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Study of the toxicokinetics of 3,4-dimethylmethcathinone (3,4-DMMC) in Wistar Rats

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STUDY OF THE TOXICOKINETICS OF 3,4-DIMETHYLMETHCATHINONE (3,4-DMMC) IN WISTAR RATS

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Abstract

3,4-Dimethylmetcathinone (3,4-DMMC) is a new psychoactive substance (NPS) belonging to the first group of synthetic cathinones that were detected via the EU Early Warning System in 2010. Its recreational use and marketing have been banned in several countries. However, the drug remains readily available for purchase on the internet. In addition to the growing number of reports on its abuse published in forums and drug use blogs, the number of intoxications described in the scientific literature has also increased, including a fatality that has highlighted the health threats associated with this drug.

Given the need to increase the knowledge about the toxicological and toxicokinetic profile of 3,4-DMMC, the present work aimed at characterizing its biodistribution profile through the implementation and validation of a gas chromatography coupled to mass spectrometry (GC-MS) methodology.

For this purpose, adult female Wistar rats weighing 200–300 g were administered with 20 or 40 mg/Kg of 3,4-DMMC i.p.. After 1h or 24h, rats were anaesthetized, euthanized and blood, brain, liver, heart, kidneys, lungs, spleen, a portion of gut, muscle and adipose tissue, and urine (only at 24h) were subsequently collected for analysis. Blood was centrifuged at 1,600 *g* for 15 min at 4 ºC. Plasma was separated, precipitated with $HClO_4$ (5% final concentration) and centrifuged at 3,000 *g* for 10 min at 4 ºC. Organs were homogenized (1:4 m/v) in ice cold 100 mM phosphate buffer (pH 7.4) and centrifuged at 3,000 *g* for 10 min at 4 ºC. Urine was centrifuged at 3,000 *g* for 10 min at 4 ºC. All supernatants obtained from the tissue homogenates, plasma, and urine were subjected to a solid phase extraction, and the obtained residue was derivatized with trifluoroacetic anhydride prior to GC-MS analysis.

The method was fully validated in plasma using methylone as internal standard. The validation of the method consisted of the evaluation of the limit of detection and limit of quantification (0.004 µg/mL and 0.0135 µg/mL, respectively), linearity (with correlation coefficients above 0.9937 and within the concentration range 0.078–2.5 µg/mL), selectivity, inter-day and intra-day precision (CV always lower than 15%), accuracy (always between 80–120%) and recovery (78–98%). The inter-day and intra-day precision, accuracy and recovery were evaluated at three distinct concentrations

(0.078, 0.625 and 2.5 µg/mL). All these parameters accomplished with the international acceptance criteria for bioanalytical methods, indicating good linearity, recovery, precision and accuracy of the method, without interferences. A preliminary and partial validation was also performed with the remaining biological matrices under analysis with respect to linearity, recovery rate, and study of interferences.

Analysis of the biological samples showed that, after 1h, 3,4-DMMC was distributed to all matrices analysed, reaching higher concentrations in the spleen, lungs, kidneys and brain. At the same time point, two metabolites (tentatively identified as nor-dimethylmethcathinone and 3-hydroxymethyl-4-methyl-methcathinone or 3-methyl-4-hydroxymethyl-methcathinone) were detected and identified in all matrices. After 24h, analysis of the biological samples only indicated the presence of 3,4-DMMC and five metabolites in the urine samples (tentatively identified as nor-dimethylmethcathinone, 3-hydroxymethyl-4-methyl-methcathinone or 3-methyl-4-hydroxymethyl-methcathinone, nor-3-methyl-4-hydroxymethyl-methcathinone, nor-3-hydroxymethyl-4-methyl-methcathinone, and 3-methyl-4-carboxy-dihydro-methcathinone or 3-carboxy-4-methyl-dihydro-methcathinone). The proposed identification for the metabolites found showed differences in the metabolic pathways between rats and humans, as compared to the data described in the literature. After enzymatic hydrolysis, it was concluded that 3,4-DMMC and most of the metabolites were also excreted as glucuronide or sulphate conjugates.

With the present study we can conclude that 3,4-DMMC has a rapid and extensive biodistribution, as noted with amphetamines. These data and the development of a fully validated analytical method in plasma will not only help to understand the toxicological effects of 3,4-DMMC, as may also assist in future clinical and forensic investigations.

Keywords: 3,4-Dimethylmethcathinone (3,4-DMMC). Synthetic cathinone. New psychoactive substance (NPS). Gas Chromatography-Mass Spectrometry (GC-MS). Biodistribution. Metabolism.

Resumo

A 3,4-dimetilmetcatinona (3,4-DMMC) é uma nova substância psicoativa (NSP) que pertence ao primeiro grupo de catinonas sintéticas detectadas através do Sistema de Alerta Precoce da UE, em 2010. O seu uso recreativo e comercialização foram proibidos em vários países. No entanto, esta droga permanece facilmente disponível para compra na internet. Juntamente com o crescente número de relatos de abuso publicados em fóruns e *blogs* sobre consumo de drogas, o número de intoxicações descritas na literatura científica também aumentou, incluindo uma fatalidade que evidenciou as ameaças à saúde associadas a esta droga.

Atendendo à necessidade de aprofundar o conhecimento sobre o perfil toxicológico e toxicocinético da 3,4-DMMC, o presente trabalho teve como objectivo a caracterização do seu perfil de biodistribuição, através da implementação e validação de uma metodologia de cromatografia gasosa acoplada à espectrometria de massa (CG-EM).

Para este efeito, fêmeas de ratazana Wistar adultas, com cerca de 200–300 g, foram administradas com 20 ou 40 mg/Kg 3,4-DMMC *i.p.*. Após 1h ou 24h, as ratazanas foram anestesiadas e sacrificadas, tendo sido posteriormente colhidos para análise sangue, cérebro, fígado, coração, rins, pulmões, baço, uma porção de músculo, intestino e tecido adiposo, e urina (apenas às 24h). As amostras de sangue foram centrifugadas a 1.600 *g* durante 15 min, a 4 ºC. O plasma foi separado, precipitado com $HClO_4$ (concentração final de 5%) e novamente centrifugado 3.000 *g* durante 10 min, a 4 ºC. Os órgãos foram homogeneizados (1:4 m/v) em tampão fosfato 100 mM (pH 7,4) e centrifugados a 3.000 *g* durante 10 min, a 4 ºC. A urina foi centrifugada a 3.000 *g* durante 10 min, a 4 ºC. Todos os sobrenadantes obtidos dos homogeneizados de tecidos, plasma e urina, foram submetidos a uma extração em fase sólida e o resíduo obtido foi derivatizado com anidrido trifluoroacético, previamente à análise por CG-EM.

O método foi totalmente validado no plasma utilizando metilona como padrão interno. A validação do método consistiu na avaliação do limite de detecção e limite de quantificação (0,004 μg/mL e 0,0135 μg/mL, respectivamente), linearidade (com coeficiente de correlação acima de 0,9937 e dentro do intervalo de concentração 0,078–2,5 μg/mL), seletividade, precisão inter-dia e intra-dia (CV sempre menor que 15%), exatidão (sempre entre 80–120%) e recuperação (78–98%). A precisão, exatidão e

recuperação foram avaliadas em três concentrações distintas (0,078, 0,625 e 2,5 μg/mL). Todos estes parâmetros cumpriram os critérios de aceitação internacional para métodos bioanalíticos, indicando boa linearidade, recuperação, precisão e exatidão do método, sem interferências. Foi ainda realizada uma validação preliminar e parcial nas as restantes matrizes biológicas em análise no que respeita à linearidade, taxa de recuperação e estudo de interferências.

A análise das amostras biológicas mostrou que, após 1h, a 3,4-DMMC se distribui a todas as matrizes analisadas, atingindo concentrações mais elevadas no baço, pulmões, rins e cérebro. Após o mesmo período, foram detetados e identificados dois metabolitos em todas as matrizes (cujas identificações propostas foram nor-dimetilmetcatinona e 3-hidroximetil-4-metil-metcatinona ou 3-metil-4-hidroximetil-metcatinona). Após 24h, a análise das amostras biológicas apenas indicou a presença de 3,4-DMMC e de cinco metabolitos nas amostras de urina (cujas identificações propostas foram nor-dimetilmetcatinona, 3-hidroximetil-4-metil-metcatinona ou 3-metil-4-hidroximetil-metcatinona, nor-3-metil-4-hidroximetil-metcatinona, nor-3-hidroximetil-4-metil-metcatinona e 3-metil-4-carboxi-di-hidro-metcatinona ou 3-carboxi-4-metil-di-hidro-metcatinona). A identificação proposta para os metabolitos encontrados evidenciou diferenças nas vias metabólicas entre ratazanas e humanos, por comparação aos dados descritos na literatura. Após hidrólise enzimática, concluiu-se que a 3,4-DMMC e a maior parte dos metabolitos foram também excretados na forma de conjugados de glucuronídeo e/ou sulfato.

Com o presente estudo, podemos concluir que a 3,4-DMMC possui uma biodistribuição rápida e extensa, tal como ocorre para as anfetaminas. Esta informação e o desenvolvimento de um método de analítico completamente validado em plasma não só ajudarão a entender os efeitos toxicológicos da 3,4-DMMC, como poderão também auxiliar em futuras investigações clínicas e forenses.

Palavras-chave: 3,4-Dimetilmetcatinona (3,4-DMMC). Catinona sintética. Nova substância psicoativa (NSP). Cromatografia Gasosa acoplada à Espectrometria de Massa (CG-EM). Biodistribuição. Metabolismo.

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List of Abbreviations

2-DPMP: 2-Diphenylmethylpiperidine

2,3-DMMC: 2,3-Dimethylmethcathinone

2,4-DMMC: 2,4-Dimethylmethcathinone

3-MMC: 3-Methylmethcathinone or metaphedrone

3,4-DMMC: 3,4-Dimethylmethcathinone

4-MEC: 4-Methylethcathinone

4-MMC: 4-Methylmethcathinone or mephedrone

5-HT: Serotonin

5-HT$_x$: Receptor of serotonin

α_x: Receptors of noradrenalin

α-PVP: α-Pyrrolidinopentiophenone

MDMC: 3,4-Methylenedioxy-N-methylcathinone or methylone or βk-MDMA

CE: Capillary electrophoresis

CI: Confidence Interval

C_{max}: Maximum (or peak) concentration that a drug achieves in a body compartment

CV: Coefficient of variation

D_2: Receptor of dopamine, type 2

DA: Dopamine

DAT: Transporter of dopamine

DBA: Dibenzylamine

DMB: Dimethylbuphedrone

DMP: Dimethylpentedrone

E_{max}: Activation efficacy

EMCDDA: European Monitoring Centre for Drugs and Drug Addiction

EU-EWS: European Union Early Warning System

GC-MS: Gas chromatography-mass spectrometry detection

HPLC-UV: High performance liquid chromatography-ultraviolet detection

HR ESI-MS: High resolution electrospray ionization-mass spectrometry detection

IC_{50}: Half maximal inhibitory concentration

IR: Infrared

IS: Internal standard

LC-HRMS: Liquid chromatography-high resolution mass spectrometry detection

LC-MS: Liquid chromatography-mass spectrometry detection

LC-MS/MS: Liquid chromatography-tandem mass spectrometry detection

LC/QTOF-MS: Liquid chromatography quadrupole time-of-flight-mass spectrometry detection

LLE: Liquid-liquid extraction

LOD: Limit of detection

LOQ: Limit of quantification

MBTFA: N-Methylbistrifluoroacetamine

MDA-D5: Methylenedioxyamphetamine deuterated

MDEA-D6: 3,4-Methylenedioxy-N-ethyl-amphetamine deuterated

MDMA: 3,4-Methylenedioxymethamphetamine or “ecstasy”

MDMC: 3,4-Methylenedioxy-N-methylcathinone or methylone

MDPV: Methylenedioxypyrovalerone

MDPV-D8: Methylenedioxypyrovalerone deuterated

MPA: Methiopropamine

MS: Mass spectrometry

MSTFA: N-Methyl-N-trimethylsilyl-trifluoroacetamine

MXE: Methoxetamine

NA: Noradrenalin

NAT: Transporter of noradrenalin

NEB: N-Ethylbuphedrone

NMR: Nuclear magnetic resonance

NPS: New psychoactive substance

PMSF: Phenylmethanesulfonyl fluoride

QuEChERS: Quick, easy, cheap, effective, rugged, and safe extraction

R^2: Squared correlation coefficient or coefficient of determination

RE: Relative error

RSD: Relative standard deviation

RT: Room temperature

S: Slope

SD: Standard deviation

SERT: Transporter of serotonin

SFC-UV: Supercritical fluid chromatography-ultraviolet detection

SIM: Selected ion monitoring

SPE: Solid-phase extraction

SWGTOX: Scientific Working Group for Forensic Toxicology

TAAR: Amine-associated receptors

TFAA: Trifluoroacetic anhydride

UHPLC: Ultra high performance liquid chromatography

UHPSFC: Ultra high performance supercritical fluid chromatography

UNODC: United Nations Office on Drugs and Crime

UV: Ultraviolet

WHO: World Health Organization

Chapter I - General Introduction

1.1 Introduction

In recent years, the production and consumption of new psychoactive substances (NPS) have undergone an unprecedented expansion in the drug abuse market. Synthetic cathinones, which are frequently sold on the Internet, are among the most prevalent NPS. According to the United Nations Office on Drugs and Crime (UNODC), in 2015, 75 NPS were reported for the first time, compared to the 66 new substances that emerged in 2014 (UNODC, 2016). Of these, 20 NPS corresponded to new synthetic cathinones. In Europe, 130 synthetic cathinones were detected in 2017, including 12 that appeared for the first time (EMCDDA, 2017). Other evidence that supports the growth of synthetic cathinones in the NPS market is the increasing number of seizures in the past years. Between 2013 and 2014, seizures of synthetic cathinones tripled worldwide, reaching 1.3 tons. Most seizures occurred in Europe and in East and Southeast Asia (UNODC, 2016). More recently, statistics from the 2017 European Drug Report indicate that the number of seizures of synthetic cathinones in 2016 was 33% of all NPS, as compared to 45% of synthetic cannabinoids (23,000 and 32,000 seizures, respectively), although the amount of seized synthetic cannabinoids was lower (1.5 tons compared to 1.9 tons of synthetic cathinones) (EMCDDA, 2017). Also, statistics from the EU authority on drug monitoring indicate that 3% of the European students aged 15–16 consumed NPS in 2017, and synthetic cathinones were the group of NPS with the highest prevalence (EMCDDA, 2018).

Synthetic cathinones are structurally similar to cathinone, a natural psychostimulant found in the *khat* plant (Fowble et al., 2018; Richeval et al., 2018; Valente et al., 2014). These drugs are comprised in the phenylethylamine family, being chemically β-ketoamphetamines, and therefore are often referred to as ‘bk-amphetamines’.

Synthetic cathinones strongly inhibit dopamine (DA), serotonin (5-HT) and noradrenaline (NA) reuptake, increase the release of DA, NA, and 5-HT, activate α-adrenergic pre- and post-synaptic receptors, and induce tolerance and dependence (Shima et al., 2013). Therefore, these psychotropic substances were originally synthesized as a legal alternative to obtain the stimulant effects of illicit phenylethylamines, but were eventually also banned (Fowble et al., 2018; Richeval et al., 2018). This phenomenon of ‘emerging drugs’ is very dynamic and, as legal control is exerted over some cathinones whose popularity and abuse have spread, new variants

are synthesized by molecular alterations that are sometimes very subtle (bioisosterism). In this context, mephedrone (4-methylmethcathinone; 4-MMC) became a popular substitute for 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), being along with methylone the first NPS that attained widespread use. As the production and trafficking of mephedrone was forbidden in most European countries, the search for other drugs with similar stimulant effects proceeded, and other mephedrone-like substitutes emerged quickly on the NPS market. Accordingly, after the mephedrone ban in 2010, a second generation of drugs showed up as an alternative to the recently controlled cathinone, including methylone (βk-MDMA or 3,4-methylenedioxy-N-methylcathinone, MDMC), 3-methylmethcathinone (3-MMC or metaphedrone), 4-methylethcathinone (4-MEC), methylenedioxypyrovalerone (MDPV), and 3,4-dimethylmethcathinone (3,4-DMMC) (Figure 1) (Zaami et al., 2018).

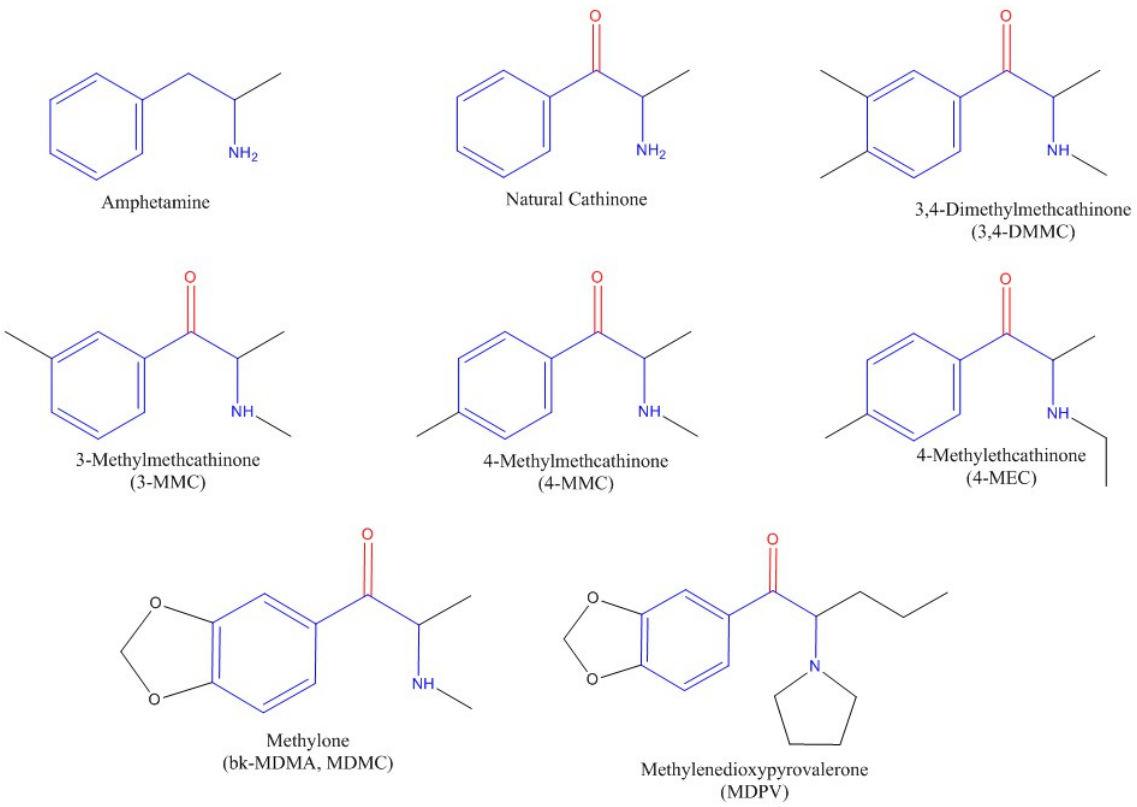

Figure 1: Chemical structure of amphetamine, natural cathinone and some synthetic cathinones frequently abused. The phenethylamine core is highlighted in blue, and the ketone group of cathinones in red.

The 3,4-DMMC cathinone derivative was first identified in 2010 in Hungary (EMCDDA, 2010), and in 2012 in Portugal through the EU Early Warning System (EU–EWS). Its 2,4-isomer was reported for the first time in Poland, in 2015 (EMCDDA, 2015a). Currently, the drug is consumed throughout the world, particularly in Europe, Australia and Japan (Locos and Reynolds, 2012; Odoardi et al., 2016; Zancajo et al., 2014). Along with the increasing number of anecdotal reports on 3,4-DMMC abuse published in drug forums and blogs (Bluelight, 2010; Drugs-forum, 2011), also the number of poisonings described in the literature sprung (Usui et al., 2014; Zaami et al., 2018; Zellnera et al., 2017), including one fatality that made clear the hazard associated with 3,4-DMMC (Usui et al., 2014). This substance is also chemically and structurally similar to the classic amphetamines (Figure 1). Thus, similar toxicological effects are produced (Valente et al., 2014). Yet, it is distinct enough to evade drug control tests in many countries were the drug is legally framed. Similar to other NPS, the identification and quantification of 3,4-DMMC, both in counterfeit products and in biological samples, is challenging since it requires the availability of adequate standards, knowledge on the metabolites excreted in the relevant matrices (e.g. blood and urine) and the constant updating of detection and quantification methods. In this regard, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) methodologies have been developed for the analysis of 3,4-DMMC in various biological samples, but the application of these analytical protocols is often hindered by the scarcity of information regarding its metabolism and excretion patterns (Grapp et al., 2016; Locos and Reynolds, 2012).

The present introduction attempts to provide an overview of the available data on 3,4-DMMC physicochemical properties and detection methods, patterns of abuse and prevalence, biological effects, pharmacodynamics, pharmacokinetics, and mechanisms of toxicity.

1.2 Physicochemical properties

As mentioned, 3,4-DMMC belongs to the phenylethylamine family, being chemically a β-ketophenylethylamine. The properties of this N-alkylated cathinone derivative, which can be presented as a free base or as an halogen salt (e.g. hydrochloride salt), are listed in Table 1 (Tyrkko et al., 2013; Valente et al., 2016; Valente et al., 2014).

Table 1: Properties of 3,4-dimethylmethcathinone (3,4-DMMC).

Name	3,4-Dimethylmethcathinone (free base)	3,4-Dimethylmethcathinone hydrochloride
Chemical Formula	$C_{12}H_{17}NO$	$C_{12}H_{18}ClNO$
CAS Number	1082110-00-6	1081772-06-6
IPUAC Name	1-(3,4-Dimethylphenyl)-2-(methylamino)propan-1-one	1-(3,4-Dimethylphenyl)-2-(methylamino)propan-1-one hydrochloride
Other Names	3,4-Dimethyl-methcathinone 3,4-DMMC	3,4-Dimethyl-methcathinone hydrochloride 3,4-DMMC.HCl
Molar Mass	191.269 g/mol	227.732 g/mol
Melting Point	Not Determined	216.4 ºC
Appearance	Crystalline solid	White powder

Compared with amphetamines, the ketone group present in all cathinones increases the polarity of the substance, resulting in a decreased ability to cross membranes and the blood-brain barrier, so consumers use higher doses to obtain compensatory effects. In comparison with mephedrone, the methyl-substitution of the aromatic ring increases the lipophilicity of the molecule, inducing faster and more intense peak effects (Gaspar et al., 2018; Valente et al., 2016).

The spectroscopic characteristics of this cathinone were thoroughly investigated after a drug seizure from the Australian Customs Service (Locos and Reynolds, 2012). The mass spectra fragmentation was typical of the cathinone family. The molecular ion at m/z 191 was barely present and the base peak at m/z 58, which corresponds to an immonium íon formed from the α-cleavage of the benzylic bond, was indicative of the N-methylphenethylamine structure. Additional ions at m/z 133, m/z 105, and m/z 77 indicate the 3,4-dimethylbenzoyl cation, the xylene cation, and the further loss of an acetylene group, respectively. A minor ion at m/z 176 results from an α-cleavage, causing the loss of the α-methyl group (Locos and Reynolds, 2012). In this study, the

chemical shifts of carbons and hydrogens were assigned by one- and two-dimensional Nuclear Magnetic Resonance (NMR) techniques. A molar absorptivity (ε) of 12,000 was calculated and an UV maximum absorption was observed at 266 nm, producing an UV spectrum that is identical to mephedrone. Similarly, the IR spectrum was very similar to that of mephedrone (Locos and Reynolds, 2012).

Using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC/QTOF-MS), the stability of 3,4-DMMC in blood was evaluated at different concentrations (1,000 ng/mL and 100 ng/mL) and temperatures (32 ºC, 20 ºC, 4 ºC and -20 ºC) (Glicksberg and Kerrigan, 2017). The authors concluded that, after 180 days, the stability of the drug does not depend on its concentration, since at concentrations of 1,000 ng/mL and 100 ng/mL it decreased in the same proportion. On the contrary, different temperatures affected its stability. After 4 days at room temperature (RT, 20 ºC) and 1 day at elevated temperature (32 ºC), 20% of 3,4-DMMC degrades. A similar degradation rate is observed when the drug is refrigerated (4 ºC) for 19 days or frozen (-20 ºC) for 101 days (Glicksberg and Kerrigan, 2017). Studies of this kind are important and necessary for the correct interpretation of the ante- and post-mortem analytical results, as they can be strongly impacted by the storage conditions of biological samples.

1.3 Analytical methods for the identification and quantification of 3,4-DMMC

Various studies have been performed for the identification and quantification of synthetic cathinones, including 3,4-DMMC, in several biological matrices and/or seized products. In most cases, the identification and/or quantification rely on liquid or gas chromatographic analysis coupled to mass spectrometry (Couto et al., 2018). These methods are summarized in Table 2.

Table 2: Analytical techniques for the identification and quantification of 3,4-dimethylmethcathinone (3,4-DMMC).

Reference	Substances analysed	Matrix	Extraction method	Reagent of derivatization	Internal Standard	Analytical method	LOQ	LOD	Recovery	Linearity	Repeatability (RSD) and Accuracy (A)	Results
(Tyrkko et al., 2013)	3,4-DMMC; 2-DPMP; α-PVP; MPA	Drug abusers urine	SPE			LC/QTOF-MS GC-MS						3,4-DMMC: 0.53 and 1.4 mg/L
(Usui et al., 2014)	3,4-DMMC	Human urine and blood	QuEChERS		Diazepam-d_5	LC-MS	5.38 ng/mL for urine; 5 ng/mL for blood	1.3 ng/mL for urine; 1.03 ng/mL for blood	95.8-101% for urine; 85.9-89.4% for blood	R^2=0.998 for urine; R^2=0.994 for blood	5/50/400 ng/mL: RSD % Intra-day: 3.0/6.4/7.1 for urine; 5.3/5.5/4.8 for blood. Inter-day: 2.4/8.5/4.1 for urine; 3.8/8.1/6.6 for blood. A (RE%) Intra-day: 0.1/-1.1/0.9 for urine; -0.5/5.3/-4.8 for blood. Inter-day: 0.2/1.7/-2.2 for urine; 0.78/7.2/-2.2 for blood	7.6 mg/L for urine; 27 mg/L for blood
(Gaspar et al., 2018)	3,4-DMMC; DMB; DMP; methylone; NEB	Human $HepG_2$ cells and seized samples	SPE			GC-MS NMR HR ESI-MS						
(Molnar et al., 2016)	3,4-DMMC and another cathinones	Human urine		MSTFA		GC-MS	15-24 µg/mL		97-99%	R^2=0.9976-0.998	RSD: 1.43-5.44%	
(Odoardi et al., 2016)	3,4-DMMC and another cathinones; synthetic cannabinoids	Seized sample		2,2,2-trichloroethyl chloroformate		GC-MS LC-HRMS NMR						
(Locos and Reynolds, 2012)	3,4-DMMC	Seized samples	LLE		Bupivacaine	GC-MS LC-MS NMR IR UV						
(Uralets et al., 2014)	3,4-DMMC and metabolites	Drug abusers urine	LLE	TFAA		GC-MS						

Table 2: Analytical techniques for the identification and quantification of 3,4-dimethylmethcathinone (3,4-DMMC) (cont.).

Reference	Substances analysed	Matrix	Extraction method	Reagent of derivatization	Internal Standard	Analytical method	LOQ	LOD	Recovery	Linearity	Repeatability (RSD) and Accuracy (A)	Results
(Boumba et al., 2017)	3,4-DMMC; synthetic cannabinoids; amphetamines and piperazines	Human hair	LLE		MDA-D5 MDEA-D6	LC-MS		0.01 ng/mL				
(Concheiro et al., 2015)	3,4-DMMC; amphetamines and piperazines	Human urine	Solid phase cation exchange extraction		MDPV-D8	LC-MS	5 µg/mL		15 µg/mL: 93.9% 30 µg/mL: 97.8% 400 µg/mL: 93.9%	(R^2±SD) 0.9908±0.0064	RSD Intra-day: 15 µg/mL:9.6% 30 µg/mL: 11.9% 400 µg/mL:6.6%	
(Fontanals et al., 2017)	3,4-DMMC and another synthetic cathinones	Influent and effluent sewage and river water	SPE			LC-HRMS	1-5 ng/L	0.1-0.5 ng/L	70-100%			
(Glicksberg and Kerrigan, 2017)	3,4-DMMC and another synthetic cathinones	Human blood	SPE		Methylone-D3; Ethylone-D5; Bytlone-D3; Mephedrone-D3; Eutylone-D5; Pentylone-D3, α-PVP-D8; MDPV-D8; Naphyrone-D5	LC/QTOF-MS	5 ng/mL	5 ng/mL				
(Mohr et al., 2012a)	3,4-DMMC and another synthetic cathinones	Internet-purchased samples				GC-MS NMR CE						
(Taschwer et al., 2014)	3,4-DMMC and another synthetic cathinones and amphetamines	Internet-purchased samples	No special sample preparation			HPLC-UV NMR						
(Mohr et al., 2012b)	3,4-DMMC and another synthetic cathinones	Internet-purchased samples	LLE			HPLC-UV						

Table 2: Analytical techniques for the identification and quantification of 3,4-dimethylmethcathinone (3,4-DMMC) (cont.).

Reference	Substances analysed	Matrix	Extraction method	Reagent of derivatization	Internal Standard	Analytical method	LOQ	LOD	Recovery	Linearity	Repeatability (RSD) and Accuracy (A)	Results
(Shima et al., 2013)	3,4-DMMC and metabolites	Drug abusers hurine	LLE	TFAA	DBA	GC-MS LC-MS			3,4-DMMC: 102% Nor- DMMC: 99% Dihydro-DMMC: 100% Nor-dihydro-DMMC: 97%	3,4-DMMC: R^2=0.998; Nor-DMMC: R^2=0.999; Dihydro-DMMC: R^2=0.997; Nor-DMMMC: R^2=0.997	At 300/3000ng/mL: Intra-day: 3,4-DMMC: RSD:4.8/6.1% and A:4.9/5.2%; Nor-DMMC: RSD:11.8/5.7% and A: -4.1/-3.5%; Dihydro-DMMC: RSD: 5.5/4.9% and A:7.2/5.1%; Nor-dihydro- DMMC: RSD:7.2/6.9% and A:-4.0/5.0% Inter-day: 3,4-DMMC: RSD: 5.3/7.2%; Nor-DMMC: RSD: 5.2/4.9%; Dihydro-DMMC: RSD:8.9/8.2%; Nor-dihydro- DMMC: RSD:9.2/6.3%	3,4-DMMC: 4410 ng/mL; 3,4-DMMC glucuronide 640 ng/mL; Nor-DMMC: 920 ng/mL; Nor-DMMC glucuronide:180 ng/mL; Dihydro-DMMC: 3790ng/mL; Dihydro-DMMC glucuronide: 180 ng/mL; Nor-dihydro-DMMC: 2270 ng/ mL; Nor-dihydro-DMMC glucuronide: 1000 ng/ml
(Carnes et al., 2017)	3,4-DMMC and another synthetic cathinones	Internet-purchased samples	LLE			GC-MS UHPSFC, UHPLC						

Table 2: Analytical techniques for the identification and quantification of 3,4-dimethylmethcathinone (3,4-DMMC) (cont.).

Reference	Substances analysed	Matrix	Extraction method	Reagent of derivatization	Internal Standard	Analytical method	LOQ	LOD	Recovery	Linearity	Repeatability (RSD) and Accuracy (A)	Results
(Geryk et al., 2016)	3,4-DMMC, another synthetic cathinones, benzofury and amphetamines	Internet-purchased samples				SFC						Partial enantioseparation for 3,4-DMMC
(Rowe et al., 2017)	3,4-DMMC and another synthetic cathinones	Obtained from Cayman Chemical				UHPSFC, UHPLC						
(Freni et al., 2019)	3,4-DMMC and another synthetic cathinones	Human hair	SPE		mephedrone-D3	LC-MS/MS	1 pg/mg	0.3 pg/mg	100%	R^2=0.99	RSD Intra-day: 20.0% at 1 pg/mg; 14.6% at 20 pg/mg; 1.8% at 250 pg/mg Inter-day: 11.8% at 1 pg/mg; 16.2% at 20 pg/mg; 12% at 250 pg/mg A 12.2% at 1 pg/mg; 3.5% at 20 pg/mg; 0.2% at 250 pg/mg	Segment 0–2.5 cm: 2800.0 pg/mg Segment 2.5–5 cm: 572.6 pg/mg

α-PVP: α-Pyrrolidinopentiophenone; **2-DPMP:** 2-Diphenylmethylpiperidine, **A:** Accuracy**; CE:** Capillary electrophoresis; **DBA:** Dibenzylamine; **Dihydro-DMMC:** Dihydro-dimethylmethcathinone; **DMB:** Dimethylbuphedrone, **DMP:** Dimethylpentedrone, **GC-MS:** Gas chromatography-mass spectrometry detection; **HPLC-UV:** High performance liquid chromatography-ultraviolet detection; **HR ESI-MS:** High resolution electrospray ionization-mass spectrometry detection; **IR:** Infrared spectroscopy; **LC-HRMS:** Liquid chromatography-high resolution mass spectrometry detection; **LC-MS:** Liquid chromatograph-mass spectrometry detection; **LC-MS/MS:** Liquid chromatography-tandem mass spectrometry detection; **LC/QTOF-MS:** Liquid chromatography-quadrupole time-of-flight mass spectrometry detection; **LLE:** Liquid-liquid extraction**; LOD:** Limit of detection; **LOQ:** Limit of quantification; **MDA-D5:** Methylenedioxyamphetamine deuterated**; MDEA-D6:** Fentanyl-D5-3,4-methylenedioxy-*N*-ethylamphetamine deuterated; **MDPV:** Methylenedioxypyrovalerone; **MDPV-D8:** Methylenedioxypyrovalerone deuterated; **MPA:** Methiopropamine, **MSTFA:** N-Methyl-N-(trimethylsilyl) trifluoracetamide; **NEB:** N-Ethylbuphedrone, **NMR:** Nuclear magnetic resonance*;* **Nor-DMMC:** Nor-dimethylmethcathinone; **Nor-dihydro-DMMC:** Nor-dihydro-dimethylmethcathinone; **QuEChERS**: Quick, easy, cheap, effective, rugged, and safe extraction; **R^2:** Squared correlation coefficient**; RE:** Relative error; **RSD:** Relative standard deviation; **SD:** Standard deviation; **SFC-UV:** Supercritical fluid chromatography-ultraviolet detection; **SPE:** Solid-phase extraction; **TFAA:** Trifluoroacetic anhydride; **UHPLC:** Ultra high performance liquid chromatography; **UHPSFC**: Ultra high performance supercritical fluid chromatography; **UV:** Ultraviolet spectroscopy.

1.4 Patterns of consumption, prevalence and legal status

In countries such as France, Germany, Hungary, Ireland, Lithuania, Portugal, Turkey, UK, Belarus, Russia and Japan, consumption, possession and sale of 3,4-DMMC are controlled (Serpelloni G, 2013), but the drug is still sold on the internet in the form of a fine white powder (Figure 2) labelled as 'bath salts', 'research chemicals', 'plant fertilizers' and 'not for human consumption', with prices varying between 10–40 € per gram, depending on the degree of purity (Brunt et al., 2017; Valente et al., 2014; Zaami et al., 2018; Zancajo et al., 2014). 3,4-Dimethylmethcathinone can also be found on the streets in the form of pills, allegedly containing 500 mg of drug, under the alias '*Ocean'* and '*M2'*. Often, consumers report dose escalation up to 1 g in a single session (within 5–8 h) (Bluelight, 2010).

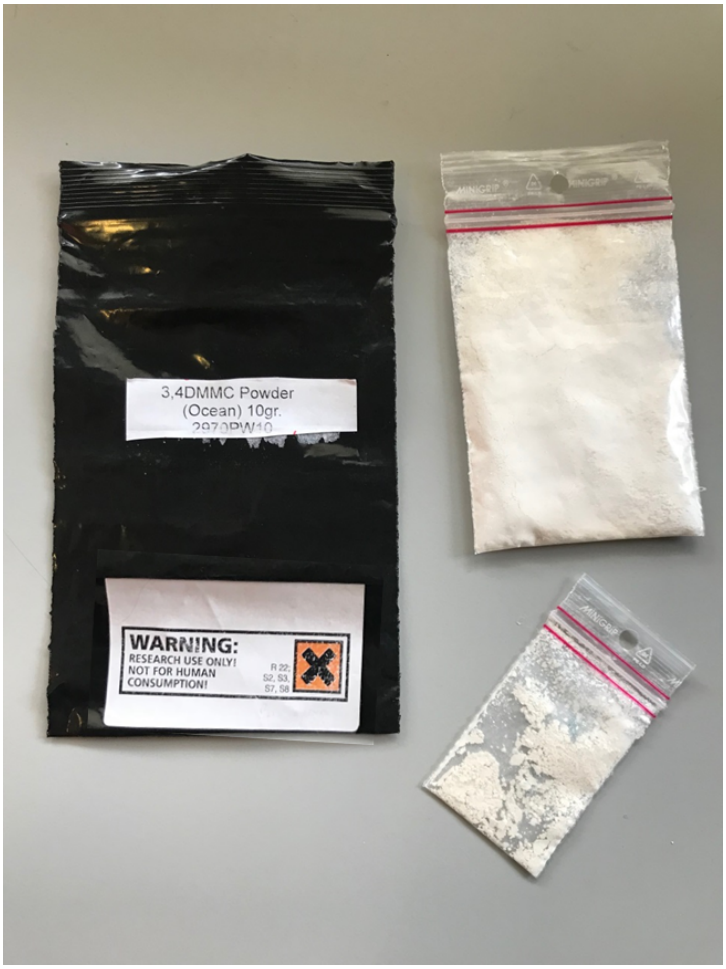

Figure 2: 3,4-Dimethylmethcathinone (3,4-DMMC) is sold online as a fine powder, under designations such as '*Ocean*', and labelled 'research use only' and 'not for human consumption'.

In 2013, 3,4-DMMC seizures represented 2% of all cathinones seized in Europe (EMCDDA, 2015b). In accordance, a study carried out in Italy that focused on the drugs of abuse confiscated in the country between 2013 and 2015, identified synthetic cathinones as the group with the highest number of substances apprehended and at larger quantities, and 3,4-DMMC was identified in the 162 seizures of drugs either from internet sales confiscation or police raids (Odoardi et al., 2016).

Drug users consider 3,4-DMMC a good drug for combinations, preferably with the dissociative hallucinogen methoxetamine (MXE) and 4-MEC, but drug intake along with benzodiazepines, alcohol, and codeine were also reported (Drugs-forum, 2011). In fact, most 3,4-DMMC consumers use drugs very often and deliberately take multiple substances simultaneously, mixing street drugs with alcohol, tobacco, illicit and prescription drugs, among others (Tyrkko et al., 2013; UNODC, 2016). In addition, drug sellers also blend several drugs in the same preparation. In 2015, European countries reported more than 110 NPS products containing a combination of up to 7 different NPS (UNODC, 2014), in spite of these were sold as one single substance, and synthetic cathinones represented more than 25% of those NPS products. In addition to NPS products containing mixtures of substances belonging to the same group, synthetic cathinones were also the class most frequently identified in products combining NPS of different groups (UNODC, 2014).

1.5 Subjective effects in humans and other biological effects

Drug use testimonies indicate that nasal intake of 100 mg of 3,4-DMMC produces effects after 10 min, and these are comparable to those elicited by the same dose of mephedrone, but slightly longer (Bluelight, 2010; Drugs-forum, 2011). Among them, a state of slight euphoria and empathy accompanied by increased blood pressure and accelerated heart rate were described (Bluelight, 2010; Drugs-forum, 2011; Serpelloni G, 2013). High doses (up to 1 g) of 3,4-DMMC produce insomnia but, unlike mephedrone, chest pain, cold hands and feet, and '*heart racing*' are not experienced. Comedown effects include severe headache and intense rhinorrhoea (Drugs-forum, 2011). Most consumers report that 3,4-DMMC is very short acting and intake together with other stimulants is required to achieve '*exceptional experiences*', with more pleasant euphoric states, energy and excitation. The drugs 3-MMC and 4-MEC are

listed among the preferred drugs for combination with 3,4-DMMC. Other users also admit mixing 3,4-DMMC with benzodiazepines to lessen the stimulant effects. In general, users also consider that tolerance sets quickly (e.g. "*you can only get high of it like 30 minutes once a week*") (Researchchemicals, 2016).

1.6 Pharmacodynamics

Upon reaching the brain, 3,4-DMMC exerts its effects on monoamine transporters and receptors (Figure 3).

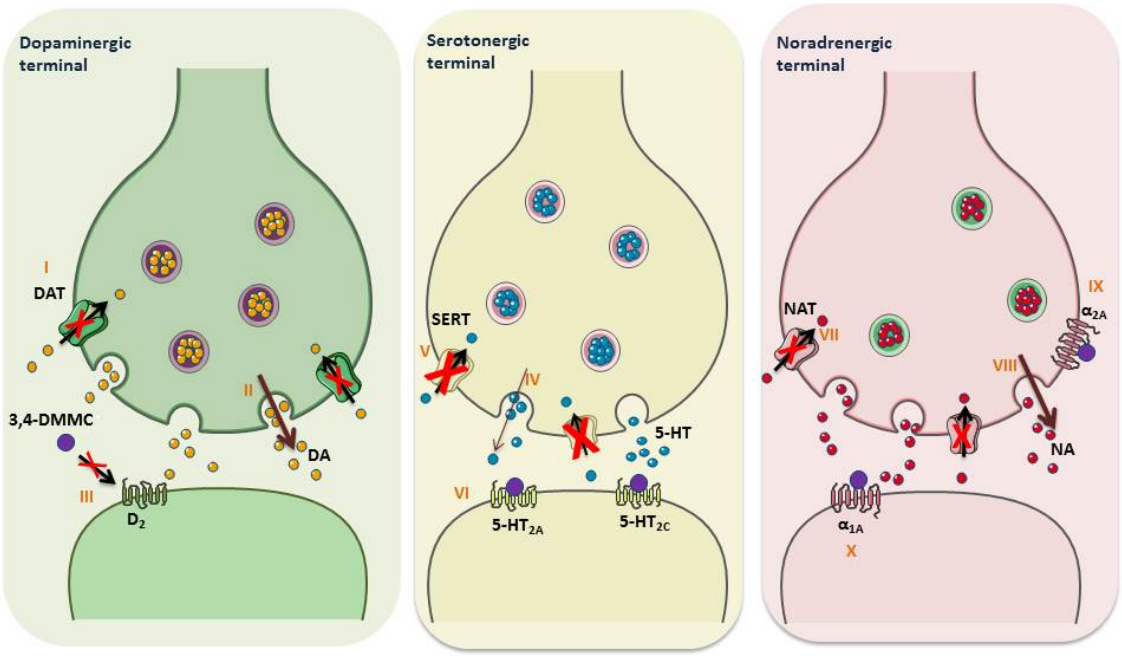

Figure 3: Pharmacodynamics of 3,4-dimethylmethcathinone. The drug promotes sustained increase of DA in the dopaminergic synapse by inhibiting uptake from DAT (I) and promoting strong DA release from the axon terminal (II). Nevertheless, the drug seems to have no affinity for the D_2 receptor (III). In the serotonergic terminal, 3,4-DMMC induces a slighter release of 5-HT (IV) but more strongly inhibits the monoamine reuptake (V), also activating postsynaptic 5-HT_{2A} and 5-HT_{2C} receptors (VI). At the noradrenergic synapse, 3,4-DMMC also inhibits the reuptake from NAT (VII), and is a potent releaser of NA (VIII). 3,4-Dimethylmethcathinone has affinity to both α_{2A} presynaptic (IX) and α_{1A} postsynaptic (X) adrenergic receptors. **DA**: Dopamine; **DAT**: Dopamine transporter; **5-HT**: Serotonin; **SERT**: Serotonin transporter; **NA**: Noradrenaline; **NAT**: Noradrenaline transporter.

Luethi et al. (2018) characterised the in vitro pharmacology of new mephedrone analogues, including 3,4-DMMC and its 2,3-DMMC and 2,4-DMMC isomers, by determining the potency of inhibition of the transporters of noradrenaline (NAT), dopamine (DAT) and serotonin (SERT), as well as the binding affinity to the receptors and transporters of these monoamines (Table 3).

Table 3: Monoamine transport inhibition and monoamine transport and receptor binding affinities of 3,4-dimethylmethcathinone (3,4-DMMC), 2,3-dimethylmethcathinone (2,3-DMMC), 2,4-dimethylmethcathinone (2,4-DMMC), 3-methylmethcathinone (3-MMC), and mephedrone (4-MMC).

		2,3-DMMC	2,4-DMMC	3,4-DMMC	3-MMC	4-MMC
NAT	**IC50** (μM)	0.53	1.5	0.45	0.27	0.26
	(95% CI)	(0.36-0.78)	(1.1-2.0)	(0.33-0.60)	(0.21-0.36)	(0.19-0.35)
	Ki (μM)	8.4±0.3	>26	12±2	5.6±1.5	>26
DAT	**IC50** (μM)	7.4	83	9.4	2.6	5.7
	(95% CI)	(5.4-10.1)	(65-105)	(7.6-11.7)	(2.0-3.3)	(4.5-7.2)
	Ki (μM)	4.2±0.6	>26	7.6±0.6	3.2±0.6	2.9±0.2
SERT	**IC50** (μM)	1.2	1.5	1.1	9.5	3.6
	(95% CI)	(1.0-1.4)	(1.0-2.2)	(0.9-1.4)	(6.9-13.2)	(2.8-4.6)
	Ki (μM)	6.1±0.5	17±1	5.7±0.3	>22	>22
RATIO	**DAT/SERT**	0.16	0.02	0.12	3.7	0.63
	(95% CI)	(0.10-0.26)	(0.01-0.03)	(0.08-0.18)	(2.1-6.6)	(0.39-1.02)
D_2	**Ki** (μM)	>11	>11	>11	>12	>11
α_{1A}	**Ki** (μM)	3.0±0.1	3.0±0.3	3.5±0.2	1.1±0.1	11±1
α_{2A}	**Ki** (μM)	0.78±0.10	0.16±0.02	1.9±0.3	7.9±0.2	1.1±0.1
5-HT_{1A}	**Ki** (μM)	>17	15±3	>17	4.8±0.5	>17
5-HT_{2A}	**Ki** (μM)	0.64±0.19	1.3±0.1	1.9±0.3	3.4±0.8	1.6±0.2
	EC_{50} (μM)	0.13±0.02	>10	>10	>20	0.36±0.19
	E_{max}	84±12				79±20
5-HT_{2B}	**EC_{50}** (μM)	>10	>10	>10	>20	>10
5-HT_{2C}	**Ki** (μM)	2.4±0.9	1.3±0.3	1.5±0.2	3.6±1.0	8.1±5.4
TAAR1	**Ki Rat** (μM)	1.2±0.1	0.59±0.08	2.6±0.2	5.7±1.4	5.0±0.1
	Ki Mouse (μM)	0.88±0.06	3.1±0.2	4.5±0.4	10±1	12±1

NAT: Noradrenaline transporter; **DAT:** Dopamine transporter; **SERT:** Serotonin transporter; **5-HT_x:** Serotonin receptor; **CI:** Confidence interval; **DAT/SERT ratio** = 1/DAT IC_{50} : 1/SERT IC_{50}; **E_{max}:** Activation efficacy, given as percentage of maximum±standard deviation; **IC_{50}:** Half maximal inhibitory concentration; **TAAR1:** Trace amine-associated receptor 1. Adapted from Luethi *et al.*(2018).

All tested drugs strongly inhibited NAT on transporter-transfected human embryonic kidneys cells (IC_{50} 450 nM for 3,4-DMMC), which likely ends up in sympathomimetic stimulation. Unlike 3-MMC, 3,4-DMMC more potently inhibited SERT than DAT (DAT/SERT ratio of 0.12 for 3,4-DMMC) and the drug displayed a high affinity for 5-HT_{2A} and 5-HT_{2C} receptors. This monoamine transporter inhibition profile is similar to that of MDMA, but contrary to the amphetamine, 3,4-DMMC did not cause a significant 5-HT efflux. This difference in the release of 5-HT may explain the different entactogenic properties elicited by both drugs. It should be noted that 2,3-DMMC significantly activated the 5-HT_{2A} receptor, suggesting hallucinogenic properties. 3,4-Dimethylmethcathinone was a potent releaser of DA and NA but did not interact with the D_2 receptor. In addition, the drug interacts with amine-associated receptor (TAAR) and has low micromolar affinity for adrenoreceptors α_{1A} and α_{2A}, known to play opposite roles in behavioural modulation. These data suggest that different inhibition of

the monoamine transporters and receptor interactions mediate the distinct psychoactive effects of DMMC isomers (Luethi et al., 2018; Valente et al., 2016).

1.7 Pharmacokinetics

The most common routes of 3,4-DMMC administration are oral ingestion and nasal insufflation, but some users acknowledge preference of oral intake to snorting due to the corrosive effect of the drug at the nasal mucosa (Bluelight, 2010; Drugs-forum, 2011). There is also suspicion of intravenous administration in the only reported fatality that was attributed to 3,4-DMMC alone (Usui et al., 2014). The analytical findings of the quantitative analysis of 3,4-DMMC in the blood and urine specimens obtained from this fatal intoxication revealed that the concentration of 3,4-DMMC was higher in the peripheral blood (27 mg/L) than in urine (7.6 mg/L) (Usui et al., 2014). Additionally, some metabolites, including 3,4-dimethylcathinone (3,4-DMC) and β-keto-reduced metabolites (β-OH-DMMC and β-OH-DMC) were detected in the victim's blood and urine (Usui et al., 2014).

Previous studies from Tyrkko et al. (2013) and Shima et al. (2013) had already identified 3,4-DMMC metabolites in the urine of regular drug users and signposted N-demethylation, β-keto-reduction, hydroxylation, and oxidation as the major reactions involved (Figure 4). The metabolites undergoing successive hydroxylation + reduction + oxidation (3-methyl-4-carboxy-dihydro-methcathinone and 3-carboxy-4-methyl-dihydro-methcathinone) and N-demethylation + hydroxylation (nor-3-methyl-4-hydroxymethyl-methcathinone and nor-3-hydroxymethyl-4-methyl-methcathinone) appear less frequently and at smaller amounts in the urine, whereas the unchanged compound is the most prevalent, followed by its β-keto-reducted metabolite nor-dihydro-dimethylmethcathinone. Since enzymatic hydrolysis increased the detected amounts of 3,4-DMMC, dihydro-dimethylmethcathinone, nor-dimethylmethcathinone, and nor-dihydro-dimethylmethcathinone, the presence of the respective conjugates with glucuronic acid was hypothesized. The phase I metabolic pathways for mephedrone are similar to the described herein for 3,4-DMMC, and studies in vitro demonstrated that the metabolism of mephedrone is mainly catalysed by CYP2D6 (Pedersen et al., 2013). Other isoforms of cytochrome P450, notably CYP1A2, CYP2B6, CYP2C8, CYP2C18, CYP2C19, and CYP3A4, seem to be also involved, but with lower relevance (Pedersen

et al., 2013). Given the similar structure, one may hypothesise that the same enzymes are involved in the metabolism of 3,4-DMMC.

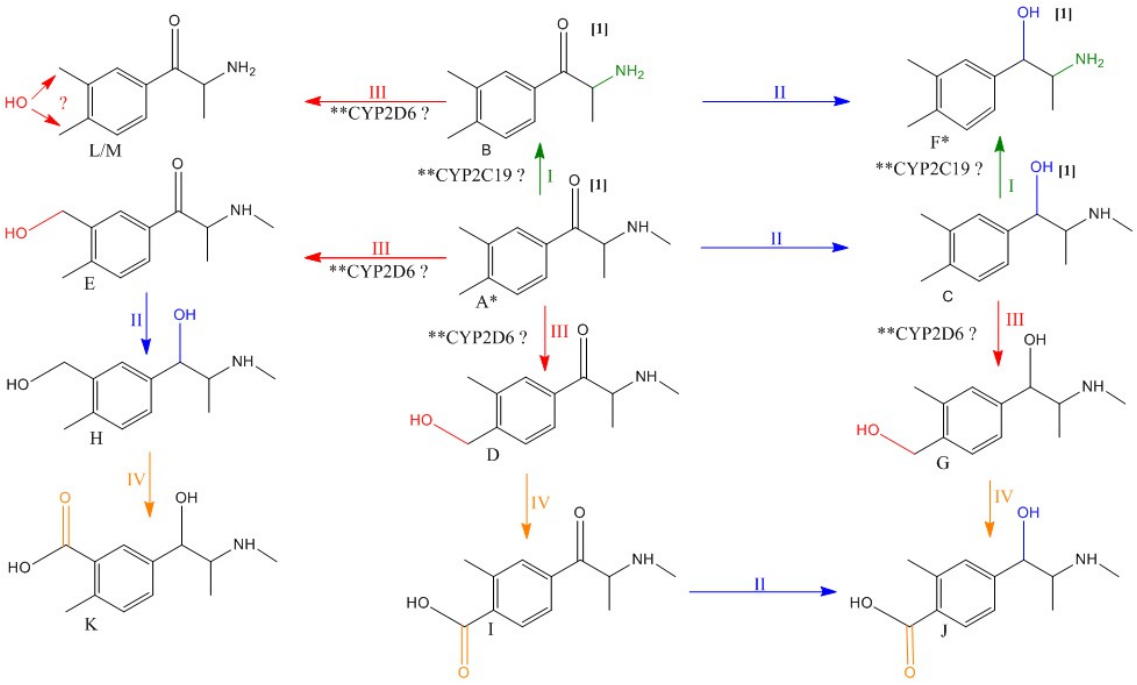

Figure 4: Metabolism of 3,4-dimethylmethcathinone (3,4-DMMC). **I:** N-demethylation. **II:** β-Keto-reduction. **III:** Hydroxylation. **IV:** Oxidation. **A:** 3,4-Dimethylmethcathinone. **B:** Nor-dimethylmethcathinone. **C:** Dihydro-dimethylmethcathinone. **D:** 3-Methyl-4-hydroxymethyl-methcathinone. **E:** 3-Hydroxymethyl-4-methyl-methcathinone. **F:** Nor-dihydro-dimethylmethcathinone. **G:** 3-Methyl-4-hydroxymethyl-dihydro-methcathinone. **H:** 3-Hydroxymethyl-4-methyl-dihydro-methcathinone. **I:** 3-Methyl-4-carboxy-methcathinone. **J:** 3-Methyl-4-carboxy-dihydro-methcathinone. **K:** 3-Carboxy-4-methyl-dihydro-methcathinone. **L:** Nor-3-methyl-4-hydroxymethyl-methcathinone. **M:** Nor-3-hydroxymethyl-4-methyl-methcathinone. **[1]** Conjugation with glucuronic acid. *Abundant in human urine. **Enzyme that catalyses this metabolic reaction for mephedrone. Adapted from Tyrkko et al. (2013) and Shima et al. (2013).

Many of the cytochrome P450 isoenzymes (CYP2D6, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2E1, and CYP3A) are inhibited by synthetic cathinones (Calinski et al., 2019; Diestelmann et al., 2018). Thus, given the risk of pharmacokinetic and pharmacodynamic drug interactions, the concomitant use of these drugs becomes dangerous. According to Calinski et al.(2019) and Diestelmann et al. (2018) the consumption of synthetic cathinones, including 3,4-DMMC, is associated with the consumption of antidepressants and other stimulants, opioids, benzodiazepines and other sedatives. In these cases, metabolic competition may arise with 3,4-DMMC, which is likely to alter the pharmacokinetic profile of the co-administered drugs (Calinski et al., 2019). For example, opioids and benzodiazepines are metabolized by CYP2D6 and CYP3A4, respectively, with subsequent glucuronidation. The extension of this interaction is however, unknown (Diestelmann et al., 2018).

1.8 Clinical toxicology

In general, 3,4-DMMC is considered more harmful and produces more severe side effects than mephedrone (Bluelight, 2010). Drug abuse testimonies indicate that nasal intake of 100 mg of 3,4-DMMC produces severely burned nostrils, throat corrosion, tear over-secretion and nose bleeding (Drugs-forum, 2011). Signs and symptoms of synthetic cathinone poisoning are consistent with sympathomimetic toxicity and include hypertension, tachycardia, dehydration, seizures, and psychomotor agitation (Couto et al., 2018; Weinstein et al., 2017). These symptoms have all also been noted for 3,4-DMMC and are in line with users' reports (e.g. '*chest tightening and pain'*, *'pressure inside my head'*) (Bluelight, 2010; Drugs-forum, 2011). Muscle spasms and palpitations have also been reported, as well as tremors, hallucinations, and confusion (Coppola and Mondola, 2012; Valente et al., 2014).

1.9 Toxicity

1.9.1 Lethal cases

In the literature, only one case report exists mentioning solely 3,4-DMMC on the death certificate (Usui et al., 2014). In this case, a man was found dead at home with a disposable syringe next to his left arm. In the house, various packages containing a white powder were found. After testing, the substance present both in the packages and in the syringe was identified as 3,4-DMMC. Based on the police investigation and the autopsy findings, it was possible to estimate the post-mortem interval as 1.5 days. During the autopsy, no significant pathological abnormalities were found, and urine and blood from the iliac vein were collected for subsequent toxicological analysis. The concentrations of 3,4-DMMC found in the biological samples were 27 mg/L in blood and 7.6 mg/L in urine. Given the absence of other published fatal reports, no conclusions could be drawn on potentially toxic and/or lethal concentrations for 3,4-DMMC. However, the concentrations reported in this fatality were much higher than those reported on fatal poisonings with other structurally identical drugs (Table 4).

Table 4: Concentration of synthetic cathinones detected in biological samples from fatal cases.

Analyte	Matrix	Concentrations	Reference
3,4-DMMC	Blood (external iliac vein)	27 mg/L	(Usui et al., 2014)
	Urine	7.6 mg/L	
4-MMC	Urine	198 mg/L	(Dickson et al., 2010)
	Blood	0.50 mg/L	
	Blood	5.5 mg/L	(Adamowicz et al., 2013)
	Blood	0.13–2.24 mg/L	(Maskell et al., 2011)
	Femoral blood	1.2; 3.3; 5.7 mg/L	(Torrance and Cooper, 2010)
MDPV	Peripheral blood	1 mg/L	(Kesha et al., 2013)
	Heart blood	0.7 mg/L	
	Femoral blood brain, muscle, lungs and cerebrospinal fluid	0.4–0.6 mg/L	(Wyman et al., 2013)
	Urine	0.67 mg/L	(Murray et al., 2012)
	Serum	0.082 mg/L	
	Urine	0.14 mg/mL	(Borek and Holstege, 2012)
Methylone	Heart blood	0.060; 0.118; 0.740; 1.12 mg/L	(Cawrse et al., 2012)
	Blood	0.7 mg/L	(Carbone et al., 2013)
	Peripheral blood	3.13 mg/L	(Barrios et al., 2016)
	Central blood	6.64 mg/L	
	Peripheral blood	0.56; 0.84; 3.3 mg/L	(Pearson et al., 2012)
	Heart blood	0.58 and 1 mg/L	
Ethylone	Femoral blood	1.7 mg/L	(deRoux and Dunn, 2017)
	Blood	0.038–2.572 mg/L	(Lee et al., 2015)

3,4-DMMC: 3,4-Dimethylmethcathinone; **4-MMC:** Mephedrone**; MDPV:** Methylenedioxypyrovalerone

1.9.2 Mechanisms of toxicity

The toxicological mechanisms of 3,4-DMMC are scarcely studied, and their clarification is very important to estimate the risks to which these consumers are exposed to, as well as to assist in the diagnosis and treatment of intoxications. In general, due to their similarity to amphetamines, it is anticipated that many of the toxicological mechanisms of synthetic cathinones are shared with these classic drugs. As observed by Valente et al.(2016) in an in vitro study with primary rat hepatocytes and human hepatoma cell lines, cathinones trigger oxidative stress processes, leading to

mitochondrial dysfunction and apoptosis. Accordingly, 3,4-DMMC promotes the increase of reactive oxygen and nitrogen species and consequently decreases the intracellular levels of reduced glutathione, an important endogenous antioxidant, while increasing oxidized glutathione (Roque Bravo et al., 2016). This oxidative stress contributes to mitochondrial dysfunction, also influenced by the decrease in intracellular levels of ATP. In response to this event, the mitochondria trigger apoptotic processes, leading to cell death (Roque Bravo et al., 2016). In addition, 3,4-DMMC was the most toxic drug among a series of synthetic cathinones tested for 24h, inducing concentration-dependent apoptosis in human hepatocellular carcinoma HepG2 cells, and thus denoting its high potential for inducing liver damage (Gaspar et al., 2018).

1.10 Treatment

To date, treatment for cathinone poisoning, including 3,4-DMMC, is limited to supportive measures to calm down the patient and alleviate the physiological and psychotic effects of the drug, which include hyperthermia, hypertension, tachycardia and agitation (Calinski et al., 2019). To treat poisoning, opioids and benzodiazepines are often administered as a first-line approach to control agitation and aggressiveness, or reduce effects such as panic attacks, anxiety, disorientation and persecution mania (Calinski et al., 2019; Diestelmann et al., 2018). However, given the potential pharmacokinetic interactions mentioned above, special caution should be taken when these pharmacological therapies are administered to the intoxicated patients.

Chapter II- Objectives

In the last decades, the production and consumption of synthetic cathinones have undergone a strong expansion, and they are now among the best-selling NPS on the internet. In this regard, 3,4-DMMC is a cathinone derivative that was first identified in 2010 in Europe, and whose reports on abuse and poisoning have recently peaked. Since synthetic cathinone products do not undergo safety assessment and quality control, research to elucidate the toxicological effects of 3,4-DMMC is of utmost importance. Unfortunately, there are insufficient data in the scientific literature related to the acute health effects in humans caused by the consumption of this NPS, and information on chronic effects and studies in animals are absent. Also, information on the pharmacokinetics and methods for the detection and quantification of 3,4-DMMC in biological samples is scarce. Therefore, studies on these aspects are very much needed and of extreme relevance to help forensic scientists, pathologists, and emergency room clinicians on their practice.

In this context, the present study aimed at:

- Fully validating a GC-MS methodology for the quantification of 3,4-DMMC in plasma, and partially validating the developed method for additional quantification of the drug in other biological matrices (liver, kidneys, heart, brain, lungs, spleen, gut, muscle, adipose tissue and urine);

- Evaluating and characterising the biodistribution of 3,4-DMMC in Wistar rats, by quantifying the drug in biological tissues and plasma, after i.p. administration;

- Characterising the in vivo metabolic profile of 3,4-DMMC in Wistar rats.

Chapter III- Material and Methods

3.1 Chemicals and Reagents

All chemicals and reagents were of analytical grade. Trifluoroacetic anhydride (TFAA), phenylmethanesulfonyl fluoride (PMSF), type 2 β-glucuronidase from *Helix pomatia,* N-methylbistrifluoroacetamine (MBTFA), N-methyl-N-trimethylsilyl-trifluoroacetamine (MSTFA), sodium chloride and trifluoroacetic acid were obtained from Sigma Aldrich (St. Louis, Missouri). Methanol, sodium acetate, ethyl acetate, 70% perchloric acid ($HClO_4$) and acetonitrile were obtained from Fisher Chemical (Loughborough*,* United Kingdom). 25% Ammonia solution (NH_3) was obtained from PanReac Applichem (ITW Reagents, Darmstadt, Germany). Potassium dihydrogen phosphate (KH_2PO_4) and 37% hydrochloride acid (HCl) were obtained from Merck (Darmstadt, Germany). Methyl orange was obtained from Koch-Light Laboratories (Colnbucks Buck, UK). Methylone (IS) and 3,4-dimethylmethcathinone were acquired online at sensearomatics.net on March 2013 and their chemical purity and identity were verified by NMR and MS methodologies. Analytical data were consistent with the assigned structures with about 99% purity.

3.2 Biological specimens

This study was performed at the highest standards of ethics after approval by the local Ethical Committee for the Welfare of Experimental Animals (University of Porto-ORBEA; project 251/2018) and by the Portuguese national authority *Direção Geral de Alimentação e Veterinária* (DGAV). Housing and all experimental procedures were performed by investigators accredited for laboratory animal use in accordance with the Portuguese and European legislation (Decree-law n. º113/2013; Directive 86/609/EEC; Directive 2010/63/EU). Female Wistar Han rats with a body weight of 200–300 g were kept in sterile facilities under controlled temperature (20±2°C), humidity (40–60%), and lighting (12 h-light/dark cycle) conditions, and fed with sterile standard rat chow and tap water *ad libitum*. On the day of the experiments, at least six animals were administered i.p. with 0.9% sodium chloride (saline) or 20 mg/Kg or 40 mg/Kg 3,4-DMMC (prepared in saline). During the experimental period, animal behaviour was carefully monitored. After 1h or 24h, the animals were anaesthetized and euthanized. Rat anaesthesia was induced by an i.p. injection of a combination of 20 mg/Kg xylazine (Rompun® 2%, Bayer HealthCare, Germany) and 100 mg/Kg ketamine (Clorketam®

1,000, Vétoquinol, France), and maintained through inhalation of isoflurane vapour (IsoVet® 1,000 mg/g, B. Braun VetCare, Germany). After euthanasia, the blood, brain, liver, heart, kidneys, lungs, spleen, and a portion of muscle, gut and adipose tissue were systematically selected and collected. In the case of animals treated for 24h, rats were kept in metabolic cages and the urine was also collected into reservoirs placed on ice and added with 200 µL of 100 mM PMSF, a protease inhibitor. Total blood was collected by puncturing the inferior vena cava with a heparinized syringe, transferred into EDTA tubes, and centrifuged at 1,600 *g* for 15 min at 4 °C. Plasma was separated, precipitated with $HClO_4$ (5% final concentration) and centrifuged at 3,000 *g* for 10 min at 4 ºC. Organs were homogenized (1:4 w/v) in ice cold 100 mM KH_2PO_4 buffer (pH 7.4) and centrifuged at 3,000 *g* for 10 min at 4 °C. Urine was centrifuged at 3,000 *g* for 10 min at 4 °C. All supernatants were stored at -80 °C prior to sample preparation for GC-MS analysis.

3.3 Calibrators and quality control samples

3,4-Dimethylmethcathinone standards and internal standard (IS; methylone) solutions were prepared in methanol at 1 mg/mL, and stored at -20 ºC. All intermediate solutions were prepared by diluting these stock solutions in methanol. Working calibrators were prepared by spiking 500 µL of blank matrices with 3,4-DMMC stock solution to obtain the final concentrations of 0.078, 0.156, 0.3125, 0.625, 1.25 and 2.5 µg/mL for plasma; 0.3125, 5 and 10 µg/mL for urine; 0.3125, 1.25 and 5 µg/mL for liver, kidneys, brain, spleen, gut and lungs; 0.156, 0.625 and 2.5 µg/mL for heart; 0.078, 1.25 and 5 µg/mL for muscle; and 0.078, 0.3125 and 5 µg/mL for adipose tissue. All blank samples used to prepare the calibrators were obtained from the biological samples of control animals, and were prepared as described above.

3.4 Extraction and derivatization

Five hundred µL of each sample or calibrator were added with 500 µL of 0.2 M sodium acetate buffer (pH 5.2) and 50 µL of 125 µg/mL IS, and applied onto a 1 cm^3 (30 mg) OASIS MCX SPE column from Waters (Milford, Massachusetts) subjected to vacuum pressure through Supleco Visiprep™ SPE Vacuum Manifold (Sigma Aldrich, St. Louis, Missouri). The sample was eluted, and further washed with 2 mL of 0.1 M HCl and 2

mL of methanol. Finally, the compounds of interest were eluted into a glass tube using 2 mL of 5% NH_4OH in methanol, which was evaporated to dryness at 50 ºC, under nitrogen flow. To eliminate residual water all tubes were left in a desiccator with phosphorus pentoxide, under vacuum, overnight.

Derivatization was carried out by adding 50 µL of ethyl acetate and 50 µL TFAA to the dry residue, following incubation at 70 ºC for 30 min. After cooling to RT, the solvent was evaporated under nitrogen flow, and the obtained residue dissolved in 100 µL of ethyl acetate. The solution was used for GC-MS analysis (da Silva et al., 2010). The summary of the procedure is described in Figure 5.

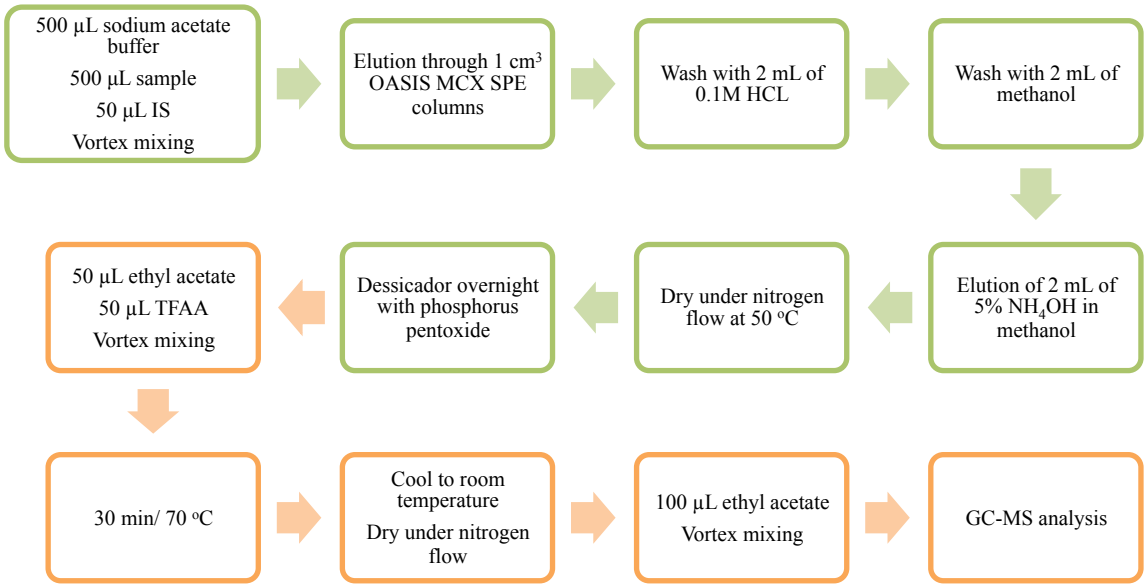

Figure 5: Summary of the sample preparation procedure. The solid phase extraction (SPE) protocol is displayed in green and the derivatization protocol in orange.

3.5 GC-MS analytical settings

The GC-MS analysis was performed with an EVOQ 436 GC system (Bruker Daltonics, Fremont, CA) coupled to a SOCION Triple Quadrupole mass detector, using a capillary column Rxi-5Sil MS (30 m x 0.25 mm x 0.25 µm) from RESTEK. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. One µL of derivatized sample was injected (split ratio was 1/50). The injector port was heated to 250 °C; the initial column temperature of 100 °C was held for 1 min, followed by a temperature

ramp of 15 °C/min to 300 °C, held for 10 min. All mass spectra were acquired in electron impact (EI) mode and the mass ranged from 40 to 600 m/z. The transfer line temperature was 260 ºC, manifold temperature was 41 ºC and EI temperature was 270 ºC. The analysis was performed in full scan mode and in selected ion monitoring (SIM). Table 5 summarizes the selected ions for identification and quantification, as well as the retention times of IS, analyte (3,4-DMMC) and metabolites, after TFAA derivatization.

Table 5: Selected ions for monitoring and retention time of internal standard (IS), 3,4-dimethylmethcathinone (3,4-DMMC), and metabolites (M1, M2, M3, M4 and M5) after derivatization with trifluoroacetic anhydride.

Analyte	Selected ions (m/z)		Retention time (min)
	Quantification	Identification	
IS	121	149, 121	9.35
3,4-DMMC	133 or 105	105, 133,154, 287 [M^+]	8.25
M1	133	105, 133, 140, 273 [M^+]	8.05
M2	154	154, 217, 245, 399 [M^+]	9.10
M3	245	140, 217, 245, 385 [M^+]	8.94
M4	245	140, 217, 245, 385 [M^+]	9.03
M5	154	154, 357, 385, 511 [M^+]	9.50

3.6 Method validation

The validation of analytical procedure for the quantification of 3,4-DMMC was performed in plasma according to the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines (SWGTOX, 2013). The selectivity/specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, recovery and stability were evaluated. For the remaining biological matrices (urine, brain, liver, heart, kidneys, lungs, spleen, gut, muscle and adipose tissue), only specificity/selectivity, linearity (only at three calibration levels), and recovery were evaluated.

3.6.1 Specificity/selectivity

To evaluate the specificity of the method, blank samples obtained from control animals were extracted, injected and the results analysed carefully to detect possible co-eluting chromatographic peaks that could interfere with the retention time of analyte, IS or metabolites. For the plasma, twenty blank samples were analysed, while for the remaining matrices only three blank samples were analysed per matrix.

3.6.2 Linearity

For plasma, linearity was determined by preparing and analysing, in five different days, a blank (no 3,4-DMMC) and six calibrators at 0.078, 0.156, 0.3125, 0.625, 1.25 and 2.5 μg/mL (one calibration curve per day). For the remaining biological matrices, the linearity was only evaluated in three different days, with a blank and three calibrators. For urine, the 3,4-DMMC concentrations tested were 0.3125, 5 and 10 μg/mL; for the liver, kidneys, brain, spleen, gut, and lungs the concentrations tested were 0.3125, 1.25 and 5 μg/mL; for the heart the concentrations tested were 0.156, 0.625 and 2.5 μg/mL; for the muscle the concentrations tested were 0.078, 1.25 and 5 μg/mL; and for the adipose tissue the concentrations tested were 0.078, 0.3125 and 5 μg/mL. The linearity of the method was evaluated by the regression curves (ratio between analyte peak area and IS peak area *versus* analyte concentration) and by the coefficient of determination (R^2).

3.6.3 Limits of detection (LOD) and quantification (LOQ)

The LOD and LOQ were calculated using the standard deviation (SD) of the signal of twenty blanks and the slope (S) of the calibration curve of the analyte, as follows: LOD=3SD/S and LOQ=10SD/S.

3.6.4 Precision

The intra-day and inter-day precision of the method and of the instrument were determined at three levels of the plasma calibration curve: 0.078, 0.625 and 2.5 μg/mL (low, medium and high concentration level, respectively). Precision is expressed as the coefficient of variation (CV), given by CV=mean/SDx100, and was calculated for each concentration of the calibrators. A CV ≤15% was considered satisfactory.

3.6.4.1 Intra-day precision of the method

The intra-day precision of the method was evaluated by independently preparing and injecting five times the same calibrator, on the same day.

3.6.4.2 Intra-day precision of the instrument

The intra-day precision of the instrument was evaluated by injecting five times the same calibrator, on the same day.

3.6.4.3 Inter-day precision of the method

The inter-day precision of the method was evaluated by independently preparing and injecting the same calibrator over five consecutive days.

3.6.4.4. Inter-day precision of the instrument

The inter-day precision of the instrument was evaluated by injecting the same calibrator, at five consecutive days.

3.6.5 Accuracy

The accuracy of the method is the percentage deviation between the calculated value from the calibration curve and the nominal value [Accuracy=(mean calculated concentration/nominal concentration)x100], and was assessed by independently preparing and analysing five replicates of each calibrator, at the same concentration levels used for the evaluation of precision.

3.6.6 Extraction recovery

The extraction recovery was evaluated at the same low, medium and high concentration levels used in determination of precision, by comparing results from addition of 3,4-DMMC to the calibrators before extraction with those from addition of 3,4-DMMC after extraction (before evaporation to dryness). Each concentration was analysed in five replicates. The recovery was calculated by comparing the peak area ratios of analyte to IS for extracted and non-extracted samples [recovery=(mean ratio of analyte *versus* IS

peak areas of extracted/ mean ratio of analyte *versus* IS peak areas of non-extracted)x100]. Values of SD ≤20% and recovery ≥75% were considered satisfactory.

The IS recovery was also evaluated by comparing IS addition values before and after extraction. Five non-extracted replicates were analysed. This recovery was calculated by the ratio of the extracted and non-extracted IS area [recovery=(mean area IS extracted/mean area IS non-extracted)x100]. Values of SD ≤20% and recovery ≥75% were considered satisfactory.

3.6.7 Stability of the analytes in plasma

The temperature and time stability of 3,4-DMMC and IS were evaluated at the low, medium and high concentration levels previously used for determination of precision, accuracy, and extraction recovery. One replicate of each calibrator was extracted, derivatized and injected, immediately after preparation. The remaining replicates were stored at RT (approximately 15 ºC) or refrigerated (4 ºC) or frozen (-20 ºC) for 1, 2, 3 or 4 weeks. At the end of the storage period, the replicates were extracted and injected according to the previously described protocol. The results obtained were compared with the results from the samples analysed on the day of preparation.

3.7 Biodistribution of 3,4-DMMC in Wistar rats

Organs, plasma and urine samples were prepared and analysed as previously described. The ratio of the peak area of the analyte to the peak area of the IS was calculated and the concentration of 3,4-DMMC in the samples interpolated from the equation of the calibration curve (y = mx + b) of the respective biological matrix.

The presence of 3,4-DMMC metabolites was hypothesized and the ratio metabolite to parent drug was calculated as [metabolite peak area (selected ion for the metabolite) / IS peak area (ion m/z 121)] / [parent drug peak area (ion m/z 133 or 105) / IS peak area (ion m/z 121)].

3.8 Derivatization with N-TFA-O-TMS

In urine samples from two control animals and from two animals administered with 20 mg/kg 3,4-DMMC, an additional N-TFA-O-TMS derivatization was performed with the purpose of tentatively identifying the structure of the proposed metabolites depicted in the analysis of the full scan chromatograms obtained with the N,O-TFA derivatization. Samples were extracted according to the protocol previously described, and to the dried residue, 50 µL of 200 ppm methyl orange solution (in acetonitrile : trifluoroacetic acid, 60:40) were added. Then, MSTFA was added until a yellow colour appeared (≈100 µL), and the sample was incubated for 5 min at 80 ºC. After cooling to RT, 10 µL of MBTFA were added and re-incubated at 80 ºC for 10 min. If in any of the previous steps the colour of the mixture turned to red, sufficient drops of MSTFA were added to obtain again a yellow colour. After cooling to RT, the sample was injected for GC-MS analysis. The summary of this derivatization procedure is described in Figure 6.

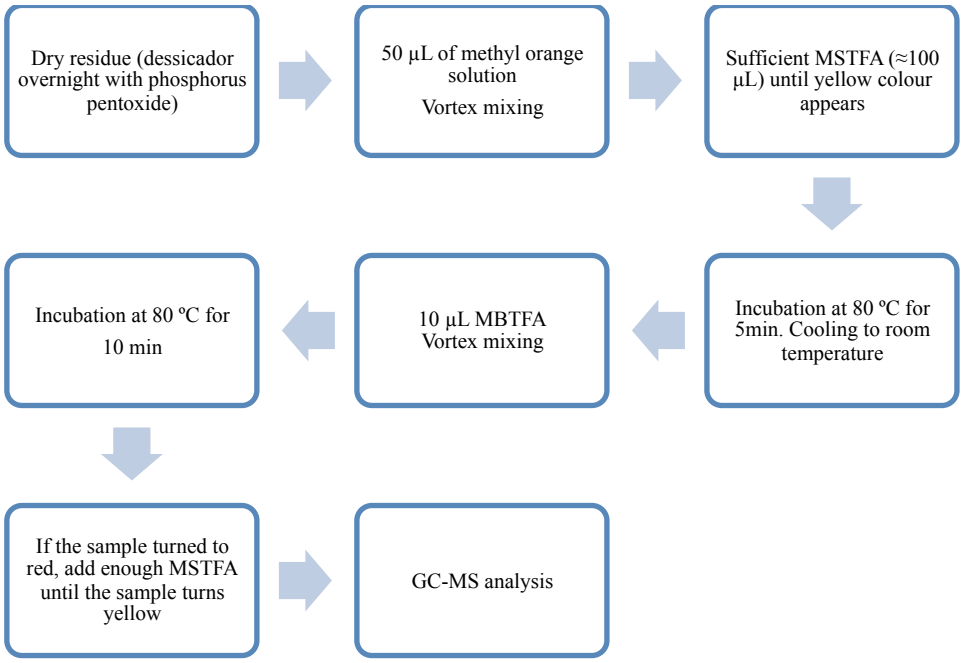

Figure 6: Summary of the procedure for the N-TFA-O-TMS derivatization.

3.9 Glucuronide and sulphate conjugates of 3,4-DMMC and metabolites in urine

Hydrolysis of glucuronide and sulphate conjugates of 3,4-DMMC and/or its metabolites was performed in urine samples by transferring 1 mL aliquots to a 5 mL glass tube with 1 mL of 0.2 M sodium acetate buffer (pH 5.2) and 50 μL of type 2 β-glucuronidase from *H. pomatia*. After incubation at 37 ^{o}C for 24h, the samples were extracted, derivatized and analysed as previously described. The conjugation extent is given by the ratio of the analyte without hydrolysis to the analyte following hydrolysis, i.e. conjugation=[(peak area analyte without hydrolysis/peak area IS)/(peak area analyte with hydrolysis/peak area IS)x100]).

3.10 Statistical analysis

All data were analysed using Microsoft Excel 2010. Data normality was assessed using the Kolmogorov-Smirnov test. Statistical comparisons between groups were performed by the Student's unpaired t-test and differences considered significant at $p<0.05$. All statistical calculations were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

Chapter IV- Results

4.1 Preparation of calibrators and GC-MS analysis

The sample preparation and GC-MS conditions for the determination of 3,4-DMMC resulted in well resolved peaks, eluting in less than 10 min. The acquisition of all mass spectra of the complete chromatogram (full scan mode) guaranteed the identification of all peaks in each chromatogram. As shown in Figure 7, the retention times for the analyte and IS were about 8.25 and 9.4 min, respectively. The total analysis time was approximately 25 min.

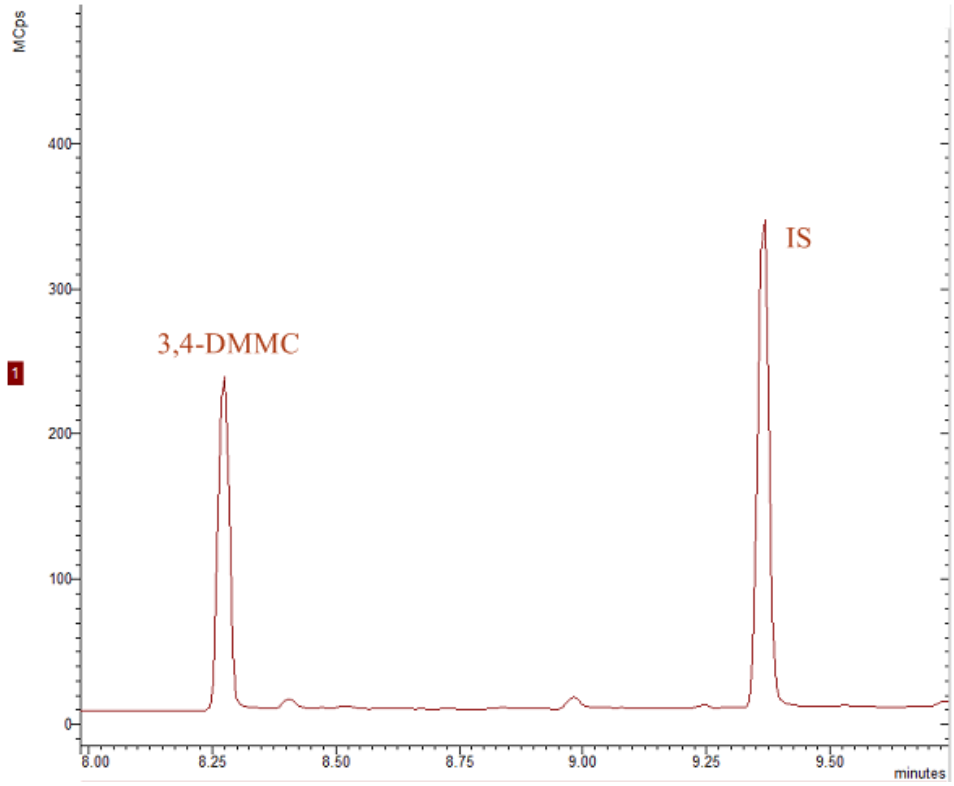

Figure 7: Representative full scan chromatogram of a urine calibrator spiked with 5 µg/mL 3,4-dimethylmethcathinone (3,4-DMMC) and 6.25 µg/mL internal standard (IS; methylone).

The selected ions for quantification were m/z 121 for IS, and m/z 105 for the liver, kidneys, and urine or m/z 133 for all other matrices, for 3,4-DMMC (Figures 8 and 9). The IS ion m/z 149, in spite of being the most abundant, was not used in the current analysis as peak interferences were observed at 9.4 min in all the matrices analysed. In the liver, kidneys, and urine, ion m/z 133 also presented an interference at the 3,4-DMMC retention time.

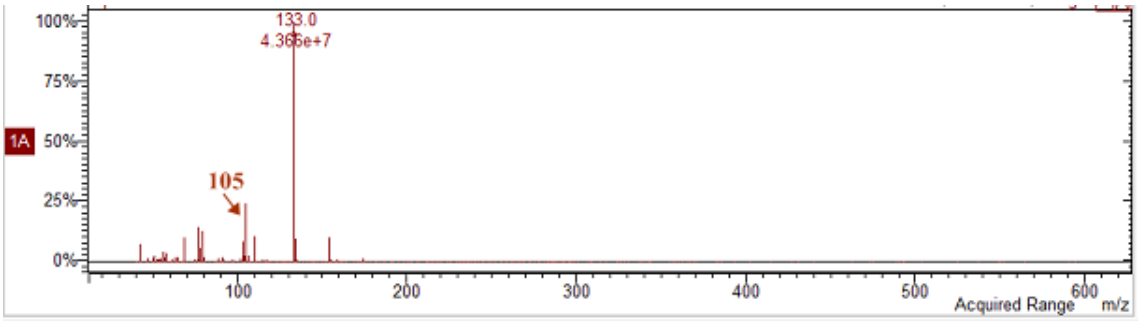

Figure 8: Mass spectrum of 3,4-dimethylmethcathinone (3,4-DMMC) after derivatization with trifluoroacetic anhydride. The selected ions for quantification were m/z 105 for the liver, kidneys, and urine or m/z 133 for plasma, brain, heart, lungs, spleen, gut, muscle, and adipose tissue.

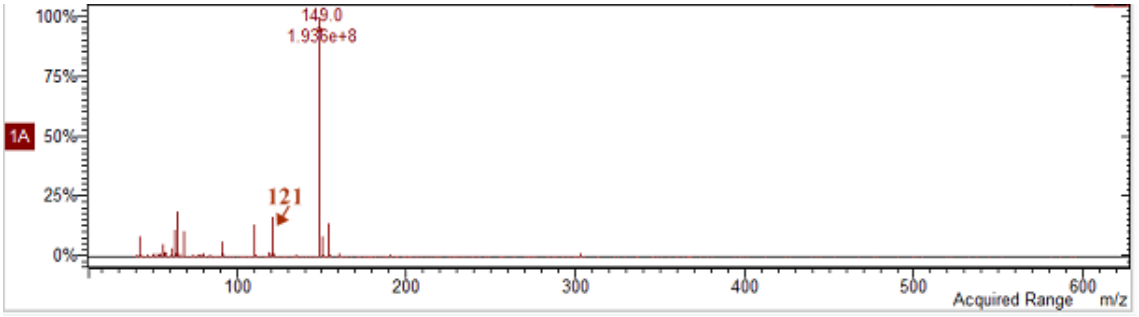

Figure 9: Mass spectrum of internal standard (IS; methylone) after derivatization with trifluoroacetic anhydride. The selected ion for quantification was m/z 121 due to the matrix interferences found for the most abundant ion m/z 149.

4.2 Validation of the GC-MS method for quantification of 3,4-DMMC in plasma

4.2.1 Specificity/selectivity

Twenty blank plasma extracts were analysed to evaluate chromatographic interferences. No interference peaks were detected in the retention times of IS or 3,4-DMMC for the ions selected for quantification (Figure 10A and B, respectively).

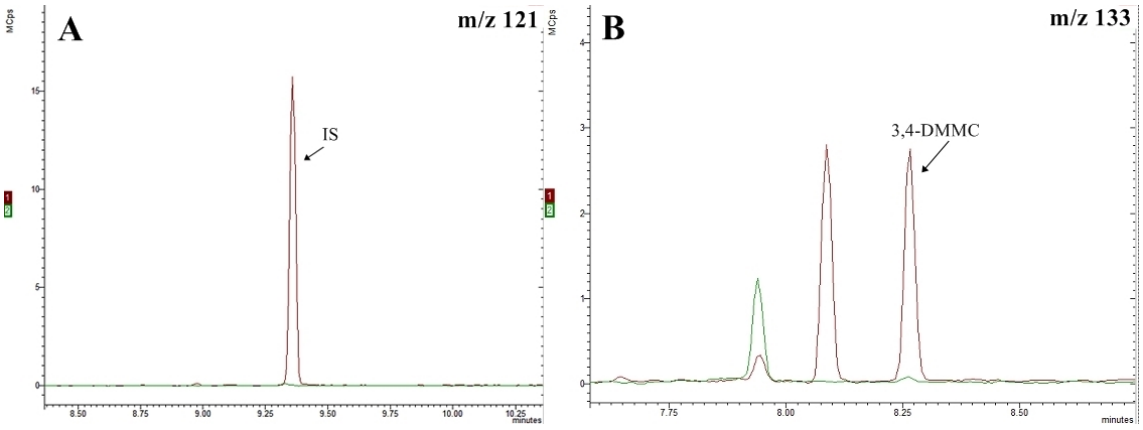

Figure 10: Representative Selective Ion Monitoring (SIM) chromatogram of blank plasma (in green) showing no interference peaks at the retention times of internal standard (IS, methylone) for m/z 121 **(A)** or 3,4-dimethylmethcathinone (3,4-DMMC) for m/z 133 **(B)** after derivatization with trifluoroacetic anhydride. The red line represents the chromatogram of a plasma sample obtained 1h after the i.p. administration of 40 mg/Kg to Wistar rats 3,4-DMMC, and fortified with 6.25 µg/mL IS.

4.2.2 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

The linear regression equation and the squared correlation coefficient (R^2) were calculated for five independent calibration curves, prepared in five consecutive days, within the concentration range of 0.078–2.5 µg/mL 3,4-DMMC. A blank (0 µg/mL) was always included. The R^2 were always higher than 0.99, indicating a linear relationship between the analyte/IS peak areas and the analyte concentration.

Following the SWGTOX guidelines (SWGTOX, 2013), the calculated LOD and LOQ values were 4 ng/mL and 13.5 ng/mL, respectively. These parameters were calculated using the mean slope of the independent curves of the five consecutive days. The linearity, LOD and LOQ data are summarized in the Table 6.

Table 6: Summary of 3,4-dimethylmethcathinone (3,4-DMMC) plasma calibration curves data (n=5), LOD (n=20) and LOQ (n=20).

	Equation* (µg/mL)	**R^2**	**LOD** (µg/mL)	**LOQ** (µg/mL)
Day 1	y=0.5893x-0.0016	0.9994	-	-
Day 2	y=0.5666x-0.0255	0.9951		
Day 3	y=0.5607x-0.037	0.9940		
Day 4	y=0.525x-0.0329	0.9923		
Day 5	y=0.5097x-0.0262	0.9924		
Mean curve	y=0.5074x-0.0201 **95% Confidence Intervals:** Slope: 0.4611 to 0.5536 Y-intercept: -0.07052 to 0.0304 X-intercept: -0.06375 to 0.1317	0.9938	0.0040	0.0135

*y=mx+b

4.2.3 Precision, accuracy and recovery

Precision, accuracy and recovery of analyte data are compiled in Table 7. Intra-day and inter-day precision of the method and of the instrument showed CV always below 15%, which means that the method is precise. Also, the results for accuracy were always between the acceptable range for this parameter (100±20%), and the values of CV ≤10% support the accuracy of the method. In what concerns the recovery of the analyte, the overall extraction efficiencies were always higher than 75%, with a CV lower than 15%, showing that there are no significant losses of analyte during the extraction process, at the three levels of concentration tested. The IS recovery was 93%, with a CV of 19%.

Table 7: Inter-day and intra-day precision, accuracy, and recovery data in plasma (n=5).

Concentration (µg/mL)	**Accuracy %** (CV %)	**Precision (CV %)**				**Recovery %** (CV %)
		Inter-day		**Intra-day**		
		Instrument	**Method**	**Instrument**	**Method**	
0.078	108 (10)	13	14	4	11	94 (14)
0.625	84 (7)	14	9	1	15	78 (8)
2.5	104 (9)	3	6	1	5	83 (7)

4.2.4 Stability

At the lowest concentration (0.078 µg/mL), the analyte does not show stability in plasma since after one week losses of 29, 39 and 58% for storage at -20 ºC, 4 ºC and RT, respectively, were noted. Over time, at this concentration, the analyte always presents losses of over 40%, contrary to the intermediate and higher concentrations, at which even after four weeks it presents losses of less than 20% at 4 ºC and at -20 ºC. In view of these results, it is recommended that the analysis of the samples are conducted in a relatively short period. When this is not possible, the sample storage should be done at -20 ºC. The results of the stability study are summarized in Table 8.

Table 8: Stability of 3,4-dimethylmethcathinone (3,4-DMMC) in plasma over time, when stored at room temperature (RT) or refrigerated (4 ºC) or frozen (-20 ºC).

	Concentration (µg/mL)	**% Drug loss**		
		RT	**4 ºC**	**-20 ºC**
Week 1	**0.078**	58	39	29
	0.625	10	6	1
	2.5	11	9	1
Week 2	**0.078**	n.d.	48	43
	0.625	n.d.	6	0
	2.5	n.d.	8	7
Week 3	**0.078**	n.d.	46	44
	0.625	n.d.	16	4
	2.5	n.d.	9	1
Week 4	**0.078**	n.d.	59	51
	0.625	n.d.	17	7
	2.5	n.d.	13	8

n.d. not determined.

4.3 Specificity/selectivity, linearity and recovery of the GC-MS method for the quantification of 3,4-DMMC in other biological matrices

In the remaining biological matrices (liver, kidneys, heart, brain, spleen, lungs, intestine, muscle, adipose tissue and urine) only specificity/selectivity, linearity, and recovery were evaluated (Table 9).

Table 9: Linearity and recovery of the GC-MS method for the quantification of 3,4-dimethylmethcathinone (3,4-DMMC) in several biological matrices (n=3).

Biological matrix	Equation*	R^2*	Concentration (µg/mL)	Recovery % [CV %]
Liver	y=0.113x-0.0083	0.999	0.3125 1.25 5	98 [3] 87 [9] 94 [9]
Kidneys	y=0.0944x-0.0157	0.9941	0.3125 1.25 5	94 [9] 80 [1] 95 [6]
Brain	y=0.3626x-0.0062	0.9987	0.3125 1.25 5	89 [3] 86 [12] 93 [3]
Heart	y=0.3915x-0.0097	0.9986	0.156 0.625 2.5	98 [0] 88 [5] 95 [3]
Urine	y=0.1206x-0.0017	0.9996	0.3125 5 10	87 [13] 89 [12] 82 [11]
Spleen	y=0.574x-0.0907	0.9926	0.3125 1.25 5	96 [4] 87 [11] 88 [3]
Muscle	y=0.4453x-0.0243	0.9992	0.078 1.25 5	72 [10] 97 [3] 94 [13]
Gut	y=0.5652x-0.1022	0.9931	0.3125 1.25 5	97 [7] 83 [3] 98 [2]
Lungs	y=0.4258x-0.047	0.9974	0.3125 1.25 5	89 [6] 91 [12] 92 [5]
Adipose tissue	y=0.2551x+0.0048	0.9999	0.078 0.3125 5	80 [15] 82 [11] 75 [3]

*y=mx+b (mean equation obtained from three independent calibration curves and the corresponding mean R^2 values)

Blank extracts (n=3) were analysed to evaluate the chromatographic interferences (Figures 11 and 12). As previously mentioned, in liver, kidneys and urine chromatograms, for the m/z 133 ion, which characterizes the analyte, there was a slight interference at the 3,4-DMMC retention time. This did not occur for the other characteristic m/z 105 ion, so the latter was selected for the analysis in these matrices. In the remaining biological matrices, the m/z 133 ion was used, as no interference peaks were detected in the retention time of the analyte. For the IS, the m/z 121 ion was selected for all biological matrices.

The mean linear regression equation and the squared correlation coefficient (R^2) were calculated using three independent calibration curves plotted with three concentrations; R^2 were always higher than 0.99, indicating a linear relationship between analyte/IS peak areas and analyte concentration. For all the analysed concentration levels, the overall extraction efficiencies were higher than 72%, with a CV lower than 15%, which shows that there are no significant losses of analyte during the extraction process.

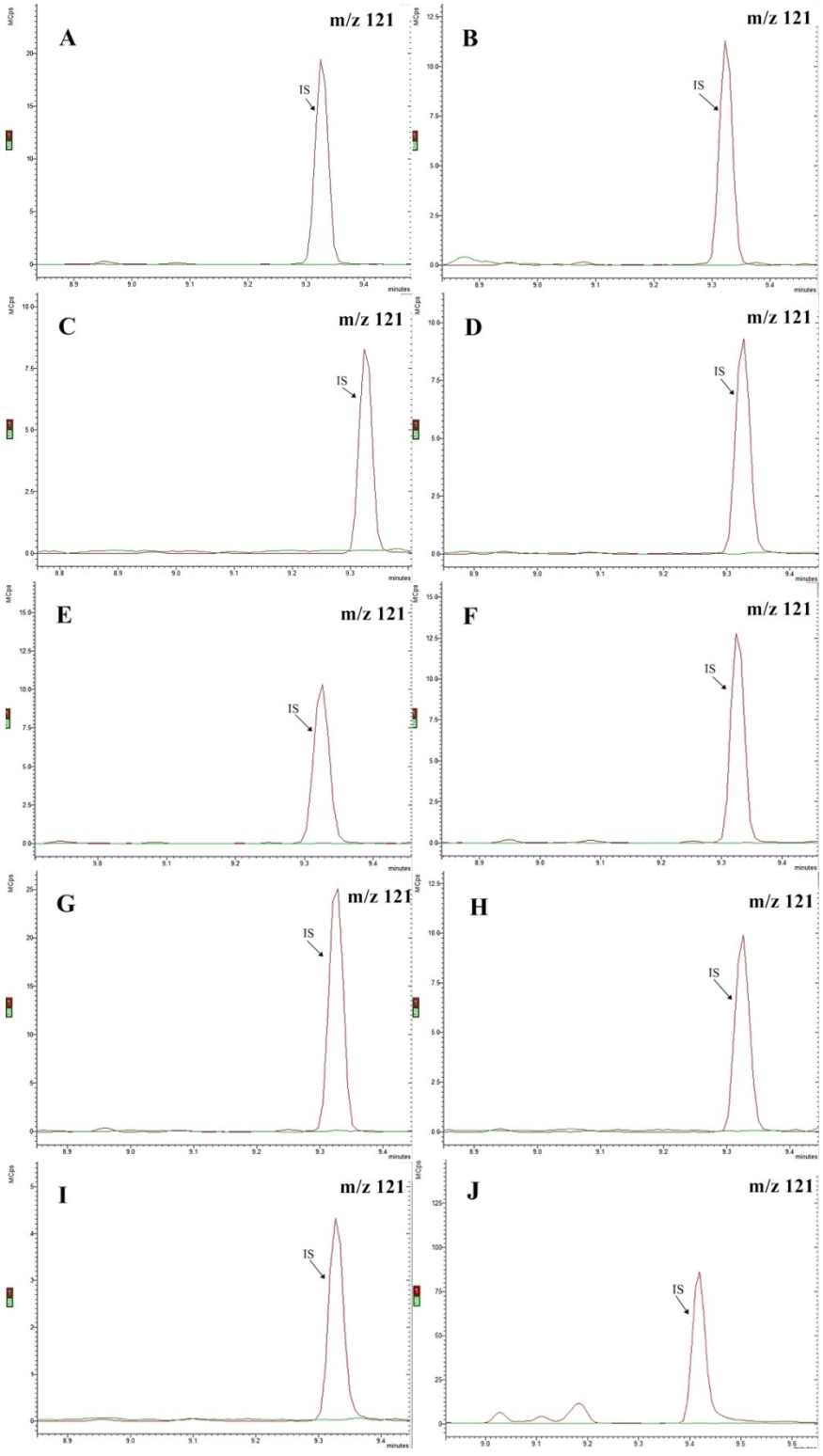

Figure 11: Representative Selective Ion Monitoring (SIM) chromatograms of blank (in green) liver **(A)**, kidneys **(B)**, heart **(C)**, brain **(D)**, lungs **(E)**, spleen **(F)**, gut **(G)**, muscle **(H)**, adipose tissue **(I)** and urine **(J)** showing no interference peaks at the retention times of the internal standard (IS, methylone) for the selected characteristic ion m/z 121 after derivatization with trifluoroacetic anhydride. The red line represents the chromatogram of the corresponding matrix sample obtained 1h (liver, kidneys, heart, brain, lungs, spleen, gut, muscle, and adipose tissue) or 24h (urine) after the i.p. administration of 20 mg/Kg 3,4-DMMC to Wistar rats, and fortified with 6.25 µg/mL IS.

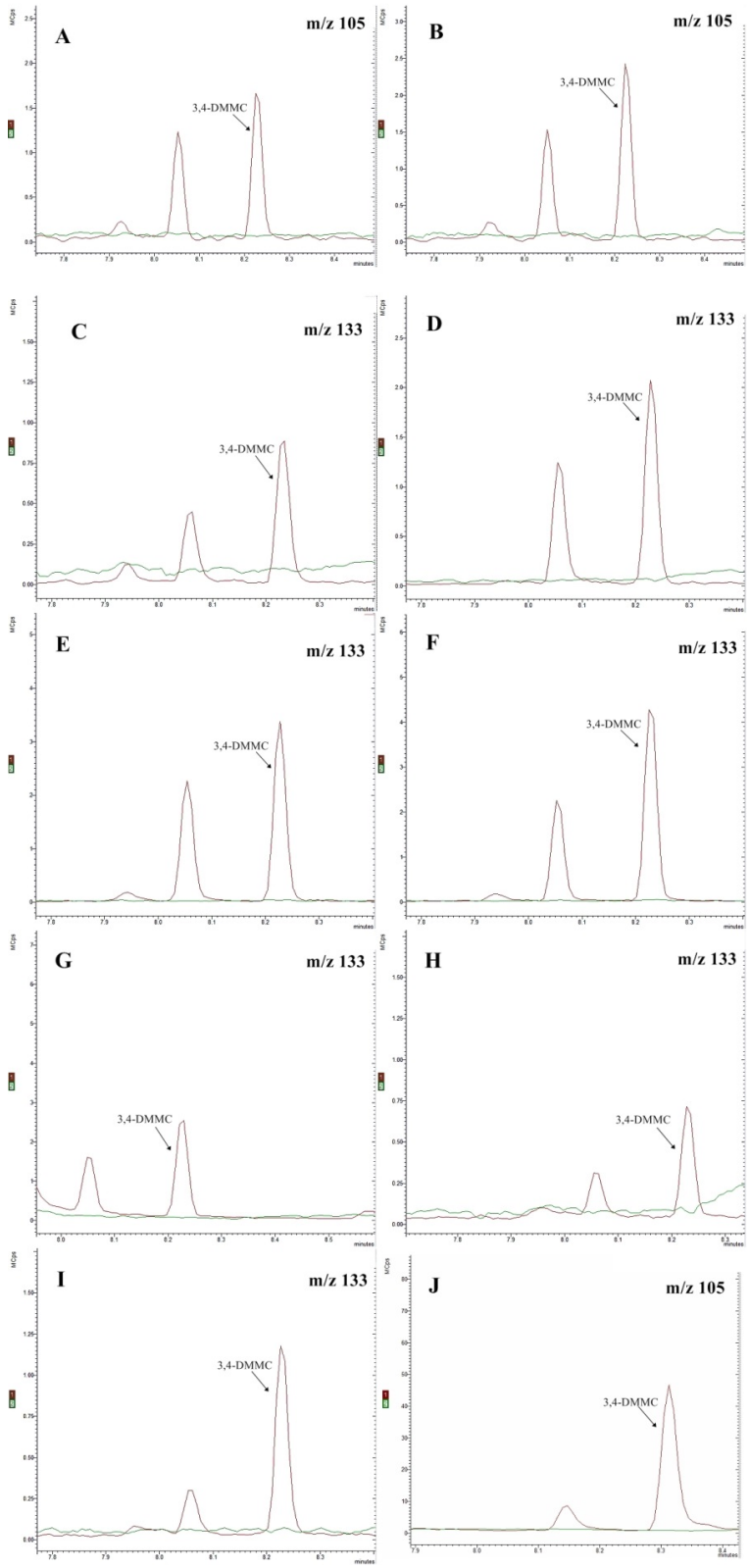

Figure 12: Representative Selective Ion Monitoring (SIM) chromatograms of blank (in green) liver **(A)**, kidneys **(B)**, heart **(C)**, brain **(D)**, lungs **(E)**, spleen **(F)**, gut **(G)**, muscle **(H)**, adipose tissue **(I)** and urine **(J)** showing no interference peaks at the retention times of 3,4-dimethylmethcathinone (3,4-DMMC) for the selected characteristic ions m/z 105 for the liver, kidneys, and urine or m/z 133 for heart, brain, lungs, spleen, gut, muscle, and adipose tissue after derivatization with trifluoroacetic anhydride. The red line represents the chromatogram of the corresponding matrix sample obtained 1h (liver, kidneys, heart, brain, lungs, spleen, gut, muscle, and adipose tissue) or 24h (urine) after the i.p. administration of 20 mg/Kg 3,4-DMMC to Wistar rats, and fortified with 6.25µg/mL IS.

4.4 Biodistribution of 3,4-DMMC in Wistar rats

3,4-Dimethylmethcathinone rapidly and extensively distributed to the rat body tissues. After 1h of drug administration, 3,4-DMMC was found in all collected organs (Figures 13 and 14).

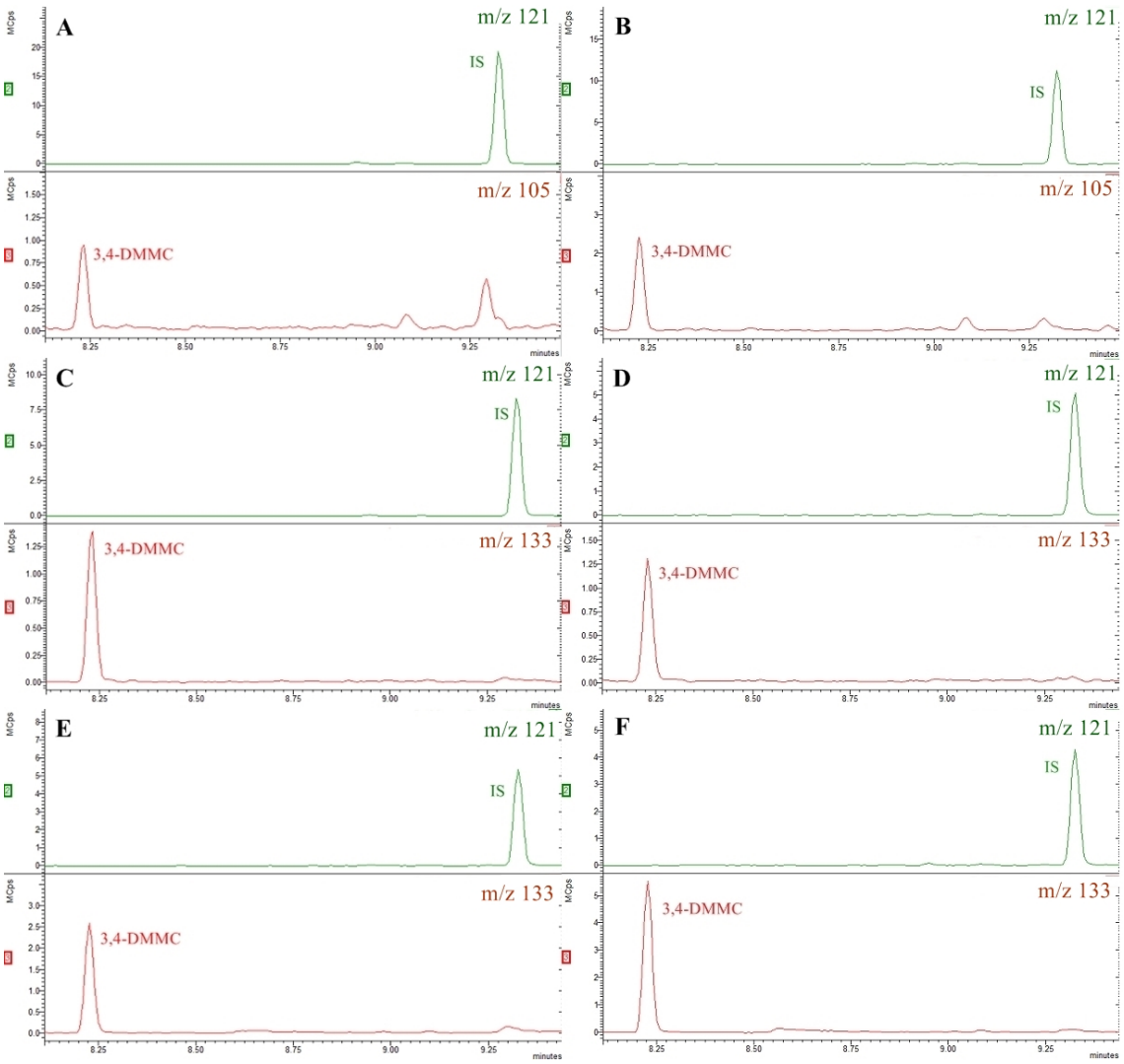

Figure 13: Representative Selective Ion Monitoring (SIM) chromatograms of the liver **(A)**, kidneys **(B)**, heart **(C)**, brain **(D)**, lungs **(E)** and spleen **(F)** at the retention times of 3,4-dimethylmethcathinone (3,4-DMMC) and internal standard (IS; methylone), for the selected IS characteristic ion m/z 121 (in green) and 3,4-DMMC characteristic ion m/z 105 for the liver and kidneys or m/z 133 for heart, brain, lungs, and spleen (in red). Matrices were obtained 1h after the i.p. administration of 20 mg/Kg 3,4-DMMC to Wistar rats, and fortified with 6.25µg/mL IS after derivatization with trifluoroacetic anhydride.

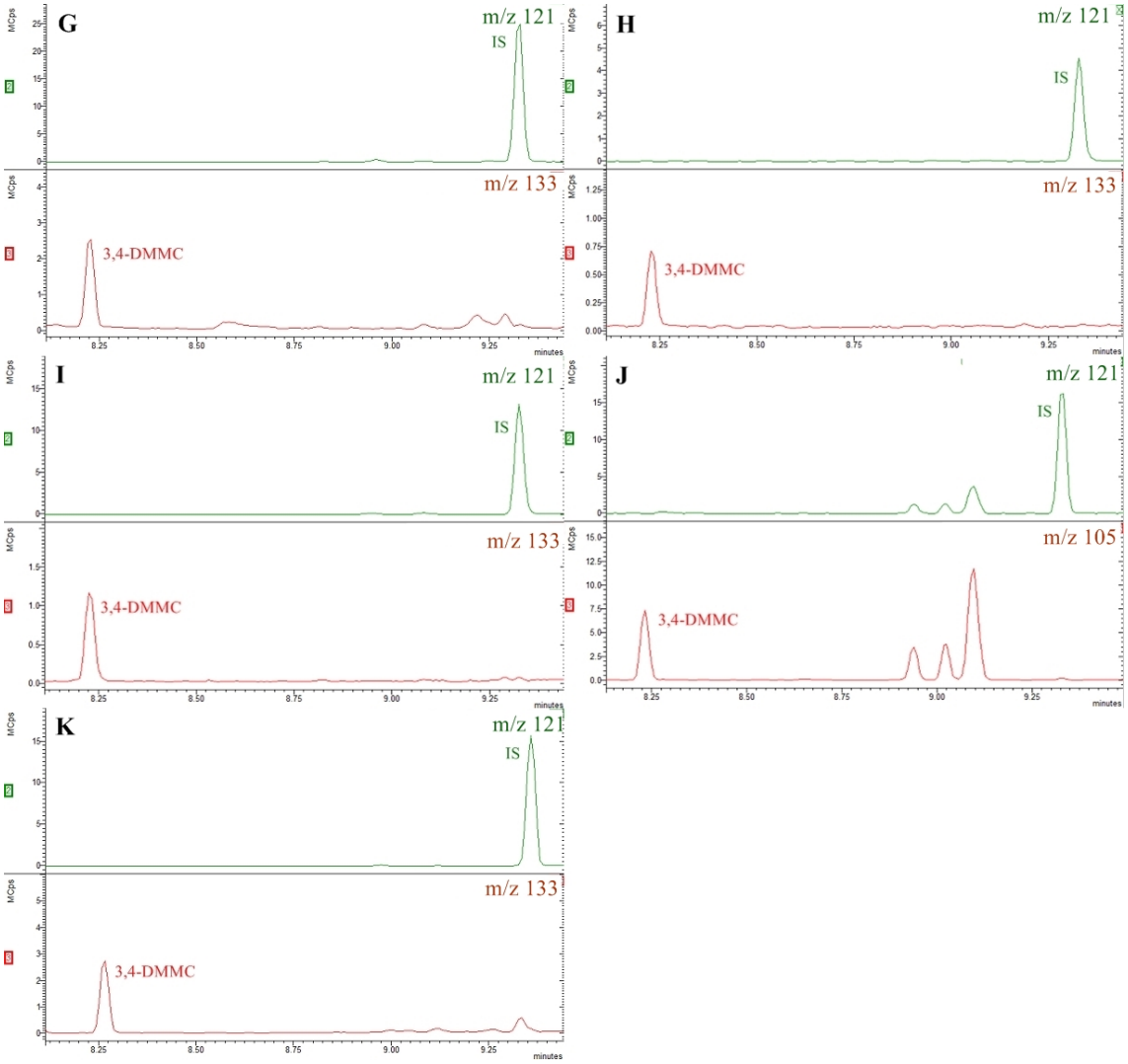

Figure 14: Representative Selective Ion Monitoring (SIM) chromatograms of the gut **(G)**, muscle **(H)**, adipose tissue **(I)**, urine **(J)** and plasma **(K)** at the retention times of 3,4-dimethylmethcathinone (3,4-DMMC) and internal standard (IS; methylone), for the selected IS characteristic ion m/z 121 (in green) and 3,4-DMMC characteristic ion m/z 105 for urine or m/z 133 for gut, muscle, adipose tissue, and plasma (in red). Matrices were obtained 1h (gut, muscle, adipose tissue, and plasma) or 24h (urine) after the i.p. administration of 20 mg/Kg 3,4-DMMC to Wistar rats, and fortified with 6.25µg/mL IS after derivatization with trifluoroacetic anhydride.

As observed in Figure 15, tissue concentrations of the drug nearly doubled from 20 mg/Kg to 40 mg/Kg rat dosing, in lungs (p<0.05), kidneys (p<0.0001) and brain (p<0.05). For the adipose tissue, spleen, muscle, plasma, liver, gut and heart, the tissue concentrations were 4.2, 4.1 (p<0.05), 3 (p<0.01), 2.9 (p<0.01), 2.7 (p<0.01), 2.5 (p<0.05), and 1.3 times higher, respectively, after administration of the highest dose. Spleen, lungs and kidneys were the organs in which the highest drug concentrations were achieved, with up to 2.67 µg/g organ (spleen) after a dose of 40 mg/Kg rat body

weight, and up to 675 ng/g organ (kidneys) after a dose of 20 mg/Kg rat body weight. In contrast, plasma presented the lowest drug amounts after 1h-administrations (80 ng/g for 20 mg/Kg; 234 ng/g for 40 mg/Kg). Elimination of 3,4-DMMC from tissues also seems to occur quickly, since after 24h the drug was only detected in urine (1.11 µg /mL urine) after a dose of 20 mg/Kg.

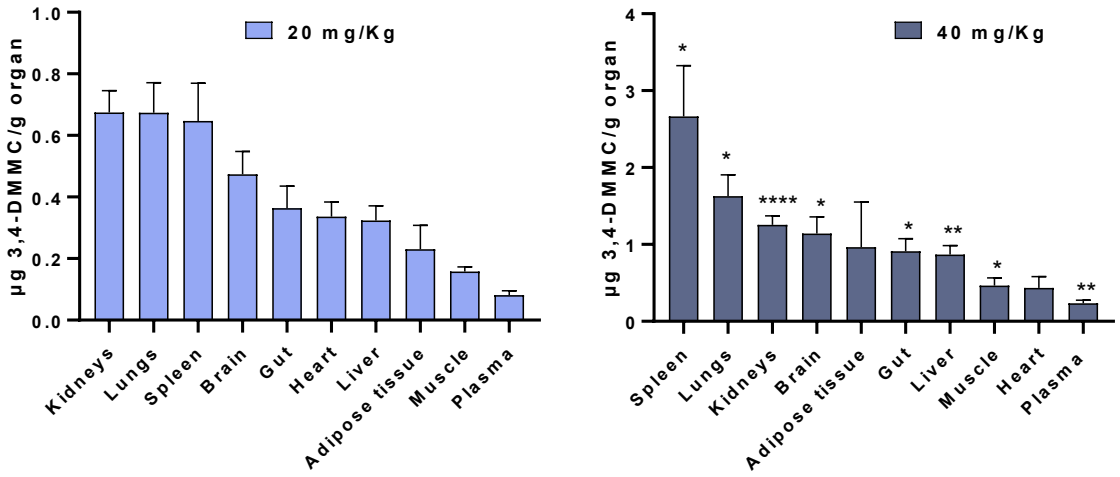

Figure 15: Quantification of 3,4-dimethylmethcathinone (3,4-DMMC) in body tissues and plasma after 1h of drug administration to Wistar rats. Animals were dosed with 20 mg/Kg or 40 mg/Kg 3,4-DMMC i.p.. The plasma density was considered 1.04 g/cm^3. *p<0.05; **p<0.01; ****p<0.0001, vs. 20 mg/Kg 3,4-DMMC.

4.5 Metabolic profile of 3,4-DMMC in Wistar rats

The analysis of the chromatograms of the tissue samples obtained 1h after the administration of 3,4-DMMC (Figure 16) revealed the presence of a peak with a retention time of approximately 8.1 min (eluting before 3,4-DMMC) that did not appear in the chromatograms obtained from the tissue samples of control animals.

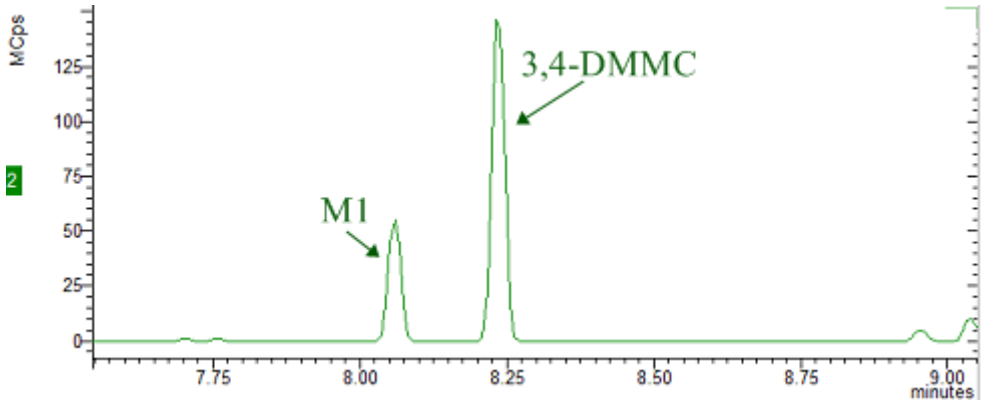

Figure 16: Representative Selective Ion Monitoring (SIM) m/z 133 chromatogram of the heart, 1h after the i.p. administration of 20 mg/Kg 3,4-dimethylmethcathinone (3,4-DMMC) to Wistar rats, revealing a potential metabolite (M1), after derivatization with trifluoroacetic anhydride.

The corresponding mass spectrum presented identical ions to those characteristic of 3,4-DMMC, i.e. m/z 133, m/z 105 and m/z 140, after derivatization with TFAA (Figure 17). Therefore, it was hypothesized the presence of a 3,4-DMMC metabolite (M1).

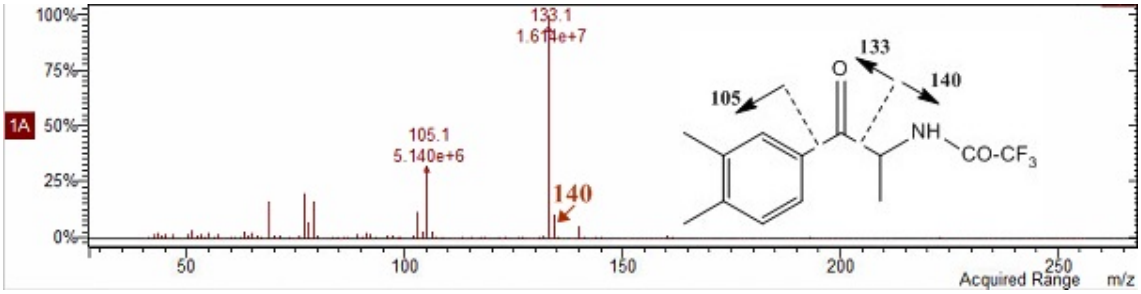

Figure 17: Mass spectrum of Metabolite 1 (M1) and proposed chemical structure after derivatization with trifluoroacetic anhydride.

After 1 h of 3,4-DMMC administration, the liver was the organ presenting the highest ratio of M1 (ion m/z 133) to parent drug, followed by plasma and heart for the dose of 20 mg/Kg, and brain and kidneys for the dose of 40 mg/Kg (Figure 18). In liver and plasma, the ratio of M1 to parent drug was similar for both doses administered, i.e. 0.56 and 0.42 respectively. This was not the case of the other biological matrices, for which the ratio of M1 to parent drug was higher at 40 mg/Kg, doubling in the case of kidneys (p<0.05), lungs (p<0.0001), spleen (p<0.001) and gut (p<0.01), while in the case of brain and muscle was 3.5 (p<0.05) and 3.6 (p<0.0001) times higher, respectively. The

heart (p<0.01) was the exception, with higher M1 to 3,4-DMMC ratio observed at 20 mg/Kg (0.35 *versus* 0.15 at 40 mg/Kg). For the adipose tissue, M1 was not detected at 20 mg/Kg, and when the dose was doubled, the ratio M1 to parent drug was low (0.04).

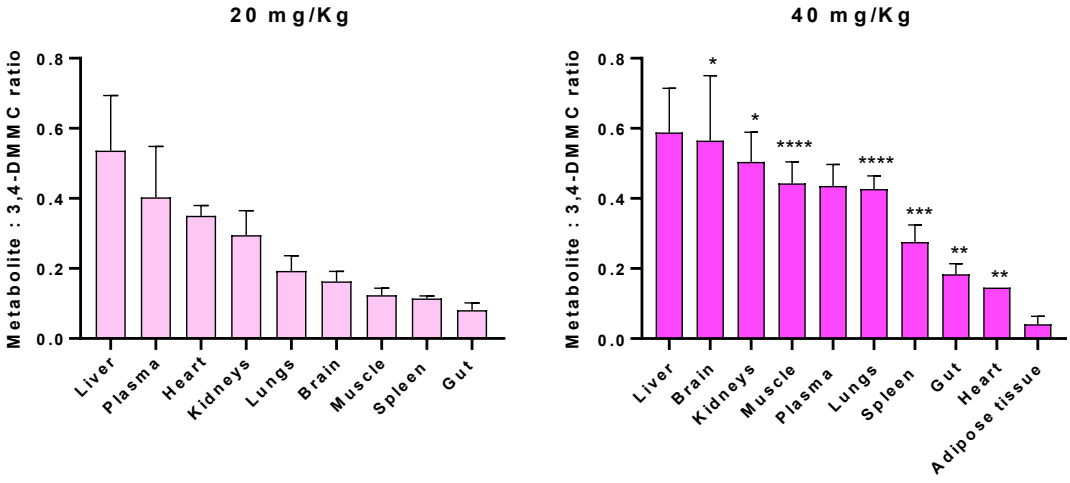

Figure 18: Ratio M1 to 3,4-dimethylmethcathinone (3,4-DMMC) in body tissues and plasma after 1h of drug administration to Wistar rats. Animals were dosed with 20 mg/Kg or 40 mg/Kg 3,4-DMMC i.p.. The M1 to 3,4-DMMC ratio was calculated as follows: [(peak area of M1 ion m/z 133 : peak area of IS ion m/z 121) / (peak area of 3,4-DMMC ion m/z 105 or m/z 133: peak area of IS ion m/z 121)]. Peak area of 3,4-DMMC ion m/z 105 was used for liver and kidneys and ion m/z 133 for plasma, heart, lungs, brain, muscle, spleen, gut, and adipose tissue. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, vs. 20 mg/Kg 3,4-DMMC.

After 24h of 3,4-DMMC administration, the ratio M1 to parent drug in urine was 0.51. At this time point, no M1 was detected in the organs analysed.

Full scan analysis of the chromatograms of the samples obtained 1h after drug administration also revealed the presence of a second peak, in this case higher than that for 3,4-DMMC, non-existent in the controls, with a retention time of approximately 9.1 min (Figure 19), whose characteristic ion was m/z 154 after derivatization with TFAA (Figure 20). Therefore, the presence of another metabolite of 3,4-DMMC (M2) was considered.

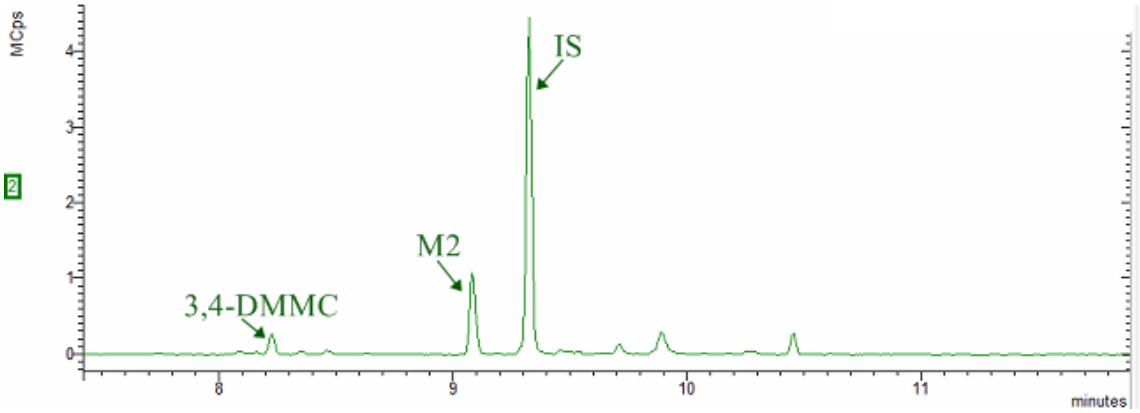

Figure 19: Representative Selective Ion Monitoring (SIM) m/z 154 chromatogram of the lungs, 1h after the i.p. administration of 20 mg/Kg 3,4-dimethylmethcathinone (3,4-DMMC) to Wistar rats, revealing a potential metabolite (M2), after derivatization with trifluoroacetic anhydride. **IS:** Internal standard (methylone).

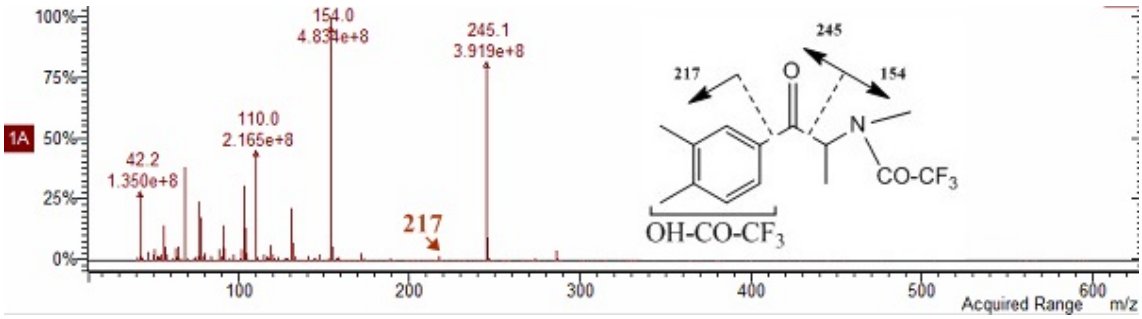

Figure 20: Mass spectrum of Metabolite 2 (M2) and proposed chemical structure after derivatization with trifluoroacetic anhydride.

For both doses (20 and 40 mg/Kg), after 1h of 3,4-DMMC administration, the liver was the organ presenting the highest ratio of M2 (ion m/z 154) to parent drug, followed by kidneys, plasma and spleen (Figure 21). In plasma, spleen, gut, muscle ($p<0.01$) and heart ($p<0.05$), the ratio M2 to parent drug doubled from the dose 20 mg/Kg to 40 mg/Kg. In the brain, lungs, liver and kidneys the ratio M2 to parent drug was 5.5 ($p<0.05$), 5.5 ($p<0.001$), 4.4 and 2.6 times higher, respectively, for the higher dose. As with M1, M2 was not detected in the adipose tissue at 20 mg/Kg, and at 40 mg/Kg the ratio M2 to parent drug was very low (0.04).

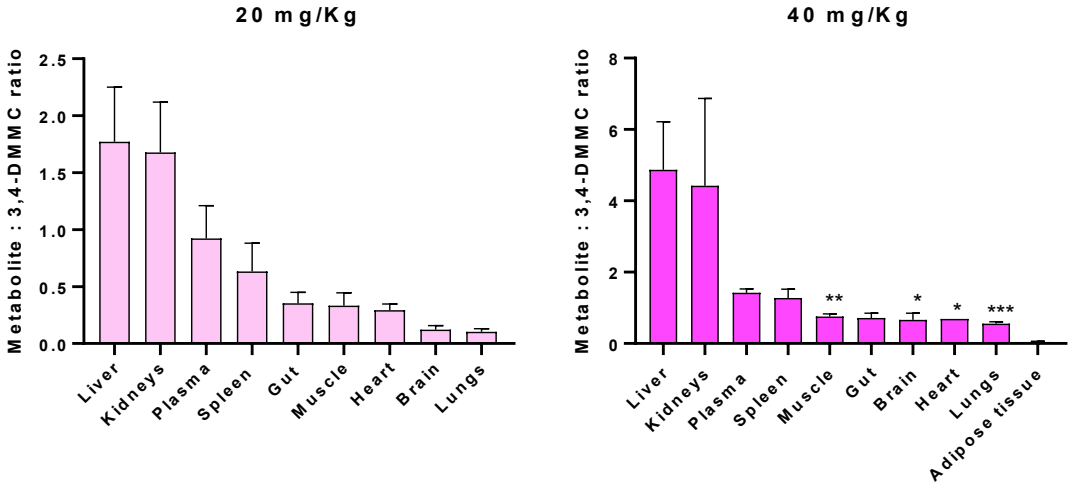

Figure 21: Ratio M2 to 3,4-dimethylmethcathinone (3,4-DMMC) in body tissues and plasma after 1h of drug administration to Wistar rats. Animals were dosed with 20 mg/Kg or 40 mg/Kg 3,4-DMMC i.p.. The M2 to 3,4-DMMC ratio was calculated as follows: [(peak area of M2 ion m/z 154 : peak area of IS ion m/z 121) / (peak area of 3,4-DMMC ion m/z 105 or m/z 133: peak area of IS ion m/z 121)]. Peak area of 3,4-DMMC ion m/z 105 was used for liver and kidneys, and ion m/z 133 for plasma, heart, lungs, brain, muscle, spleen, gut, and adipose tissue. **p*<0.05; ***p*<0.01; ****p*<0.001, vs. 20 mg/Kg 3,4-DMMC.

After 24h of 3,4-DMMC administration, the ratio M2 to parent drug in urine was 40.32. For the remaining biological matrices, M2 was not detected.

At this time-point, full-scan analysis of urine samples also revealed the existence of three additional peaks eluting after 3,4-DMMC, which did not exist in the urine of the control animals (Figure 22).

So, it was hypothesized the presence of three other metabolites, presenting retention times of approximately 8.94 (M3), 9.03 (M4) and 9.50 min (M5), with characteristic ions at m/z 245 and m/z 140 for M3 (Figure 23) and M4 (Figure 24), and m/z 154 for M5 (Figure 25), after derivatization with TFAA. The selected ions for monitoring and retention time of all metabolites after derivatization with TFAA are summarized in Table 5.

The ratio metabolite to parent drug for metabolites M3 (ion m/z 245), M4 (ion m/z 245) and M5 (ion m/z 154) were 4.70, 4.33 and 1.8, respectively.

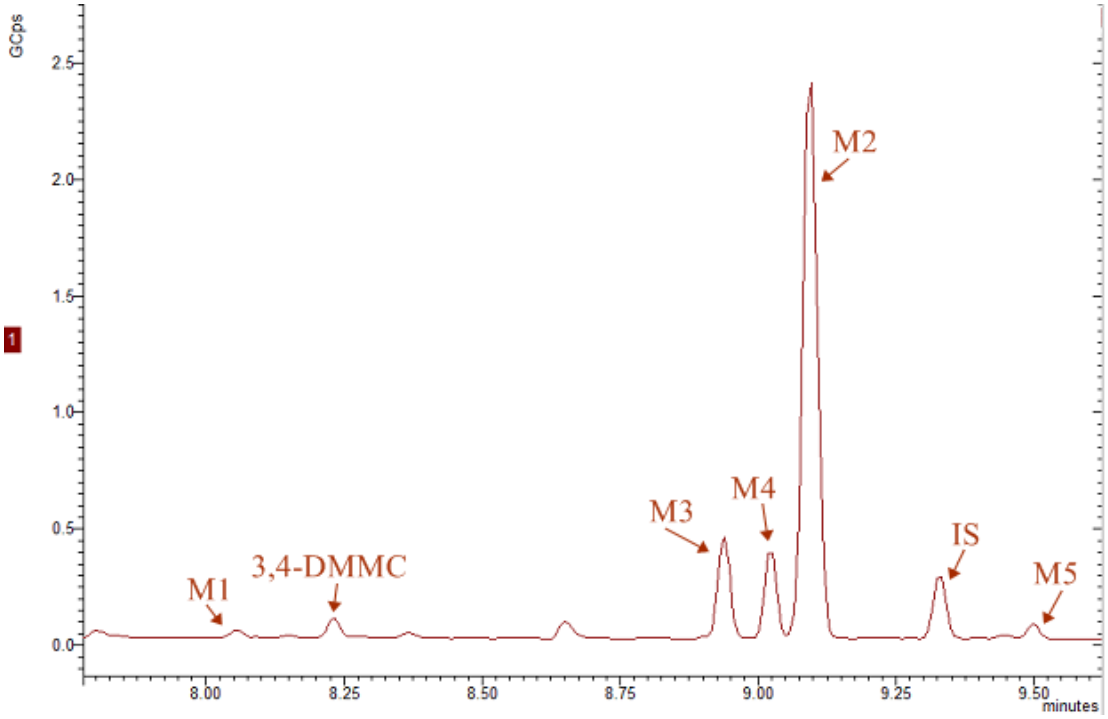

Figure 22: Representative full scan chromatogram of urine samples obtained 24h after the i.p. administration of 20 mg/Kg 3,4-dimethylmethcathinone (3,4-DMMC) to Wistar rats, revealing five potential metabolites (M1, M2, M3, M4 and M5), after derivatization with trifluoroacetic anhydride. **IS:** Internal standard (methylone).

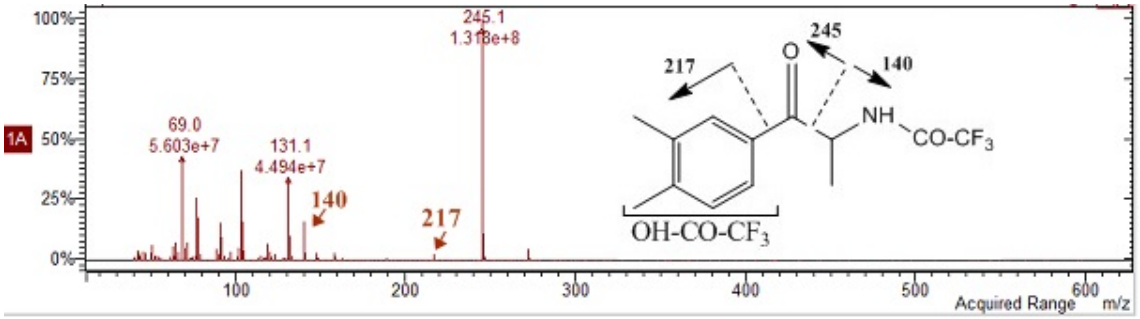

Figure 23: Mass spectrum of Metabolite 3 (M3) and proposed chemical structure after derivatization with trifluoroacetic anhydride.

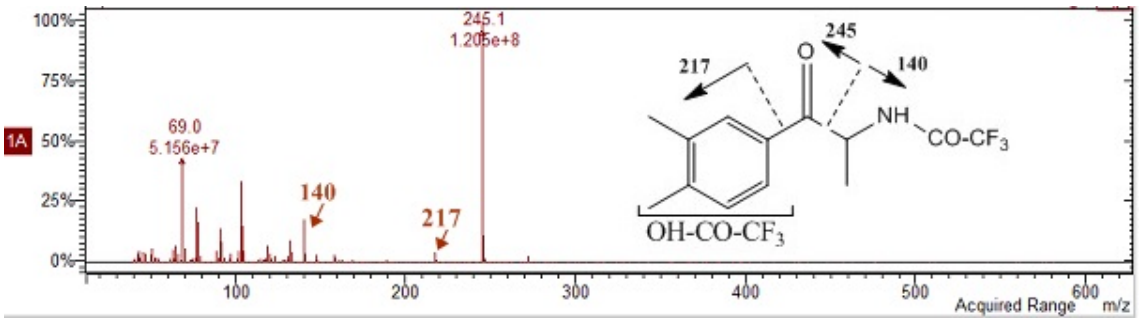

Figure 24: Mass spectrum of Metabolite 4 (M4) and proposed chemical structure after derivatization with trifluoroacetic anhydride.

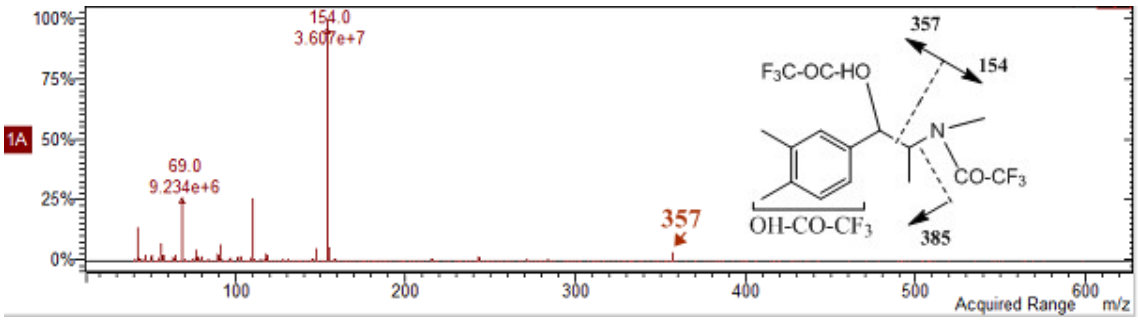

Figure 25: Mass spectrum of Metabolite 5 (M5) and proposed chemical structure after derivatization with trifluoroacetic anhydride.

4.5.1 Derivatization with N-TFA-O-TMS

As can be observed in Figure 26, after the N-TFA-O-TMS derivatization, the existence of additional peaks eluting after 3,4-DMMC that did not appear in the urine of control animals was also noted. These peaks exhibited retention times of approximately 10.08 (peak 1), 10.33 (peak 2), 10.41 (peak 3), 10.67 (peak 4), 9.09 (peak 5) and 10.17 min (peak 6), with characteristic ions m/z 235 and m/z 154 for peaks 1, 2, 3, and 4, and m/z 221 and m/z 140 for peaks 5 and 6.

Therefore, the presence of 3,4-DMMC metabolites was supported with this additional derivatization. The mass spectra of the suspected metabolites are depicted in Figures 27 and 28.

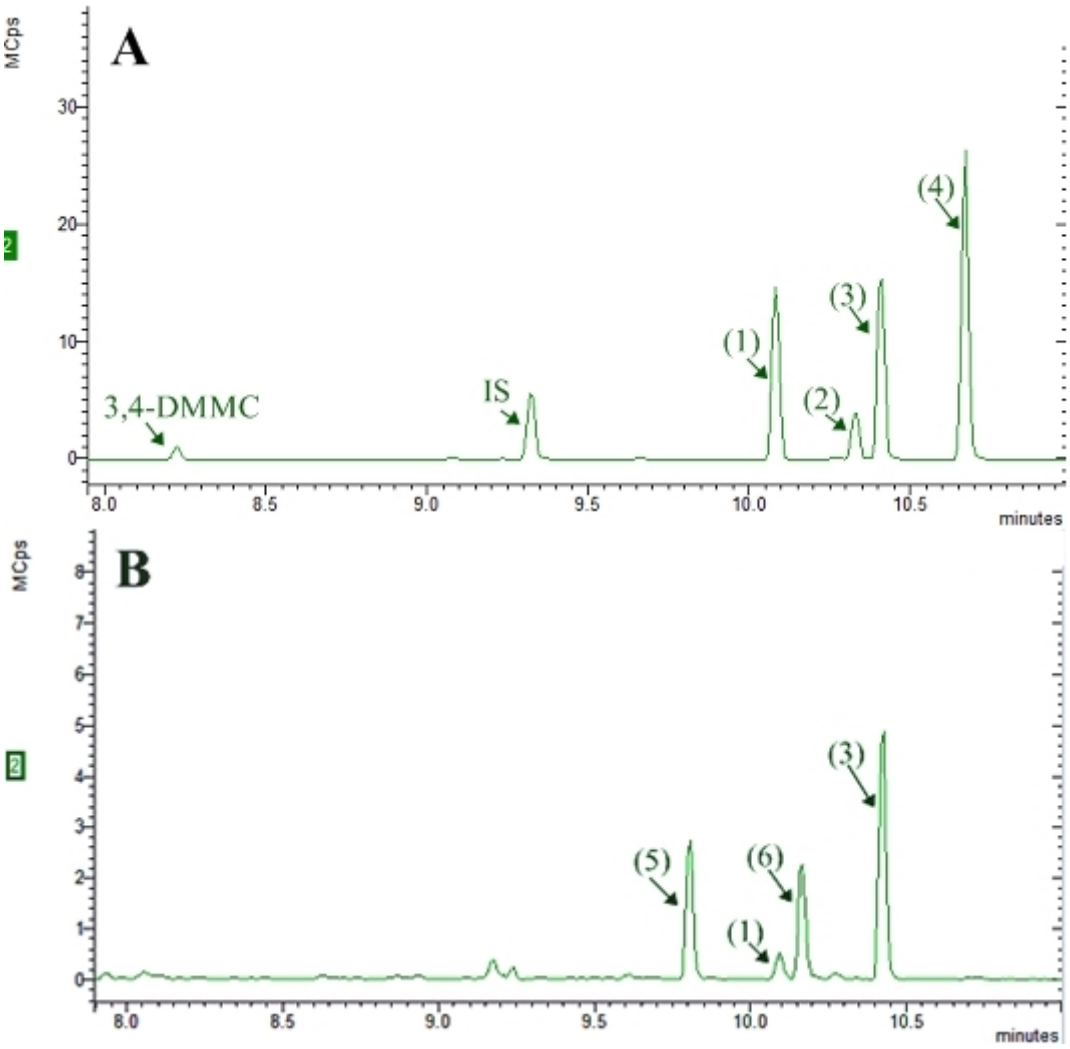

Figure 26: Representative Selective Ion Monitoring (SIM) m/z 154 **(A)** and m/z 140 **(B)** chromatograms of the urine, 24h after the i.p. administration of 20 mg/Kg 3,4-dimethylmethcathinone (3,4-DMMC) to Wistar rats, revealing potential metabolites (peaks **1–6**), after fortification with 6.25 µg/mL IS and derivatization with N-TFA-O-TMS. **IS:** Internal standard (methylone).

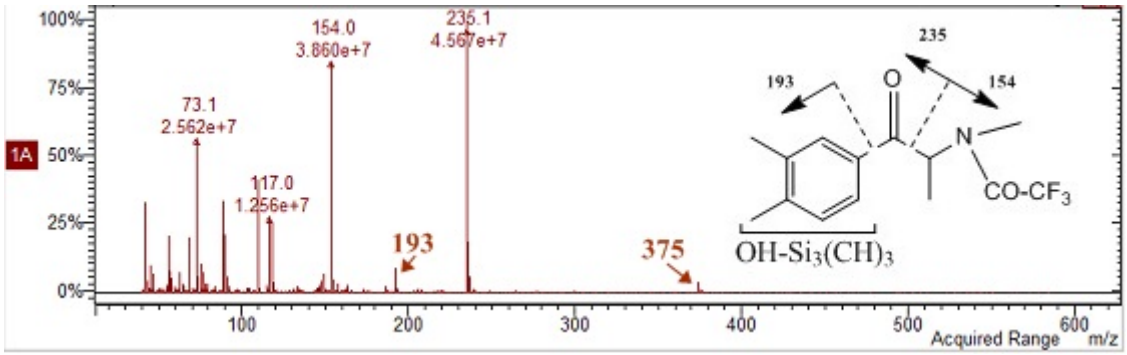

Figure 27: Mass spectrum of peaks 1, 2, 3 and 4, and proposed chemical structure after N-TFA-O-TMS derivatization.

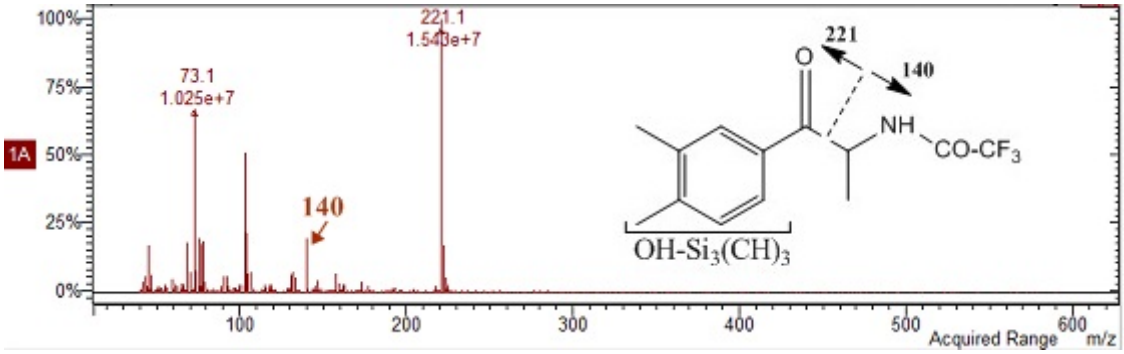

Figure 28: Mass spectrum of peaks 5 and 6, and proposed chemical structure after N-TFA-O-TMS derivatization.

4.5.2 Conjugation of 3,4-DMMC and metabolites

To evaluate if 3,4-DMMC and its main metabolites are excreted in urine as sulphate or glucuronide conjugates, cleavage of the conjugates through acidic or enzymatic hydrolysis is necessary prior to sample extraction. Type 2 β-glucuronidase from *H. pomatia* presents both sulfatase and glucuronidase activities. The results of 3,4-DMMC quantification obtained for urine samples extracted and derivatized with (11.90±2.48 µg drug/mL) or without previous cleavage of conjugates (5.53±1.02 µg drug/mL) show that 48.39±4.01% of the parent drug is excreted as sulphate or glucuronide conjugates. Likewise, 57.43±3.35% of M1, 43.9±5.16% of M2, 65.83±8.89% of M3, 57.94±8.17% of M4 are also excreted in urine in the conjugated form. Noteworthy, for all metabolites and parent drug, results were very reproducible among urines from different animals (Table 10).

Table 10: Percentage of conjugation* of 3,4-dimethylmethcathinone (3,4-DMMC) and metabolites (M1, M2, M3 and M4), for all urine samples**, after derivatization with trifluoroacetic anhydride.

	3,4-DMMC	M1	M2	M3	M4
Sample 1	54.74	53.43	47.73	6483	45.88
Sample 2	58.59	66.38	58.78	46.32	69.41
Sample 3	51.84	45.20	36.67	68.72	43.89
Sample 4	36.84	51.67	64.26	112.56	98.86
Sample 5	40.83	61.82	32.36	37.51	65.89
Sample 6	60.48	69.99	39.91	67.75	42.28
Sample 7	34.88	53.51	27.61	63.15	39.37

* % conjugation = [(peak area analyte without hydrolysis/peak area IS) : (peak area analyte with hydrolysis/peak area IS) x 100])

**After 24h of i.p. administration of 20 mg/Kg 3,4-DMMC to Wistar rats.

For M5, no differences were observed between results from samples with and without β-glucuronidase hydrolysis, which likely means that this metabolite does not suffer phase II metabolism to a significant extent (Table 11).

Table 11: Peak area ratio of M5 to IS with or without β-glucuronidase hydrolysis for urine samples* after derivatization with trifluoroacetic anhydride.

	With hydrolysis	**Without hydrolysis**
Sample 1	1.34	1.50
Sample 2	1.68	1.73
Sample 3	1.94	2.16
Sample 4	1.41	1.76
Sample 5	0.19	0.25
Sample 6	0.66	0.98
Sample 7	0.38	0.39

*After 24h of i.p. administration of 20 mg/Kg 3,4-DMMC to Wistar rats.

Chapter V - Discussion

The proposed GG-MS method was successfully developed and validated for the quantification of 3,4-DMMC in plasma, in accordance with the SWGTOX guidelines (SWGTOX, 2013) (Tables 6 and 7). Given the reduced number of validated analytical methodologies for quantification of this cathinone in biological samples other than plasma and urine, it was imperative to develop a method that would allow to obtain reliable results to assist clinical and forensic investigations. Validated GC conditions resulted in good resolution peaks, with 3,4-DMMC, metabolites, and IS presenting distinct retention times. In addition, all compounds eluted before 10 min, which also represents an advantage. This methodology proved to be robust, with a large range of linearity, low LOD and LOQ, and good precision, accuracy, and recovery efficiency. The high recovery rate ensures that there were no significant losses of analyte and IS during the extraction process. In a LC/QTOF-MS methodology previously developed to evaluate the stability of synthetic cathinones in blood, the calculated LOD (5 ng/mL) was slightly higher than that calculated in our study (4 ng/mL), but no other validation parameters were evaluated (Glicksberg and Kerrigan, 2017). In a LC-MS methodology developed for the analysis of the only fatal case described in the literature involving 3,4-DMMC alone, the calculated LOD (1.03 ng/mL) and LOQ (5 ng/mL) were lower than those calculated in our study, with a similar extraction efficiency (between 85.9–89.4%) (Usui et al., 2014). Most of the analytical methodologies described in the literature for the analysis of 3,4-DMMC use LC (Boumba et al., 2017; Carnes et al., 2017; Concheiro et al., 2015; Fontanals et al., 2017; Freni et al., 2019; Glicksberg and Kerrigan, 2017; Locos and Reynolds, 2012; Mohr et al., 2012b; Odoardi et al., 2016; Rowe et al., 2017; Shima et al., 2013; Taschwer et al., 2014; Tyrkko et al., 2013; Usui et al., 2014), and present lower LOQ values than that calculated in our study. In spite of these unequivocal advantages of LC-MS methodologies, our GC-MS method is still a very interesting alternative in laboratories that lack a LC-MS equipment and presents the advantage of being a less expensive analytical technique, ensuring operational ease and requiring less maintenance. To the best of our knowledge, the method described herein is the first GC-MS method validated for 3,4-DMMC quantification in plasma. Nevertheless, in a study using a GC-MS methodology for the quantification of 3,4-DMMC and other synthetic cathinones in human urine (Molnar et al., 2016), the reported LOQ (15–24 μg/mL) was higher than ours, demonstrating that, for the same analytical method, we present a good LOQ. A LC-MS methodology employing previous liquid-liquid extraction (LLE) for the quantification of 3,4-DMMC and its

metabolites was validated in the urine with excellent results of precision (4.8–12.1%), accuracy (100±10%) and recovery (95–102%) (Shima et al., 2013). However, in what concerns sample preparation, the LLE presents limitations since it is not as selective as SPE, is time-consuming and requires the use of large amounts of organic solvents. These limitations are important, especially when applied to complex matrices such as biological samples.

Glicksberg et al. (2017) systematically evaluated the concentration, temperature and analyte-dependent stability of synthetic cathinones, including 3,4-DMMC, in blood. Cathinones stability was investigated at two concentrations (0.1 and 1 μg/mL) in samples kept at elevated temperature (32 ºC), RT (20 ºC), refrigerated (4 ºC) or frozen (-20 ºC). Although no concentration-dependent stability differences were observed, cathinone stability was highly temperature- and analyte-dependent. 3,4-DMMC was one of the most unstable cathinones, presenting 20% of degradation after 4 days at RT (20 ºC) and after 1 day at elevated temperature (32 ºC). A similar rate of degradation was observed when the drug was refrigerated (4 ºC) for 19 days or frozen (-20 ºC) for 101 days. Based on our results, 3,4-DMMC stability depends on concentration, time, and temperature. After 1 week at RT there was more than 50% of loss at the lowest concentration tested (0.078 μg/mL). The same occurred at lower temperatures (4 ºC and -20 ºC), but only after 4 weeks. At higher concentrations (0.625 and 2.5 μg/mL), 3,4-DMMC showed greater stability, with losses below 20% at 4 ºC and -20 ºC, after 4 weeks. Based on these findings, we suggest that cold storage of clinical and forensic samples should be guaranteed as soon as possible. In addition, a cautious interpretation of the ante- and post-mortem analytical results should be made, since they may be strongly impacted by the storage conditions of the biological samples.

In the thorough and extensive analysis of all blank samples, no interferences were found for any of the analytes of interest in this study. In our biodistribution study, such interferences were not expected, given the standardised diet, strictly controlled experimental conditions, and genetic homogeneity of the animal model. However, it should be noted that no possible interferences were evaluated with regard to other drugs of possible relevance for the analysis of human samples. Even though the analytical performance of the method indicates that it could be easily applied to situations of clinical and/or forensic relevance, in those cases, possible interferences from drugs of abuse and/or common therapeutic use should be further investigated. Additionally,

should this method be applied to human intoxications, another IS would have to be selected, since methylone, which was very well suited for our biodistribution study purposes, could be ingested together with 3,4-DMMC in a drug abuse scenario (Drugs-forum, 2011; UNODC, 2016).

Despite the small number of validated parameters for all other analysed matrices, we were nevertheless able to guarantee a reliable quantification through good linearity and high recovery percentage. Therefore, we believe that the present analytical method can be applied to predict and clarify the pharmacokinetics of 3,4-DMMC.

Given the lack of studies for 3,4-DMMC, the dose of 20 mg/Kg was initially selected based on in vivo studies performed with MDMA and other cathinones (da Silva et al., 2010; Grecco et al., 2017; Lopez-Arnau et al., 2014). Compared with amphetamines, the presence of the ketone group in 3,4-DMMC increases the polarity of the substance, resulting in a decreased ability to cross the membranes and the blood-brain barrier, which causes consumers to use higher doses to obtain compensatory effects. Accordingly, consumer reports indicate the ingestion of doses above 1 g (Drugs-forum, 2011; Shima et al., 2013). Therefore, a second dose of 40 mg/Kg (Grecco and Sprague, 2016; Sprague et al., 2003) was chosen with the purpose of mimicking this high dose administration scenario. However, due to the increased animal discomfort noted, experiments at this dose were only conducted for 1h.

The obtained data clearly show that 3,4-DMMC presents a wide and rapid distribution, since it is present in plasma, as well as in all other matrices collected, after 1h of the drug administration (Figures 13 and 14). However, after 24h of administration, the drug was only found in urine (Figure 22). This wide and rapid distribution is also observed for amphetamines. In a study in which methamphetamine (METH) was administered to baboons, the authors verified through positron emission tomography (PET) analysis a rapid distribution to organs such as the heart, lungs, kidneys and liver (Fowler et al., 2007). More recently, in a similar study conducted in humans, the authors showed that amphetamine was accumulated in various organs, particularly the lungs, liver, brain and kidneys (Volkow et al., 2010).

In our study, after 1h of administration, the organs that had the highest concentrations of 3,4-DMMC at both doses were the lungs, kidneys, spleen, and brain. The highest influx

of 3,4-DMMC into these organs might be attributed to their high perfusion. The lungs and brain also displayed the highest concentrations of 4-MMC in rats s.c. administered with 5 mg/Kg of this drug, while the liver presented a much lower concentration (Sichova et al., 2017), as also occurred in our study with 3,4-DMMC. A brain concentration five times higher than that found in serum after 30 min of administration of 10 mg/Kg of methylone to rats was also described (Stefkova et al., 2017). In our study, the liver is among the tissues with lower concentration of the drug, and the plasma presented the lowest drug concentration. This may be explained by weak binding to plasma proteins, as verified after oral administration of 30 mg/Kg 4-MMC (structurally identical to 3,4-DMMC) to rats, in which the authors found a protein binding of about 20% (Martinez-Clemente et al., 2013); or by active transporting of the drug into tissues through influx pumps, as proposed by Shimshoni et al. (2015) and Diestelmann et al. (2018). One of the causes that can favour the low concentration of drug in the liver is the high metabolism that occurs in this organ, which is corroborated by the high ratio Metabolite to parent compound that we observed in the liver (Figures 18 and 21).

Comparing the concentrations found in each biological matrix at the two doses administered (Figure 15), a possible saturation of drug degradation enzymes at the dose of 40 mg/Kg may be suggested to explain the increased tissue accumulation of the drug, as verified by Martinez-Clemente et al. (2013). These authors found that following oral administration of two different doses of 4-MMC to rats, metabolic clearance decreased from 77% of total plasma clearance at 30 mg/Kg to 20% at 60 mg/Kg and, in view of the presence of phase I metabolites in the plasma, a possible saturation of metabolic enzymes was hypothesised. In that study, the Cmax of 4-MMC was found after 0.5–1h of administration, and the drug was no longer detectable 4h after i.v. administration (10 mg/Kg) and 9h after oral administration (30 and 60 mg/Kg), which supports the rapid distribution and elimination of this type of drug (Martinez-Clemente et al., 2013). In another pharmacokinetic study, in which a dose of 5 mg/Kg 4-MMC was administered to rats, followed by sacrifice by decapitation after 30, 60, 120, 240 or 480 min, the authors concluded that maximum concentrations of the drug in all analysed matrices (serum, brain, liver and lungs) were reached 30 min after dosing, and after 4h the drug almost disappeared from all samples analysed (Sichova et al., 2017). This corroborates the present findings, since 3,4-DMMC was not detected in any of the biological samples

collected 24h after administration, except in urine. Also, in a study with healthy drug users administered with an oral dose of 200 mg of 4-MMC, the rapid drug distribution was demonstrated by a Cmax occurring 1h after drug administration (Papaseit et al., 2016). The drug was also undetectable in plasma 24h after dosing. Autopsy results in fatal MDMA poisonings revealed elevated concentrations of this amphetamine primarily in the lungs (10.9 μg/g), brain (2.25 μg/g), heart (1.73 μg/g), kidneys (4.06 μg/g) and liver (6.66 μg/g), and always with the lowest concentrations found in plasma (1.13 mg/L) (Carvalho et al., 2012; Dams et al., 2003), which is also consistent with the data herein reported.

After 1h of drug administration, in addition to the peak of the parent drug, two peaks were observed in the chromatograms of all analysed matrices that could correspond to metabolites. These peaks of potential metabolites were not found in the chromatograms from tissues and plasma obtained 24h after drug administration. Sichova et al. (2017) also detected a metabolite of mephedrone, nor-mephedrone, in serum, lungs, brain and liver 1h after administration of a 5 mg/Kg dose of the drug to rats. However, after 6h, the levels of that metabolite in those matrices were only slightly above the level of detection (Sichova et al., 2017). Together with the two metabolites (M1 and M2) previously found in all the matrices analysed after 1h of administration, three other chromatographic peaks were consistently found in the 24h urines collected after drug administration (putative M3, M4, and M5 metabolites). The MS characteristics of all hypothetical metabolites were summarized in Table 5 and their chemical structures were tentatively identified from the corresponding mass spectra (Figures 17, 20 23, 24 and 25).

The M1 metabolite was identified as the nor-dimethylmethcathinone (Figure 4B), showing the same ions of the parent compound after TFAA derivatization (m/z 133 and m/z 105), and also the m/z 140 ion that is consistent with the demethylation at the amine group of 3,4-DMMC (Figure 17). The same metabolite was described by Tyrkko et al. (2013) and Shima et al. (2013), when analysing urine of 3,4-DMMC consumers.

On the other hand, the characteristics of the metabolite M2 shown in Table 5 and Figure 20 are consistent with those of the N-O-TFA derivatives (derivatization with TFAA) resulting from benzylic hydroxylation (i.e. 3-methyl-4-hydroxymethyl-methcathinone or 3-hydroxymethyl-4-methyl-methcathinone, compounds D and E, respectively, in

Figure 4) or possibly from aromatic hydroxylation (at C2, C5 or C6). Apparently, this metabolite did not suffer demethylation since the ion characteristic of this reaction (m/z 140) is not present, while presenting the ion m/z 154 instead. This hypothesis was further confirmed by a second derivatization with N-TFA-O-TMS derivatization, which resulted in four peaks with identical mass spectra (Figure 26, peaks 1–4), whose characteristics cold be assigned to the hypothesized metabolite (Figure 27).

Two additional peaks (Metabolites 3 and 4) with identical mass spectra (Table 5 and Figures 23 and 24) were observed in the retention times of 8.94 and 9.03 min (Figure 22). It was hypothesized that these were N-demethylated metabolites due to the presence of the characteristic ion of this reaction (m/z 140) instead of m/z 154. However, only with TFAA derivatization, it was not possible to conclude about their structure, since the characteristic ions (m/z 245 and 140) indicate either a possible metabolite resulting from benzylic hydroxylation (Figure 4, compounds L or M) or a metabolite resulting from the reduction of the ketone group (Figure 4, compound F). The only difference that would allow identification would be the molecular ion, m/z 385[M^+] or m/z 371[M^+], respectively, but none of these ions appeared in these spectra. With the results of the N-TFA-O-TMS derivatization (Figure 26B, peaks 5 and 6), and given the characteristics of the mass spectrum (Figure 28), showing the ions m/z 221 and m/z 140 that characterize an α-β cleavage and the absence of the ion m/z 207 that is characteristic of metabolites resulting from the reduction of the ketone group, it was concluded that this was most likely a metabolite resulting from the benzylic or aromatic hydroxylation after N-demethylation of the secondary amine. The presence of the m/z 361[M^+] confirmed this hypothesis. The two peaks can be explained by hydroxylation at different positions of the aromatic ring (at C2, C5 or C6), or at one of the methyl substituents of the aromatic ring (for example: nor-3-methyl-4-hydroxymethyl-methcathinone or nor-3-hydroxymethyl-4-methyl-methcathinone). In the study by Shima et al. (2013) carried out in urine of consumers, the metabolite resulting from the reduction of the ketone group (dihydro-dimethylmethcathinone, compound C in Figure 4) was identified, which may imply that the main paths of metabolism are different between humans and rats. In this line, Shima et al. (2013) additionally found the metabolite resulting from the reduction of the side chain ketone group after demethylation of the secondary amine (Figure 4, compound F) as a major metabolite in human urine, and therefore this is probably a major metabolic pathway in humans. In

the rat the predominant route appears to be the aromatic hydroxylation and/or benzylic hydroxylation. Other studies (Tyrkko et al., 2013) conducted in urine of drug abusers also revealed a high abundance of the metabolites resulting from the reduction of the ketone group (Figure 4, compounds C and F). However, they also reported the presence of compounds resulting from benzylic hydroxylation (Figure 4, compounds D and E), in agreement with our study.

In turn, the metabolite M5 presents ions that suggest that it derives from reactions involving a β-keto-reduction and a benzylic hydroxylation followed by oxidation. The presence of the m/z 154 ion confirms that the N-demethylation of the amine group did not occur, and together with the m/z 357, indicates the cleavage of the α-β chain (Figure 25). Thus, depending on the location of the benzylic hydroxylation, M5 may be 3-methyl-4-carboxy-dihydro-methcathinone or 3-carboxy-4-methyl-dihydro-methcathinone (Figure 4, compounds J and K, respectively). Nevertheless, all these hypotheses need analytical confirmation that can only be provided with proper reference standards. According to Papaseit et al. (2017), who summarised all published data on 4-MMC, more metabolites were found in studies conducted in animal models than those found in studies with humans. A similar finding stems from the comparison of the present study, where five possible metabolites other than 3,4-DMMC were identified in urine of rats administered with 3,4-DMMC, with the study of Shima et al. (2013), in which only three metabolites were identified, in addition to the parent compound, in urine of drug abusers.

β-Glucuronidase hydrolysis increased the ratio analyte to IS of 3,4-DMMC and its metabolites, except for M5, which means that these compounds are also excreted in urine in the form of glucuronides or sulphates. Concerning M5, given the similarity of the results obtained from samples subjected or not to hydrolysis, our results suggest the absence of conjugation. The conjugation of 3,4-DMMC and its metabolites in the form of glucuronide/sulphates was also verified in humans, with the increase of 3,4-DMMC and its metabolites (nor-dimethylmethcathinone, dihydro-dimethylmethcathinone and nor-dihidro-dimethylmethcathinone) after acid/enzymatic hydrolysis (Shima et al., 2013). Similar results were already reported for other cathinones, such as 4-MMC and methylone (Pedersen et al., 2013; Stefkova et al., 2017). In our study, the extent of conjugation was very similar among the various animals tested (Table 10), which was to be expected. On the contrary, in humans, a higher inter-individual variability is

expected (Papaseit et al., 2017) due to environmental variables, including diet, as well as genetic variability.

Chapter VI- Conclusions

With the increased consumption of 3,4-DMMC and the scarcity of pharmacokinetic studies on this drug, the evaluation of its biodistribution and metabolic profile, as well as the validation of methodologies for its identification and quantification in distinct biological matrices, became mandatory and urgent. To our knowledge, this is the first biodistribution study of 3,4-DMMC. Our investigation revealed that this cathinone has an extensive and rapid biodistribution, as it occurs with the amphetamines and with other synthetic cathinones, since after 1h of drug administration, 3,4-DMMC was present in all biological matrices analysed, while after 24h it was only present in urine.

Also, it became apparent that this drug has a rapid metabolism, with two metabolites appearing in all matrices analysed after 1h and three more, in addition to these, in the urine produced during 24h. Given the increased concentration of 3,4-DMMC and its metabolites in the urine following enzymatic hydrolysis with β-glucuronidase, we can also conclude that they likely undergo phase II metabolism, conjugating in the form of glucuronide/sulphates.

This whole study was possible due to the development of a methodology that allowed the extraction, identification and quantification of 3,4-DMMC in the various biological matrices.

The implemented GC-MS methodology to quantify 3,4-DMMC in plasma was successfully validated, proving to be selective, accurate, precise, with good recovery and linearity. In this sense, it proved to be a good alternative, which was fully optimise, representing un advantage compared to many other studies previously published in the literature.

Finally, the method seems to be adequate for the purpose of 3,4-DMMC analysis and can be easily adapted to routine laboratory procedures for the identification and quantification of 3,4-DMMC. In addition, clarification of the biodistribution of the drug is essential to allow the identification of the most appropriate analytical matrices, as well as the metabolites that may have the widest detection windows. All this knowledge will definitely help improve methods of drug detection in biological matrices, which will ultimately aid in clinical and forensic investigations.

Chapter VII- References

Adamowicz P, Tokarczyk B, Stanaszek R and Slopianka M (2013) Fatal mephedrone intoxication--a case report. *Journal of analytical toxicology* **37**:37-42.

Barrios L, Grison-Hernando H, Boels D, Bouquie R, Monteil-Ganiere C and Clement R (2016) Death following ingestion of methylone. *International journal of legal medicine* **130**:381-385.

Bluelight (2010) 3,4-DMMC. http://www.bluelight.org/vb/threads/537782-3-4-dmmc. Access date 18 December 2018.

Borek HA and Holstege CP (2012) Hyperthermia and multiorgan failure after abuse of "bath salts" containing 3,4-methylenedioxypyrovalerone. *Annals of emergency medicine* **60**:103-105.

Boumba VA, Di Rago M, Peka M, Drummer OH and Gerostamoulos D (2017) The analysis of 132 novel psychoactive substances in human hair using a single step extraction by tandem LC/MS. *Forensic science international* **279**:192-202.

Brunt TM, Atkinson AM, Nefau T, Martinez M, Lahaie E, Malzcewski A, Pazitny M, Belackova V and Brandt SD (2017) Online test purchased new psychoactive substances in 5 different European countries: A snapshot study of chemical composition and price. *The International journal on drug policy* **44**:105-114.

Calinski DM, Kisor DF and Sprague JE (2019) A review of the influence of functional group modifications to the core scaffold of synthetic cathinones on drug pharmacokinetics. *Psychopharmacology* **236**:881-890.

Carbone PN, Carbone DL, Carstairs SD and Luzi SA (2013) Sudden cardiac death associated with methylone use. *The American journal of forensic medicine and pathology* **34**:26-28.

Carnes S, O'Brien S, Szewczak A, Tremeau-Cayel L, Rowe WF, McCord B and Lurie IS (2017) Comparison of ultra high performance supercritical fluid chromatography, ultra high performance liquid chromatography, and gas chromatography for the separation of synthetic cathinones. *Journal of separation science* **40**:3545-3556.

Carvalho M, Carmo H, Costa VM, Capela JP, Pontes H, Remiao F, Carvalho F and Bastos Mde L (2012) Toxicity of amphetamines: an update. *Archives of toxicology* **86**:1167-1231.

Cawrse BM, Levine B, Jufer RA, Fowler DR, Vorce SP, Dickson AJ and Holler JM (2012) Distribution of methylone in four postmortem cases. *Journal of analytical toxicology* **36**:434-439.

Concheiro M, Castaneto M, Kronstrand R and Huestis MA (2015) Simultaneous determination of 40 novel psychoactive stimulants in urine by liquid chromatography-high resolution mass spectrometry and library matching. *Journal of chromatography A* **1397**:32-42.

Coppola M and Mondola R (2012) Synthetic cathinones: chemistry, pharmacology and toxicology of a new class of designer drugs of abuse marketed as "bath salts" or "plant food". *Toxicology letters* **211**:144-149.

Couto RAS, Goncalves LM, Carvalho F, Rodrigues JA, Rodrigues CMP and Quinaz MB (2018) The Analytical Challenge in the Determination of Cathinones, Key-Players in the Worldwide Phenomenon of Novel Psychoactive Substances. *Critical reviews in analytical chemistry* **48**:372-390.

da Silva DG, de Pinho PG, Pontes H, Ferreira L, Branco P, Remiao F, Carvalho F, Bastos ML and Carmo H (2010) Gas chromatography-ion trap mass spectrometry method for the simultaneous measurement of MDMA (ecstasy) and its metabolites, MDA, HMA, and HMMA in plasma and urine. *Journal of*

chromatography B, Analytical technologies in the biomedical and life sciences **878**:815-822.

Dams R, De Letter EA, Mortier KA, Cordonnier JA, Lambert WE, Piette MH, Van Calenbergh S and De Leenheer AP (2003) Fatality due to combined use of the designer drugs MDMA and PMA: a distribution study. *Journal of analytical toxicology* **27**:318-322.

deRoux SJ and Dunn WA (2017) "Bath Salts" the New York City Medical Examiner Experience: A 3-Year Retrospective Review. *Journal of forensic sciences* **62**:695-699.

Dickson AJ, Vorce SP, Levine B and Past MR (2010) Multiple-drug toxicity caused by the coadministration of 4-methylmethcathinone (mephedrone) and heroin. *Journal of analytical toxicology* **34**:162-168.

Diestelmann M, Zangl A, Herrle I, Koch E, Graw M and Paul LD (2018) MDPV in forensic routine cases: Psychotic and aggressive behavior in relation to plasma concentrations. *Forensic science international* **283**:72-84.

Drugs-forum (2011) 3,4-DMMC (3,4-dimethylmethcathinone) Experiences. http://drugs-forum.com/threads/3-4-dmmc-3-4-dimethylmethcathinone-experiences.160183/. Access date 18 December 2018.

EMCDDA (2010) Annual Report on the implementation of Council Decision 2005/387/JHA, European Monitoring Centre for Drugs and Drug Addiction, Lisbon.

EMCDDA (2015a) Annual Report on the implementation of Council Decision 2005/387/JHA, European Monitoring Centre for Drugs and Drug Addiction, Lisbon.

EMCDDA (2015b) New psychoactive substances in Europe. An update from the EU Early Warning System, Publications Office of the European Union, Luxembourg.

EMCDDA (2017) European Drugs Report 2017: Trends and developments, Publications Office of the European Union, Luxembourg.

EMCDDA (2018) European Drugs Report 2018: Trends and developments, Publications Office of the European Union, Luxembourg.

Fontanals N, Marce RM and Borrull F (2017) Solid-phase extraction followed by liquid chromatography-high resolution mass spectrometry to determine synthetic cathinones in different types of environmental water samples. *Journal of chromatography A* **1524**:66-73.

Fowble KL, Shepard JRE and Musah RA (2018) Identification and classification of cathinone unknowns by statistical analysis processing of direct analysis in real time-high resolution mass spectrometry-derived "neutral loss" spectra. *Talanta* **179**:546-553.

Fowler JS, Kroll C, Ferrieri R, Alexoff D, Logan J, Dewey SL, Schiffer W, Schlyer D, Carter P, King P, Shea C, Xu Y, Muench L, Benveniste H, Vaska P and Volkow ND (2007) PET studies of *d*-methamphetamine pharmacokinetics in primates: comparison with *I*-methamphetamine and (--)-cocaine. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **48**:1724-1732.

Freni F, Bianco S, Vignali C, Groppi A, Moretti M, Osculati AMM and Morini L (2019) A multi-analyte LC-MS/MS method for screening and quantification of 16 synthetic cathinones in hair: Application to postmortem cases. *Forensic science international* **298**:115-120.

Gaspar H, Bronze S, Oliveira C, Victor BL, Machuqueiro M, Pacheco R, Caldeira MJ and Santos S (2018) Proactive response to tackle the threat of emerging drugs:

Synthesis and toxicity evaluation of new cathinones. *Forensic science international* **290**:146-156.

Geryk R, Kalikova K, Schmid MG and Tesarova E (2016) Enantioselective separation of biologically active basic compounds in ultra-performance supercritical fluid chromatography. *Analytica chimica acta* **932**:98-105.

Glicksberg L and Kerrigan S (2017) Stability of Synthetic Cathinones in Blood. *Journal of analytical toxicology* **41**:711-719.

Grapp M, Sauer C, Vidal C and Muller D (2016) GC-MS analysis of the designer drug alpha-pyrrolidinovalerophenone and its metabolites in urine and blood in an acute poisoning case. *Forensic science international* **259**:e14-19.

Grecco GG, Kisor DF, Magura JS and Sprague JE (2017) Impact of common clandestine structural modifications on synthetic cathinone "bath salt" pharmacokinetics. *Toxicology and applied pharmacology* **328**:18-24.

Grecco GG and Sprague JE (2016) Impact of Functional Group Modifications on Designer Phenethylamine Induced Hyperthermia. *Chemical research in toxicology* **29**:871-878.

Kesha K, Boggs CL, Ripple MG, Allan CH, Levine B, Jufer-Phipps R, Doyon S, Chi P and Fowler DR (2013) Methylenedioxypyrovalerone ("bath salts"), related death: case report and review of the literature. *Journal of forensic sciences* **58**:1654-1659.

Lee D, Chronister CW, Hoyer J and Goldberger BA (2015) Ethylone-Related Deaths: Toxicological Findings. *Journal of analytical toxicology* **39**:567-571.

Locos O and Reynolds D (2012) The characterization of 3,4-dimethylmethcathinone (3,4-DMMC). *Journal of forensic sciences* **57**:1303-1306.

Lopez-Arnau R, Martinez-Clemente J, Abad S, Pubill D, Camarasa J and Escubedo E (2014) Repeated doses of methylone, a new drug of abuse, induce changes in serotonin and dopamine systems in the mouse. *Psychopharmacology* **231**:3119-3129.

Luethi D, Kolaczynska KE, Docci L, Krahenbuhl S, Hoener MC and Liechti ME (2018) Pharmacological profile of mephedrone analogs and related new psychoactive substances. *Neuropharmacology* **134**:4-12.

Martinez-Clemente J, Lopez-Arnau R, Carbo M, Pubill D, Camarasa J and Escubedo E (2013) Mephedrone pharmacokinetics after intravenous and oral administration in rats: relation to pharmacodynamics. *Psychopharmacology* **229**:295-306.

Maskell PD, De Paoli G, Seneviratne C and Pounder DJ (2011) Mephedrone (4-methylmethcathinone)-related deaths. *Journal of analytical toxicology* **35**:188-191.

Mohr S, Pilaj S and Schmid MG (2012a) Chiral separation of cathinone derivatives used as recreational drugs by cyclodextrin-modified capillary electrophoresis. *Electrophoresis* **33**:1624-1630.

Mohr S, Taschwer M and Schmid MG (2012b) Chiral separation of cathinone derivatives used as recreational drugs by HPLC-UV using a CHIRALPAK(R) AS-H column as stationary phase. *Chirality* **24**:486-492.

Molnar B, Fodor B, Csampai A, Hidvegi E and Molnar-Perl I (2016) Structure-related related new approach in the gas chromatography-mass spectrometry analysis of cathinone type synthetic drugs. *Journal of chromatography A* **1477**:70-75.

Murray BL, Murphy CM and Beuhler MC (2012) Death following recreational use of designer drug "bath salts" containing 3,4-Methylenedioxypyrovalerone (MDPV). *Journal of medical toxicology : official journal of the American College of Medical Toxicology* **8**:69-75.

Odoardi S, Romolo FS and Strano-Rossi S (2016) A snapshot on NPS in Italy: Distribution of drugs in seized materials analysed in an Italian forensic laboratory in the period 2013-2015. *Forensic science international* **265**:116-120.

Papaseit E, Molto J, Muga R, Torrens M, de la Torre R and Farre M (2017) Clinical Pharmacology of the Synthetic Cathinone Mephedrone. *Current topics in behavioral neurosciences* **32**:313-331.

Papaseit E, Perez-Mana C, Mateus JA, Pujadas M, Fonseca F, Torrens M, Olesti E, de la Torre R and Farre M (2016) Human Pharmacology of Mephedrone in Comparison with MDMA. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **41**:2704-2713.

Pearson JM, Hargraves TL, Hair LS, Massucci CJ, Frazee CCr, Garg U and Pietak BR (2012) Three fatal intoxications due to methylone. *Journal of analytical toxicology* **36**:444-451.

Pedersen AJ, Reitzel LA, Johansen SS and Linnet K (2013) In vitro metabolism studies on mephedrone and analysis of forensic cases. *Drug testing and analysis* **5**:430-438.

Researchchemicals (2016) 3,4-DMMC experiences? Opinions? https://www.reddit.com/r/researchchemicals/comments/4mpeqa/34dmmc_experiences_opinions/. Access date 28 November 2018.

Richeval C, Wille SMR, Nachon-Phanithavong M, Samyn N, Allorge D and Gaulier JM (2018) New psychoactive substances in oral fluid of French and Belgian drivers in 2016. *The International journal on drug policy* **57**:1-3.

Roque Bravo R, Martins M, Pinto A, Valente M, Carvalho F, Bastos M, Carmo H and Dias da Silva D (2016) Emerging club drugs: in vitro hepatotoxicity of six synthetic cathinones and benzofurans. *Toxicology letters* **258:S247**.

Rowe WF, Marginean I, Carnes S and Lurie IS (2017) The role of diode array ultraviolet detection for the identification of synthetic cathinones. *Drug testing and analysis* **9**:1512-1521.

Serpelloni G MT, C Locatelli, Rimondo C, Seri C (2013) Nuove sostanze psicoattive (NSP): schede tecniche relative alle molecole registrate dal Sistema Nazionale di Allerta Precoce p 1075, Allegato al PAN-NSP 2013.

Shima N, Katagi M, Kamata H, Matsuta S, Nakanishi K, Zaitsu K, Kamata T, Nishioka H, Miki A, Tatsuno M, Sato T, Tsuchihashi H and Suzuki K (2013) Urinary excretion and metabolism of the newly encountered designer drug 3,4-dimethylmethcathinone in humans. *Forensic Toxicology* **31**:101-112.

Shimshoni JA, Britzi M, Sobol E, Willenz U, Nutt D and Edery N (2015) 3-Methyl-methcathinone: Pharmacokinetic profile evaluation in pigs in relation to pharmacodynamics. *Journal of psychopharmacology (Oxford, England)* **29**:734-743.

Sichova K, Pinterova N, Zidkova M, Horsley RR, Lhotkova E, Stefkova K, Vejmola C, Uttl L, Balikova M, Kuchar M and Palenicek T (2017) Mephedrone (4-Methylmethcathinone): Acute Behavioral Effects, Hyperthermic, and Pharmacokinetic Profile in Rats. *Frontiers in psychiatry* **8**:306.

Sprague JE, Banks ML, Cook VJ and Mills EM (2003) Hypothalamic-pituitary-thyroid axis and sympathetic nervous system involvement in hyperthermia induced by 3,4-methylenedioxymethamphetamine (Ecstasy). *The Journal of pharmacology and experimental therapeutics* **305**:159-166.

Stefkova K, Zidkova M, Horsley RR, Pinterova N, Sichova K, Uttl L, Balikova M, Danda H, Kuchar M and Palenicek T (2017) Pharmacokinetic, Ambulatory, and

Hyperthermic Effects of 3,4-Methylenedioxy-N-Methylcathinone (Methylone) in Rats. *Frontiers in psychiatry* **8**:232.

SWGTOX (2013) Standard practices for method validation in forensic toxicology. *Journal of analytical toxicology* **37**:452-474.

Taschwer M, Seidl Y, Mohr S and Schmid MG (2014) Chiral separation of cathinone and amphetamine derivatives by HPLC/UV using sulfated ss-cyclodextrin as chiral mobile phase additive. *Chirality* **26**:411-418.

Torrance H and Cooper G (2010) The detection of mephedrone (4-methylmethcathinone) in 4 fatalities in Scotland. *Forensic science international* **202**:e62-63.

Tyrkko E, Pelander A, Ketola RA and Ojanpera I (2013) In silico and in vitro metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry. *Analytical and bioanalytical chemistry* **405**:6697-6709.

UNODC (2014) World Drug Report 2014, United Nations publication.

UNODC (2016) World Drug Report 2016, United Nations publication.

Uralets V, Rana S, Morgan S and Ross W (2014) Testing for designer stimulants: metabolic profiles of 16 synthetic cathinones excreted free in human urine. *Journal of analytical toxicology* **38**:233-241.

Usui K, Aramaki T, Hashiyada M, Hayashizaki Y and Funayama M (2014) Quantitative analysis of 3,4-dimethylmethcathinone in blood and urine by liquid chromatography-tandem mass spectrometry in a fatal case. *Legal medicine (Tokyo, Japan)* **16**:222-226.

Valente MJ, Araujo AM, Bastos Mde L, Fernandes E, Carvalho F, Guedes de Pinho P and Carvalho M (2016) Characterization of Hepatotoxicity Mechanisms Triggered by Designer Cathinone Drugs (beta-Keto Amphetamines). *Toxicological sciences : an official journal of the Society of Toxicology* **153**:89-102.

Valente MJ, Guedes de Pinho P, de Lourdes Bastos M, Carvalho F and Carvalho M (2014) Khat and synthetic cathinones: a review. *Archives of toxicology* **88**:15-45.

Volkow ND, Fowler JS, Wang GJ, Shumay E, Telang F, Thanos PK and Alexoff D (2010) Distribution and pharmacokinetics of methamphetamine in the human body: clinical implications. *PloS one* **5**:e15269.

Weinstein AM, Rosca P, Fattore L and London ED (2017) Synthetic Cathinone and Cannabinoid Designer Drugs Pose a Major Risk for Public Health. *Frontiers in psychiatry* **8**:156.

Wyman JF, Lavins ES, Engelhart D, Armstrong EJ, Snell KD, Boggs PD, Taylor SM, Norris RN and Miller FP (2013) Postmortem tissue distribution of MDPV following lethal intoxication by "bath salts". *Journal of analytical toxicology* **37**:182-185.

Zaami S, Giorgetti R, Pichini S, Pantano F, Marinelli E and Busardo FP (2018) Synthetic cathinones related fatalities: an update. *European review for medical and pharmacological sciences* **22**:268-274.

Zancajo VM, Brito J, Carrasco MP, Bronze MR, Moreira R and Lopes A (2014) Analytical profiles of "legal highs" containing cathinones available in the area of Lisbon, Portugal. *Forensic science international* **244**:102-110.

Zellnera T, Diestelmannb M, Paulb LD, Pfaba R and Eyera F (2017) Acute “bath salts” intoxications: analytical findings and clinical features, in *37th International Congress of the European Association of Poisons Centres and Clinical*

Toxicologists (EAPCCT) pp 371–544, CLINICAL TOXICOLOGY, Basel, Switzerland.

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