thaw cycles, with the exception of PVP and 2C-P, which were not stable from day 7, dragonFLY from day 20 and methylone from day 28 (Table 11).

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

Analytes	Spiked Concentration (ng/mL)														
	5			10			40			200			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 (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)
Cathinone	5.4 ± 0.1	2.1	7.4	10.0 ± 0.8	7.8	-0.2	42.5 ± 0.5	1.1	6.3	211.4 ± 8.0	3.8	5.7	509.8 ± 11.3	2.2	2.0
Flephedrone	-	-	-	10.2 ± 0.4	3.8	2.3	41.2 ± 1.3	3.2	3.0	219.2 ± 9.5	4.3	9.6	508.5 ± 6.3	1.2	1.7
Buphedrone	5.3 ± 0.2	4.0	6.7	10.3 ± 0.3	3.3	3.4	44.0 ± 3.2	7.3	9.9	219.0 ± 14.4	6.6	9.5	512.3 ± 5.4	1.1	2.5
4-MTA	5.5 ± 0.4	6.6	9.5	10.7 ± 0.8	1.8	7.0	43.7 ± 1.5	3.5	9.5	211.0 ± 4.7	2.2	5.5	511.5 ± 19.2	3.8	2.3
PVP	-	-	-	-	-	-	39.4 ± 2.1	5.4	-1.6	216.1 ± 7.3	3.4	8.1	531.0 ± 6.6	1.2	6.2
Methylone	5.3 ± 0.2	4.1	5.6	-	-	-	39.4 ± 1.6	4.0	-1.4	219.8 ± 9.5	4.3	9.9	482.1 ± 30.8	6.4	-3.6
2C-P	5.2 ± 0.4	8.1	3.6	10.4 ± 0.5	4.3	4.4	40.2 ± 1.2	2.9	0.5	211.3 ± 11.0	5.2	5.6	517.6 ± 16.5	3.2	3.5
Ethylone	-	-	-	10.9 ± 0.2	2.1	9.0	40.3 ± 1.5	3.7	0.7	213.6 ± 7.3	3.4	6.8	500.0 ± 9.2	1.8	0.0
Pentylone	-	-	-	10.4 ± 0.5	4.4	3.9	41.1 ± 0.8	2.0	2.7	213.0 ± 5.9	2.8	6.5	518.9 ± 15.4	3.0	3.8
MDPV	-	-	-	10.0 ± 0.6	5.6	0.2	41.8 ± 1.5	3.7	4.4	211.8 ± 7.2	3.4	5.9	540.8 ± 31.0	5.7	8.2
DragonFLY	5.5 ± 0.2	4.0	9.2	11.0 ± 0.2	1.9	9.6	39.6 ± 1.3	3.4	-0.9	206.1 ± 8.3	4.0	3.1	513.3 ± 18.9	3.7	2.7

Table 9 - Intermediate precision and trueness (n=6).

Analytes	Spiked Concentration (ng/mL)														
	5			10			40			200			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 (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)
Cathinone	5.3 ± 0.4	7.7	6.6	-	-	-	-	-	-	209.3 ± 17.5	8.4	4.6	509.6 ± 43.8	8.6	1.9
Flephedrone	-	-	-	10.7±0.8	7.1	6.8	-	-	-	213.7±11.1	5.2	6.9	513.9±14.6	2.8	2.8
Buphedrone	5.4 ± 0.4	6.8	7.5	-	-	-	-	-	-	209.9 ± 10.8	5.1	5.0	510.8 ± 36.2	7.1	2.2
4-MTA	5.4 ± 0.4	7.2	7.8	-	-	-	-	-	-	212.6 ± 8.2	3.9	6.3	531.1 ± 22.0	4.1	6.4
PVP	-	-	-	-	-	-	42.0 ± 2.6	6.3	5.1	207.5 ± 11.0	5.3	3.7	514.6 ± 19.4	3.8	2.9
Methylone	5.3 ± 0.3	4.9	5.0	-	-	-	-	-	-	204.7 ± 13.6	6.7	2.3	516.4 ± 33.1	6.4	3.3
2C-P	5.1 ± 0.3	5.7	3.0	-	-	-	-	-	-	214.7 ± 10.7	5.0	7.3	526.6 ± 16.7	3.2	5.4
Ethylone	-	-	-	10.8 ± 0.6	6.0	7.6	-	-	-	213.4 ± 18.2	8.5	6.7	523.8 ± 26.3	5.0	4.8
Pentylone	-	-	-	10.6 ± 0.5	4.5	6.1	-	-	-	213.7 ± 7.8	3.6	6.9	531.2 ± 19.4	3.6	6.2
MDPV	-	-	-	10.6 ± 0.9	8.2	5.9	-	-	-	209.4 ± 14.8	7.0	4.7	512.6 ± 26.9	5.2	2.5
DragonFLY	5.4 ± 0.4	7.1	7.1	-	-	-	-	-	-	214.5 ± 9.5	4.4	7.2	529.7 ± 20.4	3.9	5.9

Table 10 - Extraction efficiency.

Analyte	Extraction efficiency (mean values ± standard deviation) (n=3)				
	5	10	40	200	500
	(ng/mL)				
Cathinone	83.6±7.3	-	-	93.9±2.1	95.3±1.3
Flephedrone	-	82.0±0.9	-	93.8±2.0	91.4±1.5
Buphedrone	89.8±6.7	-	-	98.6±9.2	95.7±5.6
4-MTA	86.6±1.2	-	-	95.2±7.8	84.2±5.6
PVP	-	-	116.6±6.6	95.3±9.3	87.2±7.3
Methylone	92.8±8.6	-	-	95.4±1.0	95.9±3.7
2C-P	70.9±5.5	-	-	84.5±7.7	74.6±6.8
Ethylone	-	89.4±0.3	-	88.8±5.2	96.3±3.1
Pentylone	-	71.5±3.1	-	89.6±2.6	95.3±3.8
MDPV	-	103.3±5.4	-	95.6±5.8	83.6±7.1
DragonFLY	70.3±0.9	-	-	88.3±4.5	71.0±4.3

Table 11 - Stability data (% difference) after storage at room temperature 24h and after 3 freeze-thaw cycles.

Compounds	Bench-top 24 h (Room temp)		Freeze-thaw (-15 ºC) 7 days (3 cycles)		Freeze-thaw (-15 ºC) 20 days (3 cycles)		Freeze-thaw (-15 ºC) 28 days (3 cycles)	
	40	200	40	200	40	200	40	200
	(ng/mL)		(ng/mL)		(ng/mL)		(ng/mL)	
Cathinone	6.5	-12.8	-0.7	14.1	-9.1	17.5	-12.9	-19.2
Flephedrone	4.8	-16.9	8.0	-3.2	-6.2	-7.4	-14.3	-0.3
Buphedrone	-9.4	-8.9	16.3	11.2	-5.9	-2.1	-8.8	-6.1
4-MTA	-3.9	7.8	7.7	-5.8	-3.7	11.4	1.8	10.4
PVP	17.4	-2.1	28.1	-21.0	-	-	-	-
Methylone	-3.5	-2.9	-18.8	-12.9	-18.7	10.2	-51.5	54.5
2C-P	-16.2	-17.2	-41.6	-70.5	-	-	-	-
Ethylone	9.0	-19.4	7.0	-15.0	-3.7	3.7	-17.2	17.2
Pentylone	-12.9	-13.3	2.5	-15.7	-18.9	11.3	-16.8	5.0
MDPV	5.8	-14.2	-7.1	-8.0	-17.5	-11.0	-17.7	-15.4
DragonFLY	-14.5	-18.6	10.7	-18.8	-26.9	-21.2	-	-

Application to authentic samples

The developed methodology was applied to authentic samples resulting from autopsies performed at the Forensic Services of Clinical and Pathology of the INMLCF and from subjects under psychiatric evaluation and traffic legislation, but no positive cases were found yet.

Discussion

Several studies have been made regarding this class of substances, allowing to compare the results obtained in this assay with those from other authors.

Sørensen presents on his work LLOQs of 10 ng/mL for the cathinones: comparing with the limits achieved with this methodology, it demonstrates that our results are satisfactory (41). Comparing with Pasin et al., they developed a methodology for the analysis of new designer drugs utilizing UHPLC-Q-TOF-MS, and the LOQs obtained for the analysed cathinones ranged between 50 and 100 ng/mL (24). Also, Ambach et al., utilizing the same volume of blood and extraction procedure, yet using LC-MS/MS, achieved LOQs for 2C-P, 4-MTA and cathinone similar to those obtained in this work (28). In the work of Glicksberg et al., despite the values of LOD being slightly lower for some substances (ethylone, MDPV and methylone present values of 2 ng/mL), they utilized the triple of blood volume compared to the present study and they performed the instrumental analysis on a quadrupole time of flight equipment, which should present more sensitivity on the detection of the substances (32). Mercolini et al., developed an assay that, despite the low volume of sample used, presented similar or higher values for LOQ were obtained comparing to our work. They achieved LOQs of 10 ng/mL for methylone, ethylone and MDPV, while we have obtained lower values for methylone (33). In another study using dried blood spots, Ambach et al., developed a method for the screening of 64 NPS. The LOD values obtained were in some cases slightly lower compared to our results. In their study they achieved values of 2,5 ng/mL for methylone and 5 ng/mL for flephedrone (versus those obtained in this work, of 5 and 10 ng/mL respectively). However, it is important to take into consideration that they used a liquid chromatographer for instrumental analysis, an equipment normally more sensitive compared to a GC. Besides, the LOD for the detection of cathinone is 10 ng/mL, a value higher than ours for that same substance (5 ng/mL) (27).

Relatively to the results obtained in this assay for the study of the recovery, they are in agreement with other procedures developed. Comparing the values of recovery achieved by Glicksberg *et al.* (83-88%), similar or higher recoveries were obtained for the same substances with our assay (32). Besides that, also Sørensen *et al.* in his work achieved similar recoveries (96-102%) to ours (41). Vaiano *et al.*, with the development of a methodology for a screening of 64 NPS, obtained recoveries ranging between 72 and 110% for all substances. However, analysing the substances in common with this assay, it is verified that, between Vaiano *et al.* study and the present work, the values obtained are similar, or even higher, as in case of buphedrone using our assay (23). Pasin *et al.*, presented a procedure for the detection and quantitation of 37 new designer drugs: their methodology allowed them to achieve recoveries ranging from 71 to 100%, which is in accordance with the work here presented. Comparing the substances in common with our study (cathinone, buphedrone, flephedrone, methylone, ethylone, pentylone, PVP and MDPV) all recoveries are similar, with the exceptions of

methylone, which is significantly lower (82-84%) compared to our work (92-95%), and ethylone and pentylone which presentes slightly higher values (24). The group of Montesano *et al*. have developed a method for the identification of a broad group of NPS and the recoveries obtained were over 75% which is compatible to our study since our smallest value is slightly lower (70% for dragonFLY) (26).

Concerning stability, according to other authors which studied some of the compounds, it was demonstrated that the results achieved fulfil the criteria of stability for these samples at room temperature. Glicksberg et al. refers that flephedrone, buphedrone, ethylone, methylone, pentylone, α-PVP and MDPV appeared to be stable during several days at 20 ºC in blood, until a 20% loss of the analyte was observed (74). Tang et al. developed a study of a large group of conventional and emerging drugs of abuse in urine samples, which refers that substances as 4-MTA, bromo-dragonFLY, cathinone, ethylone and methylone, are stable for 1 day at room temperature (45). Also Habrdova et al., in addition to the other substances, shows that 2C-P is stable for 3 hours when left at room temperature (70).

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Conclusions

An analytical methodology was developed and validated to detect and quantify a group of eight synthetic cathinones (cathinone, flephedrone, buphedrone, α-PVP, methylone, ethylone, pentylone and MDPV) and three phenethylamines (4-MTA, 2C-P and dragonFLY) using a mixed-mode solid phase extraction for sample preparation followed by a time saver derivatization process consisting on the utilization of a domestic microwave, to extract the analytes from 500 µL of whole blood. The qualitative and quantitative analysis determination was made by a gas chromatographer coupled to single quadrupole mass spectrometer equipped with a source of electronic impact (GC/MS-EI).

The methodology developed was validated following the international guidelines of the Scientific Working Group for Forensic Toxicology (SWGTOX) and parameters as selectivity, linearity, limits of detection and quantitation, precision, accuracy, extraction efficiency, stability and dilution factor were evaluated.

The developed method was linear between 5 to 500 ng/mL to cathinone, buphedrone, 4-MTA, methylone, 2C-P and dragonFLY, 10 to 500 ng/mL to flephedrone, ethylone, pentylone and MPDV and 40 to 500 ng/mL to α-PVP, with adequate precision and accuracy for all substances according to the mentioned guidelines. Despite the limits obtained for some substances, high values of recovery were obtained for all substances (between 70 and 116%). In addition, all substances demonstrated to be stable at the autosampler for 72 hours, at bench-top for 24 hours and except for α-PVP and 2C-P (unstable at day 7), dragonFLY and methylone (unstable starting day 20 and 28, respectively), all the other substances were stable at the end of 28 days for the study of freeze/thaw stability.

According to the obtained results, it is safe to admit that developed methodology utilizing this type o sample preparation can be applied as an alternative to conventional methods, with the main advantage of a fast derivatization procedure.

It should be noticed too that this is the first study which this procedure is applied for these group substances in blood samples.

Attachments

The present dissertation was disseminated in some congresses in the area of chemistry and toxicology, as well was submitted to publication.

Presentation in congress:

OTIMIZAÇÃO DE UMA METODOLOGIA PARA A DETEÇÃO DE CATINONAS E FENILETILAMINAS EM AMOSTRAS BIOLÓGICAS POR GC/MS-EI (Poster)
Pedro Almeida, Alice Castanheira, João Franco, Francisco Corte Real, Eugenia Gallardo, Cláudia Margalho
XVI Congresso Nacional de Medicina Legal e Ciências Forenses, Coimbra, Novembro 2017

DESENVOLVIMENTO DE UMA METODOLOGIA PARA A IDENTIFICAÇÃO DE NOVAS SUBSTÂNCIAS PSICOATIVAS EM AMOSTRAS DE SANGUE (Poster)
Pedro Almeida, Alice Castanheira, Eugenia Gallardo, Cláudia Margalho
VI Encontro Nacional de Estudantes de Química, Covilhã, Março 2018

ANALYTICAL APPROACH TO DETERMINE NEW PSYCHOACTIVE SUBSTANCES IN WHOLE BLOOD USING SOLID PHASE EXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY (Poster)
Pedro Almeida, Alice Castanheira, Francisco Corte Real, Eugenia Gallardo, Cláudia Margalho
The International Association of Forensic Toxicologists, Ghent (Belgium), August 2018