Psychoactive substances present in "legal highs" acquired in "smartshops" or via Internet

Chemical characterization and *in vitro* cytotoxicity studies of synthetic cathinones

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- **Araújo, Ana Margarida; Bastos, Maria de Lourdes; Carvalho, Márcia; Carvalho, Félix; Gaspar, Helena; Cardoso Matias, Ana; Guedes de Pinho, Paula.** (2013) Identification of psychoactive substances in "legal highs" marketed in Portugal. *Toxicology Letters, Abstracts*. 221S: S59-S256.

ABSTRACT

The world's culture on recreational drugs has recently changed and the consumption of new psychoactive substances, known as "legal highs", is continuously growing, especially among young people. These drugs are typically sold via Internet or "smartshops" as legal alternatives to controlled substances, being announced as "bath salts" and "plant feeders" and claiming to be not intended for human consumption. The astonishing speed at which these compounds appear makes detection, identification and the associated health risks and potential harm difficult. Information about the actual content of these products is still very scarce, raising concern of consumer safety. Although the illusion of being substances more pure and safe for consumption, the number of fatalities has increased, with clinical patterns comparable to those reported for better-studied stimulant drugs like amphetamines.

The purpose of this study is to chemically characterize commercial products marketed as "plant feeders" in Portuguese "smartshops", using highly powerful methodologies, such as GC-MS (Gas Chromatography coupled to Mass Spectrometry) and NMR (Nuclear Magnetic Resonance) analysis. It is intended to further evaluate the hepatotoxic effects of two commercial "legal high" products and individual cathinone derivatives, using HepaRG cells and primary rat hepatocytes cultures as *in vitro* models.

NMR spectroscopy, the most useful analytical technique for determining the structure of molecules, in combination with the highly sensitive analytical methodology GC-MS were applied to unveil the real composition of these products. The main active compounds identified in methanolic solutions of the marked products are synthetic cathinones, namely methedrone, methylone, buphedrone, flephedrone, pentedrone, 4-methylethylcathinone (4-MEC), ethcathinone, methylenedioxypyrovalerone (MDPV), but also aminoalkyl benzofurans derivatives, cocaine analogs (dimethocaine) and caffeine. The content differs between the products; some containing only one active compound, while others have a greater variety. Qualitative and quantitative variability was found between identical products sold in different "smartshops".

In cytotoxicity studies, cells were exposed to the test drugs (0.05 – 5mM), at 37°C for 48 hours. MDMA was used as a reference for comparison effects. Results show that for all agents tested there is an increase of cell death dependent of chemical concentration. In both cell models, methylone proved to be the least hepatotoxic individual agent and MDPV the most potent. The commercial "legal highs" studied ("Bloom" and "Blow") induced a severe hepatotoxicity, with EC_{50} values significantly lower or equal to MDMA, respectively. Mathematical models of independent action (IA) and concentration addition (CA) were applied for modeling the expected effects of "legal highs" mixtures; the exposure of primary hepatocytes

to "Bloom" shows an obvious synergism, while "Blow" reveals an additive effects. Primary rat hepatocytes showed to be more sensitive for hepatotoxicity evaluation since the HepaRG are metabolically less competent.

In synopsis, these results show a miscellany of psychoactive compounds present in "legal high" products marketed in Portugal, with hepatotoxic effects extremely pronounced. In addition, potential harmful interactions among synthetic cathinones are expected when these drugs are taken concomitantly. These data contributes to unveil the health hazards likely to arise from exposure to these emerging psychoactive drugs and may influence behavioral changes in consumers worldwide.

Key words:

Legal highs – Synthetic cathinones – GC-MS – NMR – Hepatotoxicity – Primary rat hepatocytes – HepaRG cell line

A cultura do mundo das drogas recreativas mudou recentemente e o consumo de novas substâncias psicoativas, conhecidas como "drogas legais", tem vindo a crescer continuamente, especialmente entre os jovens. Estas novas drogas são normalmente vendidas através da Internet ou "smartshops" como alternativas legais para substâncias controladas, sendo anunciadas como "sais de banho" e "fertilizantes para plantas", afirmando não serem destinadas ao consumo humano. A velocidade estonteante a que estes compostos aparecem torna a sua detecção, identificação e avaliação dos riscos de saúde associados e dano potencial difíceis. Informações sobre o conteúdo real destes produtos são ainda muito escassas, conduzindo a um aumento da preocupação com a segurança dos consumidores. Embora exista a ilusão de serem substâncias mais puras e seguras para o consumo, o número de mortes aumentou, com padrões clínicos comparáveis aos relatados para drogas estimulantes bem estudadas, como é o caso das anfetaminas.

Um dos objectivos deste estudo passa por caracterizar quimicamente produtos comercializados como "fertilizantes para plantas" à venda em "smartshops" portuguesas, utilizando metodologias altamente poderosas, tais como a análise por GC-MS (cromatografia gasosa acoplada à espectrometria de massa) e RMN (espectroscopia por ressonância magnética nuclear). O trabalho destina-se ainda a avaliar os efeitos hepatotóxicos de dois produtos comerciais adquiridos em "smartshops" e de derivados de catinonas vendidos como agentes individuais através da Internet, usando células HepaRG e cultura primária de hepatócitos de rato como modelos *in vitro*.

A espectroscopia por RMN, uma técnica analítica útil na determinação da estrutura de moléculas, em combinação com uma metodologia analítica altamente sensível, o GC-MS, foram aplicadas para desvendar a composição real destes produtos. Os principais compostos ativos identificados em soluções metanólicas foram catinonas sintéticas, nomeadamente a metedrona, metilona, bufedrona, flefedrona, pentedrona, 4-metiletilcatinona (4-MEC), etcatinona, metilenodioxipirovalerona (MDPV), mas também derivados dos aminoalquil benzofuranos, análogos da cocaína (dimetocaína) e cafeína. O conteúdo entre os produtos mostrou-se variável; alguns contendo apenas um composto ativo, enquanto outros têm uma maior variedade. Foi encontrada variabilidade qualitativa e quantitativa entre produtos idênticos vendidos em "smartshops" diferentes.

Nos estudos de citotoxicidade, as células foram expostas a drogas teste (0.05 – 5mM) a 37°C, durante um período de incubação de 48h. A MDMA foi usada como composto de referência para comparação de efeitos de hepatotoxicidade. Os resultados mostram que para todos os agentes testados existe um aumento da morte celular dependente da concentração química. Nos

dois modelos celulares, a metilona mostrou-se o agente individual menos hepatotóxico e o MDPV o mais potente. As "drogas legais" estudadas ("Bloom" e "Blow") induziram severa hepatotoxicidade, com um valor de EC_{50} consideravelmente inferior e similar aos da MDMA, respectivamente. Modelos matemáticos de ação independente (IA) e adição da concentração (CA) foram aplicados para calcular os efeitos expectáveis das misturas de "drogas legais"; a exposição dos hepatócitos primários à mistura de "Bloom" mostra um evidente sinergismo, enquanto que o "Blow" revela uma situação de aditividade de efeitos. Os hepatócitos primários de rato mostraram ser mais sensíveis na avaliação da hepatotoxicidade, uma vez que as HepaRG parecem ser metabolicamente menos competentes.

Em resumo, estes resultados mostram uma miscelânea de compostos psicoativos presentes nos produtos de "drogas legais" comercializados em Portugal, com efeitos hepatotóxicos muito pronunciados. Mostram ainda a existência de interações potencialmente perigosas entre catinonas sintéticas administradas concomitantemente. Esta informação é crucial para revelar os riscos para a saúde susceptíveis de resultar da exposição a estas drogas psicoativas emergentes, podendo também influenciar mudanças de comportamento de consumidores de todo o mundo.

Palavras-chave:

Drogas Legais – Catinonas Sintéticas – GC-MS – NMR – Hepatotoxicidade – Hepatócitos primários de rato – Linha celular HepaRG

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Abbreviations List Index

1D – First dimension

1H NMR – Proton NMR

2D – Two-dimensional

3,4-DMMC – 3,4-Dimethylmethcathinone

3-MEC – 3-Methylethcathinone

4-MEC – 4-Methylethcathinone

4-MMC – Mephedrone

5-APB – 5-(2-Aminopropyl)benzofuran

5-APDB – 5-(2-Aminopropyl)2,3-dihydrobenzofuran

5-HT – 5-hydroxytryptamine or serotonin

6-APB – 6-(2-Aminopropyl)benzofuran

13C NMR – Carbon 13 NMR

°C – Celsius degrees

°C/min – Celsius Degrees per minute

µA – Microampere

µg/mL – Microgram per milliliter

µL – Microliter

µm – Micrometer

µsec - Microsecond

% - Percentage

€/g – Price in euros per gram

A – Available

AMT – Alpha-methyltryptamine

amu – Atomic mass units

APT – Attached proton test

BZP – Benzylpiperazine

CA – Concentration addiction

$CaCl_2.2H_2O$ – Calcium chloride dihydrate

C.A.C.T.I. – Support center for scientific and technological research (Centro de apoio científico e tecnológico à investigação)

CDU/mg – Collagen digestion units per milligram

Cells/cm^2 – Number of cells per square centimeter

cm – Centimeter

cm^2 – Square centimeter

CNS – Central Nervous System

COSY – Correlation spectroscopy

CV – Coefficient of variation

CYP – Cytochrome P450

DA – Dopamine

DAT – Dopamine transporter

DIC – Disseminated Intravascular Coagulation

DMSO - Dimethylsulfoxide

EC_{50} – Half maximal effective concentration

EC_{max} – Maximum effective concentration

EDTA – Ethylenediamine tetraacetic acid

EGTA – Ethylene glycol tetraacetic acid

EI – Electron impact ionization

EI-GC-MS – Gas chromatography-Electron impact ionization-Mass spectrometry

EMCDDA – European monitoring center for drugs and drug addiction

ENT – Ear-Nose-Throat

EU – European Union

EWS – Early warning system

FBS – Fetal bovine serum

FTIR – Fourier transform infrared spectroscopy

g – gram

GC – Gas chromatography

GC-MS – Gas chromatography coupled to mass spectrometry

h – Hour(s)

HBSS – Hank's buffered salt solution

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMBC – Heteronuclear multiple-bond correlation spectroscopy

HSQC – Heteronuclear sigle-quantum correlation spectroscopy

IA – Independent action

IC_{50} – Half maximal inhibitory concentration

IS – Internal standard

KCl – Potassium chloride

KH_2PO_4 – Potassium dihydrogen phosphate

L - Liter

LC – Liquid chromatography

LC-QTOF-MS – Liquid chromatography hybrid quadropole time-of-flight mass spectrometry

m – Meter

MAO – Monoamine oxidase

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MBDB – N-methyl-1,3-benzodioxolylbutanamine

MDAI – 5,6-Methylenedioxy-2-aminoindane

MDMA – 3,4-Methylenedioxymethamphetamine

MDPBP – 3,4-Methyelenedioxy-alpha- pyrrolidinobutiophenone

MDPPP – 3,4-Methylenedioxy-alpha- pyrrolidinopropiophenone

MDPV – Methylenedioxypyrovalerone

MeOD – Deuterated methanol

MeOH - Methanol

mg – Milligram

mg/Kg – Milligram per Kilo

mg/mL – Milligram per milliliter

$MgSO_4.7H_2O$ – Magnesium sulfate heptahydrate

MHz – Mega hertz

min - Minutes

mL/min – Milliliter per minute

mm – Millimeter

mM - Millimolar

MPBP - Methyl-alpha-pyrrolidinobutiophenone

MPPP – Methyl-alpha-pyrrolidinopropiophenone

mRNA – Messenger ribonucleic acid

MS – Mass spectrometry

MTBE – Methyl Tertiary Butyl Ether

MTT – 3-[4,5-Dimethylthiazol]-2,5-diphenyltetrazolium

m/z – Mass to charge ratio

N – NIST library

N/A – Not available

NA – Noradrenaline

Na_2HPO_4 – Sodium phosphate dibasic

NaCl – Sodium chloride

$NaHCO_3$ – Sodium hydrogen carbonate

NAT – Noradrenaline transporter

ng/mL – Nanogram per milliliter

NIST – National Institute of Standards and Technology

nM – Nanomolar

nm - Nanometer

NMR – Nuclear Magnetic Resonance spectroscopy

NRG - Naphyrone

PFPA – Pentafluoropropionic anhydride

pFPP – para-Fluorophenylpiperazine

R^2 – Squared correlation coefficient

rpm – Revolutions per minute

SD – Standard deviation

S.E.M. – Standard error of mean

SERT – Serotonin transporter

SW – SWGDRUG library

SWGDRUG – Scientific working group for the analysis of seized drugs

TFAA – Trifluotoacetic anhydride

TFMPP – 1-[3-(trifluoromethyl)phenyl]piperazine

TH – Tryptophan hydroxylase

UK – United Kingdom

U/mL – Enzyme unit per milliliter

UN – United Nations

USA – Unites States of America

v/v – Volume concentration, Volume/Volume

$VMAT_2$ – Monoaminic vesicular carrier

1 *General Introduction*

Chapter 1 – The "Legal Highs"

In recent years, the world's drug culture has changed substantially and, nowadays, includes a group of products known as "legal highs", "legal drugs" or "designer drugs", referring to a range of natural or artificial psychotropic substances that are marketed as "incense", "plant feeders" and "bath salts", although this is not their legitimate practice. These products are purchased to be used as recreational drugs that mimic the effects of illegal drugs of abuse, as cocaine and 3,4-methylenedioxymethamphetamine (MDMA or "*Ecstasy*"), acting as their legal alternatives, being easily purchased at Internet or through specialized shops – called "smartshops" or "headshops" [1-3].

The packages are very attractive with suggestive names – "Bliss", "Bloom", "Blast", "Kick" – as a marketing strategy to attract consumers (Figure 1). All packages are labeled as "not for human consumption" in order to circumvent the laws that govern the sale of psychoactive substances [4-5].

Figure 1 – "Legal highs" sold in Portuguese "smartshops" (Magic mushroom and Euphoria)

1.1 The problem of "legal highs"

These new recreational substances are very similar to the controlled drugs in their chemical structure and/or pharmacodynamics [6]. The term "legal highs" is defined as psychoactive substances whose production, sale and possession, are not covered by the law, not being prohibited [7]. This seems to be the ideal situation from the point of view of producers, sellers and consumers of these new drugs; however, there are a number of problems associated, as will be discussed in this thesis.

Despite some legislative initiatives on these products, there are evidences suggesting that little has changed. Consequently, banned substances are still being sold under a new guise. It is not known how many and which of these products contain substances known as legal or illegal substances [5, 8-10]. In addition, new products are also produced through the modification of the chemical structure of substances that have been enacted [11-13]. The emergence of these new substances is extremely challenging, not only due to the large number of different compounds that are theoretically possible to exist, but also due to the number of those that are actually manufactured, distributed and consumed [14]. The new alternative products are immediately promoted, being the Internet an effective medium for its dissemination, once it responds quickly to legal changes. Thus, the online market is an important tool to identify the new trends of drugs of abuse [15]. In view of this panorama, as the law is always a step behind, manufacturers and consumers continue to explore legislative gaps to constantly create new "legal drugs", continuing the "cat and mouse game".

Another problem is that these drugs are sold in packages highly variable and inconsistent concerning its content. This has been verified for products with the same brand name and identical packages. The variability found refers to significant qualitative as quantitative differences. This inconsistency increases the risk of toxicity and injury associated with its use. In accordance, the information provided to consumers on the composition, dosage, duration and even the effects is generally limited or imprecise [6, 8-9, 16].

Despite all the problems associated with these new psychoactive substances, its popularity keeps growing due to the illusory idea of security that was installed on a misinformed public. In addition, other reasons are associated with their incessant demand, where users suggest a decline in purity of illegal drugs, replacing them by these new synthetic drugs. These new substances produce similar effects to illicit drugs and are easily purchased, but the biggest advantage lies on the absence of criminal sanctions [8, 9, 11, 13].

In contrast to what happens with traditional drugs of abuse, there is little or no information available about these new substances, both in terms of their chemical composition and toxicity or long-term effects associated with its use, representing a serious challenge for public health authorities [3, 9, 12].

Based in medico-legal information, excessive use of drugs of abuse is a social danger respecting self-harm and crimes committed under the influence of such substances. The easy access to new recreational products lead to great social burden, as exemplified by the disruption of personality, mental disorders and deterioration of social relations, leading users to a social marginalization [17].

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The fact that most of these new drugs are legal in many countries, lead many consumers to believe they are harmless substances. Unfortunately, the consumption of these unknown substances is very risky and many of them appear associated with adverse physical effects and fatalities [18-21]. To increase the problem, it is common for consumers to create "designer mixtures" in order to enhance its effects, which consequently become unpredictable, making it difficult to evaluate the effects of specific substances [17].

Hence, it is necessary and urgent for health care providers to be familiar with the effects of these new psychoactive substances, as well as it is important that the forensic toxicological research community develops strategies for fast and effective recognition of the consumption of new "legal highs" [22].

For correct application of the law and for benefit of the general community, it is vital that a forensic laboratory is able to correctly identify the compounds present in these new drugs and determine if the sample is a controlled substance under the legal system of the country concerned. The process of detection and identification of new drugs is not easy, because many substances exhibit identical chemical, chromatographic and spectral properties, since the structural modifications of a controlled substance may include only the addition of a small chemical group or the formation of isomeric structures. Furthermore, the absence of standards and reference spectra in available bibliotheca data will increase the complexity of identification, being hardly achieved through routine analytical protocols. Thus, sophisticated methods of analysis are needed to test these unknown products, often through complex and expensive processes [6, 11].

1.2 The new drugs evolution

The market of new drugs is characterized by the extreme speed of producers' reaction to the imposition of control measures and monitoring, providing alternatives to banned products. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), between 2005 and 2012, more than 230 new psychoactive substances were notified through the early warning system (EWS). The rhythm at which these drugs appear on the market reached record values in recent years: 24 new substances have emerged in 2009, 41 in 2010, 49 in 2011 and 73 in 2012 (Figure 2). Currently, about two-thirds of notified substances belong to cannabinoids and synthetic cathinones group [23-25].
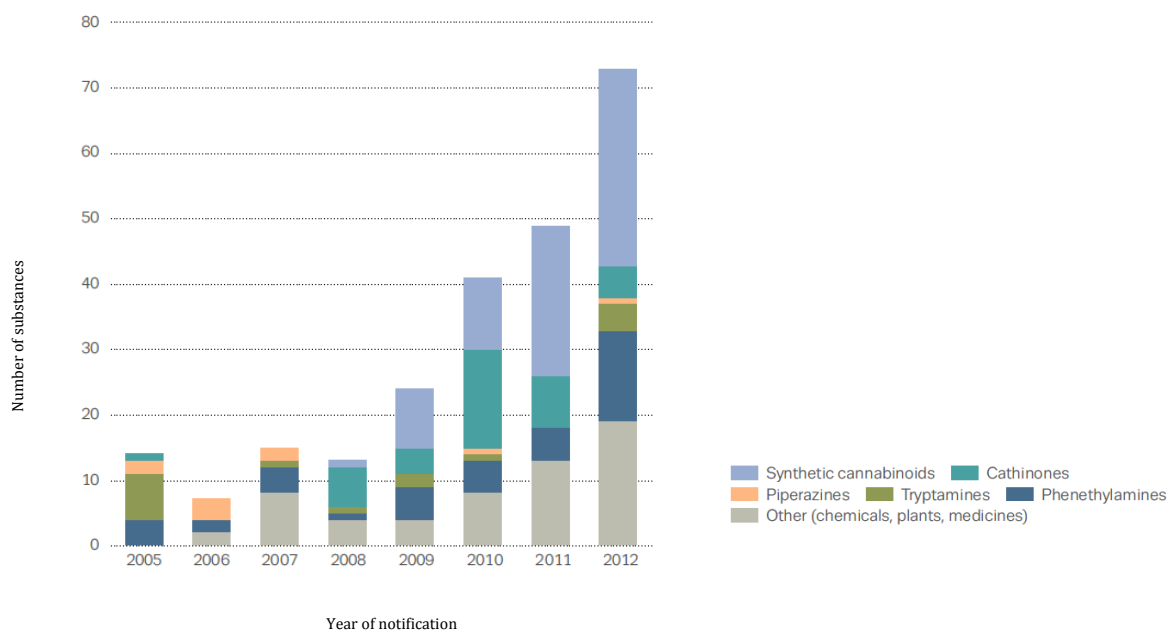
Figure 2 - New psychoactive substances notified to EMCDDA in 2005-2012 [adapted from 25]

Until 2008, the new psychoactive substances that have emerged in the European Union (EU) drug market belong to a restricted number of chemical classes, being the phenethylamines and tryptamines the most accounted for [23]. However, the market has undergone drastic changes in recent years. The increasing number of substances that has been reported corroborates this fact.

In the last years, a new chemical class – the synthetic cathinones – with stimulant properties emerged in Europe's drug market. These substances are structurally related to cathinone, the main natural psychoactive substance of khat plant [26]. In 2010, 15 of the 41 novel derivatives detected belonged to synthetic cathinones group, being now the second largest family of drugs monitored by EWS [23].

The newly identified substances list also includes a diverse group of plant-derived and synthetic substances, including indanes, benzodifuranyls and synthetic derivatives of cocaine, ketamine and phencyclidine [6].

1.3 Prevalence

In Europe, studies on the prevalence of use of licit alternatives are scarce and, often, are accompanied by methodological limitations such as lack of representative samples [24, 27].

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During 2008, Polish researchers conducted a study in 1250 students with 18-19 years and concluded that 3.5% of these young people had consumed this type of substances at least once in their lifetime. In 2010, a follow-up study found an increase to 11.4%. When asked about the consumption of licit drugs over the last 12 months, in 2008, 2.6% responded affirmatively and this number increased to 7.2% in 2010. This fact can be explained by the exponential increase of selling points in Poland, rising from 40 "smartshops" in 2008 to 1500 in 2010 [23].

In 2011, a survey was carried out at European countries, including more than 12000 young people (15-24 years), about their attitudes to the new generation of drugs. The analysis of results estimated that 5% of the young surveyed have used "legal highs" (Figure 3). The highest percentages came from Ireland (16%), followed by Latvia, Poland and United Kingdom (10% each). Italy, Finland and Greece were the countries with the lowest values [28].
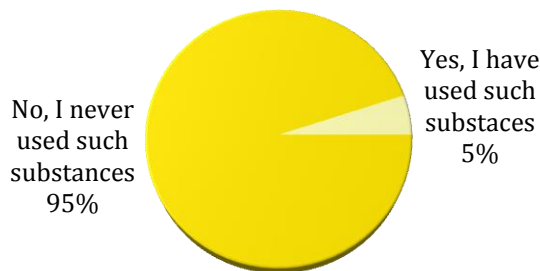
Figure 3 - Percentage of young Europeans who consumed, any time during life, new licit drugs
[adapted from 28]

In the same inquiry, it was asked to young people how they purchased these products. The vast majority of respondents (54%) indicated that friends offered them and 36% mentioned that they have been obtained at parties. About a third of participants (33%) admitted having purchased the substances in specialized shops and only 7% via the Internet (Figure 4) [28].
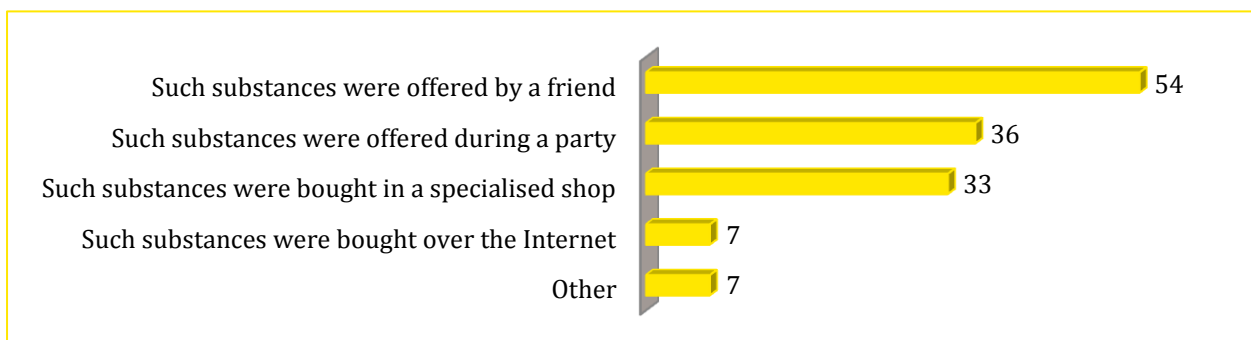
Figure 4 – Market availability of new psychoactive products. [Adapted from 28]

After approval of mephedrone control, the British Crime Survey results, obtained from 2010/2011, showed that in England and Wales, the general population (16-59 years) consumed, during this period, the same levels of mephedrone and *ecstasy* (1.4%). In a more restricted age group (16-24), the prevalence increased considerably, equaling the values of cocaine powder (4.4%). Note that most of the subjects who reported using mephedrone also assume the use of other illicit drugs, in particular cannabis, cocaine or *ecstasy*. This study also shows that men consume about twice more (2%) this type of drugs when compared with females (0.8%) [29].

1.4 Identification of active components of "legal highs"

The composition of the "legal highs" is one of the problems associated with these products, being extremely important and fundamental to know which active compounds are present in the different products, for a more effective performance of medical entities. For this purpose, analytical procedures should be developed in order to reveal their real composition.

1.4.1 Chromatographic techniques

Gas chromatography coupled to mass spectrometry (GC-MS) is an analytical method used in most laboratories for qualitative analysis of diverse psychoactive drugs [6, 8, 16, 30-35]. Most active components of "legal highs" can be conveniently subjected to analysis and characterized by GC due to their low molecular weight [12]. The advantage of this instrumentation is that it is relatively inexpensive, can be automated, provides rapid analysis and achieves good resolution, sensitivity, specificity and repeatability [36].

Another chromatographic technique frequently used is the liquid chromatography (LC) that allows the study of substances of higher molecular mass and lower volatility [6-7, 12].

These analytical techniques typically use mass spectrometry (MS) as detection technique, which allows the identification of interest analytes by characteristic ions resulting from molecule fragmentation, being separated according to their mass/charge ratio (m/z) [37]. The identification of the interest peaks is accomplished by comparison with spectra libraries (e.g. NIST Mass Spectral Library). Unfortunately, the majority of compounds present in "legal highs" are not yet referenced in this type of library, which gives a limitation to this technique [12]. Different GC and LC methodologies have been extensively used for the qualitative analysis of these new commercial products, as summarized in Table 1.

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Table 1: Chromatographic techniques used in qualitative analysis of several "legal highs"

Analytes	Sample		Method		Stationary Phase	Mobile Phase		Mass range	Reference
	Appearance	Preparation	Purpose	Instrumentation		Eluent	Program		
Different chemical classes (Cathinones, Piperazines, other substances)	Over 6000 powders, tablets and capsules	Methanolic extract and centrifuged	Substances identification	GC-MS	HP-5MS capillary column (30m x 0,25mm x 0,25 µm)	Helium at flow rate of 1mL/min	50 °C maintained for 1min., increased 10 °C/min to 280 °C maintained for 10 min.	29 to 600 amu	[6]
		Methanol mixed with water (1:1, v/v) and centrifuged		LC-QTOF-MS	Ascentis Express C18 column (7,5 cm x 2,1 mm x 2,7 µm)	Mixture of 0,1 % (v/v) formic acid in AcCN and water at flow rate of 0,3 mL/min	Gradient mode: 0 min – 5%, 11 min – 23%, 15 min – 27%, 15,2 min – 5%, 21 min – 5%	50 to 1000 amu	
Different chemical classes (Cathinones, Piperazines, Tryptamines, Canabinnoides and other substances)	2094 samples in the form of pills, tablets, gels, lollipops, powders and smoking mixes	Ultrasound-assisted extraction with methanol	Substances identification	LC-QTOF-MS	Reversed phase system using a C18 column	0,1 % formic acid (A) and 0,1 % formic acid in acetonitrile (B)	Linear gradient	100 to 1000 m/z	[7]
Different chemical classes (Cathinones, Piperazines and other substances)	35 Internet and headshops purchased "legal highs" products	Methanolic extracts (10 mg/mL) followed by centrifugation. Supernatants were filtered and diluted 100 times with methanol	Substances identification	Fast GC-MS	SLB-5MS column (10 m x 0,1 mm x 0,1 µm)	Helium at 0,5 mL/min	100 °C to 270°C at 50 °C/min and maintained for 0,6 min.	46 to 320 m/z	[30]
Different chemical classes (Piperazines, Cathinones, Caffeine)	Seven samples in the form of powders sold as "legal highs"	Methanolic extracts (10 mg/mL) followed by centrifugation. Supernatants were filtered and diluted 1000 times with methanol	Substances identification	GC-MS	DB-1MS (30 m x 0,25 mm x 0,25 µm)	Helium at 1 mL/min	150 °C for 4 min, ramp to 350 °C at 32 °C/min	40 to 300 m/z	[31]

Table 1: Chromatographic techniques used in qualitative analysis of several "legal highs" (continued)

Analytes	Sample		Method		Stationary Phase	Mobile Phase		Mass range	Reference
	Appearance	Preparation	Purpose	Instrumentation		Eluent	Program		
Cathinones derivatives	Four capsules containing white powders	Powder was extracted into a range of solvents being injected directly. Other extracts was derivatized with PFPA	Substances identification	GC-MS	HP-1 capillary column (15 m x 0,25 mm x 0,25 µm)	Helium at 58 cm/s	90 °C for 3 min, ramped at 45 °C/min to 300 °C, maintained for 1 min	40 to 450 m/z	[32]
Caffeine and other active compounds	Six novel psychoactive products ("legal highs")	Methanolic extract (1mg/mL) followed by centrifugation. The methanolic extract was combined with a internal standard solution and MTBE	Identify the active ingredients and measure the caffeine content of the product	GC-MS	---	---	80 °C for 4 min and then ramped at 40 °C/min to 290 °C and held for 10,75 min	---	[33]
Cathinones and other constituents	Eighteen samples obtained from headshops in Dublin	Methanolic extract (1mg/mL). The mixture was sonicated and centrifugated	Substances identification	GC-MS	HP-5MS column (30 m x 0,25 mm x 0,25 µm)	Helium at 1 mL/min	90 °C for 1 min, 15 °C/min to 280 °C and held for 6 min, 10 °C/min to 300 °C maintained for 13,33 min	40 to 800 m/z	[34]
Cathinone derivatives and other constituents	24 "legal highs" products obtained from 18 UK-based websites	Methanolic extract at a concentration of 0,50 mg/mL	Substances identification	GC-(EI/CI)-IT-MS	Factor four capillary column (VF-5 ms) (30 m x 0,25 mm x 0,25 µm)	Helium at 1 mL/min	100 °C for 1 min, 20 °C/min to 280 °C and held for 10 min	40 to 500 m/z	[8, 35]
Cathinones, piperazines and caffeine	Twenty six "legal highs" purchased from five different Internet sites	Batches of similar tablets were assumed to be homogenous. Methanolic extracts	Substances identification	GC-MS	SGE solgel capillary column (30 m x 0,25 mm x 0,25 µm)	---	70 °C for 4 min and ramping 40 °C /min to 280 °C	40 to 400 m/z	[16]

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1.4.2 Other techniques

Recently, a "test field" as a Raman and Infra-Red spectrometry has been developed for the detection and characterization of these new substances, but has only obtained positive results for relatively pure substances [9, 14, 38-42].

Another technique used in the qualitative analysis of these new substances is nuclear magnetic resonance spectroscopy (NMR) [9, 38, 39-40, 43-46]. The existence of a large number of positional isomers in synthetic cathinones group requires the use of powerful tools that provide structural information to allow differentiation. Still, despite being a powerful tool, the cost associated with the NMR technique as well as the necessary technical knowledge, are limitations for the application of the NMR technique in routine analysis [47].

Chapter 2 – Natural Cathinones

2.1 History

Khat (Figure 5) (Family: Celastraceae; Specie: *Catha edulis*) is a slow growing plant, native to Eastern Africa and Arabian Peninsula and has other names and spellings such as kat, chat, quat, catha, tschat, miraa, African salad, African tea, Abyssinian tea, kuses-salahin, and tohai [48].

The origin of the plant is controversial. Many believe that its origin is Ethiopian; others claim that it originated in Yemen before spreading to Ethiopia and neighboring countries. However, according to data collected by British explorers, the Ethiopian origin seems most likely [49-50].

Figure 5 – Khat (*Catha edulis*) plant

Abu Rayhan Al-Biurni, a Persian scientist, carried out the first description of this plant during the 11th century. The oldest scientific report about this specimen dates back to the 18th century, when the botanist Peter Forskal identified the plant in Yemen, naming it *Catha edulis* [51-53].

The consumption of plants, and especially of khat, to take advantage of its stimulant properties is not a new practice. According to a famous legend, the first human that experienced khat was a Yemeni herder. He observed the effects that leaves caused in his goats, deciding to experience these same effects. To this end, the herder chewed the leaves, which is currently the exclusive method for the consumption of khat [54]. Chewing leaves to take pleasure of its stimulant effects become a deeply entrenched social and cultural tradition, practiced by the inhabitants of these geographical areas (Figure 6A), where there are no laws restricting its cultivation and consumption. It is estimated that about 5 to 10 million people do so daily [53]. This habit involves picking tender leaves of khat, beginning to masticate thoroughly one at time while users engage in discussions and social interactions. During the sessions, the leaves are chewed slowly over several hours and the juice is swallowed while the residue is putted into

one side of the mouth [48].

During the last two decades, this practice has gained global prominence as a result of migration, although khat use in western countries such as the United Kingdom, Canada and United States has recently become restricted and is now classified as a controlled substance of abuse [48]. Khat has won a recognized economic value comparable to other crops such as tea, coffee and cocoa [53].

Figure 6 – (A) Khat chewing; (B) Fresh khat leaves, ready for sale and chewing

Traditionally, khat is used as a socializing drug although it is also sought by farm workers to reduce physical fatigue and by drivers and students for improving attention [54-55].

2.2 Active constituents of Khat

The alkaloids present in khat have been investigated for years, but it was only in 1930 that norpseudoephedrine or cathine [S, S (+) phenylpropanolamine] was identified in the leaves. Initially the stimulating power of khat was explained by the presence of this substance, until it was shown that its concentration in the leaves and respective potency were too low to justify the effects caused [49, 56].

Subsequently, other studies have indicated the presence of another alkaloid in the plant, with a more potent stimulant power – cathinone [S (-) alpha - aminopropiophenone], a beta-keto amphetamine analog (Figure 7), known as "natural amphetamine", since the effects are similar to those produced by other known psychostimulants as amphetamines and their congeners. This substance was found at high concentrations particularly in young leaves [49, 56-57].

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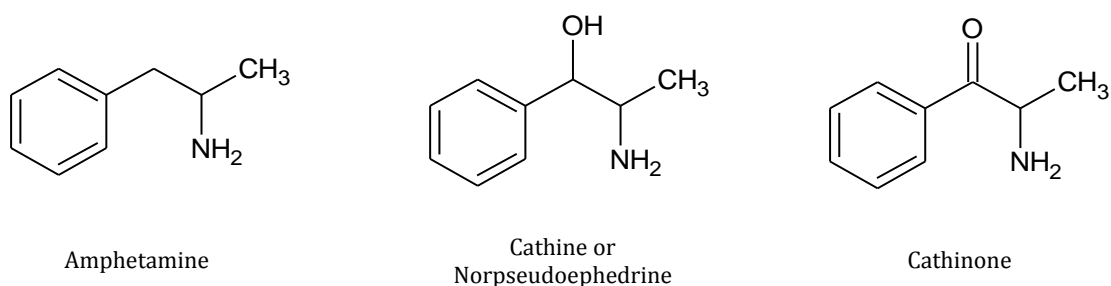
Figure 7 – Chemical structure of amphetamine, cathine and cathinone

Other researchers found that cathinone is a precursor that in khat leaves is enzymatically converted into cathine and norephedrine, the latter to a lesser extent. This transformation is fast in the adult leaves and slow in younger leaves. This can be explained presumably because in younger leaves the enzymatic development is still incomplete [49-50]. Thus, users of khat will choose, preferentially, to consume young leaves, where the amount of cathinone is high, thus obtaining the desired stimulating effects with less adverse systemic effects.

Cathinone degradation by sunlight or heat was also checked, and to delay the process, the leaves of khat when harvested for sale are usually wrapped in banana leaves (Figure 6B) to retain the moisture and preserve freshness [58].

Many other chemical components were identified in khat plant including tannins, flavonoids, sterols, carotenes, terpenoids, essential oils, amino acids, proteins, vitamins and minerals [50, 58-59].

2.3 Effects produced by Khat consumption

2.3.1 Physical sequelae

Cathinone is structurally and functionally similar to amphetamine (Figure 7). It causes the release of catecholamines originating Central Nervous System (CNS) stimulation in low doses, associated with an alertness, euphoria and sense of well-being. Cathinone has also peripheral sympathomimetic effects, such as increased respiration, body temperature, blood pressure and heart rate. Some cases of anorexia, hyperactivity and loquacity have been reported after khat consumption. Another common effect is insomnia, a condition that users try to overcome with sedatives or alcohol in order to neutralize the stimulating effect caused by khat [52, 56-57].

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The adverse physical effects in humans resulting from high doses and chronic consumption of khat have been well studied and can be briefly described according to the physiological systems involved (Table 2).

Table 2: Adverse physical effects of khat [51-53, 59-61]

Central nervous system	Dizziness, impaired concentration, memory deficit, insomnia, headaches, migraines, conjunctival congestion, impaired motor coordination, tremors, stereotyped behaviors
Respiratory system	Bronchitis, tachypnea, dyspnea, tuberculosis
Cardiovascular system	Tachycardia, arrhythmias, palpitations, hypertension, vasoconstriction, ischemia, myocardial infarction, chest pain, pulmonary edema, cerebral hemorrhage
Gastrointestinal system	Dry mouth, polydipsia, tooth decay, brownish teeth, oral cancer, periodontal disease, oral keratotic lesions, chronic gastritis, gastric ulcers, esophagitis, constipation, hemorrhoids, paralytic ileus, duodenal ulcer, anorexia, weight loss, increased risk of upper gastrointestinal malignancy
Hepatobiliary system	Cirrhosis
Genitourinary system	Spermatorrhea, change in libido, impotence, urinary retention, renal toxicity, abnormal sperm
Obstetric effects	Low birth weight, stillbirth, impaired lactation
Metabolic and endocrine effects	Hyperthermia, sweating, hyperglycaemia
Ocular effects	Mydriasis, blurred vision
Psychiatric effects	Lethargy, irritability, depression and psychotic reactions

2.3.2 Dependence, tolerance and withdrawal

According to some authors, the habit of chewing khat can cause a moderate, but permanent, psychological dependence. After continued use, mild withdrawal symptoms have been reported, including lethargy, mild depression, recurring nightmares and tremors [49]. In the literature there is little information regarding the dependency on khat and regular consumers do not report difficulties to stop consumption. However a cessation of its use results in significant improvement of sleep and appetite [56, 62].

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Tolerance is difficult to assess since the tradition defines a maximum amount (100-200 g) of khat to be consumed daily [50]. However, a certain degree of tolerance seems to be associated with an increase in blood pressure, heart rate, respiratory rate and body temperature [63].

2.3.3 Treatment of intoxications

Few reports on this topic have been enumerated. Giannini and collaborators (1992) reported two cases of addiction to khat treated with bromocriptine, following the same protocol as developed for the treatment of cocaine addiction [62].

2.3.4 Socio-economic effects

Apart the negative health impact in the communities where khat is consumed regularly, impact on socio-economic conditions have also been reported. While the consumption of khat can be indirectly associated with absenteeism and unemployment due to the hours dedicated to daily ritual, on the other hand there are those who defend that a moderate intake of khat improves performance due to its stimulant effects, decreasing fatigue. Therefore, the work is extended over hours, due to an increase in the motivation of employees, thereby increasing the productivity and the economy. Another problem arises to the fact that these people spend a large part of the salary to feed the addiction to khat consumption, which leads the idea of possible dependency. Against this background, consumers seek incessantly income to pay for khat and may resort to inappropriate behavior such as criminal behavior and prostitution. However, apart to the losses associated, economic benefits for these producer countries can also be found in the sale and export of khat [57].

Chapter 3 – Synthetic Cathinones

3.1 History

Prior the confirmation of the cathine as one of the active ingredients of khat, synthesis procedures were carried out, discovering the first two synthetic cathinones: methcathinone (1928) and mephedrone (1929) [64]. The evident structural relationship of these substances with the amphetamine has led to a strong interest in these compounds, initially for therapeutic purposes. In the 1930s the methcathinone (also known as ephedrone) has been developed as an antidepressant in the Soviet Union, but was never marketed for this purpose due to its strong addictive potential [20]; the amfepramone (or diethylcathinone) was marketed as an appetite suppressant in the late 1950s [65]; from 1970 the pyrovalerone was used for a few years in the treatment of chronic fatigue and lethargy, which was also abandoned due to dependency issues [66]; bupropion was introduced on the market in 1985 as an antidepressant and later used in pharmacotherapy of smoking cessation, being the only cathinone derivative currently used for therapeutic purposes in the United States and Europe [67-68].

For several decades methcathinone was consumed as a recreational drug without any kind of control, confirming its widespread abuse in the former Soviet Union, Russia and Western Europe, sprawling to the USA in the 1990s [20]. In 1994, the U.S. Government recommended the inclusion of methcathinone as a Schedule I controlled substance in the UN Convention on Psychotropic Substances [64]. However, a growing number of new derivatives of cathinones have emerged and its therapeutic potential has continued to be evaluated. In 1996, methylone was patented as antiparkinsonian agent and its similarity to amphetamine, particularly with MDMA was described [69].

Over the next decade, the scenario of cathinone derivatives began to change, appearing now as "legal highs" in some countries. It was the case of methylone, which in 2004 emerged in the Japanese and European market under the name of "Explosion", being one of the first products to be sold in "smartshops" and on the Internet [70]. In turn, mephedrone appeared in Israel in the early 2000s, locally nicknamed "Hagigat", though its consumption was banned in 2008 by the Israeli Government due to the large number of hospitalizations associated with their use [71]. However, other countries have shown interest in this product, starting to be marketed in Europe since 2007 [38]. Growing concerns about the safety of mephedrone and related derivatives have led to a series of restrictions in countries in which it was predominantly consumed, as it was the case of the United Kingdom that in 2010 has introduced these derivatives on a collective group of class B substances, under the Misuse of Drugs Act 1971, having a specific legislation to deal with such substances [38].

After mephedrone, other derivatives appeared on the market, such as flephedrone (4-fluoromethcathinone) and two structural isomers (2 - and 3 - fluoromethcathinone) [43], as well

as naphyrone (also known as naphthylpyrovalerone or NRG-1) and MDAI (5,6-Methylenedioxy-2-aminoindane) [8, 71].

In Portugal, mephedrone only became an illegal substance at the beginning of 2012 [72]. However, a growing number of fatal cases related to "legal highs", in particular with cathinone derivatives, led the new legislation to amend the law which now prohibit any activity related to the production, import, export, advertising, distribution and sale of new psychoactive substances, initially in the Madeira Archipelago, extended this year to the entire country [73-74].

The major events in the history of cathinone derivatives are summarized in figure 8.

1928	Synthesis of methcathinone
1929	Synthesis of mephedrone
1930	Methcathinone marketed as an antidepressant in the Sovietic Union
1950	Amfepramone introduced as an appetite suppressant
1970	Abuse of methcathinone in Sovietic Union; treatments with pyrovalerone
1985	Bupropion introduced as an antidepressant
1991	Methcathinone abuse appears in USA
1994	Methcathinone scheduled
1996	Methylone patented as an antiparkinsonian agent
2004	Methylone emerged for sale in drug market
2007	Appearance of mephedrone in drug market
2008	Appearance of flephedrone and naphyrone (NRG)
2010	Legal restrictions enforced on cathinone derivatives in several countries
2012	(March) Banning of mephedrone in Portugal
2012	(October) Ban the production and sale of derivatives cathinones in Madeira
2013	(March) Ban the derivatives cathinones products in Portugal

Figure 8 – Timeline associated with cathinone derivatives

3.2 Chemistry

The synthetic cathinones were developed taking into account the active ingredient found in the khat plant, cathinone. Its chemical structure can be considered as a prototype, from which were developed a lot of derivatives. This idea is due to the fact that all cathinones have in common the same chemical structure, comprising a benzene ring and a aminoalkyl side chain [64].

However, despite the similar central structure, derivatives can be chemically organized into four groups (Figure 9) [6]:

A. "Classic" cathinones – consisting of a benzene ring substituted or unsubstituted and a straight side chain. Examples of these cathinones are mephedrone (4-MMC) or buphedrone.

B. Pyrrolidinopropiophenone derivatives – consisting of a benzene ring substituted or not and a pyrrolidinyl ring in a side chain. This is the case of substances like MPPP (Methyl-α-Pyrrolidinopropriophenone) or MPBP (Methyl-α-Pyrrolidinobutiophenone).

C. "3,4-methylenedioxy" cathinones – are composed of a 3,4-methylenedioxyphenyl ring and a straight side chain, for example methylone, butylone and pentylone which are substances that may be similar to MDMA and are advertised as "*legal ecstasy*".

D. "Mixed" cathinones – contain a 3,4-methylenedioxyphenyl ring and a pyrrolidinyl side ring. It is the model of MDPV (3,4-methylenedioxypyrovalerone), MDPPP (3,4-methylenedioxy-α-pyrrolidinopropiophenone) and MDPBP (3,4-methylenedioxy-α-pyrrolidinobutiophenone).
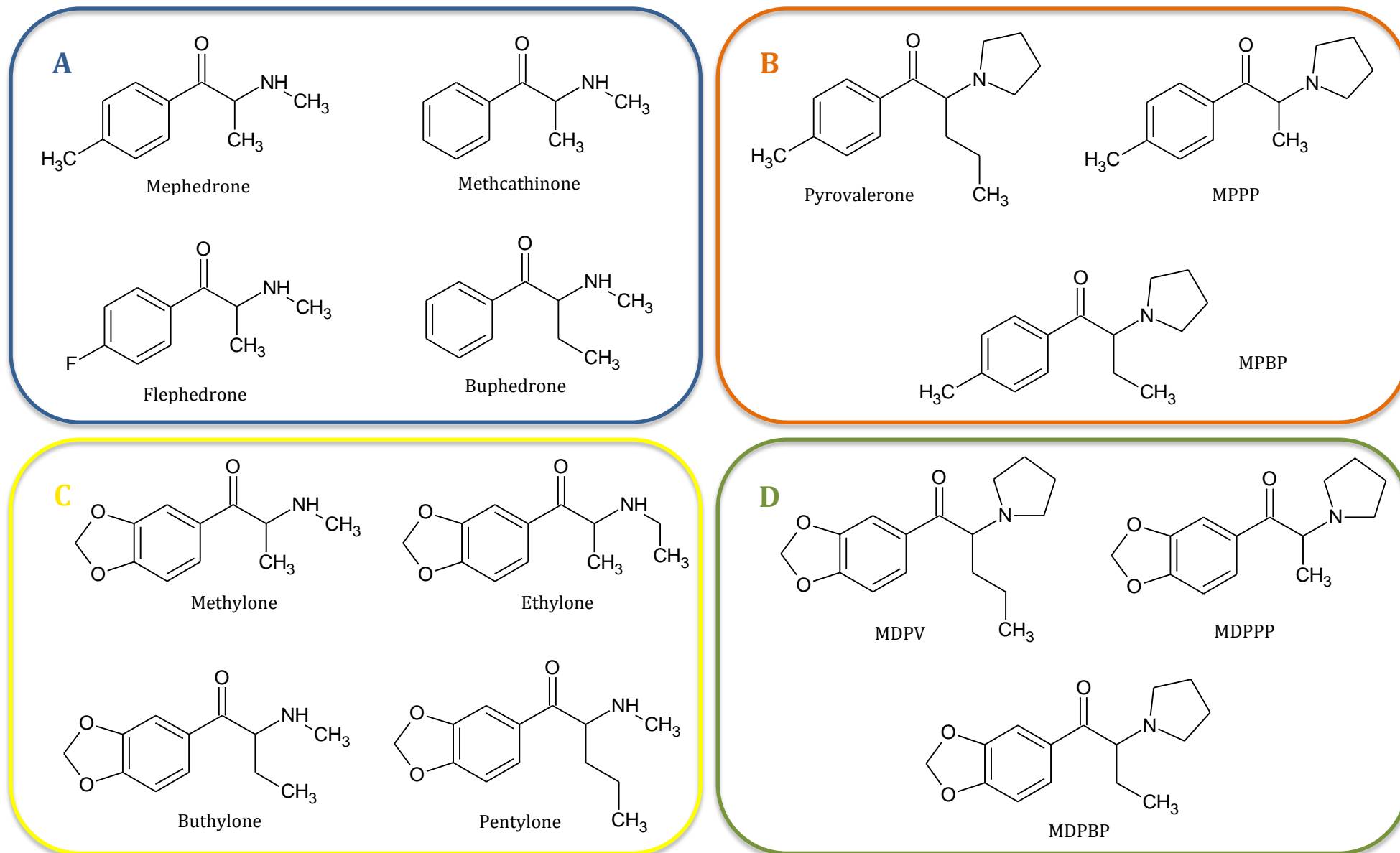
Figure 9 – Chemical structures of some elements of the class of synthetic cathinones, divided into four major groups: (A) "Classic" cathinones; (B) Pyrrolidinopropiophenone derivatives; (C) "3,4-methylenedioxy" cathinones; and (D) "Mixed" cathinones

All these new substances may be considered as phenethylamines derivatives, which also include amphetamines, with the particularity of presenting a ß-keto group on the side chain [64]. Thus, it is common cathinone derivatives being considered amphetamine analogs. This is shown in Figure 10, where it turns out, for example, that from the structural point of view, the cathinone, methcathinone and methylone are analogs of amphetamine, methamphetamine and MDMA respectively, reflecting only the difference of ß-keto fraction [75].

Cathinone

Amphetamine

Methcathinone

Methamphetamine

Mephedrone

Methylmethamphetamine

Methedrone

4'-Methoxymethamphetamine

Methylone

MDMA

Butylone

MBDB
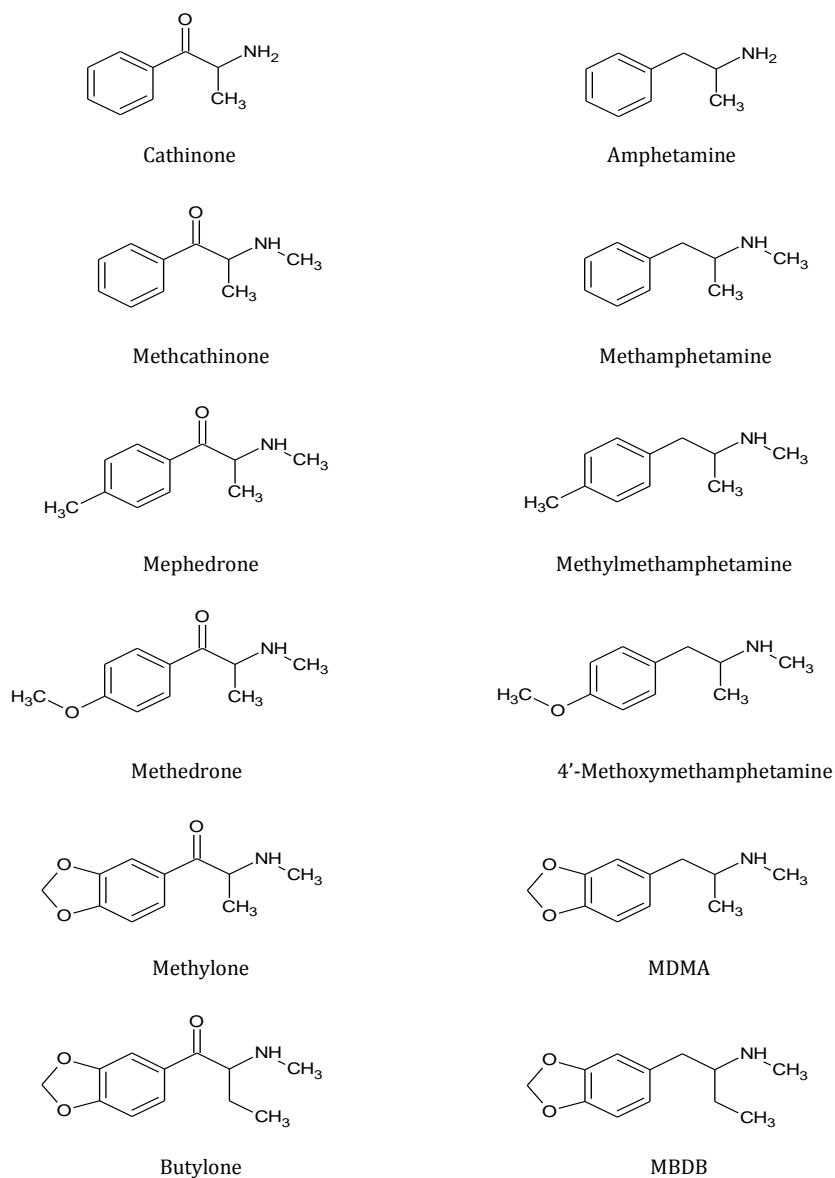
Figure 10 – Structural relationship between some cathinone derivatives and their amphetamine analogs

[adapted from 75]

These new substances, due to the ketone group that enhances its polarity, are usually more hydrophilic, decreasing its ability to cross the hematoencephalic barrier, being thus less potent than their phenethylamine analogues. Consequently, this leads to an increase in the dosage of drug consumed to induce the desired effects [1, 75-77].

However, the set of derivatives with a pyrrolidinyl ring in its structure is an exception to this rule. This group has a high lipophilicity as the nitrogen atom present in its chemical structure is linked to three carbon atoms (Figure 9), forming a tertiary amine group, which confers this property [1, 15].

3.3 Patterns of use

3.3.1 Commercial appearance

The synthetic cathinones are usually acquired as amorphous or crystalline powder, with white or brownish coloration that can occasionally be presented as capsules or tablets. Some users report that this type of synthetic drugs has a characteristic odor and flavor [1, 22, 38].

3.3.2 Routes of exposure

This kind of products can be used by multiple routes of exposure, with the nasal insufflation and oral ingestion being the most common. Yet, other means like the rectal insertion, inhalation, intramuscular and/or intravenous application or techniques such as "bombing"[1] and "keying"[2] can be used. Multiple pathways can be combined in a single session [2, 76].

3.3.3 Dosage

According to reports, typical doses of mephedrone and methylone are variable depending on the route of exposure used. According to some authors, the dose is 100 to 200mg for oral ingestion, starting the effects to appear after 30-45 minutes, persisting for two to five

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[1] Bombing is done by wrapping the cathinone powder in cigarette paper and swallowing it.
[2] Keying is done by dipping a key in powder then insufflating.

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hours, varying with the stomach content [2, 22, 38, 71]. The inhalation allows a faster onset (10-20 minutes), but on the other hand has a lower duration of action (1-2 hours), since it requires lower doses (usually varies between 25 and 75 mg) [2, 71]. Some users opt for two ways simultaneously in order to achieve the rapid onset and long duration [71]. In the case of intravenous exposure, users refer to onset and duration of symptoms extremely rapid (10-15 minutes and 30 minutes, respectively) [2]. The rapid onset of effects is also described for rectal exposure where smaller doses are required when compared to oral ingestion [71].

In the case of MDPV ingestion, lower doses (10-15 mg) and a more potent effect in a shorter period of time (15-30 minutes) are reported. There are reports of cases whose effects lasted until 7 hours [22].

Due to the unknown purity of "legal highs", as well as the exact composition, the dose is difficult to assess [2, 76]. It is impossible to determine a "safe" dosage since negative side effects can result for all dosage taken. In addition, equal dosages may lead to different results in different individuals [71].

3.4 Pharmacokinetics

The information on this topic is quite limited, being part of the knowledge acquired through user reports, surveys, clinical information or sometimes information available on websites [38].

The absorption of this type of products and the subsequent onset of desired effect is dependent upon the route of exposure. The absorption is delayed in the presence of food in the stomach, so that the effects appear later when the drug is consumed after meals [38].

Studies in rats and humans have shown that the synthetic cathinones can be metabolized by multiple pathways, which vary according to their molecular structure [69, 78-80]. Studies involving cathinones that have the ß-keto group and methylenedioxy group (Figure 11), for example the case of methylone and butylone, show that the major urinary metabolites (4-hydroxy-3-methoxymethcathinone (4'-OH-3'-MeO or HMMC) and 3-hydroxy-4-methoxymethcathinone (3'-OH-4'-MeO)) are produced by demethylenation followed by *O*-methylation of the hydroxy group on the benzene ring. The metabolite HMMC has been shown to be the most abundant in rats and humans so its analysis may become essential to prove the consumption of these substances. Another metabolic pathway, although minor is N-dealkylation to a primary amine. In these substances it may also occur the reduction of the keto group to the corresponding amino alcohol [69, 78-79, 81].
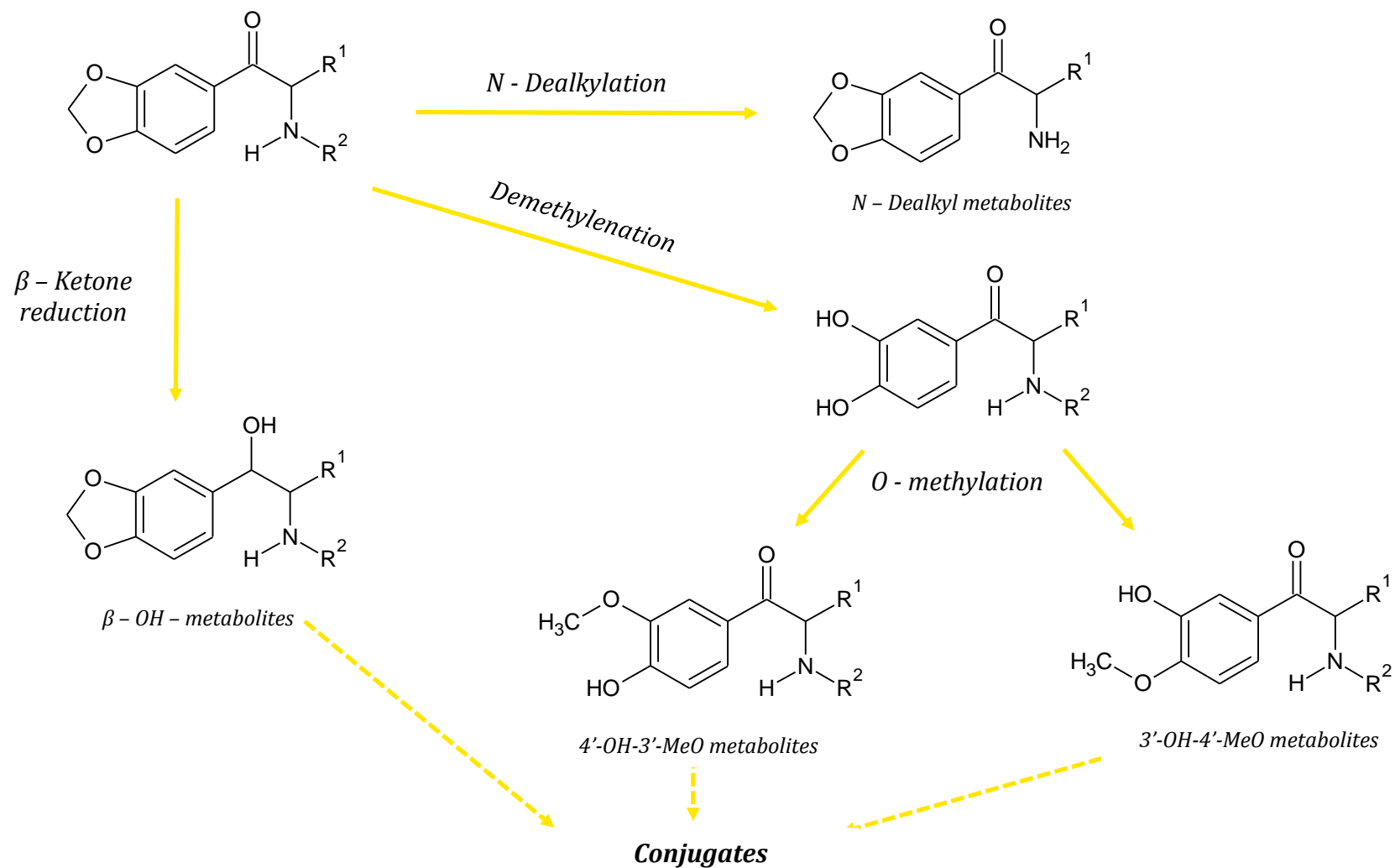
Figure 11 – Main metabolic pathways of ß-keto derivatives with 3,4-methylenedioxy ring in rats and humans [adapted from 69]

Regarding cathinones structurally similar to mephedrone, metabolic pathways differ slightly. Based on the identification of metabolites of mephedrone in the urine of rats and humans, the following metabolic pathways have been postulated (Figure 12): *N*-Dealkylation to the primary amine (metabolites nr. 2 and 3), reduction of the keto moiety to the respective alcohol (metabolites nr. 3 and 4) and oxidation of the tolyl moiety to the corresponding alcohol (metabolites 5 and 6) [80]. The metabolite represented by the number 4 has only been detected in human urine samples.
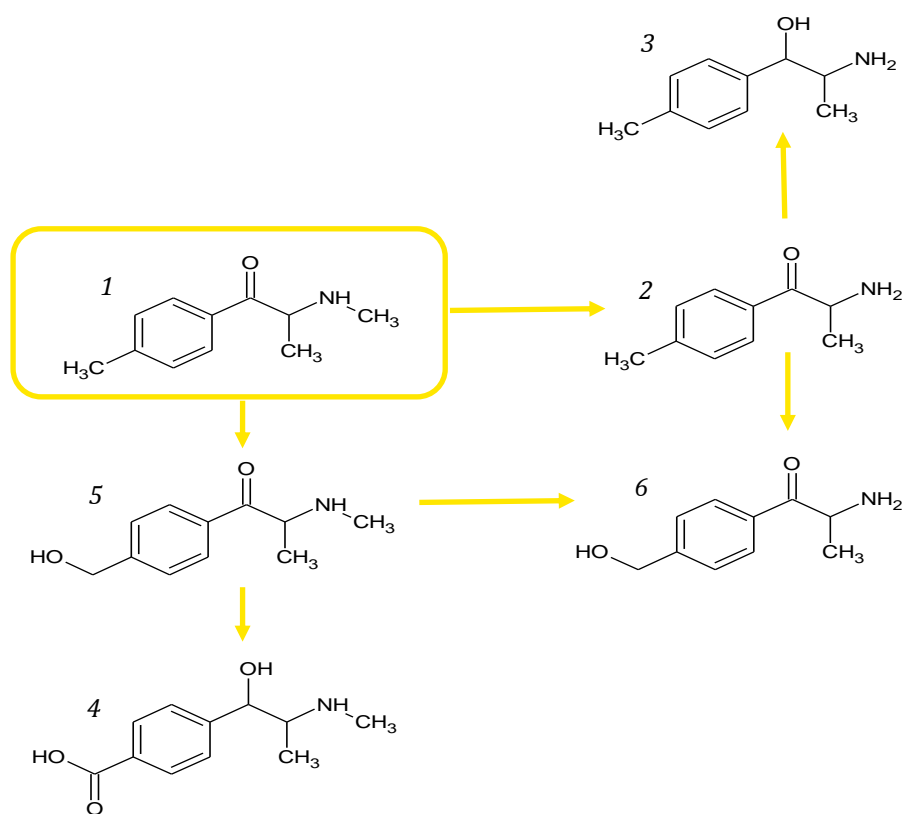
Figure 12 – Proposed scheme for the metabolism of mephedrone in rats and humans [adapted from 80].

Pyrrolidinopropiophenone derivatives and "mixed" cathinones (see topic 3.2. Chemistry), are also extensively metabolized by rats and humans [81-83]. Studies to identify the MDPV metabolites, as well as the cytochrome P450 isoenzymes involved in the process were conducted [82]. Taking into account the urinary metabolites generated, it was concluded that the major metabolic pathways in humans (Figure 13) include demethylenation (metabolite 2, 4, 6 and 7), methylation of the dihydroxy metabolites (metabolite 5, 8 and 9), hydroxylation of the propyl side chain (metabolite 6 and 9), hydroxylation of the 2'-position of the pyrrolidinyl ring

followed by dehydrogenation to the corresponding lactams (metabolite 3, 4 and 8) and finally degradation to primary amines (metabolite 7 and 10). A screening study involving the human hepatic CYPs has found that the CYP2C19, CYP2D6 and CYP1A2 isoenzymes are capable to catalyze the reaction (demethylenation) of formation the initial metabolite, the demethylenyl-MDPV, *in vitro*.
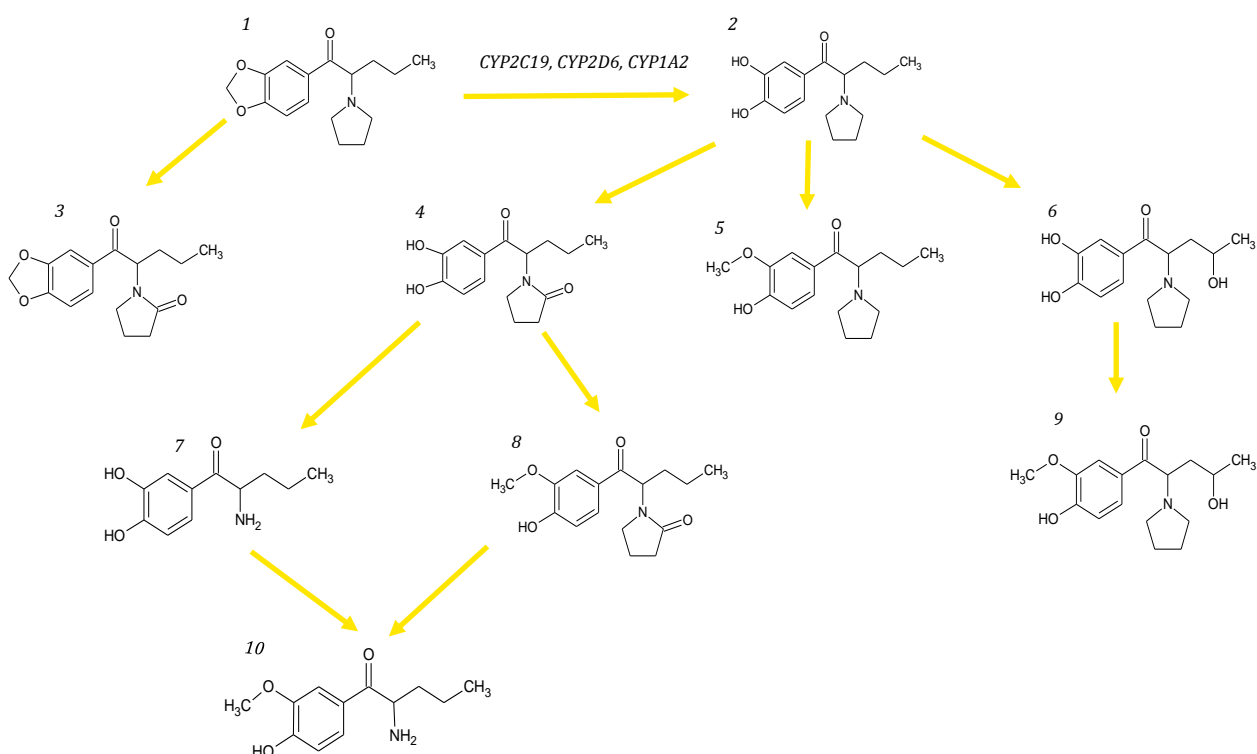
Figure 13 – Proposed scheme for the metabolism of MDPV in humans [adapted from 82].

The excretion occurs mainly by urinary track, where metabolites with a hydroxyl group are excreted in the form of glucuronide and/or sulfate conjugates and another portion is eliminated without modification [22, 79-81].

3.5 Mechanism of action

Neurotransmitters are contained whitin small storage vesicles in the neuronal cells, until it fuses with the cell membrane and expels biogenic amines (as noradrenaline (NA), dopamine (DA) and serotonin (5-HT)) into the synaptic cleft in order to exert their physiological

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effect (Figure 14) [84]. Amphetamine and other related phenethylamines exert its stimulant effects through increasing concentration of biogenic amines in the synaptic cleft. This process arises as a result of two main mechanisms (Figure 14). One of the mechanisms implies that the drug inhibits the reuptake of monoamines by membrane-bound carriers due to competition of drugs to substrate binding sites in the carriers. The second mechanism, suggests that the drug causes the release of neurotransmitters from storage vesicles. The release may be mediated by intracellular pH changes or by inhibition of a monoaminic vesicular carrier, the $VMAT_2$ [1-2, 85]. The vesicular pH is slightly acid when compared with the cytoplasmic pH. This difference creates a concentration gradient by preventing the neurotransmitters to be released into the cell cytoplasm. Drugs such as MDMA and other similar change the permeability and the physiology of intracellular vesicles and carriers. These drugs move to the cell body, merge into storage vesicles and cause a hydrogen ion buffer, increasing the vesicular pH with consequent premature release of neurotransmitters into the cytoplasm before the vesicle merge with the cell membrane [84].

Monoaminic carriers are protein structures integrated into the cell membrane, responsible for the reuptake of neurotransmitters existing in the cleft, moving them back into the cytoplasm. In turn, the $VMAT_2$ carrier is instructed to move back into the vesicles to be used again. Such carriers, when inhibited will block the entry of biogenic amines in the vesicle, staying free inside the nerve ending and subsequently in the synaptic cleft [84].

The stimulant drugs also partially inhibit the activity of the enzyme monoamine oxidase (MAO), which is responsible for the metabolism of monoamine neurotransmitters in excess [15, 84]. A schematic representation is given in Figure 14, taking MDMA as an example.
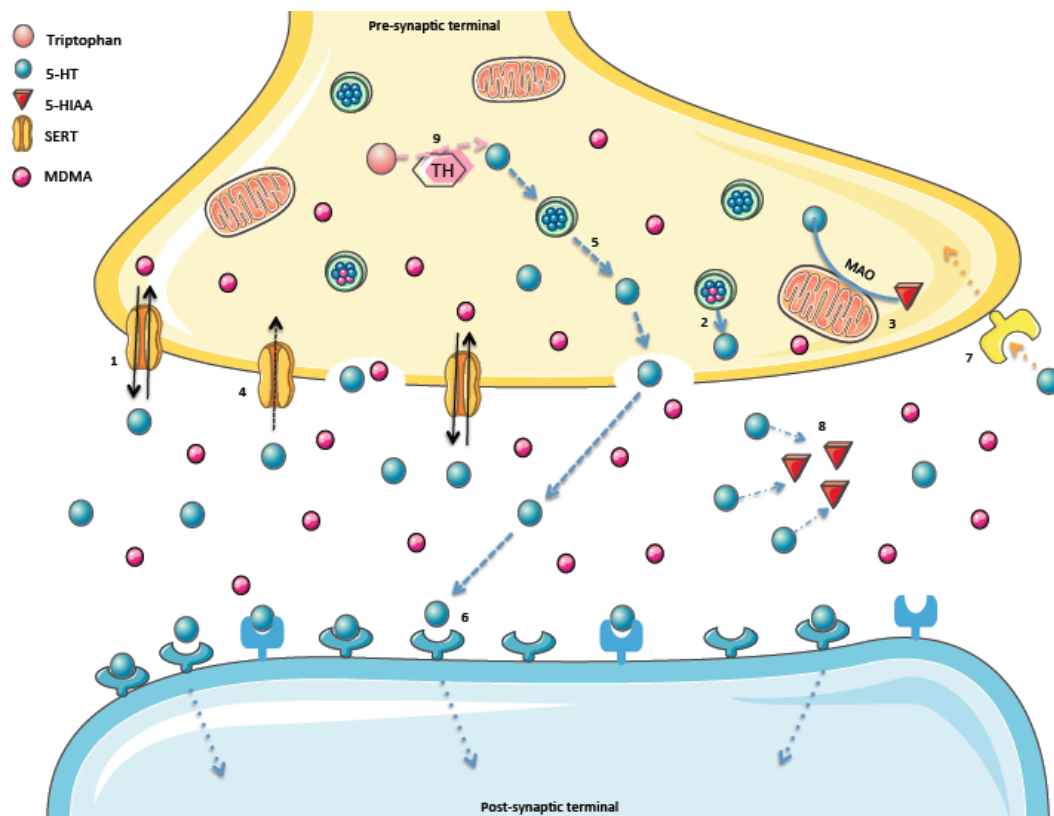
Figure 14 – Mechanism of action of MDMA at the level of the serotonergic neurons. (1) MDMA promotes the release of 5-HT by exchange at the SERT carrier. (2) At higher concentrations, MDMA penetrates into synaptic vesicles and promotes 5-HT release contained therein. Increased 5-HT is also due to inhibition of the metabolic degradation of this neurotransmitter by MAO in the mitochondria (3), inhibition neuronal reuptake system of 5-HT by SERT (4) and stimulation of 5-HT release in the synaptic cleft (5). (6) This 5-HT release will stimulate postsynaptic receptors. However 5-HT has mechanisms to avoid the excessive increase of its levels in the synaptic cleft by self-regulation of 5-HT release by presynaptic receptors (7), increased formation of metabolites and autoxidation products of 5-HT in the synaptic cleft (8). The MDMA also promotes the decrease of 5-HT reserves for causing the inhibition of biosynthesis of 5-HT by tryptophan hydroxylase (TH) (9) [adapted from 86].

Given that the information about the mechanism of action of these new synthetic drugs is limited, extrapolations are made taking into account the structural similarity with amphetamine derivatives, whereby identical mechanisms are expected. Scientific evidence supporting this hypothesis, indicate that cathinones, such as amphetamines, interact with the plasma membrane transporters of dopamine (i.e., DAT), noradrenaline (i.e., NAT) and serotonin (i.e., SERT). However, various studies *in vitro* have shown a mechanistic dichotomy between the most common synthetic cathinones, as well as a variable selectivity for carriers [4, 85, 87-88]. A study found that the methylone and the methcathinone showed a lower activity at VMAT$_2$ level

as compared with MDMA and methamphetamine, respectively [85]. *In vitro* studies with rat brain synaptosomes revealed that the mephedrone and methylone block the uptake of DA, NA and 5-HT but also act as stimulants in the release of these neurotransmitters. Table 3 summarizes the results obtained, which confirms that mephedrone, methylone and MDMA act as non-selective substrates, unlike amphetamine, which is selective for NAT and DAT. It was also found that mephedrone and methylone exhibit similar release potency in all transporters despite mephedrone is about twice as potent. In turn, substances such as cocaine and MDPV were inactive as releasers. MDPV showed a different pharmacological profile comparing to other cathinones, revealing a potent selective blocker for catecholamines carriers (DA and NA) [4, 88].

Table 3: Effects of synthetic cathinones and comparison test drugs on transporter-mediated inhibition of uptake and stimulation of release in rat brain synaptosomes [adapted from 4]

	Mephedrone	Methylone	MDPV	MDMA	Amphetamine	Cocaine
DAT uptake IC_{50} (nM ± S.E.M.)	762 ± 79	1232 ± 133	4.1 ± 0.5	1009 ± 39	93 ± 17	211 ± 19
NAT uptake IC_{50} (nM ± S.E.M.)	487 ± 66	1031 ± 162	26 ± 8	450 ± 30	67 ± 16	292 ± 34
SERT uptake IC_{50} (nM ± S.E.M.)	422 ± 26	1017 ± 59	3349 ± 305	125 ± 11	3418 ± 314	313 ± 17
DAT uptake EC_{50} (nM ± S.E.M.) [E_{max} %]	51 ± 5 [102 ± 2]	117 ± 12 [96 ± 1]	Inactive	42 ± 2 [100 ± 4]	5.8 ± 0.4 [102 ±1]	Inactive
NAT uptake EC_{50} (nM ± S.E.M.) [E_{max} %]	58 ± 11 [99 ± 4]	140 ± 17 [94 ± 2]	Inactive	53 ± 7 [95 ± 2]	6.6 ± 0.7 [92 ± 1]	Inactive
SERT uptake EC_{50} (nM ± S.E.M.) [E_{max} %]	122 ± 10 [101 ± 1]	234 ± 35 [98 ± 2]	Inactive	39 ± 5 [103 ± 4]	698 ± 71 [97 ± 2]	Inactive

Another *in vitro* study [87] with HEK 293 cells was conducted to determine the potency of different cathinone derivatives in inhibiting the transport of DA, NA and 5-HT, confirming previously published data and adding new evidence. It was verified that in addition to methylone and mephedrone, also ethylone, butylone and naphyrone act as nonselective inhibitors, but like MDMA also induce serotonin release. Moreover, cathinone, methcathinone and flephedrone, as well as amphetamine and methamphetamine, act preferentially as inhibitors of catecholamine uptake and induce the release of dopamine. Also pyrovalerone is a potent and selective inhibitor of dopamine and noradrenaline uptake, without producing the release of monoamines.

Thus, synthetic cathinones with a high affinity to a specific carrier will inhibit its actions, resulting in a higher concentration of neurotransmitter in the synaptic cleft. In this way, a greater affinity to noradrenaline transporter will increase the sympathetic effects and cause

signs and symptoms similar to those caused by ingestion of cocaine, amphetamine and methamphetamine [71]. By another hand, a greater affinity for serotonin has been associated with delusions, hallucinations and paranoia [84]. Of note the predominant action of all cathinones on the dopamine transporter is probably associated with a considerable risk of addition [4, 84, 87-88].

3.6 Toxicological effects

There are not scientific studies in humans to determine the adverse effects of these new compounds. All information is based on descriptions reported by consumers and medical entities. However, the fact that the user believes that he consumed a specific substance does not invalidate that actually has consumed a different one and this fact makes it difficult to assess this type of information. Furthermore, in the case of mixture of compounds it is difficult to attribute the adverse effect to a certain substance. Thus, caution is required when analyzing this information [44].

The clinical effects described are consistent with a sympathomimetic toxicity, denoting a clinical history similar to cocaine, methamphetamine or *ecstasy* intoxication. The desired effects reported by consumers include increased energy, sociability, euphoria, increased alertness, talkativeness and increased libido [2, 71]. Some synthetic cathinones users also report adverse effects such as sweating, palpitations, nausea, vomiting, headaches, muscle pain, muscle spasms, dizziness and short-term memory problems [2, 76]. Consumers who used the nasal route of administration also reported aggressive behavior and psychosis [76]. However, specific effects of cathinones are sometimes difficult to assess since they are often consumed in combination with other substances such as alcohol, tobacco, MDMA, cannabis and cocaine [2, 26].

These new synthetic substances may affect multiple organ systems, as it happens with natural cathinones (Table 4).

Table 4: Effects associated with the consumption of synthetic cathinones reported by consumers and medical entities [2, 5, 27, 38, 44, 71]

Cardiovascular	Palpitations, chest pain, hypertension, myocarditis, tachycardia, disseminated intravascular coagulation
Ear – Nose – Throat (ENT)	Dry mouth, nose bleeds, nasal irritation, tinnitus, tongue disorder
Gastrointestinal	Abdominal pain, loss of appetite, nausea, vomiting, liver failure, abnormal liver function tests
Skeletal Muscle	Arthralgia, changes in extremities (discoloration, numbness, tingling, cyanosis, muscle tension and cramps), increased creatinine kinase, peripheral vasoconstriction, rhabdomyolysis
Neurologic	Disorientation, altered mental status, collapse, confusion, bruxism, dizziness, headaches, memory loss, tremors, convulsions, drowsiness, dystonia, hyperreflexia, myoclonus, paresthesia
Ophthalmologic	Blurred vision, mydriasis, nystagmus, dilated pupils
Respiratory	Shortness of breath, tachypnea, respiratory distress
Psychological	Anger, irritability, aggressiveness, anxiety, visual and auditory hallucinations, depression, dysphoria, euphoria, fatigue, increased or decreased concentration, talkativeness, incoherent speech, panic, paranoia, perceptual distortions, agitation, delirium, psychosis, anhedonia, suicidal ideation, self-mutilation
Genitourinary	Anorgasmia, erectile dysfunction, increased libido
Renal	Impaired renal function, acute renal failure
Others	Body odor, dehydration, sweating, fever, insomnia, nightmares, rash, hyponatremia, hyperthermia

3.6.1 Hyperthermia

Hyperthermia is one of the characteristic effects in MDMA-induced toxicity event cases in which body temperature reaching 43°C has been related [89-91]. Such hyperthermic reactions may contribute to several complications, including rhabdomyolysis, disseminated intravascular coagulation (DIC) and acute renal failure [89-91].

Studies with experimental animals show that the effects of MDMA on thermoregulatory system are strongly conditioned by room temperature. In general, animals exposed to MDMA in low ambient temperatures (below 22°C) had hypothermia, while at high temperatures (above 28°C) MDMA causes severe hyperthermia [92-93]. Since these types of psychostimulants are generally consumed in hot and crowded environments (usually "rave" parties), the ambient temperature is frequently high. This fact, together with the prolonged motor hyperactivity, as well as the state of dehydration may potentiate the hyperthermic effects in the human thermoregulatory system [92].

Hyperthermic effects, as well as its consequent effects, are also reported in cases of consumption of synthetic cathinones (see topic 3.6 Toxicological effects). However, studies in rats after subcutaneous administration of mephedrone shows that at low temperatures (below 20°C) there is a decrease in body temperature, but in case of high ambient temperatures (in the order of 30°C) there were no changes in opposite to what has been established for MDMA exposure. In both environmental temperatures studied, it was verified an increase in locomotor activity, although it is more meaningful for higher temperatures. These results show that the mephedrone exhibits, *in vivo*, thermoregulatory properties distinct from those that are produced by "*Ecstasy*" [94]. Thus, it can be deduced that despite the structural similarity between the synthetic cathinones and MDMA, not all information relating to MDMA can be extrapolated to these new synthetic drugs.

3.6.2 Adulterant toxic effects

One of the potential factors that contribute to adverse reactions caused by recreational drugs is the concomitant use of other substances that can be intentional or through consumption of adulterants existing in marketed products [95]. Adulterants are very commonly found in illicit drugs and may be present for various reasons. To increase the drug volume and consequently the profit, manufacturers deliberately add many substances pharmacologically inactive, as the case of sucrose. But there are also pharmacologically active adulterants that are added in order to enhance and/or mimic the effect of psychotropic substances. A well known

example of an adulterant with such characteristics is caffeine. Adulterants may also be present unintentionally as a result of technical production or storage of products, taking as an example the alkaloids, microorganisms or other biological agents [96].

In the case of "legal highs", as has been already explained, information concerning the composition of commercial products is not always explicit or corresponds to reality. However, compositional analyses carried out on a wide variety of "legal highs" sold all over the world have found the presence of several adulterants, including benzocaine, lidocaine, procaine, glaucine, nicotinamide, caffeine, among others [6, 8, 16, 31].

Certain analysis have shown that caffeine is part of the composition of a wide variety of "legal highs", being the substance most commonly found in these new drugs [6]. There has been found that some of them contain only caffeine as pharmacologically active compound or often associated with other compounds but still at significant percentages (sometimes greater than 87%, corresponding approximately to the consumption of 13L of cola drink). Consequently, the recreational use of these new products can result in severe cases of toxicity [33]. In fact, previous studies have shown that caffeine (10 mg/kg) significantly increases the mortality of rats when coadministered with a high dose of MDMA (15mg/Kg), causing also a high and lasting hyperthermic response, showing that caffeine exacerbates the acute toxicity of MDMA [95]. It was also found that the coadministration of caffeine (10 mg/kg) with MDMA (10 mg/kg) induces a deep tachycardia that is not observed in the case of administration of the substances alone, suggesting the existence of interaction between them [97].

Thus, while that for many people substances as caffeine is taken as a safe substance, its intake simultaneously with these new products leads to a synergy of the stimulant effects, also exacerbating the toxicity produced by them alone, representing a risk to the health of users of this kind of recreational drugs [95].

3.7 Treatment of intoxications

The treatment of intoxications in cases of exposure to synthetic cathinones is mainly supportive, and there is no specific antidote. The available information to guide the treatment is restricted, but given the similarities of effects with amphetamines it seems likely that therapeutic strategies are also similar. Sedation is frequent and the use of large doses of benzodiazepines is required to control the agitation, seizures, hypertension and tachycardia. In the case of persistent hypertension the administration of vasodilators such as sodium nitroprusside or nitroglycerin is recommended. The internal and/or external cooling to treat

hyperthermia should be considered. The treatment of MDMA-induced hyponatremia is done through the water restriction or by administering a hypertonic saline solution [1-2, 76].

Patients should be monitored at cardiac level and body temperature. It should also be performed the evaluation of biochemical markers of muscle damage, electrolytes, renal and hepatic function, cardiac enzymes, as well as screening tests for drugs. Monitoring of patients must remain until the resolution of symptoms and stabilization of vital signs [22, 98].

3.8 Reported intoxications

A growing number of fatal poisonings, especially among the younger population, has recently been attributed to consumption of these new synthetic substances. However, the vast majority of laboratory analyses reveal the presence of multiple drugs of abuse, which complicates the assessment of the causal agent of death. Furthermore, the detection of these new substances and its metabolites in biological samples *ante* and *postmortem* does not necessarily mean that its consumption was the cause of death [1, 38].

The synthetic cathinones, according to medical literature, have been directly involved in a growing number of fatalities [4]. The first fatal case, whose cause was confirmed and attributed to simultaneous use of methylone and butylone, is recent. The victim, 24 years old, showed severe symptoms of sympathomimetic intoxication - hyperthermia, tachycardia, tachypnea, hypertension, diaphoresis, hyperreflexia, tremors and coma. Despite the efforts, the patient developed a multiple organ system failures and succumbed [18].

Fatal cases with laboratory confirmation of MDPV exposure have also been reported. The clinical condition comprised hyperthermia, delirium, and DIC [99].

There are also serious cases of poisoning that can reverse [19]. This was the case of an individual (25 years) who was transported to the emergency service for displaying a severe agitation and altered mental status after the consumption of "bath salts." The patient was hypertensive, tachycardic and hyperthermic (41.3°C, rectally). The individual cooling was immediately provided as well as the supportive treatment. On the day of his admission toxicological analysis was performed in urine, having been detected MDPV at a concentration of 140 ng/mL. Despite the quick and appropriate intervention, the patient developed hepatic and renal failure and rhabdomyolysis, but his condition was reversed after a long hospital stay. It is unknown if the organs failure was due to direct cell toxicity induced by MDPV.

In these successful cases, some individuals get a full recovery, while others have to do a prolonged follow-up of its clinical condition, with descriptions of people who became dependent on hemodialysis [100].

3.9 Addiction and withdrawal

Regular consumption of high doses of synthetic cathinones induces tolerance, dependence and withdrawal syndrome after abrupt interruption. Signs of withdrawal were reported by users of methcathinone, mephedrone and MDPV after abrupt cessation, notably including depression, anergy, anhedonia, anxiety, fatigue and sleep cycle disturbances [1]. Users report the development of tolerance to "bath salts" describing them as highly addictive [5].

3.10 Detection in human samples

Currently, there are no quick screening tests for synthetic cathinones, but techniques such chromatography coupled to mass spectrometry may be useful in the analysis of blood, urine and gastric content samples, either in *ante* or *postmortem* specimens, allowing the detection of these synthetic substances and its metabolites [2, 69, 79-81, 101-102]. There are also data in the scientific literature showing that hair samples are also valuable matrices, particularly for retrospective studies of synthetic cathinones consumption [2, 44, 103-105].

2 *Objectives*

The synthetic cathinones are representatives of a popular group of "legal highs", and there are currently about 40 known cathinone derivatives [23-25]. Some studies have been conducted for some of these individual derivatives, in particular relating to mephedrone, methylone, and butylone; however, the publications of studies related with other derivatives are limited. In addition, there are no data in the scientific literature related to acute or chronic health effects in animal and/or humans caused by the consumption of these new psychoactive drugs. In this way, further chemical and toxicological studies are necessary.

Since the actual content of the commercial products is unknown, the concerns about safety increase, so it is of great importance develop analytical procedures in order to reveal their real compositions. Thus, considering that market products are not chemically characterized, one of the main objectives of this work consists in the chemical characterization of commercial products, sold in three distinct "smartshops", announcing these new "legal highs" as "plant feeders". The aim is to reveal the new psychoactive substances that compose them, as well as potential adulterants that can also have harmful effects, using highly powerful methodologies, in particular the GC-MS and NMR analysis.

Due to the scarcity of cytotoxity studies relating to these new psychotropic substances, the second major objective of the work is to evaluate the hepatotoxic effects of two of the "legal high" products and cathinone derivatives sold as individual agent via Internet, using a HepaRG cell line and primary rat hepatocytes culture as *in vitro* models.

3 *Chemical characterization studies*

Chapter 1 - Experimental Part:

Chemical Analysis

1. *Material and Methods*

1.1 Reagents and Standards

All the reagents used were of analytical grade. Methanol (MeOH), chloroform, ethyl acetate, acetonitrile, acetone and n-hexane were obtained from Fisher Chemicals (Loures, Portugal) and diethyl ether was obtained from Pronalab (Lisbon, Portugal). Caffeine and trifluoroacetic anhydride (TFAA), 4-hydroxy-3-metoxybenzylamine hydrochloride (IS) were acquired from Sigma-Aldrich (Saint Louis, Missouri). Deuterated methanol was obtained from Cambridge Isotope Laboratories, Inc..

The reference compounds (methylone, pentedrone, 4-MEC and MDPV) were purchased from Sensearomatic website. All the standards were characterized by mass spectrometry and nuclear magnetic resonance. Methylone, 4-MEC and MDPV were also submitted to external analysis (by Instrumental Analysis Service of C.A.C.T.I., University of Vigo) in order to determine its elemental composition and consequently the molecular weight, as well as its purity.

1.2 "Legal highs" products

Twenty seven "legal highs" products were analyzed: 8 samples were purchased in Euphoria Smartshop (Porto), 9 in Magic Mushroom Smartshop (Porto), 9 in Magic Mushroom Smartshop (Lisbon) and 1 was delivered to the laboratory for analysis. Of the 27 samples, 22 were powders and 5 were tablets.

All products were acquired prior to the approval of Decree 54/2013[73], prohibiting the marketing of these products.

1.3 Qualitative Screening by GC-MS

1.3.1 Solvent selection

Powders and tablets were homogenized in a mortar. Homogenized samples were dissolved in different solvents (chloroform, methanol, ethyl acetate, acetonitrile, acetone, diethyl ether and n-hexane) at a concentration of 1 mg/mL. Two µL of each solution was injected directly in GC-MS.

1.3.2 Analysis of commercial products by direct injection and after derivatization

1.3.2.1 Standards and samples preparation

Methanolic solutions of the standards and samples were prepared at a concentration of 1 mg/mL. Two µL were injected automatically in GC-MS.

Analysis after derivatization was also performed. The derivatization procedure was executed as described by Silva et al. [106] with appropriate adaptations. Standards and samples methanolic extracts were prepared at a concentration of 5 µg/mL and 10 µg/mL, respectively and were evaporated to dryness under nitrogen flow. Fifty µL of ethyl acetate and 50 µL of TFAA were added to the dried residue. Incubation was preformed at 70°C for 30 minutes. After cooling to room temperature the solution was dried under nitrogen flow. The obtained residue was dissolved in 100 µL of ethyl acetate and 2 µL was injected on the GC-MS apparatus. A summary of the procedures is shown in figure 15.

1.3.2.2 GC-MS conditions

GC-MS analysis was performed with a Varian CP-3800 gas chromatograph (USA) equipped with a Varian Saturn 4000 Ion Trap mass selective detector (USA) and a Saturn GC-MS workstation software version 6.8. The GC was equipped with a VF-5MS (30 m x 0,25 mm x 0,25 µm) capillary column (Varian). A CombiPAL automatic auto sampler (Varian) was used for all experiments. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. Injections was performed in split mode, with a ratio of 1/40. The injector port was heated to 250 °C. The initial column temperature of 80 °C was held for 1 min, followed by a temperature ramp of 20 °C/min to 250 °C held for 2 min and 10 °C/min to 300 °C held for 20 min. Mass spectrometric detection was performed using an "ion trap" analyzer. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50 and 180 °C, respectively. The emission current was 10 µA and the maximum ionization time was 2500 µsec. Ionization was maintained off during the first 4 minutes to avoid solvent overloading. Total separation run time was 36 minutes. All mass spectra were acquired in electron impact (EI) mode and the mass ranged from 40 to 600 m/z. The injection volume was 2 µL and the analysis was performed in Full Scan mode.

For the derivatized compounds analysis, the GC-MS conditions were properly adapted. Injections were performed in splitless mode. The injector port was heated to 250 °C, the oven

temperature was adjusted of 100 °C held for 1 min, followed by a temperature ramp of 15 °C/min to 300 °C held for 10 min. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50 and 180 °C, respectively. The emission current was 30 μA and the maximum ionization time was 2500 μsec. Ionization was maintained off during the first 4 min to avoid solvent overloading. Total separation run time was 20 minutes. All mass spectra were acquired in electron impact (EI) mode and the mass ranged from 40 to 600 m/z. The injection volume was 2 μL and the analysis was also performed in Full Scan mode.

1.3.3 NMR analysis

The samples (approximately 30 to 50 mg) were dissolved in 500 μL of deuterated methanol (methanol-d4). The nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance 400 spectrometer with a frequency of 400 MHz for ^{1}H and 104 MHz for ^{13}C. The structures identification with the respective assignment of the proton and carbon signals was based on the analysis of NMR spectra obtained by 1D (^{1}H, ^{13}C, APT) and 2D (including the COSY, HMBC and HSQC experiments) techniques. The research was conduced by Dr. Helena Gaspar at the Faculty of Sciences, University of Lisbon, Portugal.

Direct Injection Analysis

A	**Extraction**
	Preparation of methanolic extracts
	- Standards (1 mg/mL)
	- Samples (1 mg/mL)
B	**GC-MS Analysis**
	2 µL of all extracts were injected in GC-MS
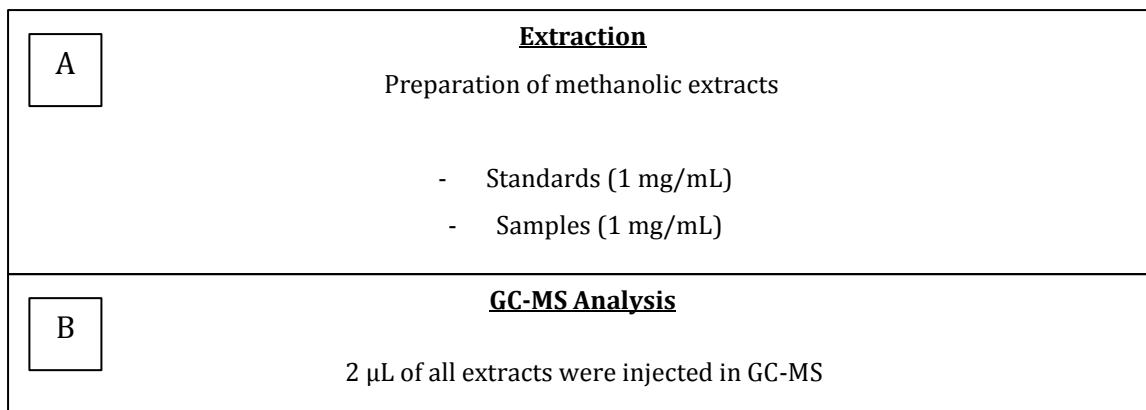
Analysis of Derivatized Samples

A	**Extraction**
	Preparation of methanolic extracts
	- Standards (5 µg /mL)
	- Samples (10 µg /mL)
B	**Derivatization**
	Dry under nitrogen flow
	↓
	50 µL ethyl acetate + 50 µL TFAA
	↓
	30 min at 70 °C
	↓
	Dry under nitrogen flow
	↓
	Dissolve residue in 100 µL ethyl acetate
C	**GC-MS Analysis**
	2 µL of all extracts were injected in GC-MS
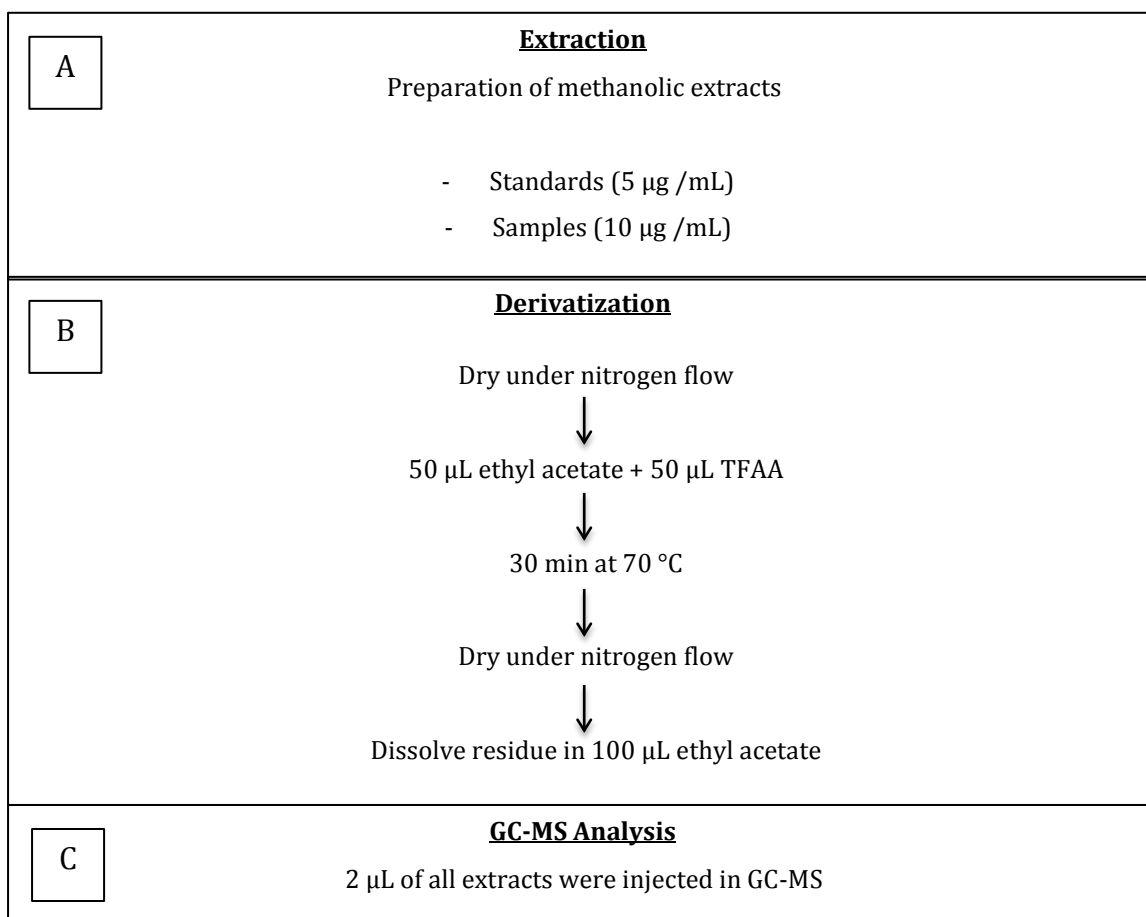
Figure 15 – Summary of procedures involved in "legal highs" characterization by GC-MS

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1.3.5 Identification process

The identification of each analyte was done by comparison of retention time and mass spectra of the tested compounds with standards injected under the same chromatographic conditions to ensure correct identification.

The EI-GC-MS spectra of unidentified peaks were searched through spectra databases supported by NIST05 (National Institute of Standards and Technology) and SWGDRUG (Scientific Working Group for the Analysis of Seized Drugs). We also made our own mass spectra library.

In order to confirm the identity of all compounds detected, derivatization with TFAA was applied. The trifluoroacylation reaction occurs in more electrophilic atoms: the nitrogen atom forming N-TFA and the oxygen atom forming O-TFA (Figure 16) [107].
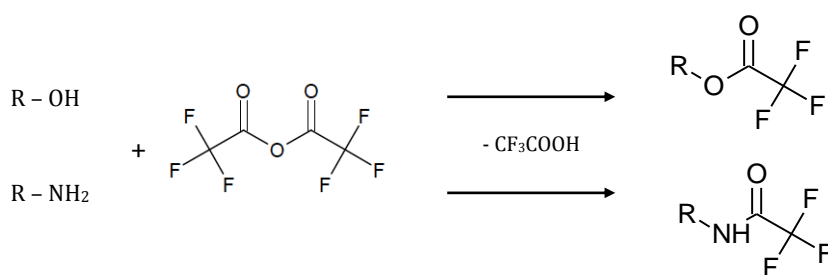
Figure 16 – Generic derivatization mechanism with the TFAA reagent to obtain the O-TFA and N-TFA derivatives

NMR spectroscopy was also applied to confirm the structure of molecules identified by GC-MS.

Thus, all information was compiled in order to reveal the real composition of the products marketed as "plant feeders" obtained in the three "smartshops". The process used for the products characterization is summarized in Figure 17.
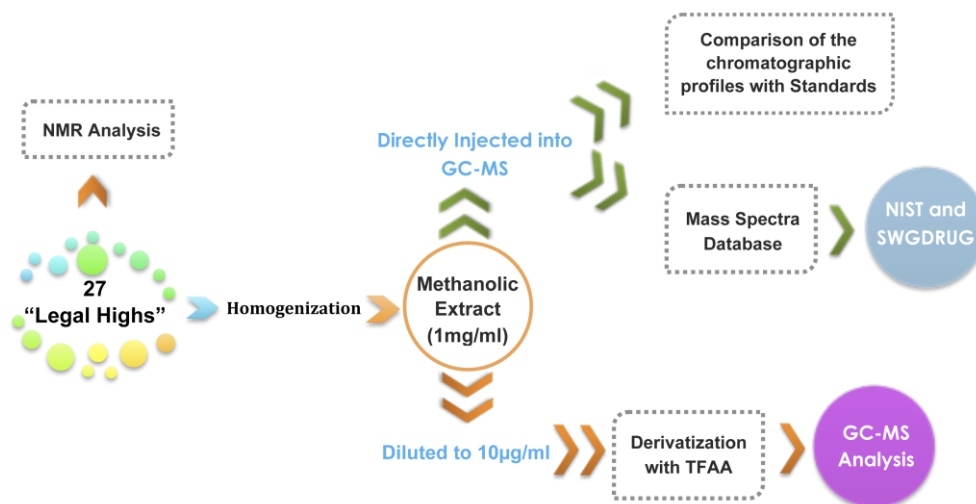
Figure 17 – Schematic representation of the procedure used in the chemical characterization of "legal highs", referring to the four crucial steps of the whole analysis

1.4 Quantitative determination of cathinones by GC-MS

1.4.1 Linearity studies

Standards (methylone, pentedrone, 4-MEC and MDPV) and internal standard stock solutions were prepared at 1 mg/mL in methanol. All intermediate solutions were prepared daily by dilution of concentrated solution in methanol; working calibrators at 10, 50, 100, 500, 1000, 2000, 5000 and 10000 ng/mL were prepared. One hundred µL of 4-hydroxy-3-methoxybenzylamine hydrochloride (IS) at a concentration of 10 µg/mL were added to each solution. The derivatization process was performed and 2 µL were injected into the GC-MS. Regression curves were obtained plotting the peak-area ratio between each analyte and the internal standard against analyte concentrations, being the method linearity evaluated by the squared correlation coefficient (R^2).

1.4.2 Reproducibility studies

Four "smartshops" products (3 powders, 1 tablet) were randomly selected to perform the semi-quantitative studies of cathinones. For each sample analyzed, three solutions were

prepared in methanol, daily, at a concentration of 1 mg/mL. For each initial solution, intermediate concentrations were prepared (5 μg/mL or 10 μg/mL) in triplicate. One hundred μL of 10 μg/mL IS was added to each solution and the derivatization process was carried out under the same conditions used in calibration curves.

The reproducibility of the method was determined, being expressed through the calculation of the coefficient of variation for each final solution, in percentage [CV % = (SD/mean) x 100].

1.4.3 Estimation of the amount of interest compounds in "legal highs" samples

Concentrations were calculated using the equation of the straight line (y = mx + b) of the respective analyte of interest and the mean was calculated for each of the independent solutions. The actual amount (mg) of the active ingredient in the four samples analyzed randomly (unknown quantities) was estimated.

1.4.4 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6 (version 6.0c) for Mac OS X. Multiple comparisons between more than two groups were performed by one or two-way ANOVA supplemented with Tukey's test. Significance was accepted at $P<0.05$.

Chapter 2 - Results and discussion of the chemical analysis

2.1 Chemical characterization of cathinone derivatives used as standards

The cathinone derivatives used as standards (Methylone, 4-MEC, Pentedrone and MDPV) were acquired through a website (Sensearomatic.com) that is able to provide highly pure chemical products at a competitive price. Although the substances sold via this website (as well as in others) entitles as "research chemicals", any person can acquire them, particularly for consumption. So the consumers of these new drugs can create, without any restriction, their own "designer mixtures", becoming more economically appealing (Figure 18).

Figure 18 – Template of one of the thousands of websites (Sensearomatic.com) from which any person can purchase several "legal highs", for consumption or other purposes, without any restriction and at a very affordable price. To circumvent all the legal aspects, there is clear information that the products are "not for human consumption" and that do not have any responsibility if the products is illegal in the country to which the intended delivery. [Image taken from http://www.sensearomatics.net/4meccrystalline/, accessed 19/07/2013]

To make sure that standards correspond to what is mentioned on the label, all were analyzed by GC-MS, whose mass spectra were compared with those already described in the literature (Figure 19) [40, 45-46, 108]. RMN analyses were also performed to determine the molecular structure of the compound. The results showed that all standards corresponded to the requested compound.

The GC-MS and NMR analyses also show that pentedrone standard contained an isomeric impurity identified as isopentedrone (1-methylamino-1-phenylpentan-2-one), a by-product of the pentedrone synthesis, in which the amino moiety and keto group change their place in the molecule [40]. Thus, this standard also served as confirmation of this minor compound.
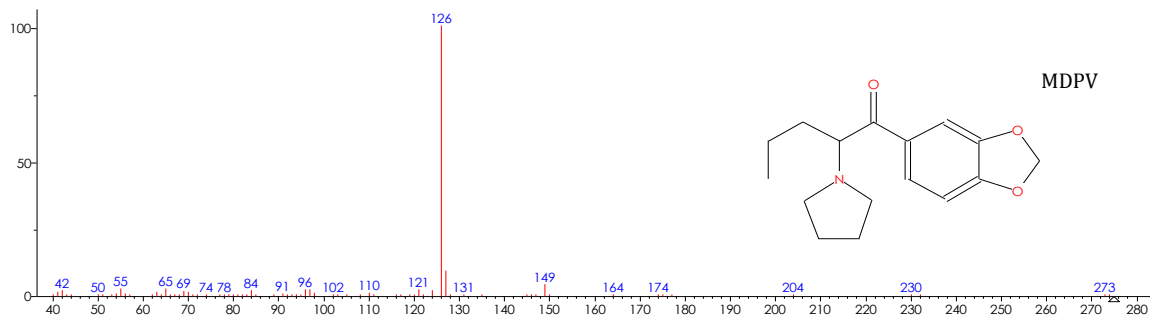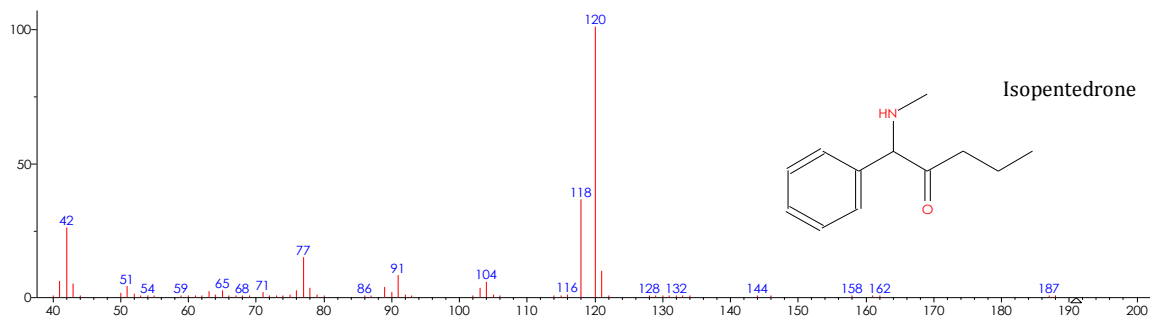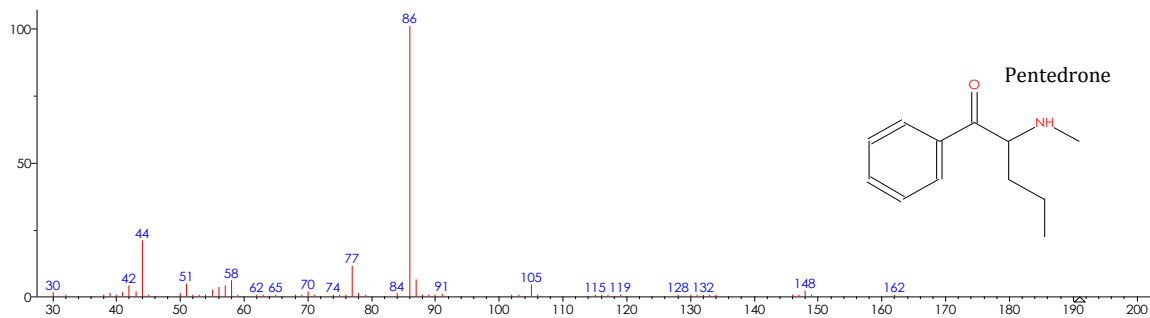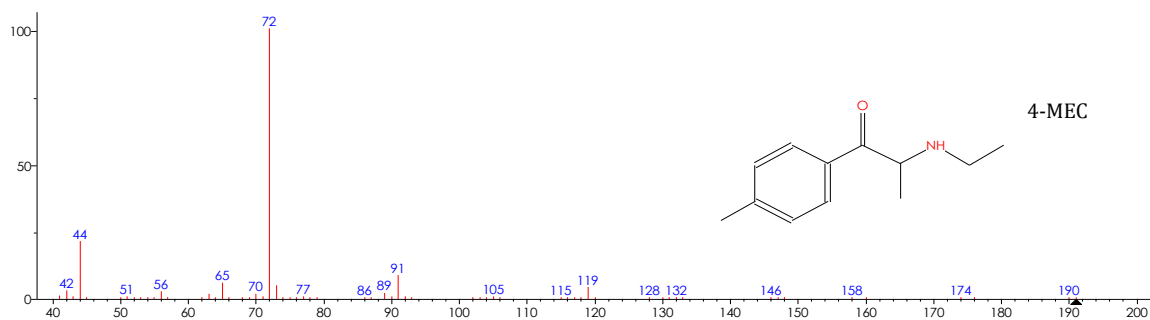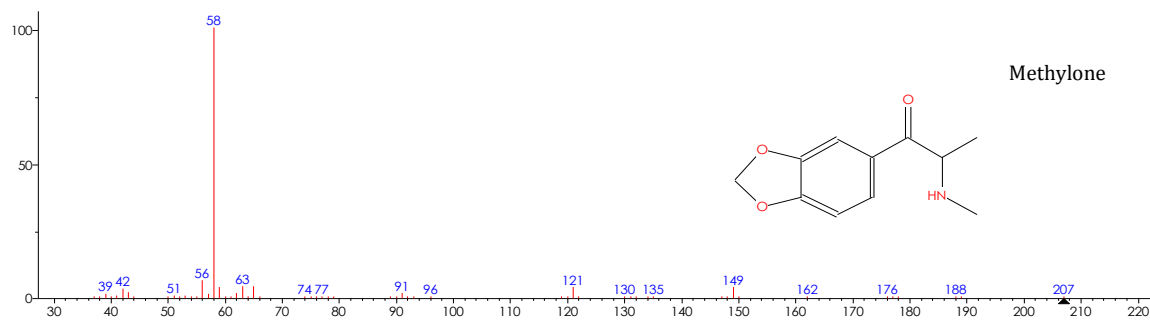
Figure 19 – EI mass spectra of methylone, 4-MEC, pentedrone, isopentedrone and MDPV, respectively (direct injection)

All cathinone derivative standards (with exception of pentedrone, which was only available in a small amount) were also subjected to elemental analysis in order to determine if they were hydrochlorides and also to determine their purity. The results of analyses carried out are described in Table 5.

Table 5: Results of external analysis of cathinone derivatives standards

Compound	% N	% C	% H	% Cl	Theoretical molecular weight	Experimental molecular weight	Proposed molecular formula	% Purity
4-MEC	6.15	63.28	7.98	15.57	227.75	221.99	$C_{12}H_{17}NO.HCl$	97.47
Methylone	5.75	54.21	4.97	14.55	243.71	238.77	$C_{11}H_{13}NO_3.HCl$	97.97
MDPV	4.50	61.62	7.13	11.37	311.84	304.40	$C_{16}H_{21}NO_3.HCl$	97.61

The results showed that the samples analyzed were actually hydrochlorides, showing all of them purity higher than 97%.

All compositions were confirmed and purities obtained were high so that all standards have been considered for the analysis of chemical characterization of commercial products.

2.2 Characterization of commercial products

A total of 27 "legal highs" were purchased in three distinct "smartshops" (Euphoria in Porto and Magic Mushroom in Porto and Lisbon). A survey of all products sold as "plant feeders" or "bath salts" in these shops was conducted, being bought at least one sample of each. Some products were purchased on a large number of units, being a random choice in order to check the possible existence of qualitative and quantitative variability between supposedly identical products, as was previously described in the literature [6, 8-9, 16].

All products were packed in sealable silver foil bags or, occasionally, in transparent plastic bags. The packages featured the commercial name of the product, the alleged composition, the product quantity, and the dosage while "plant feeders", along with the warnings "not for human consumption" (Figure 20). Some of the products presented its lot number but no other information was provided.

Figure 20 – Appearance of some commercial products

The majority of powder products (86%) had a white color, however a small part had a yellowish color (Table 6), while the predominant color of the tablets was pink (Table 7). Variation was observed in the color of products with the same commercial name, acquired in the same "smartshop" (e.g. sample 1 and 2) or in different "smartshops" (e.g. sample 1 and 3; 6 and 7; or 16, 17 and 18), which immediately raised the possibility of a different composition.

The amount of products purchased ranged between 0.5 and 1 gram in the case of the powders. All tablet products had two units per pack. The weight of each tablet was approximately 500 mg. Considering the amount of 1 g for all products, the prices for the powders varied between 30 and 38 euros, while the price of tablets varied between 14 and 20 euros (Table 6 e 7). This could be related to the product purity, leading us to consider the hypothesis that the powders are more potent than the products sold as tablets.

Regarding the composition, the ingredient names printed on the packaging were recorded (Table 6 e 7) and the spelling mistakes were not corrected (e.g. phosphoates which should read phosphates). With the exception of samples 22, 26 and 27 all others have printed "ketones", which can be interpreted as an indicator of the presence of cathinone derivatives. Samples 26 and 27 ("Bloom+") have described the presence of aminoalkyl benzofurans, which although not derived from cathinones, are structurally similar to amphetamine analogs. Most powder products also referred the presence of caffeine and glucose. In tablets, the printed composition was more elaborated once beyond the principle active other excipients related to the production of tablets were also present. The fact that some spelling errors have been detected and the composition described being extremely vague it seems that these products do not follow any kind of quality control.

Table 6: Main features of "legal highs" (powders) acquired for analysis

Sample Number	Product Name	Smartshop	Lot	Product Color	Chemical composition indicated on the label	Price per gram (€/g)
1	Bloom	Magic Mushroom (Porto)	N/A	Yellowish	Ketones, Vegetable extracts and Glucose	36
2	Bloom	Magic Mushroom (Porto)	2012 45X19P	White	94% Ketones, 5% Caffeine and 1% Glucose	36
3	Bloom	Magic Mushroom (Lisbon)	2012 45X19P	White	94% Ketones, 5% Caffeine and 1% Glucose	36
4	Blast	Euphoria (Porto)	N/A	White	89% Ketones, 10% Caffeine and 1% Glucose	36
5	Blast	Magic Mushroom (Lisbon)	2012 46X12P	White	100% Ketones	36
6	Rush	Magic Mushroom (Porto)	N/A	Yellowish	89% Ketones, 10% Caffeine and 1% Glucose	30
7	Rush	Magic Mushroom (Lisbon)	N/A	White	89% Ketones, 10% Caffeine and 1% Glucose	30
8	Invader (Crabby)	Euphoria (Porto)	2012 47X2P	White	100% Ketones	37
9	Invader (Cyclop)	Euphoria (Porto)	2012 45X2P	White	100% Ketones	37
10	Bliss	Magic Mushroom (Porto)	2013 X0619P	White	95% Ketones, 5% Caffeine and 1% Glucose	30
11	Bliss	Euphoria (Porto)	2012 37014P	White	100% Ketones	30
12	Bliss	Magic Mushroom (Lisbon)	2012 42X16P	White	100% Ketones	30
13	Charlie	Magic Mushroom (Porto)	2012 40X17P	White	100% Ketones	37
14	Charlie	Magic Mushroom (Porto)	2012 35017P	White	100% Ketones	37
15	Charlie	Magic Mushroom (Lisbon)	2012 35017P	White	100% Ketones	37
16	Blow	Magic Mushroom (Porto)	N/A	Yellowish	Ketones, Vegetable extracts and Glucose	38
17	Blow	Euphoria (Porto)	N/A	White	94% Ketones, 5% Caffeine and 1% Glucose	38
18	Blow	Magic Mushroom (Lisbon)	N/A	White	94% Ketones, 5% Caffeine and 1% Glucose	38
19	Kick	Magic Mushroom (Porto)	N/A	White	Ketones, Vegetable extracts and Glucose	30
20	Kick	Euphoria (Porto)	2012 36015P	White	94% Ketones, 5% Caffeine and 1% Glucose	30
21	Kick	Magic Mushroom (Lisbon)	2012 38015P	White	94% Ketones, 5% Caffeine and 1% Glucose	30
22	MMB	N/A	N/A	White	N/A	N/A

Table 7: Main features of "legal highs" (tablets) acquired for analysis

Sample Number	Product Name	Smartshop	Lot	Product Color	Chemical composition indicated on the label	Price per two tablets (€/2 tablets)
23	M	Magic Mushroom (Porto)	N/A	Pink	Ketones, Dicalcium, Phosphoates, Magnesium, Stearate	16
24	M	Magic Mushroom (Lisbon)	N/A	Pink	Ketones, Dicalcium, Phosphoates, Magnesium, Stearate	16
25	Bliss	Euphoria (Porto)	N/A	Pink	160 mg Ketones, 120 mg Lactose, 100 mg Corn starch, 50 mg Calcium stearate, 20 mg Magnesium stearate, 20 mg E124, 6 mg E132, 4 mg E142	14
26	Bloom +	Euphoria (Porto)	N/A	Pink	100 mg Aminoalkyl benzofurans, 120 mg Lactose, 100 mg Corn starch, 50 mg Calcium stearate, 20 mg Magnesium stearate, 20 mg E128, 5 mg E142	20
27	Bloom +	Magic Mushroom (Lisbon)	N/A	Pink	100 mg Aminoalkyl benzofurans, 120 mg Lactose, 100 mg Corn starch, 50 mg Calcium stearate, 20 mg Magnesium stearate, 20 mg E128, 5 mg E142	20

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2.3 Screening of psychoactive substances in "legal high" products

Since the products labeled as "plant feeders" or "bath salts" are supposed to contain ketones (hypothetically cathinone derivatives), we started this work by studying the chemical profiling of different commercial products. Due to difficulties in accessing spectra libraries containing the mass spectra of these new substances, we had to create our own database taking into account the information collected, which contains the molecular structure, molecular weight, mass spectrum profile including base peak and other characteristic fragmentation ions (m/z) of the different compounds.

Gas chromatography coupled to mass spectrometry technique was chosen to separate and identify the various compounds present in the "legal highs" samples.

Initially, the extraction of compounds was tested with different solvents, being the methanol the solvent chosen for all analyzes. This choice is due to the fact that the cathinone derivatives are very polar molecules due to the presence of the ketone group and thus can be easily extracted by polar solvents such as methanol.

Methanolic extracts of all samples were directly analyzed by GC-MS, resulting in different chromatographic profiles. All peaks were taken into account for the products characterization. Using the mass spectrum information of each chromatographic peak (e.g. base peak, molecular ion) we were able to establish relationships with the spectra database. By performing the intersection of information obtained from mass spectra interpretation with mass spectra database (NIST05 and SWGDRUG), and later with NMR data, we were able to identify cathinones in each commercial product.

We were able to identify methylone, 4-MEC, isopentedrone, pentedrone and MDPV in commercial products by comparing retention times and mass spectra of each chromatographic peak with the respective standards. Positive matches were obtained for all compounds that initially had been identified through the database created. Thus, these six compounds were unequivocally identified.

The other compounds were assigned potential identifications through the analysis of their mass spectra and comparison with compounds of our database. Due to the lack of standards to confirm or reject the other identifications, derivatization with TFAA was performed. Even the substances confirmed by standards were subjected to this process. Derivatization allowed us to obtain more clean chromatograms and get better separations with increased resolution and response (Figure 21).
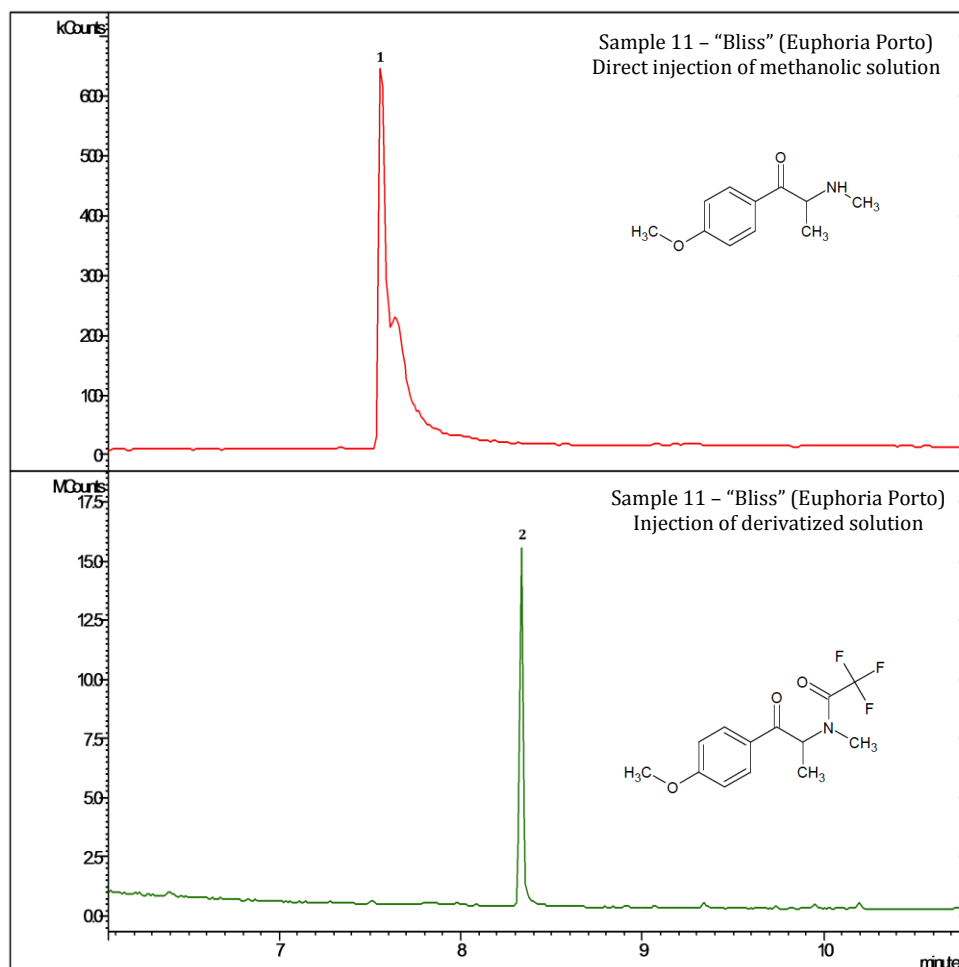
Figure 21 – Full scan chromatographic profile of methanolic extracts (Sample 11 – "Bliss") injected directly (red) into the GC-MS and injected after derivatization with TFAA (green), indicating the potential identification of compounds based on the analysis of mass spectra and new fragmentation patterns of molecules after derivatization with TFAA. The signal strength after derivatization is significantly higher, showing greater sensitivity of the method. 1 – Methedrone; 2 – Methedrone N-TFA

Knowing the TFAA affinity for binding to the active hydrogen's of amine and hydroxyl groups, it was possible to predict the chemical structures of the formed compounds whose identity was intended to determine. Knowing the molecular weight after derivatization and ions resulting from fragmentation, comparisons with the mass spectrum of the derivatized compounds (Figure 22) and derivatized standards were established in order to confirm the identification of compounds.

Of note, there are compounds without binding sites for TFAA, such as caffeine (Figure 22) and MDPV, whereby the molecular structure and consequently the mass spectrum are not altered after derivatization.

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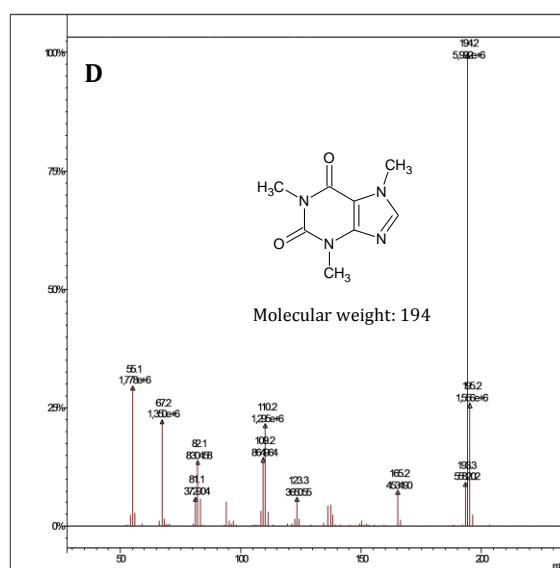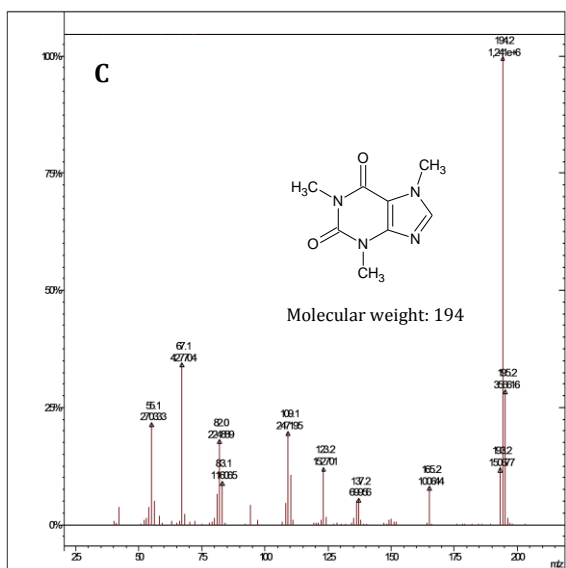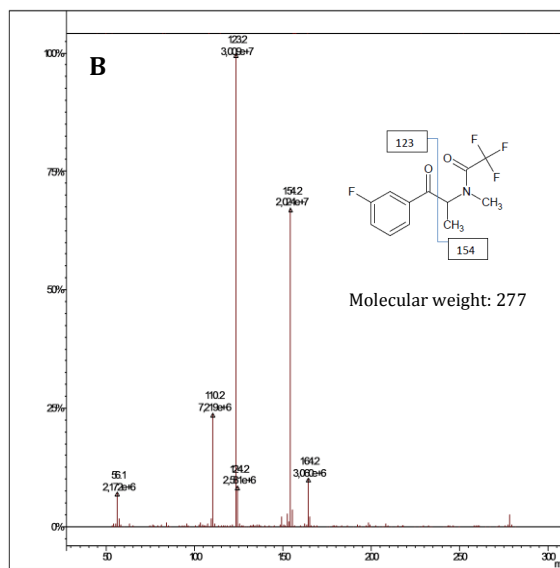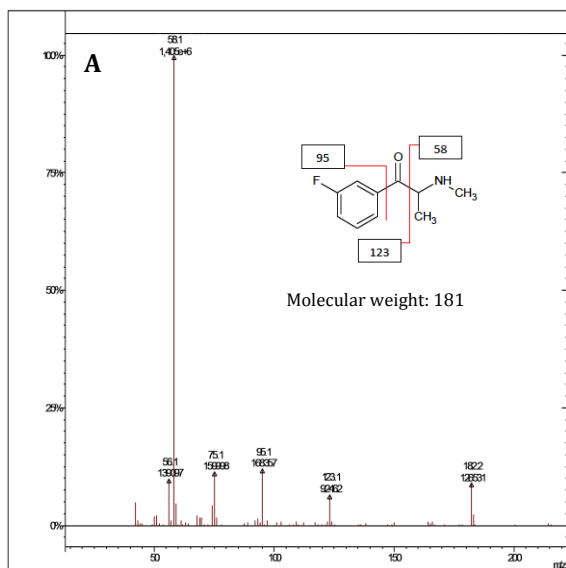
Figure 22 – EI mass spectra corresponding to compounds of the sample 4 ("Blast")
A – Flephedrone EI mass spectrum (direct injection); B – EI mass spectrum of flephedrone N-TFA; C –
Caffeine EI mass spectrum (direct Injection); D – Caffeine EI mass spectrum after derivatization

Following the entire procedure, the chemical characterizations of all products purchased in different "smartshops" are summarized in Table 8. In Table 9 are presented the retention time, base peak and other characteristic qualitative ions (m/z) for the compounds identified by GC-MS before and after derivatization.

In a later stage of the experimental work, we had access to a new mass spectra database (SWGDRUG), which includes these new synthetic substances. Thus, all chromatographic peaks (direct injection) were also screened in this new library. It should also be noted that all products have been analyzed by NMR, thus confirming the molecular structure of each compound being an informative methodology in the distinction of positional isomers. These analyses allowed for example the distinction of 3-MEC and 4-MEC isomers.

Table 8: Active ingredients detected in 27 "legal highs" acquired in three distinct "smartshops" after analysis by GC-MS and NMR. (*) "Legal high" sold in tablet form.

Product name	Smartshop	Active substances identified by GC-MS and NMR
Bloom	Magic Mushroom Porto	Isopentedrone, Pentedrone, 4-Methylethcathinone (4-MEC), Methylone, Dimethocaine
	Magic Mushroom Porto	Ethcathinone, Pentedrone, Methedrone, Caffeine
	Magic Mushroom Lisbon	Ethcathinone, Pentedrone, Methedrone, Caffeine
Blast	Euphoria Porto	Flephedrone, Caffeine
	Magic Mushroom Lisbon	Flephedrone, Caffeine
Rush	Magic Mushroom Porto	Buphedrone, Caffeine
	Magic Mushroom Lisbon	Isopentedrone, Pentedrone, Caffeine
Crabby	Euphoria Porto	3,4-Dimethylmethcathinone (3,4-DMMC)
Cyclop	Euphoria Porto	3,4-DMMC
Bliss	Magic Mushroom Porto	Isopentedrone, Pentedrone, 3,4-DMMC, Methedrone, Caffeine
	Euphoria Porto	Methedrone
	Magic Mushroom Lisbon	Methedrone
Charlie	Magic Mushroom Porto	Ethcathinone, Buphedrone, Caffeine
	Magic Mushroom Porto	Ethcathinone, Buphedrone
	Magic Mushroom Lisbon	Ethcathinone, Buphedrone
Blow	Magic Mushroom Porto	3-Methylmethcathinone (3-MEC), 4-MEC, Methylenedioxypyrovalerone (MDPV)
	Euphoria Porto	3-MEC, 4-MEC, Caffeine, MDPV
	Magic Mushroom Lisbon	3-MEC, 4-MEC, Caffeine, MDPV
Kick	Magic Mushroom Porto	Isopentedrone, Pentedrone
	Euphoria Porto	Buphedrone, Caffeine
	Magic Mushroom Lisbon	Buphedrone, Caffeine
MMB	N/A	Alpha-methyltryptamine (AMT), Dimethocaine
M (*)	Magic Mushroom Porto	4-Fluoroamphetamine
	Magic Mushroom Porto	4-Fluoroamphetamine
Bliss (*)	Euphoria Porto	Methylone
Bloom + (*)	Euphoria Porto	5-(2-Aminopropyl)benzofuran (5-APB), 6-(2-Aminopropyl)benzofuran (6-APB), 5-(2-Aminopropyl)2,3-dihydrobenzofuran (5-APDB)
	Magic Mushroom Porto	6-APB, 5-APDB

Table 9: Retention time (minutes), base peak (m/z) and other characteristic ions (m/z) of active ingredients detected directly by GC-MS and after derivatization with TFAA. Legend: A – available; N/A – not available; SW – SWGDRUG library; N – NIST05 library; m/z – mass to charge ratio

Compound	Standard	Library	Without TFAA			With TFAA		
			Retention time (minutes)	Base Peak (m/z)	Characteristic ions (m/z)	Retention time (minutes)	Base Peak (m/z)	Characteristic ions (m/z)
Isopentedrone	A	SW	6,5	120	77, 91, 191	N/A	N/A	N/A
Pentedrone	A	SW	6,7	86	77, 105, 191	7,2	140	77, 105, 182, 287
3-MEC	N/A	SW	6,8	72	91, 119, 191	7,5	119	91, 168, 287
4-MEC	A	SW	6,9	72	91, 119, 191	7,7	119	91, 168, 287
Methylone	A	SW	8,1	58	91, 121, 149, 207	9,0	149	65, 110, 303
Ethcathinone	N/A	SW	6,1	72	44, 77, 105, 177	6,7	105	110, 168, 273
Methedrone	N/A	SW	7,5	58	77, 107, 135, 193	8,3	135	176, 182, 289
Flephedrone	N/A	SW	5,7	58	95, 123, 181	6,0	123	110, 154, 277
Buphedrone	N/A	SW	6,3	72	77, 105, 177	6,9	105	71, 77, 168, 273
3,4-DMMC	N/A	SW	7,3	58	77, 105, 133, 191	7,9	133	105, 174, 182, 287
MDPV	A	SW	10,5	126	84, 121, 149, 275	11,3	126	84, 121, 149, 275
5-APB	N/A	SW	6,6	44	77, 131, 175	7,4	131	140, 158, 270
6-APB	N/A	SW	6,7	44	77, 131, 175	7,6	131	140, 158, 270
5-APDB	N/A	SW	7,2	44	77, 134, 177	8,2	160	105, 133, 273
4-Fluoroamphetamine	N/A	SW	4,3	44	83, 109, 153	5,0	136	89, 109, 248
AMT	N/A	SW/N	8,4	131	44, 77, 103, 174	8,7	226	140, 154, 366
Dimethocaine	N/A	SW/N	11,0	86	58, 120, 278	11,8	86	65, 120, 374
Caffeine	A	SW/N	8,8	194	55, 67, 82, 109	9,4	194	55, 67, 82, 109

A total of 18 different substances were detected (Figure 23), belonging the vast majority to the class of synthetic cathinones. Substances of other classes were also found, namely dimethocaine (cocaine derivative), alpha-methyltryptamine (class of tryptamine), as well as other substances derived from phenethylamines class, namely the derivatives of benzofurans (5-APB, 6-APB and 5-APDB).

In total we found 11 distinct cathinone derivatives (Figure 23), the pentedrone and buphedrone being the most frequent (9.1%) while flephedrone and methylone the less common (3%). However, despite the synthetic cathinones correspond to the representative class, the most common substance was caffeine (18.2%) (Figure 23), having the function of promoting the stimulant effects, being an adulterant.
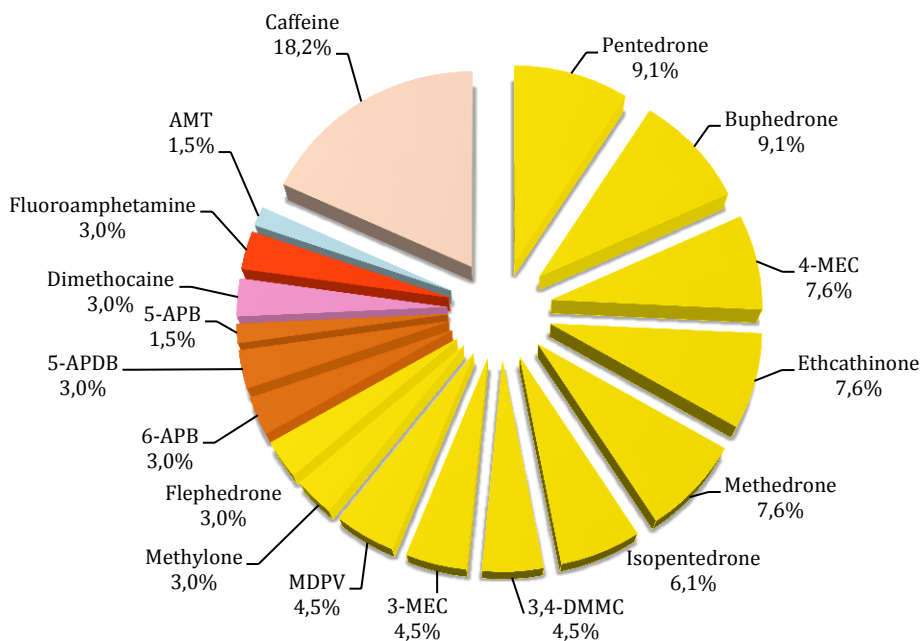
Figure 23 - Different substances found in compositional analysis of the various "legal highs". Yellow compounds are representatives of the synthetic cathinones class (66.7%), the orange are aminoalkyl benzofurans (7.6%), the red is a representative of amphetamine class (3%), while blue is of the tryptamine class (1.5%). The only cocaine derivative (3%) detected is highlighted in rose while caffeine (18.2%) is in beige.

In the study of "legal highs" characterizations performed by Sobczak et al. (2012) [7], the synthetic cathinones were also the most frequent class of compounds (47%) followed by cannabinoids (45%), being the piperazines (7.5%) and phenethylamines (3.8%) the less numerous classes. The MDPV was the cathinone most frequently detected, although caffeine was the substance most commonly present.

In turn the study conducted by Zuba et al. (2012) [6], on "legal highs" samples seized in "headshops" in Poland, showed that 449 products contained substances from different chemical classes, but active components can be classified into two major classes: cathinones and piperazines. In total, 61 substances were identified of which 14 belonged to the class of synthetic cathinones. The most common ingredients detected in "legal highs" seized were, in descending order: MDPV, caffeine, butylone, TFMPP, lidocaína, 4-MEC, mephedrone, pFPP, BZP and MDPBP.

2.4 Inconsistency in the composition of the commercial products analyzed

The number of compounds present in each "legal high" showed be variable. According to the results, there are products where only one active principle is present, under our conditions of analysis (for example sample 8, 9, 11, 12, 23, 24 and 25), but most "legal highs" are mixtures of psychoactive substances with a high number of constituents, as for example sample 1 ("Bloom") or sample 10 ("Bliss") that have five different components. Of 27 analyzed preparations, only 7 (26%) contained a single component, 10 (37%) were composed of two psychoactive ingredients, 4 (15%) presented three substances, 4 products (15%) presented four ingredients and only 2 products (7%) had five different ingredients.

It might be thought that the price of the products was related with the number of psychoactive compounds, however, according to our results it is not true since samples 8 and 9 only present an active ingredient and have about the same retail price (37€) than for example the sample 1 which features 5 different compounds (36€).

Zuba et al. (2012) [6] and Sobczak et al. (2012) [7] observed that it is more frequent the detection of a single active principle than a higher number. Sobczak et al. [7] have also found that it is more common to find samples with substances of the same class (66%), for example cathinones, than of different classes. Our results, in part, corroborate these findings since only a minority of products possesses a large number of substances. However, commercial products with two active principles are much more frequent (Table 8), in opposite to what has been reported by Zuba et al. (2012) and Sobczak et al. (2012). Our results are in agreement with those obtained by the same authors, being preferred the use of substances of the same class (e.g. the class of synthetic cathinones). The presence of mixtures of drug classes (e.g. synthetic cathinone class with cocaine derivatives) has a less expression, although in most cases the caffeine appear associated to the main class.

Taking into account the GC-MS and NMR analyses, this study revealed a large diversity in the composition of the "legal highs" acquired. The 27 samples correspond to a total of 12 different commercial products ("Bloom" (3); "Blast" (2); "Rush" (2); "Invader" (2); "Bliss" (3); "Charlie" (3); "Blow" (3); "Kick" (3); "MMB" (1); "M" (2); "Bliss" Tablet (1); "Bloom +" (2)). However, 19 different compositions were found, revealing the existence of a great compositional variability, which is an important finding from this study.

Direct analysis of methanolic extracts by GC-MS showed different chromatographic profiles among different products and, in some cases, among products with the same commercial names (Figure 24). The high variability had been considered as different colors of the same products

were found. In all these cases ("Bloom", "Rush" and "Blow") analyses corroborate our suspicious of differences in the composition. Even in cases where sample colors were similar, clearly distinct compositions were found, particularly in samples of "Bliss" (10, 11 and 12), "Charlie" (13, 14 and 15) and "Kick" (19, 20 and 21).

Apparently, a quantitative variability among products with the same composition was also detected (Figure 24 – Sample 2 and 3).
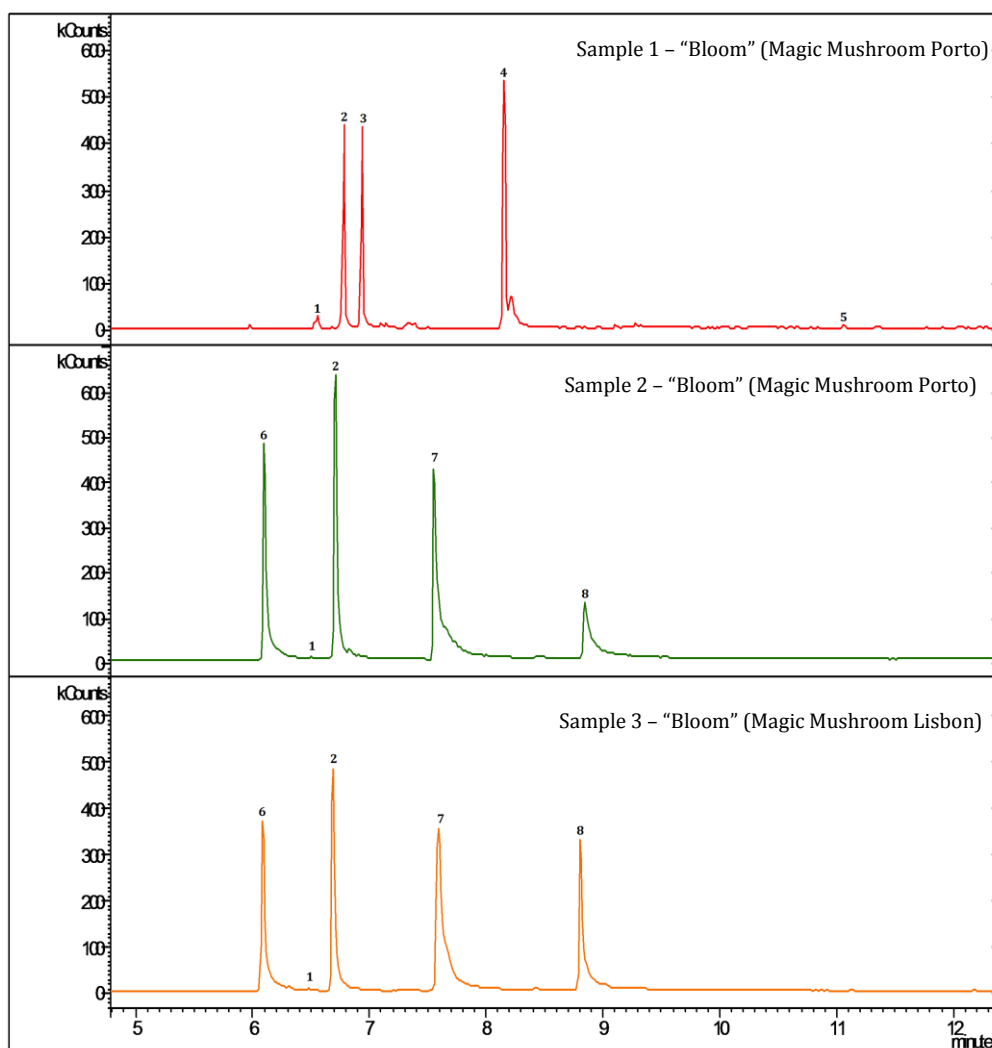
Figure 24 – Full scan chromatographic profile of methanolic extracts (Sample 1, 2 and 3 – "Bloom") injected directly into the GC-MS, indicating the potential identification of compounds based on mass spectrum analysis and revealing the qualitative and quantitative variability between products with the same commercial name. 1 – Isopentedrone; 2 – Pentedrone; 3 – 4-MEC; 4 – Methylone; 5 – Dimethocaine; 6 – Ethcathinone; 7 – Methedrone; 8 – Caffeine

Most cases of variability were observed in samples with the same name sold in different "smartshops". However, in "Bloom" and "Charlie" products, acquired in the same "smartshops",

composition was also different. All components differing in "Bloom" samples, while in "Charlie" one of the samples has caffeine as minority peak that is absent in the others.

The analyses of two samples of "Rush" and three samples of "Kick" reveal distinct situations with extremely important evidences (Figure 25). The first corresponds to the fact that a commercial product with the same name (case of "Kick" and "Rush"), distributed by the same supplier (Aurafeel), sold in "smartshops" with the same name (Magic Mushroom) but situated in different locals (Lisbon and Porto), presents a completely different composition. We also found that the same product ("Kick") sold in distinct "smartshops" (Magic Mushroom and Euphoria), in the same city (Porto), also has a different composition.

The analysis also revealed that the same mixture could also be sold as products with different trade names (Figure 25), as checked in the sample 6 ("Rush" – Magic Mushroom Porto), samples 20 ("Kick" – Magic Mushroom Lisbon) and 21 ("Kick" – Euphoria Porto). In these three samples, chemical analyzes detected isopentedrone, pentedrone and caffeine. This suggests that the distributors (in this particular case all products were distributed by the same company – Aurafeel) do not know necessarily what they are selling, just being responsible for packaging and distribution, not being associated with sample processing.

Finally, this analysis also allowed noting that products sold with the same name and same real composition (Sample 20 and 21 – "Kick") feature a clear quantitative variability with regard to amount of caffeine (Figure 25).
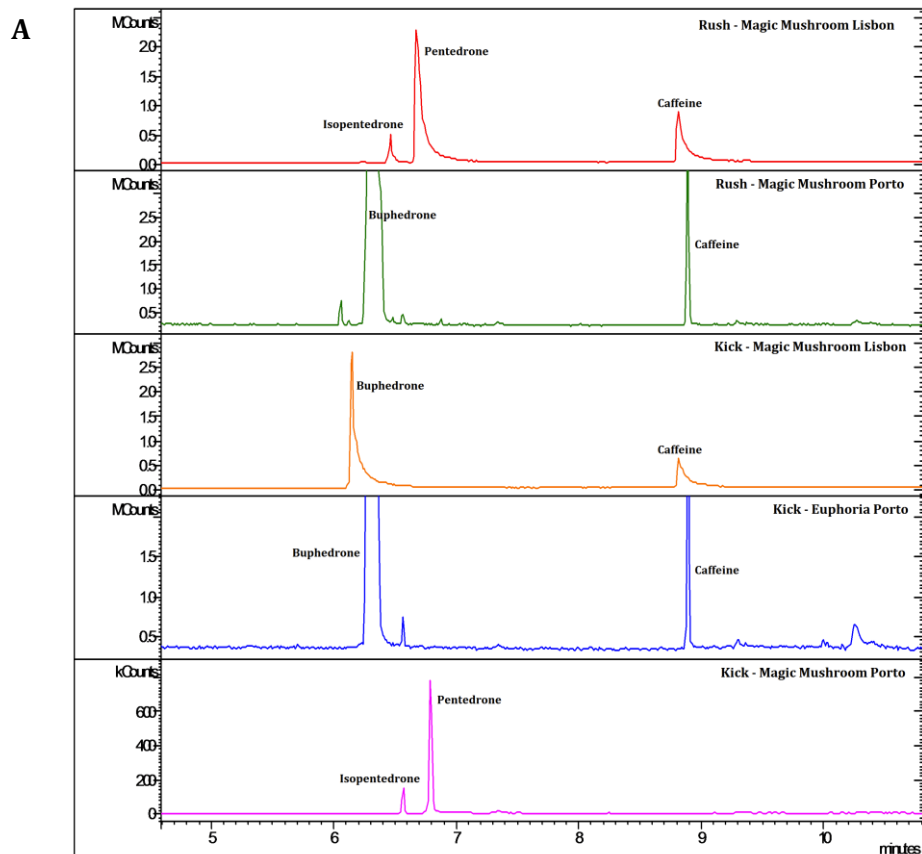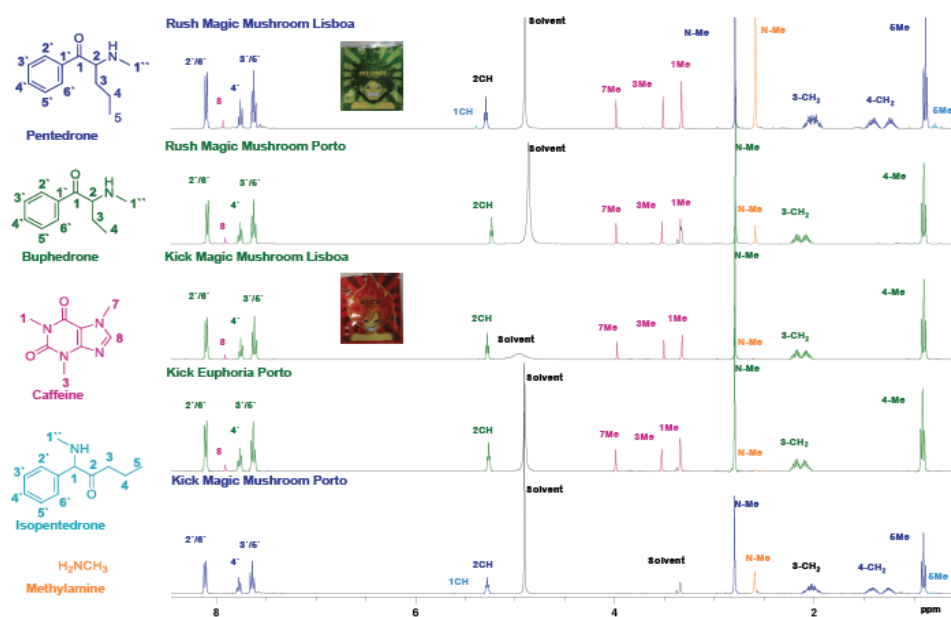
Figure 25 – Analysis of "Rush" and "Kick" samples acquired in 3 different Portuguese "smartshops": (A) Full scan chromatographic profile of methanolic solutions (1mg/mL) injected directly into the GC-MS, in parallel with the standard compounds. (B) ^{1}H-NMR in MeOD (400.13 MHz) of the samples that allowed to assign the structure of the molecules present in the "plant feeders".

The data presented by Zuba et al. (2012) [6] also indicate a high variability in the content of the products, being detected 10 different qualitative compositions in products named *"Coco"*. Some *"Coco"* products contained a single component, whereas others had mixtures. Even if the name was the same and packages were identical, the content was different. The real composition of *"Coco Jumbo 0.5g"* and *"Cherry Coco Jumbo"* was identical and the products contained six different active ingredients, whereas *"Coco Jumbo 0.25g"* contained only pentedrone.

All variability found could be explained by the fact that the products belong to different lots as in the samples 13 and 14 ("Charlie"). However, there are different lots whose composition is the same as for example the case of samples 11 and 12 ("Bliss") or 20 and 21 ("Kick"). Furthermore, in vast majority of cases we cannot draw conclusions about this topic once the lot number is not available, demonstrating once again the carelessness in the handling of these new products.

In addition, in the same study developed by Zuba et al. [6], data show differences in qualitative and quantitative compositions even among samples packed together.

In addition to the qualitative variability exploited above, in most cases it was also noted inconsistency between the "legal high" composition obtained by GC-MS analysis and what is label on the package. Caffeine was not detected in all products that appear described (e.g. sample 16). Other cases where the composition labeled was 100% ketones, chemically it was not observed (e.g. sample 5 it was found cathinones and caffeine). In relation to glucose, this was not detected in any of the samples analyzed. However we cannot conclude with certainty that this is not present in the products, because it may be at an insufficient amount to be detected or may be undetected under the conditions used in this study. This type of inconsistency has also been verified by other researchers [31], dedicated to the study of new psychoactive substances phenomenon. FTIR studies confirmed by GC-MS revealed that 6 out of 7 samples acquired did not contain the labeled drugs but large amounts of caffeine.

The lack of consistency in the qualitative composition is a serious problem. Significant variations in the contents of similarly labeled products containing psychoactive substances of variable potency and adverse effects is a serious threat for users and increases the risk of acute

harm and toxicity associated with their use. This inconsistency also hampers the assessment of the clinical state of the patient and consequently to provide appropriate treatment.

When one substance is replaced by another or by a mixture, or the amount is higher than those labeled, the effects can be significantly different. These differences could have an impact on duration of action, the time required to cause the effects, and to the multiplicity of effects and respective interactions, etc, which could result in severe intoxications. Another matter of concern that should be the subject of further studies passes through the drug-drug interactions between the different components of the mixture and in cases of polydrugs these may worsen the clinical consequences. We can speculate that terrible consequences would come from taking these "legal highs", but there are not enough evidences to state with certainty that the cause of intoxication and/or death is full responsibility of these new products.

In addition, all these inconsistencies reported throughout the study demonstrate unequivocally the lack of control associated with these new substances regarded as "safe", showing only sellers concern at the monetary impact level, putting aside the health of users.

Despite all the problems associated with the new drugs phenomenon, it should be noted that none of the analyses detected compounds present in the list of prohibited substances [72, 74], in Portugal, at the time that these products were purchased. However, there are cases in the literature where substances have been identified in these so-called "legal highs" after their ban. Brandt et al. (2010) [8] obtained 24 products in 18 UK-websites over a period of 6 weeks following the ban of mephedrone and found that over 62% of analyzed products had this banned substance. Other study [31] revealed that five samples, also acquired through a UK-website, had controlled substances (benzylpiperazine (BZP) and 1-[3-(trifluoromethyl)phenyl]piperazine (TFMPP)) combined with caffeine. Due to the sudden ban on the sale of certain substances sellers continue selling prohibited products, rebranded as "new legal highs", in order to reduce the stock. Taking into account this information, users of these "new products" are in possession of illegal substances assuming that they are legal and they are unaware of the consequent criminal and health risks.

2.5 Semi-quantitative analysis

2.5.1 Semi-quantitative variability in commercial products

By comparing the chromatographic profile of a 10 µg/mL of a "legal high" (powder vs. tablet), it was found that the amount of cathinones in the tablet samples is lower (Figure 26). Comparing the majority peaks of each sample, it is verified that the signal of the tablet samples is about two to four times lower than the powder samples, when prepared in the same concentration and under the same conditions. This finding was observed in all "legal highs" purchased in the form of tablets although the signal strength varies slightly between them.

This finding associated with the fact that the tablets are sold at a significantly lower price (about half), gives emphasis to the hypothesis previously raised of the "legal highs" tablets being less powerful/pure in comparison to the powders.
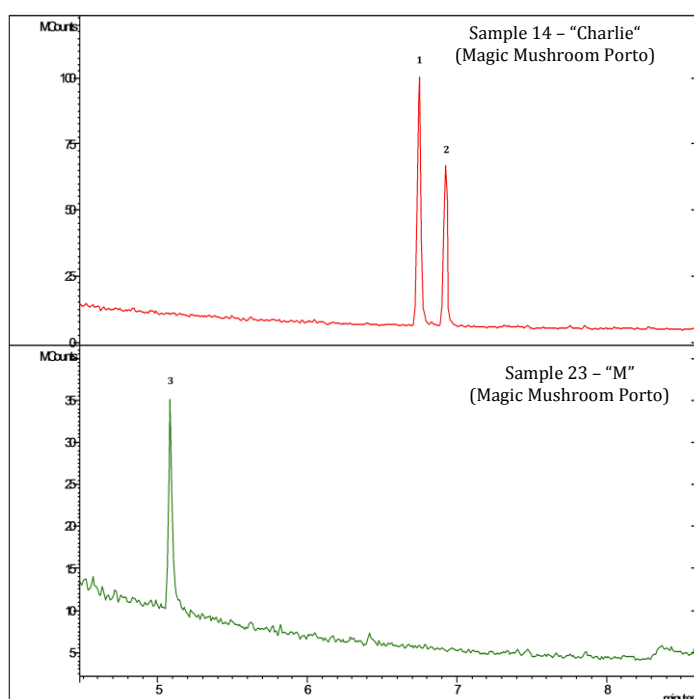
Figure 26 – Full scan chromatographic profile of derivatized solution (Sample 14 – "Charlie" in the form of powder and Sample 23 – "M" in the form of tablet) injected after derivatization with TFAA. The powder signal strength is significantly higher, may be an indicator of higher purity of product. The potential identification is indicated based on the analysis of mass spectra and new fragmentation patterns of molecules. 1 – Ethcathinone N-TFA; 2 – Buphedrone N-TFA; 3 – Fluoroamphetamine N-TFA.

By comparing the chromatographic profile of samples whose real composition is identical, it was found in some cases an apparent quantitative variability (Figure 24 and 25). However, due to the lack of robust standards, it became impossible to perform a complete and reliable quantitative determination.

Still, there are studies in the literature [6] that reveal significant differences in the quantitative composition of the products with real qualitative content identical. The content of

MDPV detected in some preparations was about 10-15 mg per package and more than 100 mg in other packages. MDPV produces weak psychoactive effects for 3 to 5 mg and severe effects after administration of higher doses (higher than 20 mg), depending our route of exposure [5]. Thus, we can infer that administration of more than 100 mg of MDPV could be a health and even life-threatening.

2.5.2 Linearity study

In our study, four samples were randomly selected in order to semi-quantify the substances that compose them. Three powders (Sample 1 – "Bloom"; Sample 16 – "Blow"; Sample 19 – "Kick") and one tablet (Sample 25 – "Bliss") were selected. In "Bloom", it was quantified the 4-MEC, pentedrone and methylone. In the sample 16 it was quantified 4-MEC and MDPV while in sample 19 only pentedrone. In turn, in the "Bliss" sample just methylone was analyzed, since it was the only component detected in qualitative analysis.

Regarding the quantification process, all samples and standards have been derivatized as it was concluded to be a more sensitive methodology.

Table 10 shows the calibration curves equations of standards tested for a given concentration range, as well as the squared correlation coefficient associated. Because the concentration found in the commercial products containing MDPV is lower than those containing other cathinones, we chose a range of lower concentrations (10-2000 ng/mL). All analytes showed high linearity for the concentrations range established, R^2 values being higher than 0.99 (Table 10).

Table 10: Calibration curves for 4-MEC, Pentedrone, Methylone and MDPV

Compound	Equation (y=mx+b)	Range (ng/mL)	R^2
4-MEC	y=0.0002x+78,442	50 – 10000	0.99781
Pentedrone	y=0.0002x-83,787	50 – 10000	0.99909
Methylone	y=0.0002x+190,11	50 – 10000	0.99555
MDPV	y=0.0005x-3,7372	10 – 2000	0.99219

2.5.3 Intra-day precision of the method

The precision of the method was determined (Table 11), and the results were satisfactory never exceeding values of 20% for intra-day determinations.

Table 11: Intra-day precision of the method for the quantification of 4-MEC, Pentedrone, Methylone and MDPV in different "legal highs" products (n=3)

Stock Solution	Compound	Bloom			Blow			Kick			Bliss		
		Mean (mg)	SD	CV (%)	Mean (mg)	SD	CV (%)	Mean (mg)	SD	CV (%)	Mean (mg)	SD	CV (%)
A	4-MEC	296,9	53,4	**10,1**	623,1	51,0	**8,2**	--	--	--	--	--	--
	Pentedrone	237,6	15,4	**6,5**	--	--	--	705,3	31,2	**4,4**	--	--	--
	Methylone	460,2	50,8	**11,0**	--	--	--	--	--	--	133,3	7,9	**5,9**
	MDPV	--	--	--	13,4	1,3	**9,4**	--	--	--	--	--	--
B	4-MEC	208,3	15,0	**7,1**	845,6	98,0	**11,6**	--	--	--	--	--	--
	Pentedrone	146,1	1,7	**1,2**	--	--	--	536,3	31,6	**5,9**	--	--	--
	Methylone	385,0	33,3	**8,6**	--	--	--	--	--	--	254,1	19,3	**7,6**
	MDPV	--	--	--	15,1	2,5	**16,7**	--	--	--	--	--	--
C	4-MEC	219,0	21,7	**9,9**	746,9	77,5	**10,4**	--	--	--	--	--	--
	Pentedrone	188,4	2,6	**1,4**	--	--	--	579,0	40,5	**7,0**	--	--	--
	Methylone	569,3	19,5	**3,4**	--	--	--	--	--	--	215,4	9,8	**4,6**
	MDPV	--	--	--	25,7	3,5	**13,6**	--	--	--	--	--	--

2.5.4 Study of homogeneity of commercial products

In order to verify the homogeneity of selected commercial samples ("Bloom", "Blow", "Kick" and "Bliss") we took from the same sample three different aliquots (stock solution A, B and C).

Figure 27 (A-D) results show the quantitative relation between the same component in the different solutions (for example 4-MEC of a "Bloom" solution A vs. 4-MEC of a "Bloom" solution B vs. 4-MEC of a "Bloom" solution C), as well as the relationship between the different solutions as a whole (e.g. solution A of "Bloom" vs. solution B of "Bloom" vs. solution C of "Bloom"). The results presented correspond to mean ± standard deviation (SD), for n=3.
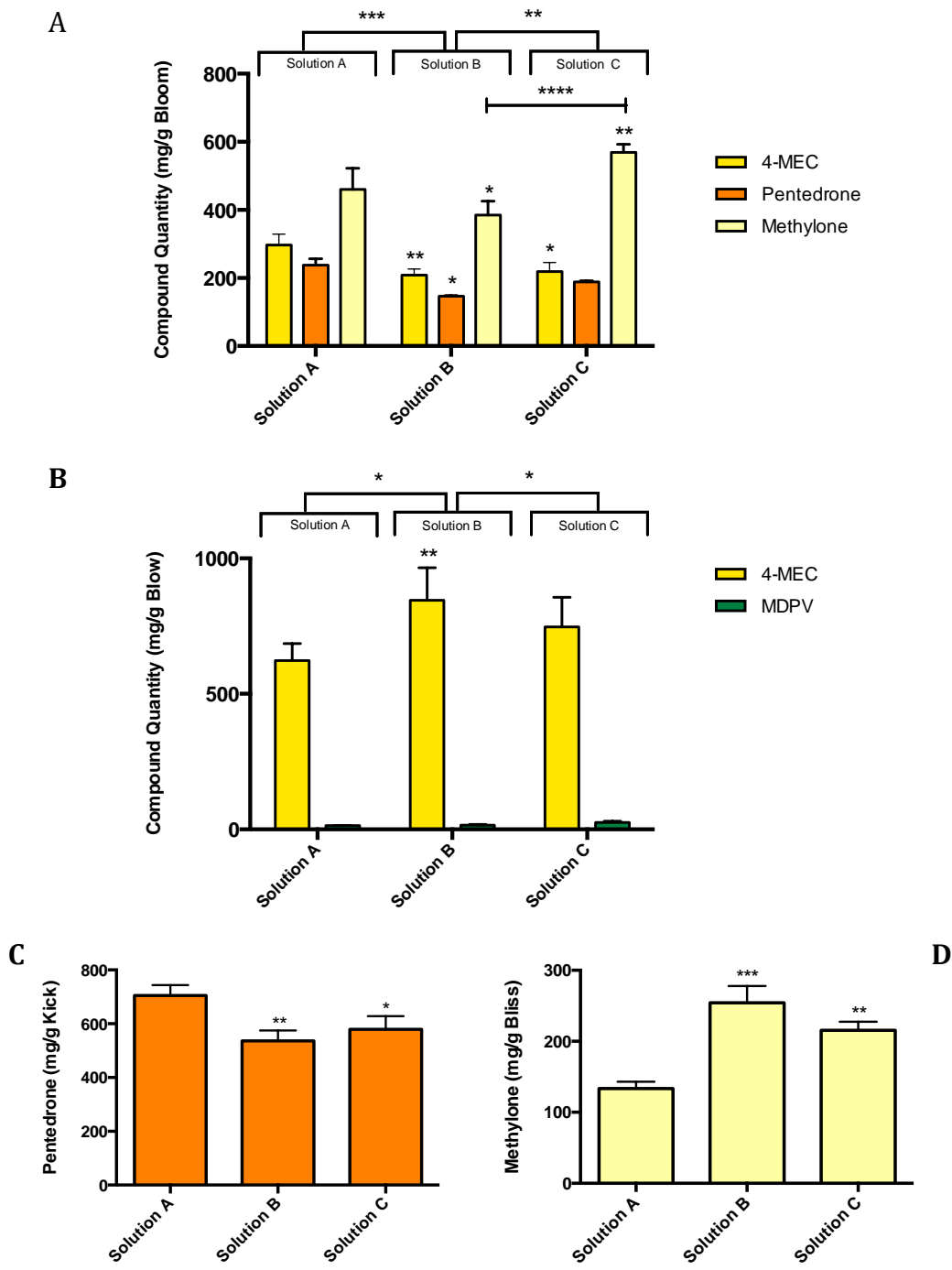
Figure 27 – Semi-quantification of some substances (4-MEC, Pentedrone, Methylone and MDPV) present in "legal high" samples randomly selected and the quantitative relation between the same compound for different solutions. The figure A expresses the amount of 4-MEC, Pentedrone and Methylone (mg) in each gram of "Bloom" (1). Graph B expresses the amount of 4-MEC and MDPV (mg) in each gram of "Blow" (16), while the graph C expresses the amount of Pentedrone (mg) in each gram of "Kick" (19). Finally, the graph D expresses the amount of Methylone (mg) in each tablet of "Bliss" (25). The results are expressed through the mean ± SD, n=3 (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$ for solution B or solution C vs. solution A). The groupings shown in figure A and B represent comparisons between the solutions as a whole.

Although the samples, visually, appear homogeneous and the same homogenization procedure was applied, it was verified that there are significant differences (P<0.05) (Figure 27) between the amounts of the compounds in the different solutions for the same product. This leads us to believe that the powders in the mixture of different "legal highs" can have different densities and/or granulometries, interfering in the reproducibility of the quantification values.

This can be reflected in different amounts of substances presents in different portions of the products if the consumer does not use the entire product at once. This happens mainly in cases where the consumer is inexperient, being advised by the sellers to consume the product splitted. Thus, if there is a lack of product homogeneity, each time the user consume a portion, will consume different substances in unequal amounts at all times, so it is expected that the intensity of effects can vary, even within the same product.

For a correct quantification of active principles and thus circumvent their heterogeneity, the ideal would be to perform a methanolic solution of the entire product and, subsequently, do all analysis from this global solution.

4 *Cytotoxicity study of cathinones*

Chapter 1 - Experimental Part:

Cytotoxicity studies

1. Material and Methods

1.1 Chemicals and supplies

All chemicals and reagents were of analytical grade. William's E medium, insulin solution from bovine pancreas, hydrocortisone hemisuccinate, collagenase from *Clostridium histolyticum* type IA ($\geq$ 600 CDU/mg solid), bovine serum albumin (fraction V), ethylene glycol-bis(ß-aminoethyl)-*N, N, N', N'*-tetraacetic acid (EGTA), sodium phosphate dibasic (Na_2HPO_4) gentamicin, dexamethasone, trypan blue solution, Triton X-100 and 3-[4,5-Dimethylthiazol]-2,5-diphenyltetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibiotic mixture of penicillin/streptomycin, fetal bovine serum (FBS), trypsin 0.05%-EDTA and Hank's buffered salt solution (HBSS) were obtained from Gibco by Life Technologies - Invitrogen (Barcelona, Spain). Sodium hydrogen carbonate ($NaHCO_3$), potassium chloride (KCl), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), potassium dihydrogen phosphate (KH_2PO_4), calcium chloride dihydrate ($CaCl_2.2H_2O$) and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Finally, sodium chloride (NaCl) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from VWR (Leuven, Belgium). HepaRG cell line was purchased from Gibco by Life Technologies - Invitrogen (France).

MDMA (HCl salt) was extracted and purified from high purity MDMA tablets that were provided by the Portuguese Criminal Police Department. Methylone, Pentedrone, 4-MEC and MDPV were the same used in chemical characterization studies, acquired through a Sensearomatic website. "Bloom" and "Blow" samples used in this study were purchased from Magic Mushroom Smartshop (Porto, Portugal). All the products were fully characterized by NMR and MS methodologies (purity higher than 97%) and stored in a container with a desiccant substance at -20°C.

1.2 Hepatocyte primary culture

1.2.1 Animals

Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research [109]. Adult male Wistar rats (Charles-River Laboratories, Barcelona, Spain), weighing 280 – 320 g were used in all experiments as a source of hepatocytes. Animals were acclimatized in polyethylene cages with wire-mesh at the top, lined with wood shavings, at an ambient

temperature of 20 ± 2 °C, humidity between 40 and 60% and 12h/12h light/dark cycle. The animals were kept in our animal facilities, having free access to standard rat chow and tap drinking water *ad libitum*.

Surgical procedures for the isolation of hepatocytes were performed under isoflurane anesthesia and carried out between 10.00h a.m. and 12.00h a.m..

1.2.2 Isolation of rat hepatocytes

The technique used for the isolation of rat hepatocytes is based on a collagenase perfusion method described by Moldéus (1978) [110] also called "two-step perfusion".

Briefly, after anesthesia, a cannula was introduced in the hepatic portal vein (Figure 28) and the liver was initially perfused *in situ* with a modified Hank solution (NaCl 2.7 M, KCl 0.1 M, $MgSO_4.7H_2O$ 16 mM, Na_2HPO_4 8.4 mM, KH_2PO_4 8.8 mM, $NaHCO_3$ 25 mM, HEPES 12.5 mM, EGTA 0.6 mM and albumin 0.67%; pH 7.4), for 10 minutes at 37 °C, in order to remove the blood from the sinusoids and to decrease the extracellular calcium concentration, to consequently cleave the hepatic desmosomes (calcium dependent). In a second stage, hepatic collagen molecules, that ensure the mechanical stability of the liver, were hydrolyzed by *ex situ* perfusion with a Hank solution supplemented with collagenase and its co-factor calcium (NaCl 2.7 M, KCl 0.1 M, $MgSO_4.7H_2O$ 16 mM, Na_2HPO_4 8.4 mM, KH_2PO_4 8.8 mM, $NaHCO_3$ 25 mM, HEPES 12.5 mM, collagenase 0.5 mg/mL and $CaCl_2$ 5.88%; pH 7.4). This last perfusion was carried out for 8 to 10 minutes at 37 °C until the liver visibly loses its plasticity when lightly pressed. During both perfusions, the solutions were continuously aerated by a stream of carbogen (95% 0_2 and 5% $C0_2$) and the flow solution was maintained at 10 mL/min.

Enzymatic dissociation was followed by a soft mechanical liver dissociation in Krebs-Henseleit buffer (NaCl 0.42 M, KCl 16.9 mM, $MgSO_4.7H_2O$ 4.2 mM, KH_2PO_4 4.2 mM, $NaHCO_3$ 25.5 mM and $CaCl_2.2H_2O$ 9.1 mM) supplemented with 12.5 mM HEPES and albumin (1%) (pH 7.4). The hepatocytes suspensions obtained have many non-parenchymal liver cells, including Kupffer cells, adipocytes, endothelial cells and non-viable parenchymal cells, being necessary to purify the suspension. Once the hepatocytes have a higher density than the non-parenchymal and dead cells, a suspension of highly enriched hepatocytes was obtained after low-speed centrifugations (300 rpm, 2 minutes) and washing with Krebs-Henseleit buffer supplemented with 12.5 mM HEPES (pH 7.4).
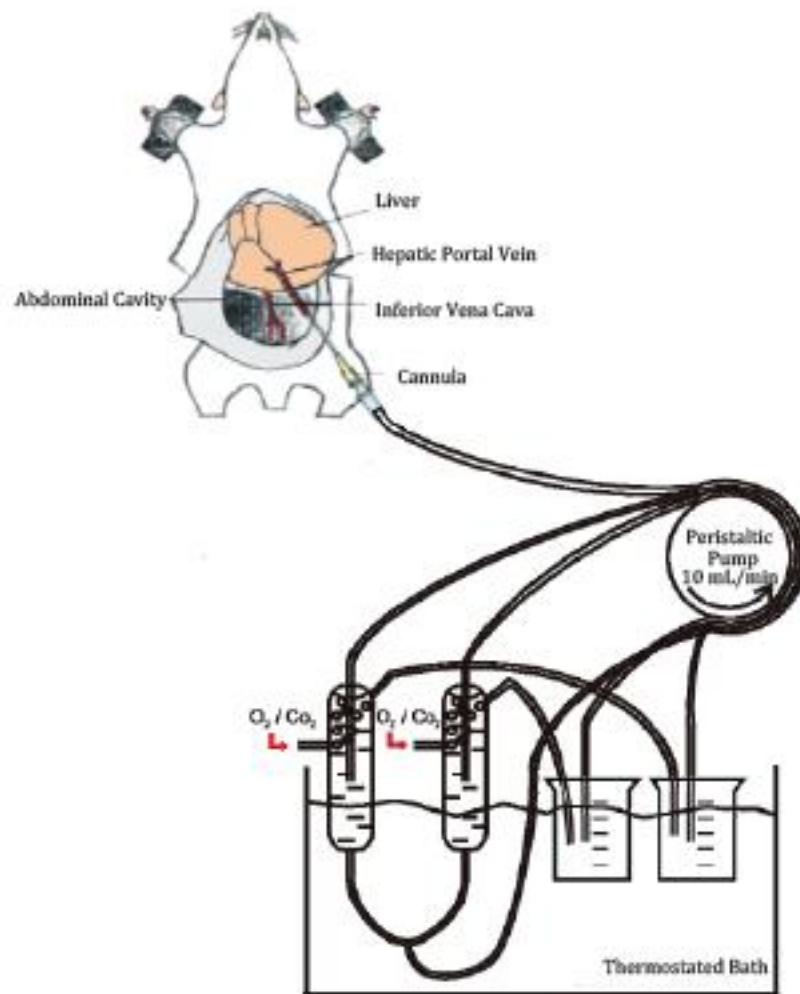
Figure 28 – Schematic representation of the hepatic portal vein cannulation and perfusion system

[Adapted from 86]

1.2.3 Determination of cell viability using the technique of trypan blue exclusion

Trypan blue is an organic dye with negative charge, which is excluded by the hepatocytes with cytoplasmic membranes intact, as a result of the maintenance of energy-dependent membrane potential. The loss of this potential due to cellular damage can cause membrane destruction, so trypan blue is included by the cells that rapidly begin to present a blue coloration, more evident in the nucleus; in turn, viable cells exhibit a spherical shape with well-defined borders [111].

The trypan blue exclusion was the method chosen for determining the viability of the initial suspension cells, for being a quick method to first assessment of cell damage. However, like all methods, trypan blue exclusion also presents some disadvantages, in particular as regards the

subjectivity visual count of cells, the different counting techniques between different operators, the uneven distribution or even due to agglomeration of the cells.

In our specific case, cell viability of the isolated rat hepatocytes, estimated through this technique, was always greater than 80%.

1.2.4 Isolated rat hepatocyte culture

A suspension of 5×10^5 viable cells/mL in complete culture medium (William's E medium supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 μg/ml gentamicin, 2ng/mL insulin and 5nM dexamethasone) was seeded in 96-well plates (BD Biosciences, Oxford, UK), in a density of 156250 viable cells/cm^2. Cells were incubated at 37 °C with 5% CO_2, overnight for cell adhesion. The day after, to the cells was added culture medium without FBS and incubation with test compounds was performed.

1.3 HepaRG cell line routine

HepaRG cells were cultured in growth medium composed of William's E medium (with L-glutamine), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL bovine insulin and 50 μM hydrocortisone hemisuccinate. The cells grew in 75 cm^2 canted-neck tissue culture flasks (Corning, NY, USA) under a humidified atmosphere of 5% CO_2/95% O_2, at 37 °C. The medium was renewed every 2 to 3 days.

After reaching 80-90% of confluence, the cells were shifted to the same medium supplemented with 2% DMSO to stimulate differentiation. Differentiation medium was also changed every 2-3 days for two weeks. Finally, differentiated cells were gently detached by trypsinization, seeded into 96-well plates at a high density of 450000 viable cells/cm^2 and incubated at 37 °C with 5% CO_2 overnight for cell adhesion.

1.4 Incubation of the primary rat hepatocytes and HepaRG cells with test compounds

Stock solutions (50 mM) of MDMA (as reference compound), pentedrone, methylone, 4-MEC and MDPV salts were prepared in ultra-purified sterile water and were at least 10 times more concentrated than the highest concentration tested to prevent media dilution.

Stock solutions of "Bloom" and "Blow" were also prepared in ultra-purified sterile water, in such a way that the concentration of main active ingredient of the commercial product (methylone and 4-MEC, respectively) also matched the 50 mM.

All stock solutions were stored at -20 °C and subsequent dilutions were freshly prepared in cell culture medium before each experiment.

Primary rat hepatocytes and HepaRG cells were seeded onto the central 60 wells of 96-well plates, in densities defined above, in a volume of 200 μL of the corresponding complete culture medium. Peripheral wells on the plate were filled with HBSS to prevent evaporation and concentration of test solutions. After adhesion, the medium was gently aspirated and the cells were exposed to the test drugs dilutions prepared in fresh culture medium, in a humidified air atmosphere containing 5% CO_2, at 37ºC, during a incubation period of 48 hours. The concentrations (0.05 – 5 mM) were selected to cover the whole effect range, from undetectable effects to maximum induced cell death. Each individual plate also included negative controls (*i.e.*, no test agents) and positive controls of cell death (culture media with 1% Triton X-100).

1.5 The MTT reduction viability assay

The cytotoxic effects of MDMA and cathinone derivatives, individually and as products sold in "smartshops", were determined using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. The mitochondrial reductases of viable cells reduce the MTT dye to purple formazan crystals that can be spectrophotometrically quantified. Tetrazolium ring is cleaved in active mitochondria, and the reaction occurs only in living cells. Therefore, the assay results will reproduce mitochondrial function and, consequently, cell viability [112].

After incubation for 48 h, the medium was removed and 200 μL/well of cell culture medium containing 500 μg/mL MTT was added to the attached cells. Plates were incubated at 37 °C, for 30 min (HepaRG cells) or 1h20 minutes (primary hepatocytes). Finally, the cell culture medium was aspirated and the formed intracellular formazan crystals were then dissolved in 100 μL/well of 100% DMSO.

The spectrophotometric analysis was run at 550 and 690 nm in a 96-well plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA). Data were obtained from five independent experiments for primary hepatocytes and three independent experiments for HepaRG cells, with each test plate containing quadruplicates of increasing concentrations of the tested drugs.

1.6 Regression modeling

Curves of normalized mortality values (effect) versus log of concentration (mM) were constructed and analyzed using the best-fit approach [113]. In the present study, the logit function was employed: $Y = \theta_{min} + (\theta_{max} - \theta_{min})/(1+exp(-\theta_1-\theta_2*log(x)))$, where θ_{min} and θ_{max} are the minimal and maximal observed effects, respectively; x is the concentration of the test drug; θ_1 is the parameter for the location and θ_2 is the slope parameter.

All of the nonlinear regression models describe sigmoidal concentration-response relationships and the plots were constructed using the GraphPad Prism 6 (version 6.0c) for Mac OS X. MTT data are presented as mean ± 95% confidence interval (CI). The EC_{50} values were determined for each individual drug and mixture, allowing for comparison between drugs.

1.7 Calculation of predicted mixture effects

After characterization of concentration-response curves of the individual agents, the effects of the "legal high" mixtures were predicted assuming additive joint responses. The expected effects were calculated using the concentration addition (CA) and independent action (IA) approaches, as described in Payne et al. [114] and used as a reference for the assessment of combination effects in terms of synergism (if the observed effects are greater than additive predictions), additivity (if the experimental mixture outcomes equal the prediction) and antagonism (if the experimental joint effects fall short of additivity) [115].

Briefly, the concept of CA is based on the assumption that the mixture constituents have similar modes of action, which means that any component can be replaced partially or totally with another without changing the overall mixture effect [116]. This means that each individual component contributes to the global joint effect by acting in proportion to its concentration, even at concentrations producing no effect. The IA alternative approach better describes combination effects of drugs with dissimilar mechanisms of action with each agent interacting at differing sites of action [117]. In this case, the fractional response of one individual component is supposed to be independent from those induced by other components, presuming that

mixture components present at zero effect concentrations will not contribute to the overall effect.

Chapter 2 - Results and Discussion of the cytotoxicity studies

2.1 Features of primary hepatocytes and HepaRG cells

In drug screening assays, the hepatotoxicity is one of the parameters of primary concern, since the liver is an organ responsible for biotransformation and elimination of toxic compounds from the human body, as well as being a primary target for allocation by xenobiotics [114]. The vast arsenal of vital biochemical functions performed by the liver requires an appropriate architecture, therefore the vast majority of all hepatic functions, including xenobiotic biotransformation, is performed by hepatocytes that constitute about 80% of the parenchymal liver cells. Hence, most of the hepatic *in vitro* models for toxicity studies are based on hepatocytes [115]. Taking this into accout, a wide range of liver-derived *in vitro* models have been developed and are now available for toxicological studies. Primary rodent hepatocyte cultures and hepatoma-derived cell lines are an important part of this *in vitro* models [116-120].

Primary cultures of rat hepatocytes (Figure 29 A) are a good alternative to human cells often used in toxicological studies because they present higher metabolic responses than the common human cell lines and the inter-donor variability can be minimized by selecting animals of the same sex, age and with similar feeding regimes [121]. Although primary hepatocytes are clinically relevant, cell lines are frequently used as alternatives. The hepatocyte cell lines present some advantages over freshly isolated hepatocytes, such as higher availability, unlimited life, stable karyotype and the fact that they are easy to handle and grow continuously [116, 118]. One disadvantage associated with hepatoma cells involves the limited extent of its biotransformative activity by some cytochrome P450 enzymes or by their low levels of when compared to a normal adult liver [118]. The HepaRG cell line has the ability to differentiate into hepatocyte-like cells and biliary-like cells, reaching maximum differentiation after 2 weeks of exposure to a 2% DMSO supplemented medium (Figure 29), expressing highly differentiated functions [117, 121-125]. For most metabolizing genes, the expression levels are associated with the presence of DMSO and the expression of CYP isoenzymes generally decreases when DMSO is removed from the medium, whereas carriers and the liver-specific factors remain unchanged [126]. Thus, it is suggested a dual effect of DMSO in cells, affecting not only their differentiation as well as gene expression in differentiated cells. Figure 29 shows HepaRG cells non-differentiated (B) and HepaRG cells after differentiation in culture medium containing 2% DMSO (C).
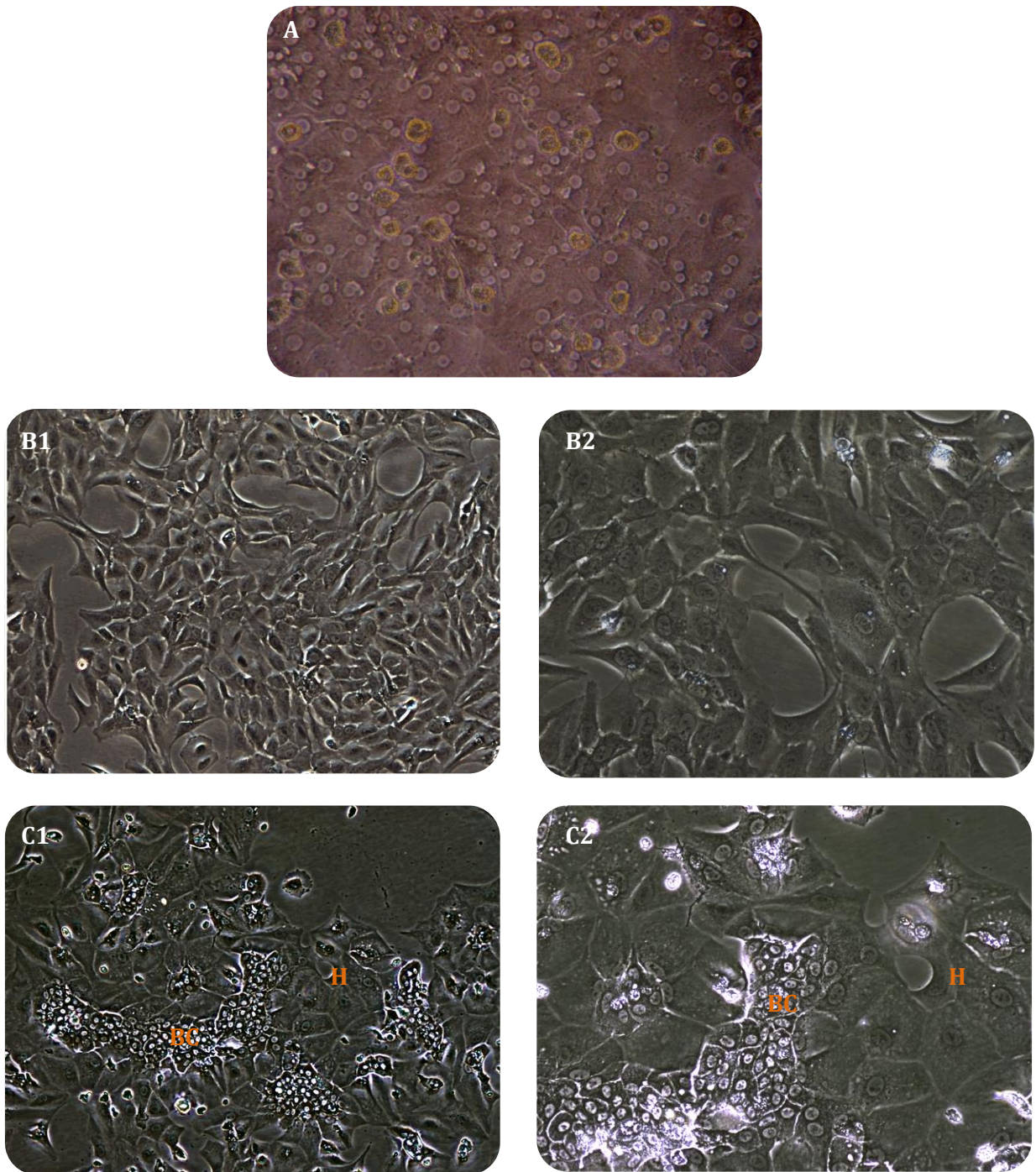
Figure 29 – (A) Microscopic appearance of freshly isolated rat hepatocytes in a total magnification of 100x. Figures B1 and B2 shows microscopic appearance of HepaRG cells to 80-90% of confluence in a magnification of 100x and 200x, respectively. Figures C shows the HepaRG cells after 18 days of culture in the presence of differentiation medium with DMSO in a magnification of 100x (B1) and 200x (B2). H – hepatocyte-like cells; BC – bili canaliculus.

2.2 Yield and viability of cell suspensions of isolated rat hepatocytes

More important than the amount of isolated hepatocytes (yield), is their quality, i.e., the percentage of viable cells in suspension. After the isolation process, it is preferable to obtain a small number of cells but intact than getting a large quantity being the vast majority non-viable. Thus, after the rat liver perfusion, mechanical dispersion of cells must be performed carefully to minimize the loss of viability of the final suspension.

The variability of cell viability was checked during the assays. The cell suspensions used for the experiments showed viability between 81 and 90.2%. These values are within the ones reported by other authors whose work presented values between 80 to 97% also obtained with Wistar rats [127-128]. The number of cells obtained in each suspension ranged between 124 to 245×10^6 cells per animal.

For good results in terms of yield and viability, the experience factor during the perfusion technique implementation also seems determinant. An efficient and rapid cannulation of the portal vein, to ensure fast and uniform perfusion of all hepatic lobes, is very important in order to obtain favorable results. Parts of the liver that does not change color immediately when perfusion start, represent a poor cannulation, indicating further areas which have not been digested by collagenase.

2.3 Test compounds

As previously described, the number of intoxications and deaths associated with synthetic cathinones consumption increased considerably [4, 18-19, 99-100]. The inconsistency of effects and their magnitude is hard to explain. However, one possible explanation for this aspect implicates a pattern of polydrug abuse, often associated with the consumption of "legal highs", as it was evident through the chemical characterization studies.

The present study aimed to evaluate the cytotoxic effects of synthetic cathinones derivatives and real mixtures of this derivatives sold as "plant feeders". Two "legal highs" were selected for cytotoxity study: "Bloom" and "Blow". The first was chosen since its major component is the methylone, the direct analogue of "*ecstasy*". In turn, the "Blow" was chosen as display in its composition 4-MEC that is structurally similar with one of the most popular synthetic cathinone (mephedrone) [108] and MDPV, which is one of the most potent derivatives due to the presence of a tertiary amine group in its molecular structure [1, 15].

In order to accurately estimate the effects of a mixture, information about the individual responses of each of the constituents is required. Thus, all the major substances present in these products were studied alone in the same conditions as the mixtures.

One of the most popular drugs in the world [129] belonging to the amphetamine-class, MDMA, was also considered for the study, since it detains structural similarities with the synthetic cathinones, allowing a comparison of the hepatotoxic potency of the different drugs.

For this purpose, stock solutions at a concentration of 50 mM for all products were prepared. Stock solutions of "Bloom" and "Blow" products were prepared in such a way that the concentration of major compound (methylone and 4-MEC, respectively) was fixed at 50 mM and the concentration of the remaining components of the mixture has been determined taking into account this main concentration. Thus, the peak area of the main active ingredient of the commercial product, obtained by gas chromatography, was equal to that obtained for the respective pure compound at desired concentration (50 mM) (Figure 30).
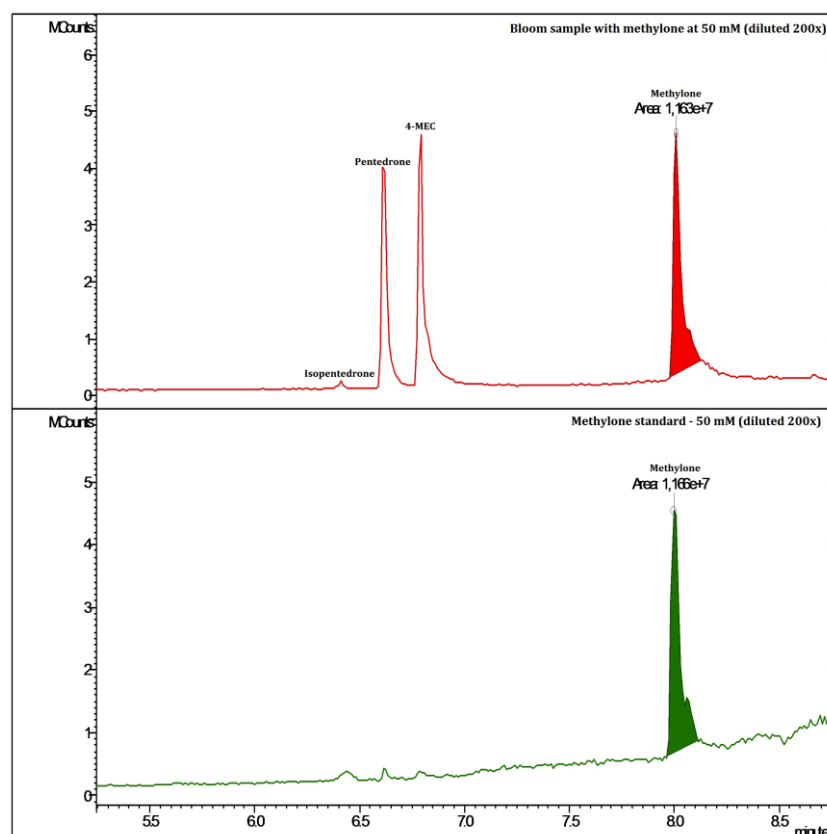
Figure 30 - Full scan chromatographic profile of "Bloom" stock solution with methylone at 50 mM and methylone standard also at 50 mM, injected directly into the GC-MS, showing equal areas and therefore equal concentrations.

Taking into account this principle, the concentration of methylone, 4-MEC and pentedrone in the "Bloom" product used for cytotoxic study is 50, 33 and 31 mM, respectively. In turn, in the "Blow" sample, the concentration is 50 mM for 4-MEC and 1.8 mM for MDPV.

Therefore, the stock solutions were diluted maintaining the ratio between each mixture constituent unchanged. Serial dilutions covered a wide range of concentrations (0.05 – 5mM) for all test substances to describe the complete response range, from 0% cell death to maximum induced cell death, when using the MTT cytotoxicity assay.

2.4 Concentration-response relationship of individual agents in primary cultured hepatocytes

In the MTT assay, all tested single agents yielded reproducible effects in a concentration-dependent fashion, resulting in increased cell death with the rising of chemical concentration (decreased percentage of cell viability). The shape of the concentration-response curves for the individual drugs tested were relatively similar, as well as their maximal effects, with the exception of pentedrone which presents values of cell death more pronounced to higher concentrations evidencing a higher slope compared to other substances. More significant differences were observed essentially in EC_{50} values as shown in figure 31.

MDMA has an EC_{50} value of 0.754 mM. Methylone with an EC_{50} of 1.18 mM shares a similar potency with 4-MEC (EC_{50} 1.29 mM), however with significantly higher values (P<0.05) than MDMA, being consequently drugs which individually offer lower cytotoxicity. In turn, there are no significant differences between MDPV (EC_{50} 0.742 mM) and pentedrone (EC_{50} 0.647 mM), being more cytotoxic than MDMA, although not significantly.

Due to the presence of the pyrrolidinyl ring and the tertiary amine group, MDPV, as previously mentioned, is more lipophilic than other cathinones, showing greater facility to cross barriers [1, 15]. Thus, it was expected that MDPV was the most cytotoxic compound in the MTT assay. Although the EC_{50} values of pentedrone and MDPV were not significantly different, the first derivative appeared to be more cytotoxic at high concentrations, whereas for lower values, MDPV showed a higher cytotoxicity. Thus, through the MTT assay we can conclude that MDPV is actually one of the most powerful cathinones, showing its maximum hepatotoxicity at concentrations below 0.5 mM, as MDMA.
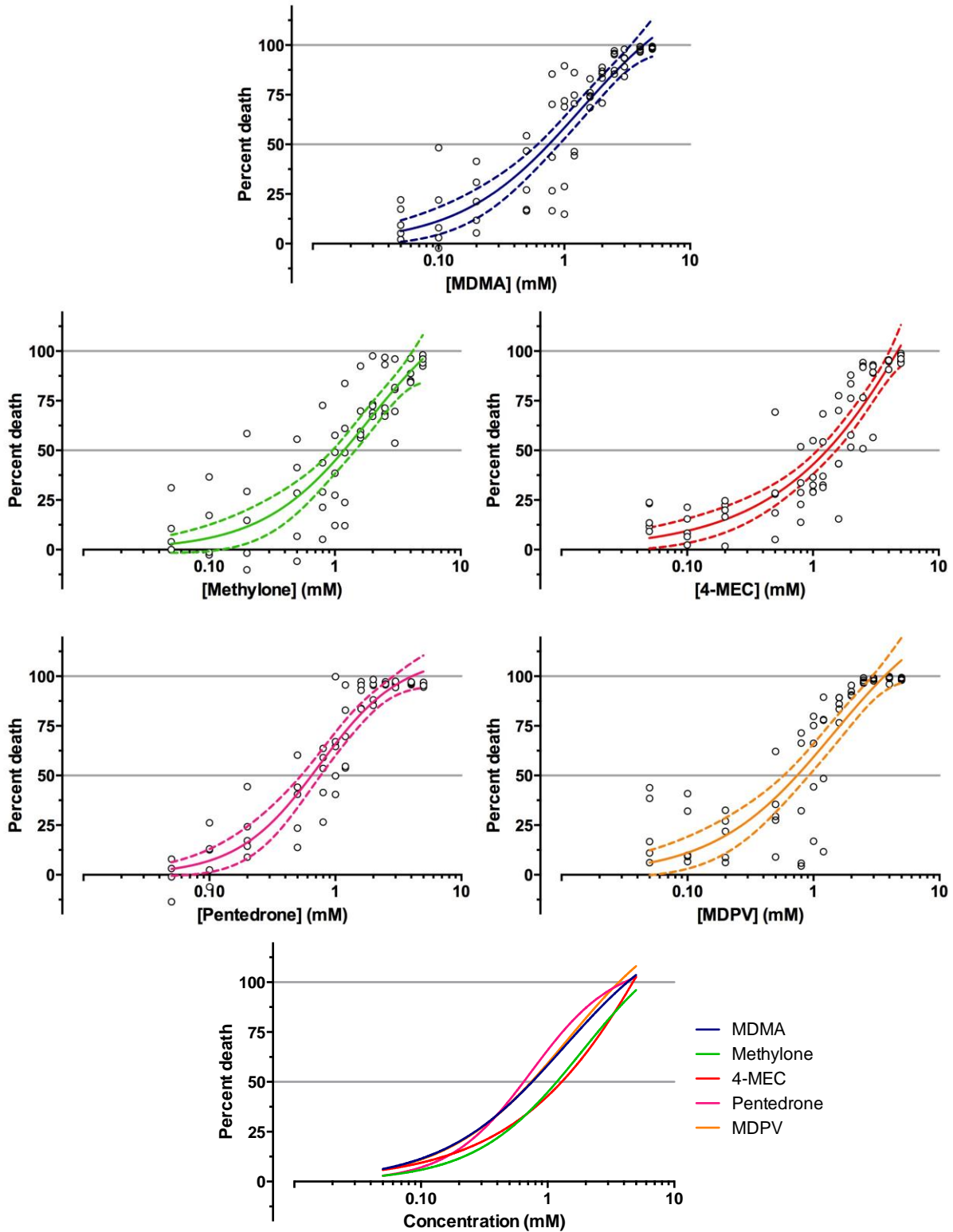
Figure 31 – Regression models for the cytotoxicity effects of individual cathinones, using isolated rat hepatocytes as a model at 37 °C. The grey line shows the EC_{50} and EC_{max} for each response curve and the dashed lines show the 95% confidence interval belt of the fit. Data were normalized to negative (untreated) and positive (1% Triton X-100) controls. Data were obtained from five independent experiments run in triplicate.

2.5 Significant synergic/additive mixture effects are observed in "legal highs"

Assessments of mixture effects in terms of synergism, antagonism or additivity depend on the determination of the expected effect of a given mixture. After characterization the concentration-response curves for the individual cathinones in terms of shape, slope and maximal effects, the CA and IA models were applied for modeling the expected effects of "legal highs" mixtures. As showed in figure 32, the two additive models produced very similar expectations and confirm that both are suitable for predicting the mixture effects.
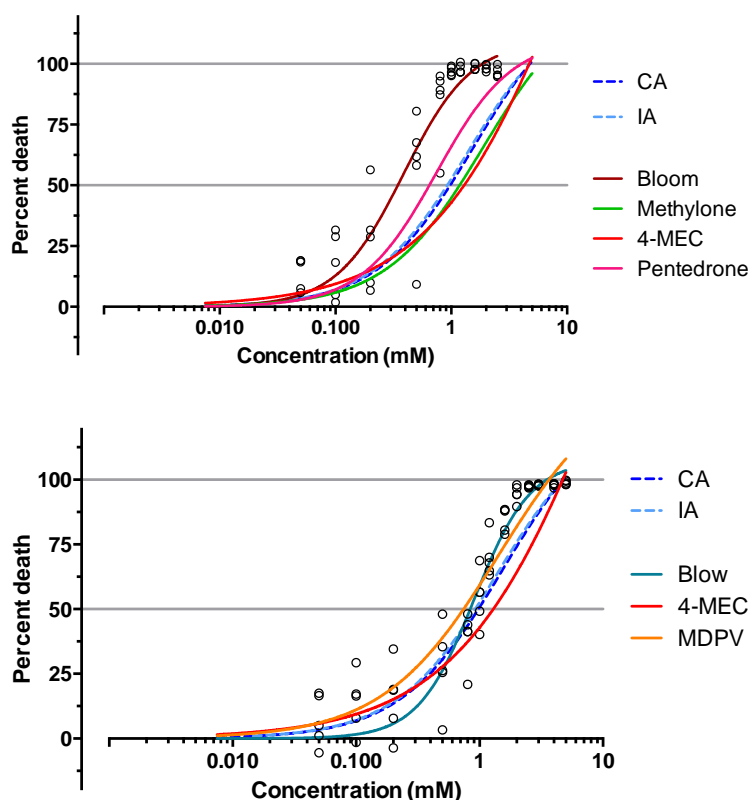
Figure 32 – Predicted and observed effects of "Bloom" and "Blow" mixtures and their individual agents in MTT assay. Circles represent individual data points of the "Bloom" and "Blow" mixtures. The dashed lines show the prediction according to concentration addition (CA) and independent action (IA). The grey lines show the EC_{50} and EC_{max} for each response curves. Experimental data derive from five independent experiments run in triplicate.

By the analysis of "Bloom" mixture effect in figure 32, it is observed that the predicted curve according to the CA and IA models are shifted to higher concentrations values, assuming that the mixture has strongest effects than expected. This displacement curves is indicative of a synergism, since smaller concentrations than expected are necessary to produce the same effects. On the other hand, for "Blow" mixture the observed and experimental EC_{50} values are similar, revealing an additivity situation, although in lower concentrations the mixture curve is slightly shifted to the right, i.e. higher concentrations than expected are required to obtain the same effects; in higher concentrations mixture is slightly shifted to the left, i.e. smaller concentrations are needed than those provided by the mathematical models for the same effects.

Despite these results, it is necessary to take into account that the studied mixtures are not pure, i.e. have other constituents which have not been taken into account in the analysis, such as dimethocaine, and that could interfere with the results predicted by mathematical models.

Concentrations that produce 50% of the maximal effect in the MTT assay (EC_{50}) were calculated by interpolation from the best-fit regression model for each product tested. A summary of the logit parameters for the best-fit regression model of each individual agent and mixture are in Table 12. These parameters were used to compute the predicted mixture effect curves shown in Figure 32.

Table 12: Parameters derived from nonlinear fits of single agents and "legal highs" mixtures concentration-response data to the asymmetric logit function, in the MTT reduction assay, for primary hepatocytes. (n = 5; * P<0.05 for each product vs. MDMA)

Compound	Estimated parameters for the best-fit regression model of each individual agent				EC50 (mM)	Fraction in the mixture
	Regression model	θ_1	θ_2	θ_{max}		
MDMA	Logit	-0.270	2.12	135	0.754	n/a
Methylone		-0.647	2.40	130	1.18*	0.439
4-MEC	Logit	-1.78	1.64	296	1.29*	0.289
Pentedrone		0.390	3.07	111	0.647	0.272
"Bloom"		1.40	3.42	110	0.346*	1
4-MEC		-1.78	1.64	296	1.29*	0.965
MDPV	Logit	-0.332	2.13	142	0.742	0.0347
"Blow"		0.192	4.39*	108	0.840	1

2.6 "Bloom" and "Blow" are potent cytotoxic products

The cathinones tested in this study share common metabolic pathways [80-83] and may compete with each other in these processes. From the analysis of the graphs in figure 32, there are synergistic/additive effects of the mixture components. Therefore, the "legal high" mixtures exhibit higher cytotoxicity than their individual components, wherein the "Bloom" and "Blow" have an EC_{50} of 0.346 mM and 0.840 mM, respectively. These values are significantly different (P< 0.0001), so we can conclude that "Bloom" is considerably more hepatotoxic than "Blow".

Figure 33 – Concentration-response curves obtained with the MTT reduction cytotoxicity assay for "legal highs" mixtures, in primary rat hepatocytes, after 48h incubation at 37 °C, and its comparison with pure MDMA in same conditions. The grey lines show the EC_{50} and EC_{max} for each response curve and the dashed lines show the 95% CI belt of the fit. Data were normalized to negative (untreated) and positive (1% Triton X-100) controls and were obtained from five independent experiments run in triplicate.

Once the synthetic cathinones holds structural similarities with MDMA, this compound was chosen to draw comparisons with "legal highs" mixtures at level of the hepatotoxic potency. If we establish a comparison between these real mixes and MDMA (figure 33), under the same conditions, we find that "Bloom" present EC_{50} value shifted to the left, i.e., lower, being more hepatotoxic than MDMA (P<0.05). Thus, in general, we can infer that a concentration of "Bloom" is potentially more hepatotoxic than the same concentration of pure *ecstasy*. However, the EC_{50} values of "Blow" and MDMA are very similar (P>0.05), and therefore have a similar hepatotoxicity, though MDMA is more toxic to lower concentrations while the "Blow" is slightly more hepatotoxic to higher values.

The additive effects of combination of amphetamines studies conducted by other authors [134] showed that the hepatotoxicity may be exacerbated by the combination of MDMA with

other amphetamines, which can be intentionally ingested or present in "*ecstasy*" pills as contaminants.

In conclusion, the exposure of hepatocytes to "legal highs" mixtures definitely results in greater hepatotoxic effects expected.

2.7 Primary rat hepatocytes are more sensitive to the cathinones effects than HepaRG cells

Studies performed on HepaRG cells under the same conditions, revealed that all concentration-response curves are shifted to the right, showing higher EC_{50} values when compared to primary rat hepatocytes. A summary of the logit parameters for the best-fit regression model of each individual agent and mixture are presented for normothermic conditions in Table 13.
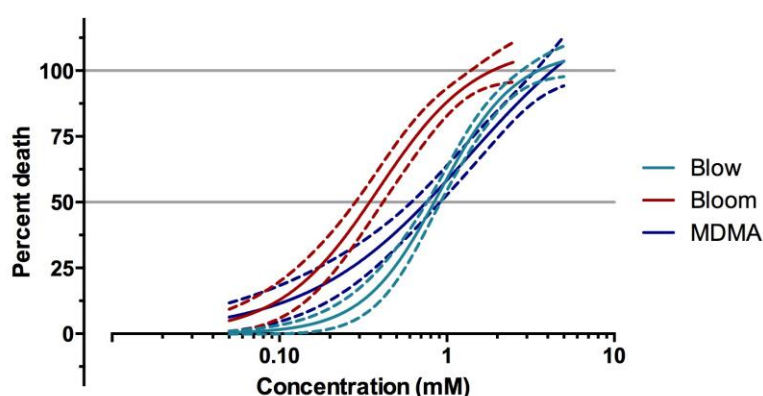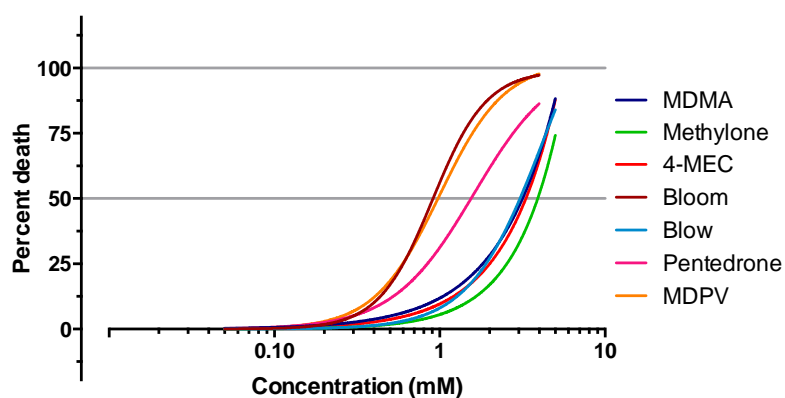

Figure 34 – Concentration-response curves obtained with the MTT reduction cytotoxicity assay for "legal highs" mixtures and all individual agents used in study, in HepaRG cells after 48h incubation at 37 °C. Data were normalized to negative (untreated) and positive (1% Triton X-100) controls and were obtained from three independent experiments run in triplicate.

In HepaRG cells, methylone remains the less toxic test compound (EC_{50} 3.91 mM) and MDPV was determined to be the most hepatotoxic (EC_{50} 0.975 mM) individual agent to the entire concentrations range. This result may be explained since recent studies show that, when compared to human hepatocytes, expression of CYP450 isoenzymes in HepaRG cells is generally lower, with the exception of a considerably higher expression of CYP3A4 and CYP7A1, as well as a relatively higher content of CYP2C19 mRNA content in this cell line [130]. Hereupon, HepaRG cells are metabolically less competent than primary human hepatocytes. It also explains the higher MDPV potency in all concentrations since this is mainly metabolized by CYP2C19 [81-82]. Some studies have also found that CYP1A2, CYP2A6 and CYP2D6 have significantly lower levels

of expression in HepaRG [123, 130], which may explain the lower hepatotoxic effects of methylone, as CYP2D6 is the primary isoenzyme involved in methylone metabolism.

Although the HepaRG cells present low levels of expression to isoenzymes involved in the metabolism of synthetic cathinones studied, recent studies [135] show that HepaRG cells are metabolically more competent than other cell lines (such as HepG2), also presenting isoenzymes with a similar expression to human hepatocytes.

Table 13: Parameters derived from nonlinear fits of single agents and "legal highs" mixtures concentration-response data to the asymmetric logit function, in the MTT reduction assay, in HepaRG cells. (n = 3; * P<0.05 for each product vs. MDMA)

Compound	Estimated parameters for the best-fit regression model of each individual agent				EC50 (mM)
	Regression model	θ_1	θ_2	θ_{max}	
MDMA		-7.947	2.87	33517	3.16
Methylone		-8.64	3.71	31517	3.91
4-MEC		-7.90	3.17	26030	3.32
Pentedrone	Logit	-0.84	4.07	104	1.55
MDPV		0.008	5.06	102.4	0.975*
"Bloom"		0.27	6.13	99.1	0.911*
"Blow"		-2.827	4.52	144.3	3.06

The effects of the individual compounds in the mixtures were also studied in HepaRG cells and "Bloom" (EC$_{50}$ 0.911 mM) remains more toxic than "Blow" (EC$_{50}$ 3.06 mM) or MDMA (EC$_{50}$ 3.16 mM). In turn, "Blow"-induced cell death in HepaRG cells was closer to that observed with MDMA, resulting in overlapping concentration-response curves. In this assay, lower concentrations of MDPV showed no influence in the mixture, as similar effects were observed with "Blow" and 4-MEC, which may be due to the low metabolic capacity of the cell model.

In synopsis, the present work clearly demonstrates that potentially harmful interactions among synthetic cathinones are expected when these drugs are taken concomitantly. Taking into account these results, evaluate combination effects of synthetic cathinones is extremely important from a toxicological point of view, since most of "legal highs" users, consciously or not, take a wide variety of cathinones and other substances on the same night. Understanding the impact of drug interactions can provide valuable information to explore the causes of

sudden lethal intoxications, as well as facilitate the diagnosis and treatment of non-fatal cases. A better understanding of these combined effects may have a considerable influence on public health, raising awareness of the potential for severe toxicity and therefore stimulating behavioral changes in consumers worldwide.

5 *Conclusions*

Conclusions of the chemical characterization part:

- For the unequivocal identification of active principles of the new drugs, in the absence of standards, the GC-MS and NMR combination proved to be a powerful tool.
- Products sold as "plant feeders" although labeled to contain ketones (hypotetical synthetic cathinones), also include substances belonging to other classes. However, the synthetic cathinones derivatives group is more representative. Caffeine is the most commonly compound detected in products.
- The diversity in composition among these products is one of the greatest problems connected with the "legal highs" phenomenon. The consumers cannot be sure what they get. As has been shown, products with the same trade name have a different content and the same mixture is sold under different trade names.
- Inconsistency in both qualitative and quantitative compositions could lead to serious consequences, because users are unware of active dose, time of duration and even the effects.

Conclusions of the cytotoxicity studies:

- All cathinones, individually or in mixtures, showed concentration-dependent decrease in cell viability, in both cell models.
- Methylone and 4-MEC were less cytotoxic, while MDPV and pentedrone are the most cytotoxic cathinone derivatives, when compared with MDMA.
- Results with HepaRG cells showed that they are less sensitive to cathinone effects than primary hepatocytes. This may be explained by the fact that HepaRG cells are metabolically less competent.
- The "legal high" mixtures exhibit cytotoxicity values higher than those of its individual components; the results show a synergistic effect for "Bloom" mixture and additive effect for "Blow" mixture.
- "Bloom" is considerably more hepatotoxic compared to "Blow".

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