Approaches to evaluate the pharmacology of new psychoactive substances

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TESI DOCTORAL UPF / 2018

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als meus pares,

als meus germans,

a la Marina.

Acknowledgements

Aquesta tesi doctoral ha estat possible gràcies a l'esforç incondicional i constant de moltes persones que han col·laborat (in)directament en la seva realització. Per tant, dedico aquest apartat a intentar transmetre el meu agraïment cap a totes i tots.

En primer lloc, la tesi ha estat viable gràcies a la impecable direcció de l'Óscar Pozo i d'en Rafael de la Torre. La contribució combinada de tots dos ha estat excel·lent i base del meu desenvolupament científic i personal. Els estic infinitament agraïda per tot el que han fet per mi. A en Rafael li agraeixo la seva paciència, la capacitat d'escolta i la confiança constants que m'ha donat sempre. Admiro la seva capacitat de veure més enllà d'allò habitual i la seva manera brillant de dirigir a bon port decisions importants. També vull destacar la seva gran bondat i la seva visió estratègica. Así mismo, a Óscar nunca le podré agradecer todo lo que me ha enseñado. Su idioma analítico y su entusiasmo incansable han hecho que aprendiera de todo lo que iba pasando a pasos de gigante. Su acertada manera de ver el mundo y su excelente capacidad científica, estratégica, analítica y vital han sido fuente de inspiración y de admiración a diario. Le agradezco las miles de conversaciones que ha compartido conmigo, haciendo de esta tesis una de las mejores experiencias de mi vida.

Una peça clau pel desenvolupament d'aquesta tesi doctoral i de la meva formació científica també ha estat en Magí Farré. Gràcies a ell vaig poder viure de molt a prop tot el món dels assajos clínics i aprendre infinitat de conceptes i rigor científic. Tanmateix, ja des dels inicis, tant l'Esther Papaseit com la Clara Pérez-Mañá sempre han estat col·laboradores i disposades a ensenyar-me tot el que calgués. Els estic molt agraïda. Així mateix, agraeixo a la unitat de recerca clínica de l'IMIM, per fer possible els assajos clínics d'una manera immillorable. Agraeixo a en Julian Mateus, a la Marta Pérez i a l'Esther Menoyo tota la seva predisposició, dedicació, alegria i

professionalitat amb què m'han acompanyat aquests últims anys. Aprofito també aquest espai per agrair a la Marta Torrens els seus comentaris encoratjadors i la seva energia incansable.

También me gustaría agradecer a Patricia Robledo, de quien he aprendido mucho con sus preguntas científicas en las *FINS-sessions* y quién hizo posible el estudio con animales y me enseñó a ser autónoma para poder llevarlo a cabo. A en Toni Pascual li vull agraïr haver-me ensenyat a plantejar-m'ho tot des de la base i no donant res per fet, imprescindible per a qualsevol científic/a!

Agradezco muchísimo todo el esfuerzo y dedicación de Mara Dierssen y de Ilario De Toma. Gracias a ellos hemos podido llegar a rincones que ni imaginábamos inicialmente y nos han enseñado a darle sentido biológico a nuestro mar de datos metabolómicos. Gracias por confirmar, una vez más, que juntos se llega más lejos.

The presented thesis was englobed in a European Project called PREDICT (Predicting Risk of Emerging Drugs with In silico and Clinical Toxicology) coordinated by Maastricht University. The project caused a really enriching collaboration with different research groups from Barcelona and from the Netherlands and my acknowledgement to all of them is endless.

The UM team (Maastricht University) encompassed with Jan Ramaekers, Kym Kuypers, Eef Theunissen and Eliza de Sousa, was pleasantly professional and greatly helpful in all situations. Thanks to them, the project was perfectly organized and scientifically excellent. It was a great gratification sharing science and fun simultaneously.

From the Trimbos Institute in Utrecht, my sincere thanks to Tibor Brunt, Annabel Vreeker and Vincent Mensen. It was an authentic pleasure to collaborate with all of them, sharing very interesting data, scientific knowledge and very good times in the Netherlands and in Barcelona.

De la companyia bioinformàtica Prous Institute, agraeixo moltíssim la col·laboració de l'Antoni Valencia, en Josep Prous i en Marcel·lí Carbó, amb les seves ganes incansables de tirar endavant amb el projecte. A en Marcel·lí li agraeixo també tot el que m'ha ensenyat sobre models farmacocinètics i sobre la docència universitària.

Amb tot, també ha estat una gran experiència treballar colze a colze amb els companys d'Energy Control. Agraeixo moltíssim haver coincidit amb la Mireia Ventura ja que la seva ajuda, el seu entusiasme i la seva dedicació van ser claus per poder fer estudis en humans. També estic molt agraïda per haver pogut compartir laboratori amb l'Iván Fornís i la Cristina Gil, qui sempre han estat molt receptius i col·laboradors.

De l'Institut de Plaguicides i Aigües de Castelló de la Plana (IUPA) m'agradaria agrair a en David Fabregat, la Maria Ibañez, en Juan Vicente Sancho i en Félix Hernández la càlida rebuda durant la meva estada a la Universitat Jaume I. Els estic molt agraïda per tot el que he pogut aprendre treballant al seu costat, tant a nivell científic com a nivell humà.

Vull mencionar també a tot l'equip del Laboratori Anti-Dopatge de Catalunya, liderat inicialment per en Jordi Segura i actualment per la Rosa Ventura, amb qui hem compartit espai, equips i temps durant la realització de la tesi.

Aquesta tesi és en gran part, contribució de tots els companys i companyes de laboratori amb qui he crescut i compartit el dia a dia durant els últims anys. Començo agraint a la Mitona Pujadas, qui em va iniciar en el fantàstic món del bioanàlisi i amb qui he compartit complicitat, aprenentatge i diversió cada dia. Agraeixo també a en Toni Pastor i a en Raul Pérez totes les històries apassionants i divertides carregades de bons moments compartits. A la Cristina Fernández tot el que m'ha ensenyat a l'estabulari amb la seva infinita paciència. A la Neus Pizarro li agraeixo i admiro la seva bondat i humanitat,

qui ha estat exemple i referència a molts nivells. A Olha Khymenets le agradezco su apoyo y animos constantes con el problemático e incansable equipo y tanto a Míriam Martínez como a Tomas Gener les agradezco sus buenos comentarios y consejos para la recta final de esta tesis. També li agraeixo a en Klaus Langohr la seva paciència per explicar-nos conceptes estadístics inimaginables.

Agraeixo moltíssim també la col·laboració de la Cristina Llop, la Laia Roca, la Mariola Vera i l'Eva Vitas, per mantenir i garantir el funcionament del laboratori així com per estar sempre disposades a fer-nos les coses més fàcils.

També estic molt agraïda d'haver coincidit amb l'Angie Soldevila i la Mariona Cabanas, les quals van venir a fer una estada amb nosaltres i les seves ganes d'aprendre i el seu entusiasme ens van deixar fascinats.

Als companys de despatx, els estic molt agraïda per totes les experiències compartides dins i fora del laboratori. A en Jordi García li agraeixo el seu entusiasme diari amb bons moments compartits i a la Laura Xicota les seves divertides anècdotes i la seva originalitat. De la Carla, admiro la seva dedicació, esforç, constància i lluita, que m'ha ensenyat durant el doctorat. També estic molt agraïda d'haver començat aquesta tesi amb l'Anna Boronat. Ja des dels inicis ha estat un suport incondicional carregat de diversió, alegria i complicitat. A la Maria Alemany li agraeixo les milers d'estones compartides amb atacs de riure incontrolats i amistat constant. La seva vitalitat contagiosa i divertida ha estat aire fresc i energia per sempre seguir endavant. Coincidir amb la Natàlia Soldevila també ha estat una incalculable alegria. Ha estat un plaer compartir i gaudir de la seva espontaneïtat, confiança i felicitat. Tanmateix, estic infinitament agraïda a en Jose Rodríguez, per ser un excel·lent referent tant a nivell científic com personal, sempre disposat a compartir i aprendre de tot el que va passant. Admiro la seva capacitat de fer fàcil i alegre qualsevol situació científica i vital. I

agraeixo també haver coincidit amb l'Àlex Gómez, qui, sense dubte, ha estat la descoberta més valuosa durant la tesi. L'admiració, germanor, agraïment i estima que li tinc és tanta que si ho intentéssim mesurar, ni amb els instruments menys sensibles ni amb totes les dilucions possibles seríem capaços de no saturar l'equip.

Finalment, estic molt agraïda de tenir la sort d'estar envoltada de persones extraordinàries amb qui comparteixo milers d'experiències, anècdotes, aprenentatges i grans amistats. Gràcies a elles, tot és millor. En especial, vull agrair a la Marina tot el que ha fet per mi. La seva paciència, intel·ligència, alegria, comprensió i estima m'han acompanyat i guiat en totes les grans aventures viscudes. M'agradaria agrair també tot el suport dels meus germans, l'Anna i l'Edu, a qui admiro, valoro i estimo infinitament. I finalment, res de tot això hagués estat possible sense els meus pares, l'Andreu i l'Anna, qui m'han fet créixer, aprendre i estimar incondicionalment, amb humilitat, esforç, dedicació, saviesa i molt d'amor.

This doctoral thesis has been funded by:

European Commission (Drugs Policy Initiatives, Justice Programme 2014-2020, contract nº. HOME/2014/JDRU/AG/DRUG/7082. The project was titled: Predicting Risk of Emerging Drugs with In silico and Clinical Toxicology (PREDICT).

Fondo de Investigación Sanitaria - Instituto de Salud Carlos III FEDER (ISCIII, FIS-FEDER PI11/01961)

DIUE de la Generalitat de Catalunya (2014SGR 680)

USADA(United States Anti-Doping Agency)

CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN) is an initiative of the Instituto de Salud Carlos III, Madrid, Spain

Red de Trastornos Adictivos RTA RD12/0028/0006

This thesis has been done in collaboration with the following institutions:

Institut Hospital del Mar d'Investigacions Mèdiques (IMIM) foundation, Neuroscience program, Department of Integrative Pharmacology and Systems in Neuroscience, Barcelona.

Doctorate school, Universitat Pompeu Fabra (UPF), Barcelona.

Barcelona Biomedical Research Park (PRBB), Barcelona.

Centre for Genomic Regulation (CRG), Barcelona.

Maastricht University (UM), Maastricht.

Trimbos Institute (TI), Utrecht, Utrecht.

Prous Institute for Biomedical Research, Barcelona.

Research Institute Germans Tries i Pujol, Badalona.

Energy Control, ABD Asociación Bienestar y Desarrollo NGO, Barcelona.

Research Institute for Pesticides and Water, Jaume I University, Castellón de la Plana.

Abbreviations

Drugs

2C-B	2,5-dimethoxy-4-bromo-phenethylamine
25I-NBOMe	2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine
2C-E	2,5-dimethoxy-phenethylamine
4-MEC	Methylethcathinone or 4-Methyl-N-ethylcathinone
5-MeO-DMT	5-methoxy-N,N-dimethyltryptamine
6-APB	6-(2-aminopropyl)benzofuran
AB-Pinaca	N-[(2S)-1-Amino-3-methyl-1-oxo-2-butanyl]-1-pentyl-1H-indazole-3-carboxamide
α-PVP	alpha-Pyrrolidinopentiophenone
AM-2201	1-(5-fluoropentyl)-3-(1-naphthoyl)indole
BZP	1-benzylpiperazine
DMT	Dimethyltryptamine
GHB	γ-hydroxybutyrate
JWH-018	Naphthalen-1-yl-(1-pentylindol-3-yl)methanone
JWH-081	4-Methoxynaphthalen-1-yl-(1-pentylindol-3-yl)methanone
mCPP	1-(3-chlorophenyl)piperazine
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MDPV	Methylenedioxypyrovalerone
MEPH	Mephedrone
METH	Methamphetamine
THC	Δ^9-Tetrahydrocannabinol

Metabolites and enzymes

3-MT	3-Methoxy-tyramine
5-HIAA	5-Hydroxytryptophan

5-HT	Serotonin
A	11-Dehydrocorticosterone
ACar	Acetyl-carnitine
ACh	Acetyl-choline
Ade	Adenosine
AED	Androstenedione
B	Corticosterone
Car	Carnitine
Ch	Choline
Cr	Creatine
Creat	Creatinine
COMT	Catechol-O-methyl transferase
COOH-MEPH	4'-Carboxy-mephedrone
DA	Dopamine
DIHYDRO-MEPH	1'-Dihydro-mephedrone
DOC	Deoxycorticosterone
DOPA	Levodopa or L-3,4-dihydroxyphenylalanine
Dopac	3,4-Dihydroxyphenylacetic acid
DOPAL	3,4-Dihydroxyphenylacetaldehyde
GABA	γ-aminobutyric acid
Glu	Glutamate
Gln	Glutamine
HVA	Homovanillic acid
Ile	Isoleucine
Leu	Leucine
LNAA	Long Neutral Amino Acids
MAO	Mono-amine Oxidase
ME	Metanephrine
NA	Noradrenaline

NM	Normetanephrine
NOR-MEPH	Nor-mephedrone
Phe	Phenetilamine
Prog	Progesterone
SUCC-NOR-MEPH	N-Succinyl Nor-Mephedrone
T	Testosterone
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

General

API	Atmospheric Pressure Ionization
BBB	Blood-Brain-Barrier
BMI	Body Mass Index
CYP 450	Cytochrome P450
DS	Dilute and Shoot
DUID	Driverd driving under the influence of drugs
EI	Electron ionization
GC-MS	Gas Chromatography coupled to Mass Spectrometry
GLT-1	Glutamate Transporter subtype I
HEK 293	Human Embryonic Kidney cells
HPLC	High-Performance Liquid Chromatography
HMDB	Human Metabolome Database
ISTD	Internal Standard
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LLOQ	Low Limit of Quantification
LOI	Limit of Identification
m/z	Mass-to-charge ratio

MSEP	Mean square error of prediction
MSTFA	N-Methyl-N-(trimethylsilyl)-trifluoroacetamide
NMR	Nuclear Magnetic Resonance
NPS	New Psychoactive Substances
NT	Neurotransmitter
MW	Molecular Weight
n.a.	Non-analized
n.d.	Non-detected
n.r.	Non-reported
PCA	Principal Component Analysis
PP	Protein precipitation
QC-H	High control
QC-L	Low control
QC-M	Medium control
QqQ	Triple quadrupol
rpm	Revolutions per minute
RSD	Relative standard deviation
Rt	Retention Time
S/N	Signal to Noise ratio
SD	Standard deviation
SIM	Selected Ion Monitoring
SPE	Solid-Phase Exctraction
SRM	Selected Reaction Monitoring
ULOQ	Upper Limit of Quantification

Pharmacokinetic parameters

AUC	Area under the curve
$Cl_{plasmatic}$	Plasmatic Clearence
Cl_{renal}	Renal Clearence

C_{max}	Peak plasma concentration
K_e	Constant of elimination
$T_{1/2}$	Half-life time
T_{max}	Time to reach peak concentration
Vd	Volum of distribution

Organizations, Projects and Programs

EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EU-MADNESS	European-wide, monitoring, analysis and knowledge dissemination of novel/emergint psychoactives
IUPAC	International Union of Pure and Applied Chemistry
NPSAD	National Programme on Substance Abuse Deaths
UNDOC	United Nations Office on Drugs and Crime
WADA	World Anti-Doping Agency

Abstract

In the last decade, the rapid emergence of a group of compounds known as novel psychoactive substances (NPS) has become a worrying problem due to their ambiguous legal status, their easy availability, their extensive consumption and their severe adverse effects. NPS are compounds designed to mimic existing established recreational drugs. The most commonly clinically encountered NPS are stimulants (such as mephedrone) and cannabinoids (such as "spice") and our knowledge of their pharmaco-toxicological profile is very limited. The evaluation of hundreds of substances in a short time period is a challenge for public health and drug policies globally. This thesis aims to contribute to the pharmacological evaluation of NPS by developing a targeted metabolomics approach (on neurotransmitters and steroids) applied to brain tissue as well as in plasma and urine as a tool to predict the pharmacological profile of NPS, circumventing limitations of the current evaluation approach. We focused on specific NPS (i.e. cathinones, synthetic cannabinoids) to demonstrate the proof of principle of the methodological approach developed. By using chromatographic techniques coupled to tandem mass spectrometry, the quantification of endogenous and exogenous compounds, presumably altered after drug intake, was achieved in different biological matrices. We studied more in depth the metabolic clearance of mephedrone in humans focusing on its pharmacokinetics and pharmacogenetics aspects regulating its disposition as well as the interactions with its pharmacodynamics in a set of multi-dose randomized double-blind clinical trials. The pharmacological predictions for novel drugs was accomplished by quantifying the neuro-metabolomic fingerprint alterations of NPS compared to those observed after the intake of classical drugs of abuse. In brief, this work has contributed to describe the human disposition of mephedrone and it also highlight the potential of using targeted metabolomics as a tool to predict the pharmacological profile of NPS.

Resum

En l'última dècada, la ràpida aparició d'un grup de compostos coneguts com a noves substàncies psicoactives (NPS) s'ha convertit en un problema preocupant a causa del seu estatus ambigu, el seva fàcil accés, el seu consum extens i els seus efectes adversos greus. Les NPS són compostos dissenyats per imitar les drogues d'abús ja establertes. Les NPS més reportades a nivell clínic són els estimulants (com la mefedrona) i els cannabinoides (com ara "spiece") i el nostre coneixement del seu perfil farmaco-toxicològic és molt limitat. L'avaluació de centenars de substàncies en un curt període de temps és un repte per a la salut pública i les polítiques de drogues a nivell mundial. Aquesta tesi pretén contribuir a l'avaluació farmacològica de les NPS desenvolupant un enfocament metabòlic dirigit (en neurotransmissors i esteroides) aplicat al teixit cerebral, al plasma i a l'orina com a eina per predir el perfil farmacològic de les NPS, evitant les limitacions de l'avaluació actual. Ens hem centrat en NPS específiques (com les catinones i els cannabinoides sintètics) per demostrar la prova del principi de l'enfocament metodològic desenvolupat. Mitjançant l'ús de tècniques cromatogràfiques acoblades a l'espectrometria de masses en tàndem, es va aconseguir la quantificació de compostos endògens i exògens, presumptament alterats després de la ingesta de drogues en diferents matrius biològiques. Es va estudiar més a fons l'eliminació metabòlica de la mefedrona en humans, centrant-nos en els seus aspectes farmacocinètics i farmacogenètics que regulaven la seva depuració, així com les interaccions amb la seva farmacodinàmica en un conjunt d'assaigs clínics aleatoritzats de dosis múltiples. Les prediccions farmacològiques de nous fàrmacs es van assolir quantificant les alteracions neuro-metabolòmiques de les NPS en comparació amb les observades després de la ingesta de drogues d'abús clàssiques. En resum, aquest treball ha contribuït a descriure la disposició humana de la mefedrona i també destaca el potencial d'utilitzar metabolòmica dirigida com a eina per predir el perfil farmacològic de NPS.

Resumen

En la última década, la rápida aparición de un grupo de compuestos conocidos como nuevas sustancias psicoactivas (NPS) se ha convertido en un problema preocupante debido a su estatus legal ambiguo, su fácil disponibilidad, su extenso consumo y sus graves efectos adversos. Las NPS son compuestos diseñados para mimetizar las drogas de abuso ya establecidas. Las NPS más reportadas clínicamente son los estimulantes (como la mefedrona) y los cannabinoides (como el "spice") y nuestro conocimiento de su perfil fármaco-toxicológico es muy limitado. La evaluación de cientos de sustancias en un corto período de tiempo es un reto para la salud pública y las políticas de control de drogas a nivel mundial. Esta tesis pretende contribuir a la evaluación farmacológica de NPS desarrollando un enfoque metabolómico dirigido (con neurotransmisores y esteroides) aplicado al tejido cerebral, al plasma y a la orina, como herramienta para predecir el perfil farmacológico de las NPS, evitando las limitaciones de los protocolos de evaluación actual. Nos hemos centrado en NPS específicos (como las catinonas y los cannabinoides sintéticos) para demostrar la prueba del principio del enfoque metodológico desarrollado. Mediante el uso de técnicas cromatográficas acopladas a la espectrometría de masas en tándem, se consiguió la cuantificación de compuestos endógenos y exógenos, presuntamente alterados después de la ingesta de drogas en diferentes matrices biológicas. Se estudió más a fondo la depuración metabólica de la mefedrona en humanos, centrándonos en sus aspectos farmacocinéticos y farmacogenéticos que la regulan, así como las interacciones con su farmacodinamia en un conjunto de ensayos clínicos aleatorizados de dosis múltiples. Las predicciones farmacológicas de nuevos fármacos se alcanzaron cuantificando las alteraciones neuro-metabolómicas de la NPS en comparación con las observadas tras la ingesta de drogas de abuso clásicas. En resumen, este trabajo ha contribuido a describir la depuración metabólica en humanos de la mefedrona y también destaca el potencial de utilizar metabolómica dirigida como herramienta para predecir el perfil farmacológico de las NPS.

Preface

Recently, the emergence of New Psychoactive Substances (NPS) has become a global issue. Hundreds of novel unknown drugs have been proliferated due its ambiguous legal status and easy availability with an increasing consume among youths. These substances mimic the effects of classical drugs of abuse (such as ecstasy or methamphetamine) but with small structural changes that can radically affect its ability to bind effectively to a given target and produce adverse medical consequences. The scientific evaluation of these substances has been thwarted due the great quantity of substances in a changing poly-consume market, which constantly adapts to be ahead of public health prevention and law-enforcement. On top of this, the collection of research data for these drugs become unfeasible considering the labor-intensive and the time-consuming costs required for a drug systematic evaluation, multiplied by hundreds of unequaled substances.

In this context, it is necessary to concentrate on the most problematic and prevalent substances and to develop predictive models to evaluate the NPS pharmacology *in vivo*.

Therefore, the presented thesis arises from the need to approach the challenge of the pharmacological evaluation of NPS.

Thesis structure

This PhD dissertation is structured in several sections as follows: firstly, an **Introduction** provides a comprehensive context of the NPS situation, emphasizing the relevance of mephedrone, one of the most popular NPS used worldwide and firstly evaluated in humans by our research group. Furthermore, highlights of analytical strategies used in the thesis are introduced with an special emphasis to metabolomics. Then, the **Objectives and Hypotheses** section presents the initial hypothesis and the aims of the presented work. The **Methods and Results** section has two differentiated parts (part I and part II) introduced with a brief summary to introduce each part. Each part includes different chapters adapted from scientific publications in peer-review journals that facilitate the thesis lecture. In **Part I**, the human metabolic disposition of mephedrone is studied. Chapter 1 and 2 focuses on the development of analytical strategies (with chromatography techniques coupled to mass spectrometry) to monitor mephedrone and its metabolites in human matrices and a broad pharmacokinetic study is presented. In chapter 3, a comprehensive study of the pharmacokinetics, the pharmacodynamics and the pharmacogenetics study at different dosages of mephedrone is presented. In **Part II**, a novel experimental approach with targeted metabolomics is developed. In chapter 4, the prediction of the pharmacological profile of two examples of NPS (mephedrone and JWH-018) was obtained by targeting monoamines neurotransmitters and steroid hormones pathways. Limitations and improvements of the model are presented in the additional information section. In chapter 5, an LC-MS/MS quantification method for amino acids neurotransmitters (such as GABA or Acetylcholine) was validated in brain and plasma with the analysis of all samples used for the targeted metabolomics study. Then a new prediction model was constructed with two synthetic cathinones

(alpha-PVP and MDPV) detailed with another additional information. Afterwards a general **Discussion** of the methodology and results achieved in the thesis, with critical comments and contextualization of the findings were discussed among future research directions. The thesis ends with the main **Conclusions** derived from the presented work and a **Supplementary material** section with additional information to dig deeper into the metabolomics results. The **Bibliography** collected all the scientific literature consulted during the thesis development. In the **Annex**, extra information about clinical intoxications of mephedrone is provided.

Six scientific papers (1 submitted, 1 under revision and 4 already published) are the result of the present PhD. The scientific publications (in peer-review journals) derived from the thesis are summarized in table 1.

Thesis section		Scientific publications	Status
Introduction		Papaseit E*, **Olesti E***, de la Torre R, Torrens M, Farré M. Mephedrone Concentrations in Cases of Clinical Intoxication. Curr Pharm Design 2017; 23(36):5511-5522. Doi:10.2174/1381612823666170704130213.	Published
Part I	Chapter 1	**Olesti E***, Pujadas-Bastardes M, Papaseit E, Pérez-Mañá C, Pozo O, Farré M, de la Torre R. GC-MS Quantification Method for Mephedrone in Plasma and Urine: Application to Human Pharmacokinetics. J Anal Toxicol 2017; 41(2): 100-106. Doi:10.1093/jat/bkw120	Published
	Chapter 2	**Olesti E***, Farré M, Papaseit E, Krotonoulas A, Pujadas-Bastardes M, de la Torre R, Pozo O. Pharmacokinetics of Mephedrone and Its Metabolites in Human by LC-MS/MS. AAPS J 2017; 19(6): 1767-1778. Doi:10.1208/s12248-017-0132-2	Published
	Chapter 3	**Olesti E***, Farré M, Carbó M, Papaseit E, Yubero-Lahoz S, Pujadas M, Pozo ÓJ, de la Torre R. Dose-dependent pharmacology study of mephedrone and its metabolites: pharmacokinetics, serotoninergic effects and CYP interaction. Clinical Pharmacokinetics.	Submitted
Part II	Chapter 4	**Olesti E***, De Toma I, Ramaekers JG, Brunt T, Carbó M, Fernández-Avilés C, Robledo P, Farré M, Dierssen M, Pozo ÓJ, de la Torre R. Metabolomics predicts the pharmacological profile of new psychoactive substances. Journal of Psychopharmacology.	Under revision
	Chapter 5	**Olesti E***, Rodríguez-Morató J*, Gómez-Gómez A, Ramaekers JG, de la Torre R, Pozo ÓJ. Quantification of endogenous neurotransmitters and related compounds by liquid chromatography coupled to mass spectrometry. Talanta; in press. Doi:10.1016/j.talanta.2018.09.034	Published

Table 1. Summary of the scientific publications presented in this thesis. With an asterisk is marked the first author of the publication.

The scientific outputs of the presented thesis were also shared with the scientific community in different international congresses in two different formats: oral and poster presentations:

Oral presentations:

- **Olesti E**, Fabregat-Safont D, Ibañez M, Sancho JV, Hernández F, de la Torre R, Pozo, Pozo OJ. Facing the problem of New Psychoactive Substances for doping control analysis. The International Association of Forensic Toxicologist (TIAFT), Ghent, Belgium. August 2018.

- **Olesti E**, Ramaekers JG, Brunt TM, Carbó M, Fernández C, Robledo P, Papaseit E, Farré M, Pozo OJ, de la Torre R. Targeted metabolomics as a novel high-throughput approach for new psychoactive substances characterization and classification. Spanish society of mass spectrometry (SEEM), Barcelona. June 2017.

- **Olesti E**, Ramaekers JG, Brunt TM, Carbó M, Fernández C, Robledo P, Farré M, Pozo OJ, de la Torre R. Metabolomics: a novel experimental approach for the pharmacology and toxicology of NPS. Dutch Neuroscience Meeting. Lunteren, The Netherlands. June 2017.

Poster presentation:

- **Olesti E**, De Toma I, Cabanas M, Ramaekers JG, Brunt TM, Carbó M, Fernández C, Robledo P, Farré M, Pozo OJ, de la Torre R. Metabolomics predicts the pharmacological profile of NPS. V International Conference on Novel Psychoactive Substances. Vienna, Austria. November 2017.

Furthermore, during the time period of the doctoral thesis the author participated in other research topics. These contributions enriched her research experience by collaborating with other European researchers, learning new techniques and topics and by acquiring new knowledge. These outputs were presented in several scientific papers:

- Haijen E, de la Torre R, Pastor A, **Olesti E**, Pizarro N, Ramaekers JG, Kuypers KP. Peripheral endocannabinoid concentrations are not associated with verbal memory impairment during MDMA intoxication. Psychopharmacology (Berl) 2017.

- Papaseit E, Pérez-Mañá C, Mateus JA, Pujadas-Bastardes M, Fonseca F, Torrens M, **Olesti E**, de la Torre R, Farré M. Human Pharmacology of Mephedrone in Comparison to MDMA. Neuropsycho-pharmacology 2016; 41(11): 2704-2713.

- Rodríguez-Morató J, Boronat A, Kotronoulas A, Pujadas-Bastardes M, Pastor A, **Olesti E**, Pérez-Mañá C, Khymenets O, Fitó M, Farré M, de la Torre R. Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol. Drug Metab Rev 2016; 48(2): 218-236.

- Pastor A, Rodríguez-Morató J, **Olesti E**, Pujadas-Bastardes M, Pérez-Mañá C, Khymenets O, Fitó M, Covas MI, Solà R, Motilva MJ, Farré M, de la Torre R. Analysis of free hydroxytyrosol in human plasma following the administration of olive oil. J Chromatogr A 2016; 1437: 183-190.

However, the content of these publications has not been included in the core thesis, as they are beside the scope of the thesis and/or the author contributed as a collaborator instead of leading the investigation.

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1. Introduction

1.1. Drugs of abuse

Since thousands of years, humans have been looking for substances capable to produce changes on the central nervous system, in order to find changes on perception, on the emotional states and alertness. These substances, present in plants or fungus, produced desired effects, but also could cause adverse effects, abuse and addiction disorders. Over time, advances in technology lead the identification, manufacturing and world-wide consumption of these drugs of abuse. Nowadays, a quarter of a billion people, 1 in 20 adults (from 15 to 64 years), consumed at least one drug of abuse in the past 2 years, being the majority of them globally illegal[1]. Recently, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) presented a global estimation of the drug-type consume, being cannabis, methamphetamine, ecstasy (MDMA), cocaine and opioids (considered as classical drugs) and new psychoactive substances (catalogued as novel substances) the most popular drugs consumed world-wide (see Figure 1)[2].

Drugs of abuse can produce stimulant, hallucinogen, euphoric or paranoia effects (among others effects), depending on their targeted side of action. Generally, the drug-interaction produces alterations in endogenous metabolic pathways such as the monoamine neurotransmitters, the endocannabinoid system, the steroid hormones, etc[3–6]. Each drug of abuse has its own target site of action with its specific pharmacology associated.

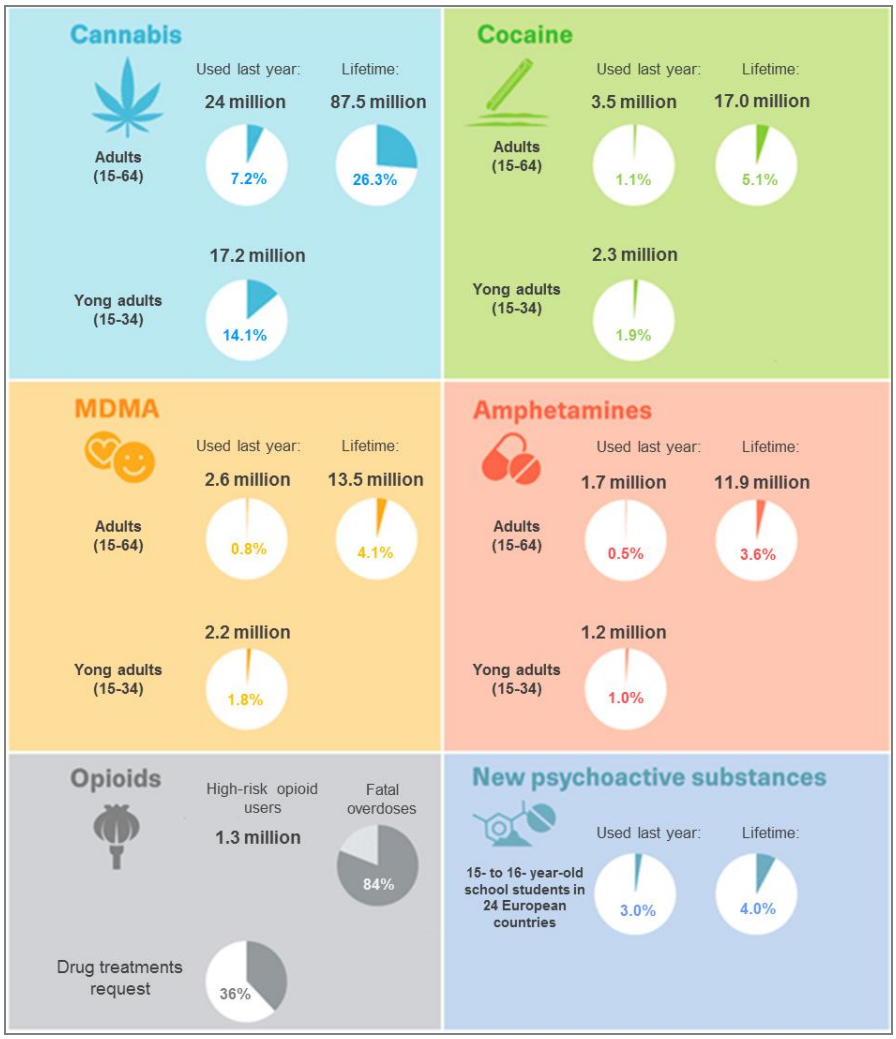


Figure 1. Estimation of drug use in the European Union during the 2018. Adapted from the European Monitoring Centre for Drugs and Drug Addiction[2].

1.1.1. New Psychoactive Substances

a. Definition

In recent years, a new group of substances, so called New Psychoactive Substances (NPS) emerged in the market as an alternative to traditional drugs of abuse. NPS were defined as those "narcotic or psychotropic drugs that are **not** controlled by the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 United Nations Convention on Psychotropic Substances, but which are liable to abuse and dependence and produce similar effects on the central nervous system (CNS) compared to Schedule I and II drugs"[7,8]. The term "new" does not necessarily refer to new drug inventions but may include substances that recently become available and consumed[9,10]. Those substances were designed to mimic the effects and to intentionally circumvent the existing controlled substances[11]. The chemical structures of many NPS are based on compounds extracted from biomedical literature or analogs of illicit drugs and prescribed medications but with an unknown pharmaco-toxicological profile[11]. Therefore, many of the NPS are analogues of endogenous neurotransmitters and their pharmacological effect relies in its interaction with the endogenous receptors[12].

b. Emergence

NPS have proliferated uncontrollably, establishing 3% of the total drug consumption worldwide[13]. The total number of such substances, already estimated to be in the hundreds, is still growing steadily due to their increment in diversity, potency and availability[1,14]. Since 2008, these novel substances have experienced a remarkable increase in popularity and by the end of 2017, the EMCDDA was monitoring over 600 NPS that have appeared on Europe's drug marked[2] (see Figure 2). The list of

substances appearing on the drug marked is still maintained, with about one new substance reported weekly in Europe[2]. Over 100 countries and territories in all regions have reported the emergence of NPS[2].

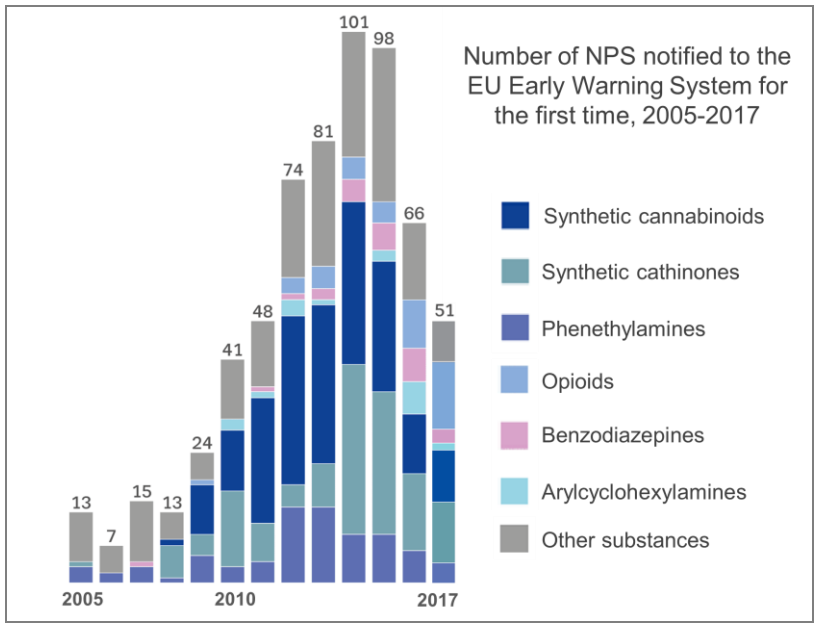


Figure 2. Number and categories of new psychoactive substances notified to the EU Early Warning System for the first time, 2005-17. Adapted from the European Monitoring Centre for Drugs and Drug Addiction[2].

In all, due to the newness substances available, their ambiguous legal situation, their ability to be undetected in toxicological test, their rapid adaptation on legal restrictions, their easy manufacturing and distribution, their potent advertising and commercial tools on the Internet and a minor public knowledge of their adverse effects, NPS has become a worrying phenomenon worldly[2,10,15].

NPS presents a serious global public health threat, since there is no quality control in their manufacturing and their biological effects are still unknown[11,16].

c. Challenges to evaluate NPS

The emergence of hundreds of NPS during the past decade unleashed a challenge to public health and drug policies at international level[10]. Synthetic drugs producers rapidly adapted by modifying chemical structures with the intention of avoiding law-enforcement and shifting drug trends[10]. In this manner, they started to sell substances not listed for control on the open market, by arguing that: "*whatever that is **not** expressly prohibited must be allowed for open sale"*[17]. Adding these substances to the controlled list obliges law enforcement to test and evaluate the potential public health harm of those substances[17]. However, technical and financial resources are not increased accordingly[17]. Furthermore, by the time that a law is updated (it can take more than a year), newer drugs appears replacing the illegal ones[17]. Indeed, analogs of classical drugs are relatively easy to synthesize and the production of stimulants, hallucinogens, entactogens or emphatogens can be rapidly distributed globally[10].

The evaluation of the potential public health risk of these substances requires a first characterization of the pharmacological effect of NPS. At the present time, *in vitro* techniques have been used to describe the mechanism of action of many NPS[18,19]. Moreover, *in vivo* studies have investigated the pharmacokinetics[20–22], clinical monitoring (e.g. heart-rate, activity levels, etc.) or toxicity[23] (among other disciplines) of some NPS. However, the speed with which scientific evaluation is obtained for a substance is still insufficient to be relevant in humans. The need of generating and accumulating research data to face the challenge of NPS has been thwarted by the large quantity of current NPS, by the complexity of their presentations (mixtures) and by its alegal status[10]. The soaring research costs and the immense amount of time required to evaluate a single substance clearly limited the pharmacological evaluation of NPS in

humans[10]. In that manner, novel high-throughput approaches are required in order to overcome these limitations. In this thesis, we have focused in the human disposition of one of the most problematic and relevant NPS, mephedrone (Part I) and we have created a targeted metabolomics prototype model able to predict the pharmacological profile of examples of NPS (Part II).

d. Consumption and users

The use of NPS was concentrated among young adults (aged 16 to 24) with around 1 in 40 (2.6%) young adults took an NPS in countries like England in 2015-16[24]. Moreover, the prevalence of NPS was major in young men than in women and poly-drug consume was present in around 85.2% of the young adults consumers of NPS[25,26].

The motivations that can incite users to try NPS instead of classical drugs, were described to be the followings[27]:

i. To skirt the law. As NPS produce similar effects to banned drugs but they are not being prohibited itself, they can be sold and consumed without criminal implications.
ii. To avoid its detection in random drug tests. As NPS are many of them undetectable in drug test, they can be an alternative to heavily monitored users (such as prisoners, athletics or public transport drivers among others).
iii. To seek new experiences. In a similar way as the early stages of ecstasy consumption during the 1970s and 1980s where consumers discovered a unique entactogenic experience, users (so called psychonauts) would consume NPS in order to find new drug effects.

iv. To potentiate currently controlled substances. Drug suppliers could sell NPS as traditional drugs in order to adulterate or potentiate currently controlled substances.

e. Adverse effects

The consumption of NPS produces reinforcing effects at recreational doses but give rise to adverse medical consequences at higher doses or after chronic exposure[28,29]. Although NPS are designed to be analogous in subjective effects to classical drugs they present off-target site of action due to their different chemical structure[16]. These unknowns' interactions of NPS could produce unexpected adverse medical consequences. General adverse effects produced by NPS include aggression, anxiety, confusion, depression, paranoia or psychosis[16,30].

Moreover, another potential threat to human health is on the production and manufacturing of NPS. These drugs are produced in clandestine laboratories with poor quality controls and unregulated conditions. As a result, these substances are synthetized without confirmation on the chemical structure or the dose distributed and without controlling the presence of adulterants and contaminants[30]. Consequently, the presence of adverse effects associated to NPS use and overdose has increased among users and it has become a growing issue globally among emergency departments and intensive care units[31]. Furthermore, many case reports of intoxication are due the combination of more than one substance and the un-knowledge of the substances or the ingested dose difficulties their possible treatment[32,33].

f. Categories of NPS

NPS may be categorized by chemical structure, by psychoactive properties and pharmacological and clinical effects (stimulants, entactogens, hallucinogens…) by biological targets (dopaminergic, serotoninergic…) or by source (plant, synthetic or semi-synthetic)[10,29]. Considering the chemical class of NPS, their pharmacological and subjective effects, the main types of NPS are: synthetic cannabinoids, synthetic cathinones, phenethylamines, piperazines, tryptamines and others[14,29]. In table 2, a brief description of the different most popular NPS types is presented according the EMCDDA and UNDOC[34,35]. Currently, synthetic cannabinoids and synthetic cathinones are the most consumed NPS world-wide[1,13].

Chemical class	Pharmacological action	Signs and symptoms	Substances
Synthetic cannabinoids	CB1 and CB2 receptor agonists displaying higher affinity, efficacy and potency compared to Δ^9-THC[28,36,37].	Mainly **sedatives**. Anxiolytic, antidepressant-like effects, euphoria paranoia, tachycardia, panic, convulsions, psychosis, visual/auditory hallucinations, vomiting and seizures[29,38].	JWH-018, JWH-081, AB-Pinaca, AM-2201
Synthetic cathinones	Sympathomimetic drugs that act on serotonin, dopamine and noradrenaline pathways[19,39,40].	Mainly **stimulants**. Agitation, aggression euphoria, restless, abdominal pain, vertigo, rhabdomyolysis, convulsions and even death[41,42].	Mephedrone, Methylone, MDPV,α-PVP, 4-MEC
Phenethylamines	Agonist of the serotoninergic receptors that cause psychedelic effects and inhibit the monoamine reuptake[43].	Mainly **stimulant**, **entactogenic** or **hallucinogenic**. Hypertension, vomiting, hyperthermia, convulsions, visual/auditory hallucinations, dissociation, respiratory deficits, liver and kidney failure and death associated[29,44].	2C-B, 2C-E, 25I-NBOMe, 6-APB
Piperazines	Inhibitors of the uptake of monoamines and promoters of the release of dopamine and noradrenaline[45].	Mainly **stimulants**. Hyperthermia, convulsions, hallucinations, kidney and heart failure and death associated[46].	BZP, mCPP
Tryptamines	Agonist of the serotoninergic receptors 5-HT_{2A} and the 5-HT reuptake inhibitors[47].	Mainly **hallucinogenic**. Alterations in sensory perception, intensification of colors, distortion of body image mood lability, depersonalization and anxiety[29,48].	DMT, 5-MeO-DMT

Table 2. Classification of substances categorized as NPS based on chemical class, pharmacological effects and subjective effects by the EMCDDA and UNODC. Adapted from Papaseit E. et al[14] and Milliano et al.[29].

1.1.2. Synthetic cannabinoids

Cannabis plants have been cultivated in Europe, Asia, Africa and the Americas for thousands of years, as a source of medical and recreational products[49]. To date, products derived from the *Cannabis* plants are the most widely produced and consumed as illicit substance worldwide[1,50].

The psychoactive effects of *Cannabis* are mainly produced by Δ^9-Tetrahydrocannabinol (Δ^9-THC or THC), a partial agonist of CB_1 (central) and CB_2 (peripheral) cannabinoid receptors[51]. Since the discovery of Δ^9-THC, characterized in the 1940s and synthetized in 1964[52,53], a wide quantity of synthetic cannabinoids were designed to pharmacologically evaluate novel therapeutics applications[54].

However, by the early 2000s, some of these compounds were manufactured and consumed to obtain a recreational marijuana-like effect but avoiding legal issues and toxicological screenings. These substances were sold in various commercial herbal blends products such as *Spice* or *K2* (see Figure 3)[28] labeled as *not for human consumption.* These packages contained synthetic cannabinoids from different chemical classes and different CB receptor affinities[28,55].

Figure 3. Spice blend herbal product. Image produced by the United States Marine Corps.

Synthetic cannabinoids englobe more than 150 compounds identified, with a broad structural diversity (naphthoylindoles or phenylacetylindoles among others)[28,56]. These products mainly bind with higher affinity to the CB_1 receptors in the brain in comparison with natural products of *cannabis*, producing an increase in the health risk associated to their consumption. Consequently, although synthetic cannabinoids produce similar effects to those of THC, these are generally accompanied by adverse and harmful effects[56–58]. Some of the most popular synthetic cannabinoids found in *Spice* products were: JWH-018, a full CB_1 agonist synthetized by John W. Huffman, or AB-PINACA, a CB_1 and CB_2 agonist, synthetized by Pfizer® in 2009 as an analgesic medication (see Figure 4).

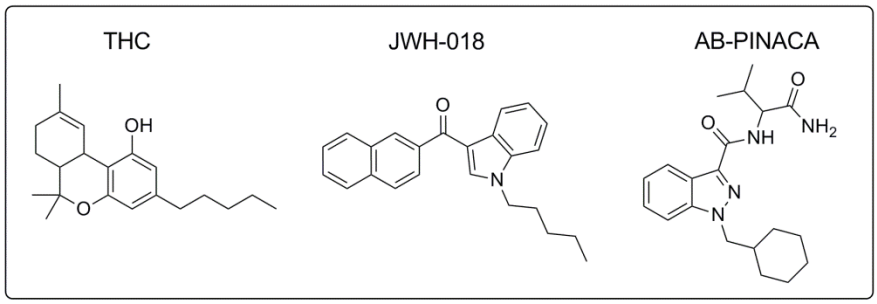


Figure 4. Chemical structures of THC (active compound of Cannabis), JWH-018 and AB-PINACA (both synthetic cannabinoids).

Recently, Theunissen E et al performed a phase I, placebo-controlled pilot study with JWH-018[55]. They found that JWH-018 produced impaired performance on neurocognitive function and subjective feelings of high[55]. Furthermore, JWH-018 presented active metabolites[59] found in urine[60] and serum[61], but not in oral fluid[62].

1.1.3. Synthetic cathinones

Synthetic cathinones are semi-synthetic derivatives of the parent compound cathinone which is a naturally psychoactive component found in the leaves of *Catha Edulis* plant[63] (see Figure 5). *Catha Edulis*, commonly known as khat is a flowering plant native to the Horn of Africa and the Arabian Peninsula. For centuries, the local communities chewed khat leaves during cultural and religious ceremonies. This tradition or social costume was practiced regularly in order to obtain stimulant, well-pleasant and euphoric effects[63,64].

Figure 5. Fresh leaves of the *Catha Edulis* plant, which belongs to the *Celastraceae* family. Image obtained from the South African National Biodiversity Institute[65].

The fresh leaves of the khat plant contain over 40 compounds including alkaloids, tannins, flavonoids, terpenoids, glycosides, etc[63]. At the end of 1880's Flückiger and Gerock detected an alkaloid which had psychoactive properties and years later it was identified as norpseudoephedrine or cathine[66]. Afterwards, in 1975 the United Nations Laboratory found a β-keto analog of cathine: the cathinone[63,67] (see Figure 6). Cathinone was 7 to 10 times more potent than cathine but with a shorted life time, which explained the costumer's need to chew fresh khat leaves in order to obtain its pharmacological activity[63,68].

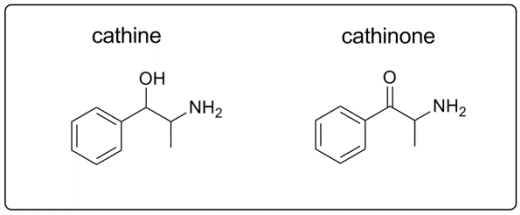


Figure 6. Chemical structures of two natural alkaloids of the khat plant.

By the early 1990s a growing number of cathinones derivatives were evaluated for their therapeutic potential purposes, mainly as antidepressants and anorectic drugs. As an example, in 1985 a cathinone derivatives called bupropion was introduced as antidepressant and it is still used nowadays. Another example of the therapeutic potential uses of cathines derivates was found in 1996, where methylone (a cathinone analogously to MDMA) was patented as an antidepressant and anti-Parkinsonian agent[66,69].

However, by the early 2000s synthetic cathinones started to appear as 'legal highs' or emerging substances for recreational purposes[66]. They were designed to mimic the pharmacological activity of compounds such as MDMA or amphetamines but avoiding the circumvolving laws[70,71].

Synthetic cathinones were usually sold as 'bath salts' products but instead of the bath salt powders, the package contained psychotropic compounds ready to be consumed[28,70]. These compounds were: 4-methylmethcathinone (mephedrone), 3,4-methylenedioxymethcathinone (methylone), α-pyrrolidinopentiophenone (α-PVP) and 3,4- methylene-dioxypyrovalerone (MDPV) among many others[66,70]. Nowadays, synthetic cathinones represent the second largest group of NPS in the street drug market[14,43,50]. Mephedrone became the headline of synthetic cathinones[70].

These groups of substances were designed to mimic the stimulants effects of forbidden drugs such as METH, MDMA or COCA (see figure 7). Indeed, all cathinones act as inhibitors of the monoamine transporters (SERT, NET and DAT) but with different affinity and in a similar manner to classical drugs of abuse[18]. The different interactions between synthetic cathinones and catecholamines' transporters could explain their distinct clinical effects and toxicities reported[18]. Nevertheless, according to their transporter interaction and users experiences, synthetic cathinones were mainly classified as follows[72]:

i) MDMA-like effects: mephedrone, methylone and buthylone among other substances. These substances have more affinity for 5-HT receptors (especially 5-HT_{2A}), in a similar manner to MDMA[19,40,73].

ii) Cocaine-like effects: MDPV and α-PVP among others. These substances potently block the dopamine and norepinephrine uptake producing dangerous cardiovascular stimulation and a highly addictive potential similar to cocaine[74–76].

iii) Methamphetamine-like effects: cathinone and methcathinone. These substances are described as potent releasers of dopamine and norepinephrine with amphetamine-like stimulants effects[77,78].

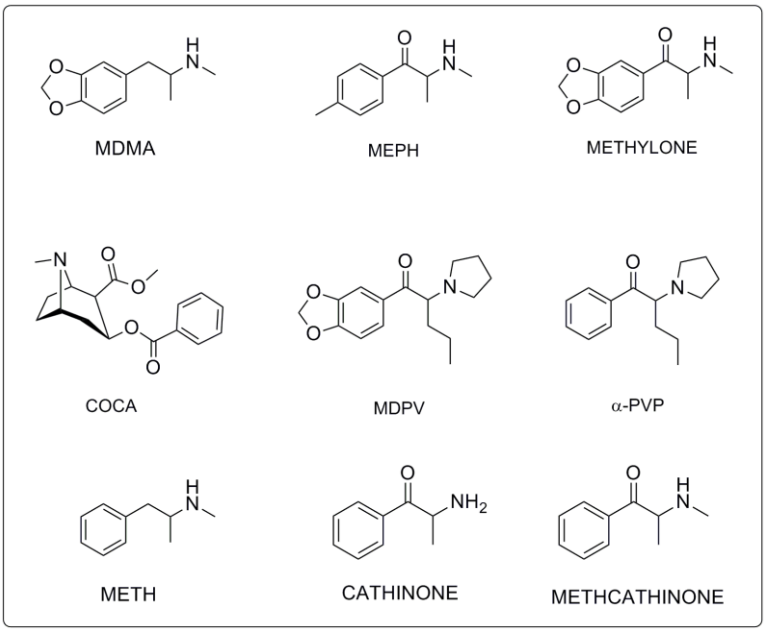


Figure 7. Chemical structures of classical drugs and their synthetic cathinones analogs.

Furthermore, considering that analogs of cathinone (such as pyrovalerone) are relatively easy and cheap to produce as they can be chemically altered in many ways to produce newer group of substances with stimulants, entactogens or empathogens effects, the synthetic cathinones products has become a worrying phenomenon. Indeed, the chemical products currently available represent a small fraction of the conceivable active structures[10]. Moreover, the cathinones studied until now have demonstrated that each compound elicited unique health risks and psycho-activities related effects[10].

1.1.4. Mephedrone

a. Physicochemical properties

Mephedrone, (RS)-2-methylamino-1-(4-methylphenyl)propan-1-one, is a ring-substituted synthetic cathinone derivative, considered one of the most popular drugs in the dynamic NPS scene[14]. It is commonly abbreviated as 4-methylmethcathinone, 4-MMC, or MEPH and it has no license for medical use[79]. MEPH is a β-keto-amphetamine stimulant drug of abuse with structural and mechanistic similarities to methamphetamine and MDMA[79,80].

The synthesis of MEPH is usually performed through precursors such as N-methylcathinone derivatives[81]. Similar equipment and chemical knowledge to that needed for the synthesis of amphetamines or MDMA is required[82]. Moreover, as MEPH also contains a chiral centre at the C-2 carbon of the propane sidechain, it can be synthetized stereo-selective. However, it is usually sold as a racemic mixture (R/S-MEPH)[83].

MEPH is known in the streets as *meow meow*, *miaou miaou*, *bubbles* or *crystal bath* (among other names) and it can be presented as a yellowish liquid (at ambient temperature) or white powder (as the hydrochloride salt)[79].

b. Emergence

MEPH was firstly synthetized in 1929 by Saem de Burnaga Sanchez but remained in obscurity for decades[84]. However, in the early 2000s, clandestine chemist began to modify the chemical structure of cathinone in order to synthetize unscheduled compounds[84]. In 2003, descriptions of MEPH's synthesis appeared online and in 2007 it started to be mentioned in online forums for recreational users[83,85,86]. In subsequent years the popularity of MEPH increased drastically being extensively consumed as

drug of abuse in the late 2008s and with a popularity still maintained nowadays[45,87].

The primary factors in MEPH's popularity were: an extended availability and its legal status. In 2008-2009, there were no restrictions to purchase online MEPH, in terms of minimum age requirements, quantities or customers identifications, and it was no necessary any knowledge of illicit markets as it is required to purchase illegal drugs[88]. MEPH was widespread available online with a very competitive pricing and with a low perception of potential harm[89]. MEPH was usually advertised and sold as 'bath salts', 'incense' or 'plant food/fertilizer' and marked as *not for human consumption* to avoid a potential legislative control (see Figure 8)[84].

Moreover, the Serious Organised Crime Agency (dependence of the UK Government) heralded a large increase in the wholesale cost of cocaine and as an immediate consequence; there was the lowest record of cocaine's purity at the street level[24,88]. The cocaine seized by the English police showed a drop in purity from over 60% in 1999 to 22% in the first quarter of 2009[24]. Additionally, at that time, the ecstasy market started to be highly instable. The purity levels of MDMA dropped drastically and MEPH was misleadingly sold by drug dealers as a legal substitute of ecstasy in countries such as the Netherlands (see Figure 8)[87,90].

So the increase in MEPH popularity was also linked not only for its availability and its legal status, but also as a response to the dissatisfaction with the quality of established illicit drugs such as cocaine and ecstasy, leading users to look for alternative substances with higher purity and easily available[86].

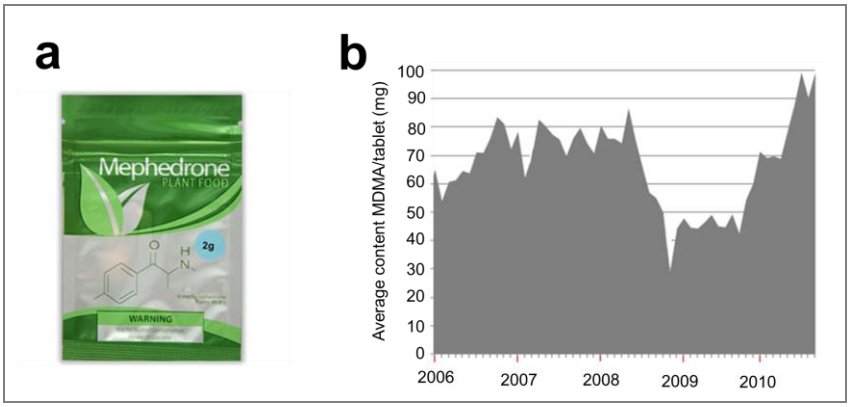


Figure 8. a) Package of MEPH sold in the Internet as bath salts (http://www.plantfeedshop.com/) b) Variations of the average of content of MDMA per tablet (in mg) through 2006 to 2010; adapted from Brunt T. et al[87].

The high prevalence use of MEPH in Europe (associated with reports of numerous hospital admissions and related overdose death consumption) produced that MEPH started to be the spotlight of regulatory agencies[84,91]. Therefore, as a consequence of the media attention and official risk assessment, in 2010 MEPH became one of the first NPS to be banned through the European Union[86,91]. Firstly it became illegal in the UK, which classified MEPH as a Class B substance under the Misuse of Drugs Act on April 2010. Afterward, other European countries and USA also adopted control measures and in 2011, MEPH was submitted to control measures and criminal penalties under the 1971 United Nations Convention on Psychotropic Substances[91,92].

c. Consumption

While global usage rates of MEPH were not documented at that time (2008) but state-level surveys such as the British crime survey showed that users consumed MEPH in a similar rate to traditional and illicit drugs of abuse as cocaine or ecstasy[93]. Indeed, the gain in population of MEPH seemed even higher among subpopulations such as regular club-goers

(with life time usage rates closer to 40% in 2010[94] and around 61% in 2011[95]) although these surveys were not-representative of the wider population of club-goers[96].

After the ban of MEPH and due the (re)emergence of quality drugs of abuse, an initial decrease on MEPH consumption was noticed[84]. Indeed, the Crime Survey for England and Wales estimated that the MEPH use among young people (16-24 years) was 0.9 % at 2015/16 and 0.3% in 2016/17; similar as previously, but down from the 4.4 % observed in 2010/11, before control measures were introduced[25,26]. From 2010, other several single-country surveys of general population or young people samples have been conducted with last year prevalence levels rates that have also between 0.1% and 5%, depending on the country[84] and in 2012, Van Hout and Brennan announced that around half of the users continued using MEPH after it had been banned while the remaining reverted to traditional illicit drugs[97].

So, despite after MEPH prohibition a decline in reported levels use was observed, MEPH still retained its popularity in countries such as UK[98,99]. Besides, the buying and selling of MEPH significantly changed. An illegal market for MEPH emerged after the ban and users continued using MEPH by buying it from street dealers[86] instead of the buying it from the internet[100].

The results of the largest worldwide survey of self-selected convenience samples (Global Drug Survey) listed MEPH in 2014, among the top 20 drugs most used in the last 12 months, with a prevalence of 7.9% in United Kingdom (n=7,326) and 1.2% in Hungary (n=3,239) even though its use was internationally prohibited[101]. During the 2014-2015 in England, there was an increase of death in which MEPH use was detected, similarly to the number of deaths associated to an established recreational

drug of abuse; MDMA[99]. These MEPH-associated deaths occurred among different subgroups of population[99]:

a. Recreational drug users who took the drug in combination with other drugs such as cocaine, amphetamine or alcohol.
b. Intravenous drug users who combined MEPH with drugs such as heroin.
c. Men who have sex with men (MSM) who combined the drug with γ-hydroxybutyrate (GHB) and methamphetamine[99].

To date, the latest official epidemiological data indicate that MEPH continues to be one of the most popular NPS in recreational settings according to indirect estimations[50,84]. MEPH is currently involved in approximately 50% of all hospital emergency presentations related to NPS misuse in Europe, particularly in England[84,102].

MEPH has remained popular in cities as London and it has become an established drug of choice in diverse sub-groups of population of drugs users (e.g. chemsex sessions[103]) alone or in combination with other psychoactive drugs[99]. So, contrary to what usually occurs with other short-lived NPS, MEPH has become established as a permanent illicit drug, probably because of its relatively cheap cost, purity, easy availability (online market and traditional black market) and effects resembling those of classical illicit psychostimulants[100,104–106].

d. Patterns of use

Typically, MEPH is sold with a very high purity (around 99%) in a white or unlighted white powder or as tablets and capsules[84]. The powder can be directly snorted by intranasal route, ingested orally (in tablets, powder or capsules), dissolved with water or saline for intramuscular or intravenous injection and dissolved in an enema or gelatin capsules for rectal

use[81,88,91]. Due MEPH physical characteristics, it is unlikely that MEPH is suitable for smoking even through some users reported its inhalation and vaporization[79,82]. Despite the different administration routes, in recreational use, MEPH is predominantly consumed by nasal insufflation (around 70%) and orally (around 30%)[79,94].

The intranasal administration route (by snorting or insufflation) avoids a possible first-pass hepatic effect or an erratic compound bioavailability, but it has many undesired effects related, such as: nasal burning, clogging and dripping of nasal cavities and even nasal bleeding[42,79,84]. Some users, after the intranasal administration of MEPH shift to oral ingestion in order to avoid the painful effects on the nasal cavity[79]. The oral administration route for MEPH can be usually performed by 4 manners: i) with a direct ingestion of MEPH in tablets or capsules, ii) with a swallowed by 'dabbing' the powder with a wet finger, iii) with wrapping a dose of powder in a paper wrap ('bombing') or iv) with mixing the powder into a beverage and drinking it quickly[42,79,84]. The combination of both administration routes (nasal and oral) during a whole session has been frequently reported as a way to maintain psychoactive effects and avoiding clinical complications. The administration intravenously route of MEPH is practiced among high-risk drug users (that normally inject other drugs such as heroin and amphetamines) or among drug-naïve injecting drug users[42,88,89].

Recreational users reported on Internet forums that MEPH is usually dosed from 15 to 300 mg for oral ingestion and 5 to 125 mg for nasal insufflation[82,107]. Intravenous/intramuscular injection has been reported as half or one third of the oral dosage and around 100 mg is an usual dose for rectal administrations[84,108]. In a similar manner to MDMA, it is common to re-dose in order to maintain the pleasurable effects of MEPH[106]. A typical MEPH session last for around 8 to 10h, consuming in total around

0.5 to 1.9g in 5-6 doses[82,109]. Moreover, it is estimated that consumers that arrive whit acute toxicity of MEPH in the healthcare services had consumed around 0.1-7g of MEPH[82].

e. Pharmacodynamics effects

The pharmacodynamics studies englobe the endogenous response to a specific compound, in terms of biochemical and physiological effects. In the case of MEPH, the knowledge of its pharmacodynamics was crucial in order to understand the drug effects induced in humans.

The pharmacodynamics effects of MEPH have been characterized *in vitro*, in experimental animals and in humans. In humans, most of the information regarding subjective and psychological effects of MEPH was reported in web sites, with questionnaires and surveys, whereas clinical acute toxicity has been provided by case reports from emergency departments[84,110]. The information of pharmacodynamics effects of MEPH in humans under controlled conditions were mainly obtained from the clinical trials performed in our research group[80,111].

The pharmacodynamics of MEPH is presented through its mechanism of action, its physiological effects, subjective and cognitive effects, adverse effects and addictive potential.

Mechanism of action

The mechanism of action of MEPH was mainly described *in vitro* (in rat synaptosomes[19,40], in recombinant human monoamine transporters[112] or in HEK 293 (Human Embryonic Kidney cells)[39] and *in vivo* (in rats[73]).

In vitro, MEPH has a (i) direct interaction with the monoamine transporters, as it compete with the endogenous substrate (DA, 5-HT or NA) to inhibit the uptake in the rat synaptosome[19], similarly to cocaine[22].

However, MEPH is also capable to (ii) reverse the normal transporter flux to induce a transporter-mediated release of monoamines, in a comparable manner to MDMA[73]. The process of release neurotransmitters is predominant versus the competing uptake inhibition[20]. In human recombinant monoamine transporters, MEPH interacted as (iii) substrates of DAT, SERT and NET in a similar manner as MDMA and METH, but with a lower potency in binding the receptor than inhibiting the uptake[79,112]. In HEK-293 cells, results were in the same direction, with a non-selective monoamine uptake and a release of 5-HT in the synaptic cleft[39] (see Figure 9).

In vivo, Baumman et al performed micro-dialysis analysis in the nucleus accumbens of the rats and MEPH produced dose-related increases in extracellular DA and 5-HT, being greater boost in 5-HT concentrations[73].

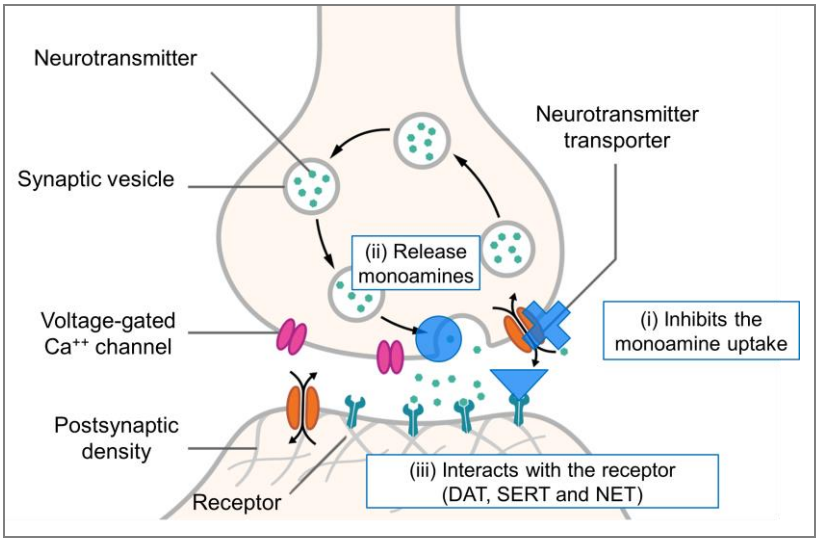


Figure 9. Graphical scheme of the mechanism of action of mephedrone in a typical chemical synapsis. Adapted from www.scistyle.com[113].

Physiological effects

In rodents, MEPH induces dose-related increases of the arterial pressure and heart rate, reverted with β-blockers such as atenolol or α-blockers as phentolamine[114]. Besides, MEPH also causes an augmentation of the locomotor activity, prevented with ketanserin of haloperidol, and it causes hyperthermia[40,73]. The acute locomotors effects of MEPH are analogous to those produced by MDMA[73].

In healthy humans, our research group described the physiological effects of MEPH after the oral ingestion of 200 mg, under controlled conditions[80]. MEPH showed significant increases in the arterial blood pressure along with boosted heart rate. Also it produced a moderate augmentation of the pupil diameter and soft changes in the oral temperature[80] (see Figure 10).

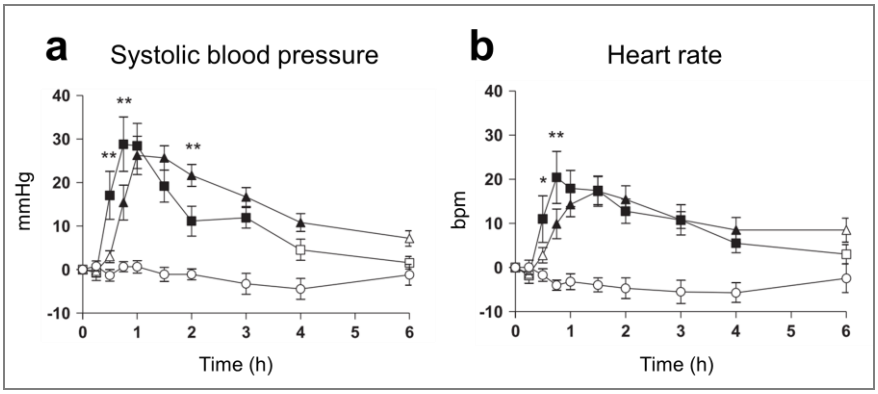


Figure 10. Time-course of physiological effects (n=12, mean, standard error); □, 200 mg of MEPH; Δ, 100 mg of MDMA; ○, placebo. *p<0.05 and **p<0.01 indicate MEPH significant differences from MDMA. Filled symbols indicate a significant difference from placebo (p<0.05). From Papaseit et al[80].

Subjective and cognitive effects

According to surveys data, consumers describe the acute effects of MEPH as typical stimulant drugs[47,48,115]. After its consumption, users reported feelings of:

a. Intense stimulation, alertness and euphoria with a reduced perception of tiredness and increased concentration.
b. Increase in sociability, empathy and feelings of closeness and talkativeness.
c. Intensification of sensory feelings.
d. Mild sexual excitement or stimulation.
e. Loss of appetite, sweating and clenched jaw.

In order to assess MEPH's acute cognitive and subjective effects, Freeman et al, performed an observational (or naturalistic) study with healthy volunteers whom ingested MEPH at their own home[115]. They measured variables such as the visual analogue scale (VAS) or working memory parameters from the intoxicated subjects (MEPH condition) versus drug free subjects (controls)[115]. They found out that MEPH induced an acute impairment in working memory and subjects described higher feelings of depression and paranoia. Remarkably, users reported a "wanting" effect with increase in the psychomotor speed and stimulant-like effects[115]. Another naturalistic study performed by Hergiz et al described that MEPH users had decreased performance in verbal learning and fluency after the drug use[116]. However, the design of these naturalistic or observational studies presented several methodological limitations. These limitations implicated unmonitored variables such as the drug purity, the dose, the possible poly-drug consume or the lack of a clear placebo condition, among others. Our research group described the subjective and cognitive effects after the oral ingestion of 200 mg of

MEPH in a single dose under controlled conditions in order to overcome these limitations[80,111].

With regard to the subjective sensations, MEPH induced stimulant-like effects, euphoria, well-being, feelings of pleasure and mild changes in perceptions. These effects were similarly presented in same volunteers whom consumed MDMA[80] (see Figure 11).

With regard to the cognitive effects, MEPH improves psychomotor performance, impairs spatial memory and seems to unaltered task of divided attention[111]. The impairment of short-term spatial memory is also observed after the administration of drugs such as MDMA[117,118], where the stimulation of 5-HT_{2A} receptors is associated with memory decrements[111,119].

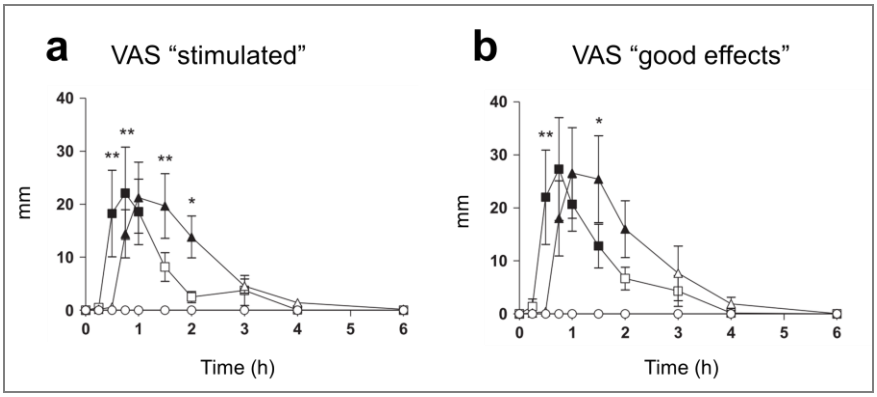


Figure 11. Time-course of subjective effects (n=12, mean, standard error); □, 200 mg of MEPH; Δ, 100 mg of MDMA; ○, placebo. *p<0.05 and **p<0.01 indicate MEPH significant differences from MDMA. Filled symbols indicate a significant difference from placebo (p<0.05). From Papaseit et al[80].

Adverse effects

In addition to pleasurable and well-being effects, recreational user's reported frequently undesirable effects link to MEPH use. These adverse effects include: jaw clenching, bruxism, sweating, palpitations, anxiety, tremor in extremities, blurred vision, shortness of breath, headache, cold or numb extremities, nausea and vomiting, agitation, anxiety, aggressiveness, paranoia, and panic[105,106,120]. In some cases, when acute "amphetamine-like" effects (cardiovascular and neuropsychiatric) are unexpected, experienced as traumatic and/or presenting relevant magnifications, they require emergency medical assistance or hospitalization. In fact, life-threatening toxicity reactions presented as hypertension, cardiac arrhythmia, chest pain, paranoia, psychosis, hallucinations, agitation, aggressive behavior, and suicidal ideation have resulted in emergency room presentations and some deaths attributed to MEPH use[121–135]. These findings are in accordance with more recent official data from Hospital emergency presentations and acute drug toxicity in Europe[102]. In the last report of the Euro-DEN study[136], MEPH was the 8th most commonly reported illicit drug among the emergency presentations registered (986 occasions in 10,956 presentations from October 2013 to September 2015) and the most common NPS involved in deaths (4/5 fatal cases). Overall, 88 presentations involving solely MEPH were in males (68.2%) from 24-33 years (mean 27 years) and MEPH in combination with alcohol (18.2%). The common effects included agitation, anxiety, palpitations, chest pain which required a hospital stay of 3.45 hours (range 2.03-7.19) before discharge, requiring critical care (2.3% cases) and fatal consequences in 1.1%[136]. In the UK the number of individuals requiring treatment for MEPH has more than doubled from 2010-2011 (n=953) to 2014-2015 (n=2,024)[137]. Since 2014, according to data from Imperial College London's Toxicology Unit, the number of

cases in which MEPH has detected in postmortem samples has increased (n=34, 1.5% of total cases) comparable to those in which MDMA is detected (n=28, 1.2% of total cases).

More information about cases of clinical intoxications of mephedrone is detailed in the annex of the thesis [adapted from Papaseit E*, Olesti E* et al. Mephedrone Concentrations in Cases of Clinical Intoxications. Review article. Current Pharmaceutical Design 23,1-12, (2017)[42]] .

Addictive potential

MEPH presents an abuse potential profile similar to MDMA, but with a rapid onset and a shorter duration of their stimulant effects[20,80]. Therefore, to prolong the desired effects, a more compulsive pattern, with several re-dosing per session, is commonly followed by consumers[80]. It is also described that MEPH taken intranasal has a potential of abuse similar to cocaine or methamphetamine[47,138]. Moreover, in heavier users a stimulant withdrawal syndrome has been documented with effects of tiredness, insomnia, impaired concentration, irritability, shivers, increased or decreased temperature, palpitations, depression, anxiety and a strong feeling of paranoia[47,138].

f. Pharmacokinetics

The pharmacokinetics studies englobes the time course of a specific substance in an organism. This information is crucial in order to i) interpret the toxicology of the substance ii) to determine the analytical targets (parents and/or metabolites) evaluating the biological effects of the drug and iii) to provide preclinical safety data[22]. Pharmacokinetics includes the process of Absorption, Distribution, Metabolism and Excretion (collectively known as ADME). The study of these processes is englobed under the *drug disposition* of the targeted drug.

With regard to mephedrone, previous studies have described its pharmacokinetics in rats models[139,140]. In humans, users reported subjective effects after the ingestion of mephedrone and toxicokinetics data was extrapolated from forensic analysis[84]. However, the time-course study of mephedrone and its metabolites was still unknown. No information about the human disposition of mephedrone and its metabolites was found in literature.

The human disposition of mephedrone was investigated in this thesis. In chapter 1 and 2, analytical strategies are developed in order to quantify mephedrone and its metabolites combined with a pharmacokinetics study. In chapter 3 a pharmacokinetics, pharmaco-dynamics and pharmacogenetics study of mephedrone and its metabolites is presented.

Absorption

As the input sources of MEPH are exogenous to the organism, the absorption process is mainly conditioned by the administered dose and the administration route.

With the regard to the administration routes, a subcutaneous administration of MEPH in rats produced maximum plasma levels of the drug within 15-30 min post drug administration[141], whereas after an oral administration of MEPH, rats achieved maximum effects at 0.5-1 h post drug administration[20].

The absolute bioavailability of MEPH fluctuated from 7.3 to 11.2 % in rats at different doses[20], which meant that from the total dose administered by oral route, only around 10% of the drug pass-through systemic circulation (see Figure 12). The bioavailability of MEPH in humans is hypothesized to be equally low, differently to other related compounds

such as MDMA which bioavailability in humans oscillates around 80%[142,143].

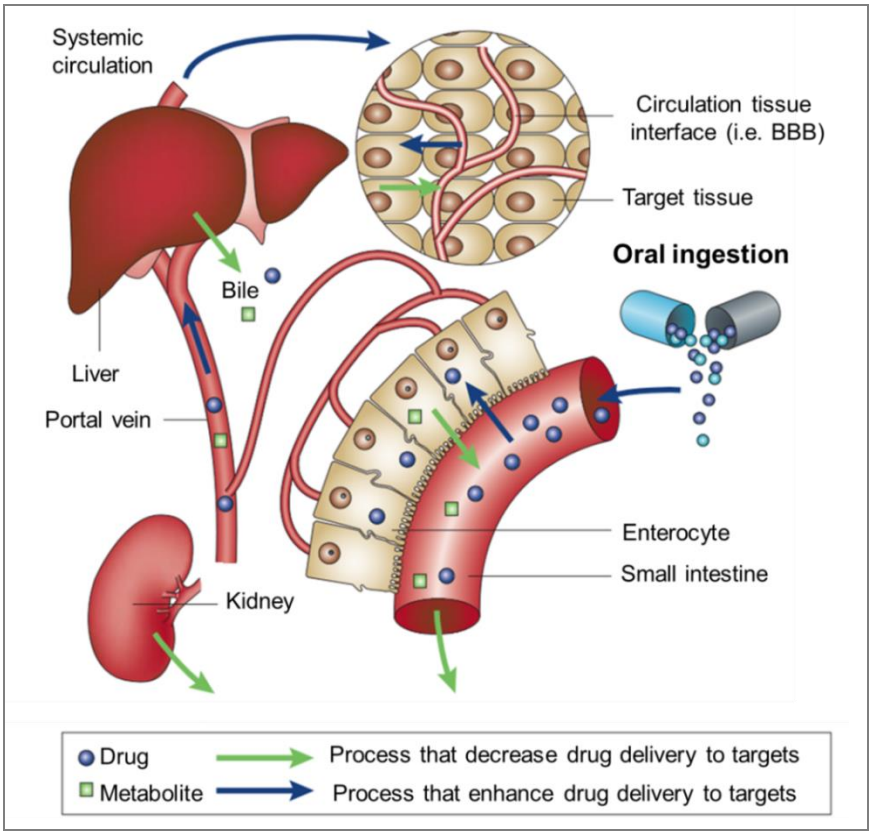


Figure 12. Schematic representation of the variables that condition the disposition of a drug delivery to target sites by oral route. Adapted from Roden D. et al[144].

Distribution

Once absorbed, MEPH is extensively distributed throughout the tissues, which fit with an open two-compartment model[20]. MEPH is described to cross the blood-brain barrier due its lipophilic characteristics to produce a stimulant-like effect[20]. However, MEPH has a lower brain penetrance in comparison with other related compounds such as MDMA or amphetamine, as its protein binding rate was about 22%[20].

Metabolism

The metabolism of MEPH was described in rats[20,145], in liver hepatocytes[146], in human liver microsomes[85] and in human samples[147]. MEPH (and its metabolites) suffered biotransformation pathways[147] through the liver metabolism (see Figure 14) in order to detoxify potentially harmful compounds and removing them from the circulation and excreting them from the body. The biotransformation pathways of the phase I metabolites were mainly mediated by the cytochrome P450 2D6, with some other contribution of NAPDH-dependent enzymes[79,85]. Our research group described several phase I and phase II human metabolites of MEPH[147] (see Figure 13).

MEPH **phase I** metabolites included:

i) Reduction of the keto function to form 1'-dihydro-mephedorne (DIHYDRO-MEPH).

ii) Hydroxylation of the methyl of the aromatic ring, to form 4'-hydroxymethyl-mephedrone (or 4'-hydroxytolyl-mephedrone) with a posterior oxidation of the benzylic methyl to carboxylic acid forming 4'-carboxymephedrone (COOH-MEPH).

iii) Hydroxylation of the propyl side chain.

iv) N-demethylation of the primary amine to form N-demethyl-mephedrone or nor-mephedrone (NOR-MEPH), with a posterior hydroxylation.

MEPH **phase II** metabolites include reactions of:

i) Glucuronidation of the alcohol to form hydroxylmephedrone-3-O-glucuronide and hydroxylnor-mephedrone-3-O-glucuronide, or the secondary amino group to form 4'-carboxy-mephedrone-N-glucuronide.

ii) Succinylation of the primary amino group of the nor-mephedrone to form N-succinyl-nor-mephedrone (SUCC-NOR-MEPH). The succinyl conjugation was reported firstly in humans by discovering SUCC-NOR-MEPH.

Occasionally, the liver metabolism may activate compounds (so the xenobiotic becomes a pro-drug) or it may produce intermediates that can induce toxicity[148]. In the case of MEPH, its metabolites 4'-hydroxymethyl-mephedrone, DIHYDRO-MEPH and NOR-MEPH were transporter substrates of DAT, NET and SERT *in vitro*. Furthermore, *in vivo*, NOR-MEPH increases extracellular dopamine and 5-HT in the brain[148].

Despite the good characterization of MEPH metabolites in humans and their biological activity, their metabolic disposition still remains unknown. In this thesis, the relevance of the metabolic disposition is investigated for the pharmaco-toxicological evaluation of MEPH.

Excretion

Human and rat studies revealed that MEPH metabolites are excreted in urine suggesting that MEPH's elimination is mediated through the kidney[20,140,147,149]. However, there is a information gap on the mephedrone's recovery and its metabolic ratio in humans.

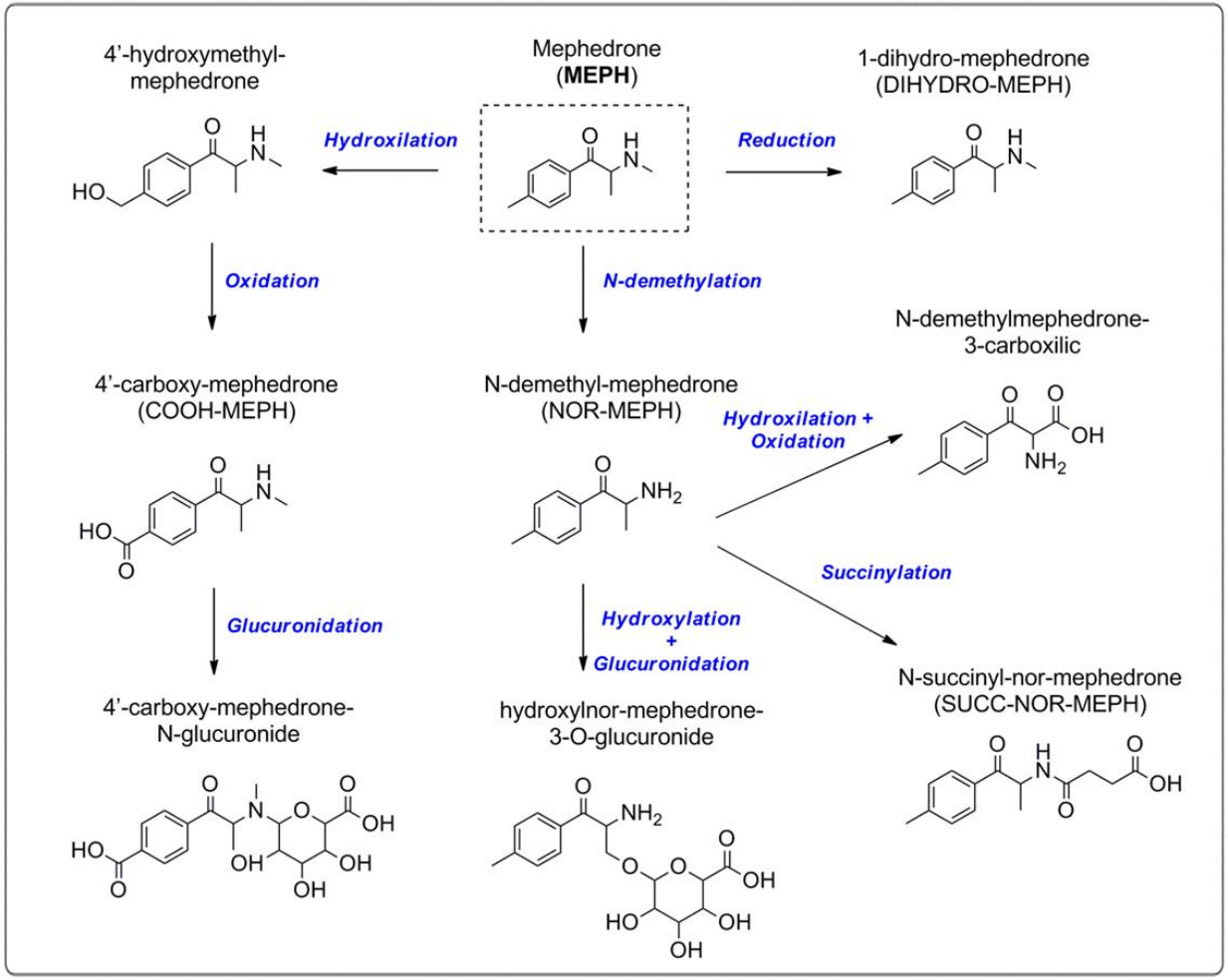


Figure 13. Human metabolism of MEPH. Adapted from Pozo et al.[147]

1.2. Analytical strategies

1.2.1. Definition

Analytical Chemistry is a scientific discipline that develops and applies methods, instruments and strategies in order to identify compounds and mixtures (qualitative analysis) or with the aim to determine the proportions of the constituents (quantitative analysis)[150,151]. The analytical strategies commonly used include spectroscopy, immunoassays, chromatography, etc[151].

Over the last decades, chromatographic techniques coupled to mass spectrometry (e.g. GC-MS or LC-MS/MS) have become powerful analytical tools able to identify and quantify small molecules with a broad range of physicochemical properties (from cations/anions to non-polar compounds) in diverse types of biological samples (plasma, urine, hair, sweat, oral fluid, etc)[152]. These small molecules include indistinctly either endogenous metabolites (e.g. neurotransmitters) or exogenous compounds (e.g. drugs of abuse, NPS)[152].

For the quantitative analytical determination of the pharmacokinetic studies (Part I) and the metabolomics analysis (Part II) of this thesis, chromatography techniques coupled to mass spectrometry were used.

a. Chromatography

The main aim of chromatography techniques is to separate different compounds of a mixture, according to the compound's relative affinities for a liquid/gaseous mobile phase and a solid stationary phase[153]. The stationary phase is hold with a chromatographic column where the compound separation takes place[154]. The compounds are separated due to their chemical properties (e.g. polarity, electrical charge or molecular size)

and recorded in a detector at the time of elution from the chromatographic system[153,155]. The effectivity of the separation critically depends on the mobile and stationary phase.

In the gas-chromatography (GC) technique, the sample is heatedly injected via a transporter gas (mobile phase, normally He, H_2 or N_2) into the chromatographic column (stationary phase, normally) and analytes are separated through a range of temperature program in the column.

In the liquid-chromatography (LC) technique, the sample is transported with the mobile phase (normally) through the chromatographic column (stationary phase, normally). The mixture of analytes is separated through a gradient elution with organic (such as methanol and acetonitrile) and aqueous solvents.

The chromatographic techniques are coupled to different detectors types, as an example: thermal conductivity receptors (for GC), ultra violet detectors (for LC) and mass spectrometers (for GC and LC)[153]. The coupling of a chromatographic technique and the mass spectrometer was used in this thesis.

b. Mass-Spectrometry

The mass-spectrometry technique (MS) is based on the formation of ions that are subsequently separated according to their mass-to-charge (*m/z*) ratio and detected[154]. The MS is composed with:

i. Ionization of the compounds at the ion source. The types of ionization in the ion source are commonly hard ionization (predominantly used in GC) and soft ionization (commonly in LC). The hard ionization technique produces multiple fragments when high-energy electrons interact with the gaseous molecules (electronic ionization, EI). There are mass spectral libraries used for

identification of EI spectra in GC-MS. In contrast, the soft ionization (with approaches such as the electrospray ionization, ESI) the fragmentation in the source is limited and the molecular ion is mainly observed. The ESI used in LC-MS converts the liquid sample into an aerosol that can be ionized by a strong electric field. The ESI is an atmospheric pressure ionization (API) interface between the liquid chromatography and the mass analyzer[156]. ESI can be performed in positive or negative mode for a wider analyte coverage[154]. The molecular species formed at the ion source are (habitually) $[M+H]^+$ in ESI+ for analytes with high proton affinity (basic) and $[M-H]^-$ in ESI- for analytes which transfer a proton to the solvent (acids). However, other molecules species or adducts can be formed such as $[M+Na]^+$, $[M+Cl]^-$ and $[M+NH_4]^+$.

ii. Mass analyzer: the ions are separated according to their *m/z*. There are different types of mechanisms to separate ions in the mass analyzer with high resolution analyzers (such as time-of-flight (TOF) or Orbitrap) and low resolution analyzers (such as the triple quadrupole)[157]. In high resolution analyzers, the mass measurements are accurate and provide detailed structural information. In contrast, low resolution analyzers are used with specific transitions in order to obtain higher sensitivity and unequivocal compound identification and quantification[157]. In this thesis, a low resolution strategy using a triple quadrupole was followed.

A quadrupole consist in four parallel rods that can selective filter a *m/z* ratio of the ions that travels through. The combination of the chromatography's techniques with a single quadrupole strategy was materialized in the LC-MS and GC-MS equipment. However, in order to gain compound specificity and sensitivity, triple quadrupoles (LC-MS/MS and GC-MS/MS equipment) were developed by adding

a collision cell and another quadrupole in the mass analyzer (QqQ). The QqQ presented different scan modes, utilized depending on the purpose: full scan, selected ion monitoring (SIM) or selected reaction monitoring (SRM), product ion scan, precursor ion scan and neutral loss scan[154]. In the SRM, a selected precursor ion of our targeted compound is isolated in the first quadrupole (Q1), then fragmented in the collision cell (q2) and specific fragments or products ions are filtered in the third quadrupole (Q2)[158]. The selection of the parent ion and the product ion is known as a transition[158]. A schematic representation of the basic LC-MS/MS functionality in SRM mode is represented in Figure 14.

iii. Registration of the ions with the detector. The detector produces a signal of the relative intensity or abundance of the recognized ions (and fragments). There are different types of detectors such as the secondary electron multiplier or the photonic conversion detector, among others. The photonic conversion detector applies a voltage to provoke the attraction and shock of the ions to an initial dynode. This shock produce multiple photons that in vacuum conditions move to the photomultiplier and the electron chain multiplies the signal[159].

The combination of chromatography techniques coupled to mass spectrometry provided separation characteristics with an increase of the compounds identification and sensitivity.

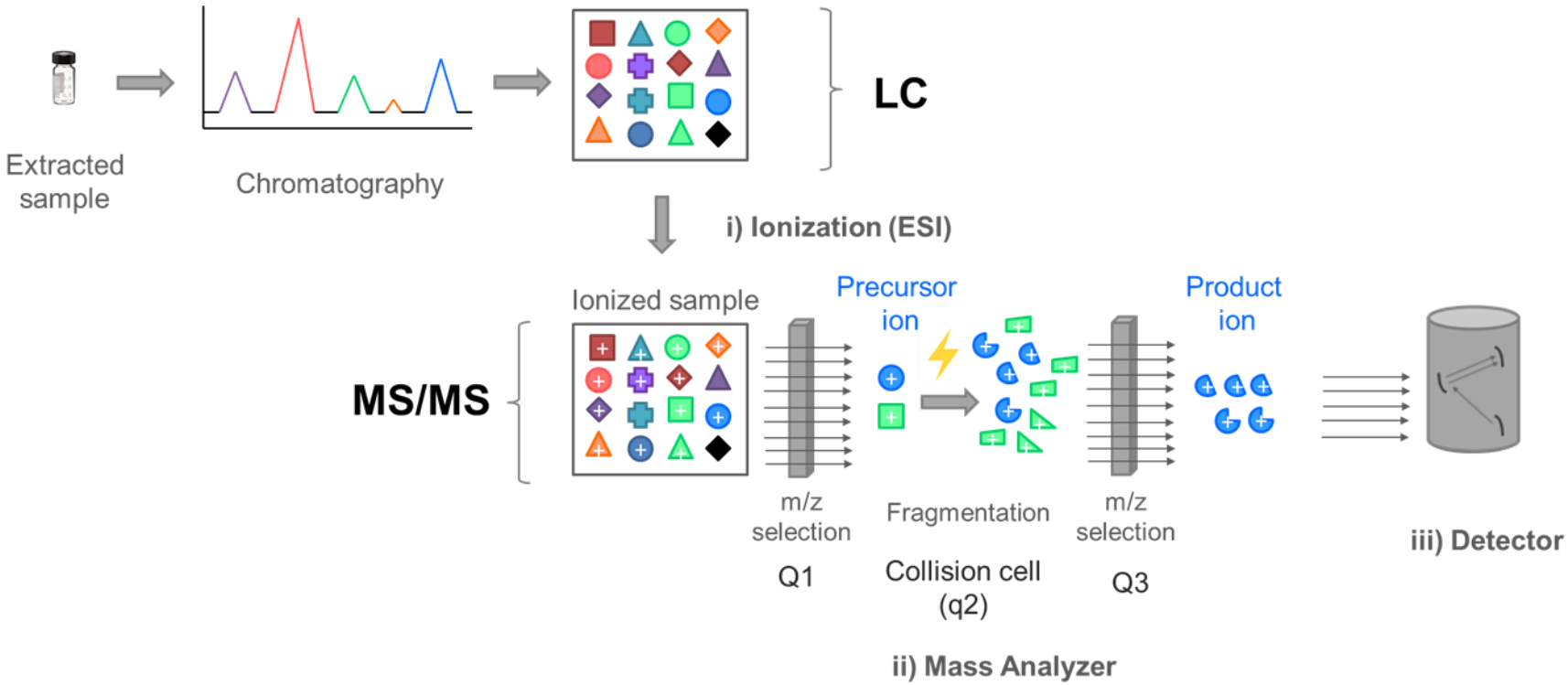


Figure 14. Schematic representation of a LC-MS/MS equipment in SRM mode. The different colorful geometrical figures represent molecules which are firstly separated in the LC, then ionized (ESI+) and filtered through the first quadrupole. Analytes are fragmented in the collision cell and selected in a second quadrupole. Finally all data is analyzed by the photomultiplier detector.

The measurement of specific compounds (exogenous and endogenous) with high- precision and accuracy has become the commonly chosen analytical strategy applied in fields of pharmacology and toxicology, in applications such as: pharmacokinetics and toxicokinetics studies[160], diseases diagnosis[161], therapeutic drug monitoring[162], forensic identifications[163] or metabolomics studies[164] among others.

In this thesis we have approached the challenges of evaluating NPS by measuring the concentrations of mephedrone in different matrices and by measuring the endogenous alterations of targeted metabolites after the administration of different NPS.

1.2.2. Exogenous compounds: mephedrone

The exogenous compounds have the peculiarity that they are originated externally from the organism. Substances such as drugs, pesticides or pollutants are considered exogenous. The measurement of drug concentrations in biological matrices (such as serum, plasma, blood, urine, and saliva) is a crucial step in order to understand its biological behavior.

The analysis of mephedrone has been mainly performed with immunoassay methods and chromatography strategies coupled to mass spectrometry:

i. The immunoassays methods had a main limitation on its use, as the technique requires the previous development of antibodies and commercial immunoassays, limiting its use for emerging substances such as MEPH. Even though, a semi-quantification commercial immunoassay for urinary cathinone detection (including MEPH) was developed but with a large negative percentage of bias[22,165].

ii. Through the use of chromatographic techniques (GC or LC) coupled to mass spectrometry (MS or MS/MS), MEPH was widely identified and

quantified by GC-MS and LC-MS/MS in a grand variety of matrices, such as: dried blood spots[166], in dried plasma[167], in dried urine[167], in blood[168,169], in urine[170] and in oral fluid[167,171,172] and hair[173–175].

However, as far as we know, there are no validated methodologies for the quantification of mephedrone and its metabolites in human plasma and urine or in rodents' brain homogenates. But, its monitorization is fundamental and necessary in order to understand de human disposition of mephedrone and its metabolites. Analytical strategies to quantify mephedrone and its metabolites in human matrices are essential to i) report the clinical and forensic behavior of MEPH and metabolites, ii) link the causative agents of the drugs to their toxicological effects, iii) enhance the interpretation of the drug performance in living humans and postmortem cases[22] and iv) to monitor its use in professional sports competition[176,177] (mediated by WADA).

The simultaneous quantification of MEPH and its metabolites supposes an analytical challenge due to the different physicochemical characteristics of mephedrone' metabolites. For instance, COOH-MEPH is a highly polar compound, whereas SUCC-NOR-MEPH has less polar affinity.

1.2.3. Endogenous compounds: metabolomics

The endogenous compounds are characterized due to their natural occurrence in biological matrices. Metabolomics covers the study of the endogenous compounds in biological matrices.

a. Definition

The term "omics" encompasses a set of techniques that simultaneously studies thousands of molecular species of specific biological matrices (in cells, tissues, organs or organisms)[178–180]. These molecular species include

gens (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics), see Figure 15. Metabolomics is the most recent research field of the so-called omics-techniques cascade. Omics technologies can be applied to increase a better understanding of physiological process, drug responses and diseases among other challenges[178,179].

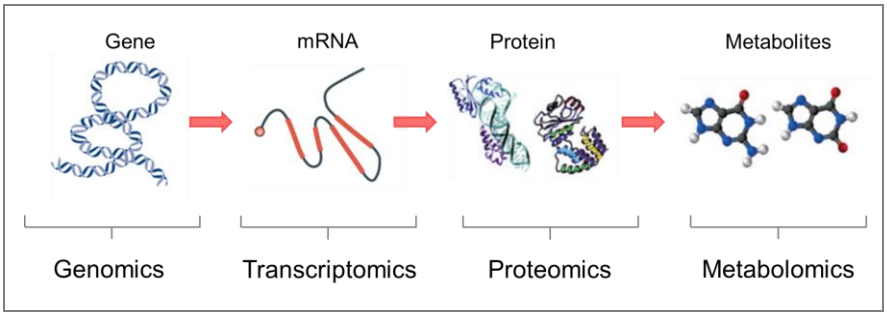


Figure 15. Schematic representation of the *omics* techniques and their subjects of study. Adapted figure from Patti G J et al.[181]

By applying the omics technologies a vast quantity of data is commonly obtained. This data is usually combined with computational and mathematical approaches in order to model a complex biological system[178]. The combination of omics data and bioinformatics (which is the core for systems biology) leave to data interpretation and comprehensive and systematic analysis.

Metabolomics refers to the systematic identification and quantification of the small molecule metabolic products (normally weighting < 1500 Da) of a biological system[181,182]. These molecules with low molecular weight include a wide range of compounds classes such as sugars, amino acids, fatty acids, nucleosides and organic acids. The complete set of small-molecules chemicals of a biological system constitutes the metabolome. Changes in the metabolome can be produced by an internal stimuli or after

the exposure to xenobiotic sources such as microorganisms, dietary or drug exposure[182].

b. Emergence

The ancient Chinese cultures (1500-2000 BC) started to recognize urine matrix as a source of health-related information, being sweet-tasting urine as indicative of a disease (nowadays known as diabetes)[183]. But, it was not until the 1971, due the technology development of techniques such as chromatography; than Linus Pauling and collaborators conceived the idea that the measurement of individual components in biological fluids could provide valuable information that would reflect the functional status of a complex biological system[184] and in 1998, Stephen Oliver[185] used firstly the term *metabolomics*. Early 2000, it was suggested the use of metabolomics as a new technique for rapid toxicity screening. Shortly after, the Consortium for Metabonomic Toxicology (COMET) was formed between five pharmaceutical companies and the Imperial College of Science, Technology and Medicine (IC), in London, UK[186] with the objective to predict liver and kidney toxicity after the exposure to several compounds though metabolomics. Additionally, in 2007 David Wishart developed the Human Metabolome Database[187], a freely electronic database containing detailed information form more than 100.000 endogenous metabolites found the human body[187] (http://www.hmdb.ca). Nowadays, HMDB presents comprehensive information about metabolites and its biological roles, physiological concentrations, diseases associations or metabolic pathways, etc[188].

From then, the interest in metabolomics has grown exponentially in the last decade (see Figure 16). The number of scientific papers related with metabolomics published in PubMed increased more than 15 times between 2007-2017 (as it is shown in Figure 16).

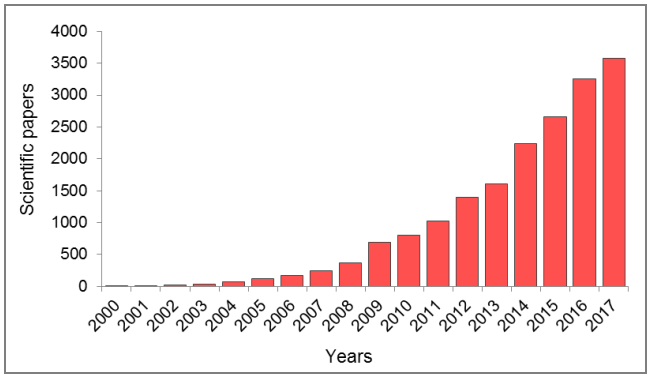


Figure 16. Growing number of scientific papers published in PubMed during 2000-2017 containing the word *metabolomics*.

c. Advantages

The principal benefit of the metabolomics approach is its close relation with the phenotype. During the last decades, the grand development of the transcriptome profiling permitted new mechanistic insights such as novel candidate genes for diseases and genetic risk scores among many others discoveries[179,180]. The genome studies described *what might happen* in the organism causing vast advances in biomedical research. However, their discriminative power to separate case and controls samples was very low[179]. The extensive advances of novel analytical techniques (such as chromatography coupled to mass spectrometry) lead the development of metabolomics as a new approach to study the gene-environment interactions in organisms. The metabolomics profiling indicate *what is currently taking place* in the organism, with a robust discrimination of cases and controls based on the gene expression profiles[179,180]. So, compared with the genome, the metabolome study (gene-derived metabolites and environmentally derived metabolites) appeared to be much closer to the phenotype, generating a quantitative molecular phenotype[189]. Therefore, the monitoring of the metabolic responses might

have more predictive power in identifying phenotype alterations as it is directly linked to genes and environment[179,180].

The metabolome is extremely dynamic as changes on a studied system are immediately reflected in the metabolome. In this manner, metabolomics can reflect a real-time status of the biological system[190] with a direct signature of biochemical activity[157].

d. Classification

The main methodologies that are used for metabolomics experimental studies are based on untargeted or targeted approaches. On Figure 17, a schematic workflow for both approaches is presented with its main characteristics and procedures[181].

Untargeted metabolomics

The untargeted approach in metabolomics aims to identify and quantify as many endogenous metabolites as possible, present in a sample without *a priori* expectation about the changes on the metabolome[181,182]. The metabolites quantified depend on the matrix, the extraction process and the analytical methodology used. The output of an untargeted metabolomics approach is a complex dataset that requires computational tools to correlate the chromatographic peaks with knowing metabolites structures (obtained from the libraries or synthetized standards) and to identify their relation in a specific metabolic pathway. The strength of the untargeted approach lies on the unbiased analysis of the sample, obtaining unexpected information without initial hypothesis required. On the other hand, a limitation on this approach is the low sensitivity achieved with the current analytical methodology and the no-absolute quantification of the metabolites[181,182]. Therefore, in the untargeted approach, only important

changes on the most prevalent metabolites are observed and either subtle alterations or less abundant metabolites can be overlooked.

Targeted metabolomics

The targeted approach in metabolomics aims to quantify a predefined set of metabolites which could be involved in a common metabolic pathway or have structural similarities. These targeted metabolites are *a priori* known, whereby analytical strategies are developed and optimized for the analysis of specific metabolites and metabolic pathways of interest[181,182]. To perform a targeted metabolomics approach a bioanalytical validation method is required (with reference standards) to confirm the robust and correct quantification of the selected analytes[181,182]. The strength of a targeted metabolomics analysis is its capability to monitor high specific and less abundant compounds. It provides a high sensitivity and selectivity analysis of the metabolites, with an initial hypothesis required. Moreover, as the metabolite quantification is previously validated the obtained concentration is robust and reliable. On the other hand, a limitation on the targeted approach is the time-cost of the validation procedure for each set of metabolites and the possible bias selecting only a specific part of the whole metabolome.

In the presented thesis, a targeted metabolomics study was performed by quantifying several metabolic pathways related with the mechanism of action of drugs of abuse. These pathways were: neurotransmitters pathways (monoamines and related amino acid neurotransmitters) and steroid hormones.

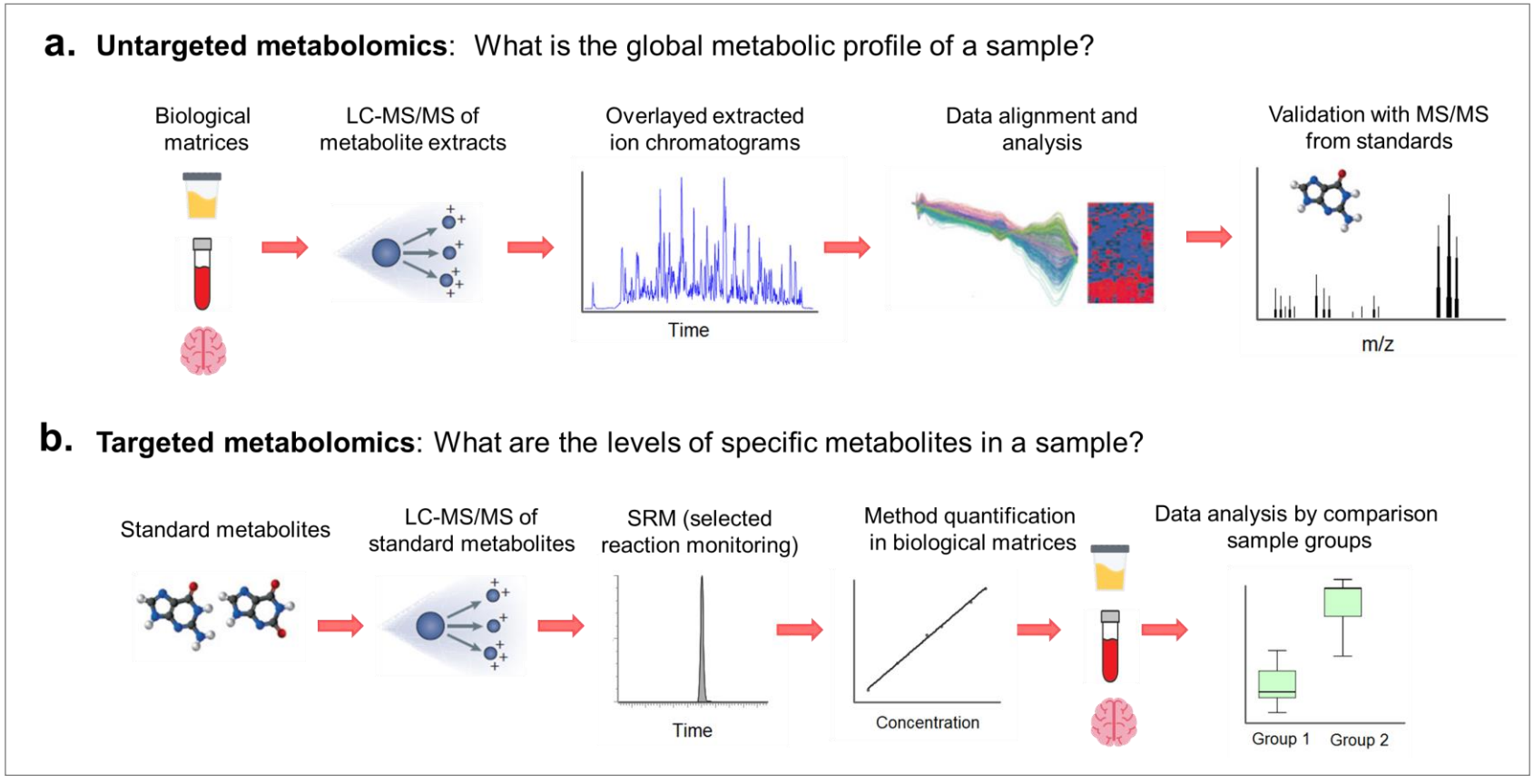


Figure 17. Schematic workflow of targeted metabolomics and untargeted metabolomics experimental design. Adapted figure from Patti G J et al.[181]

e. Strategies to quantify the (un)targeted metabolome

Due to the broad variety of molecules classified as metabolites with different physicochemical properties and abundance, it is (nowadays) impossible to monitor the entire metabolome[157]. The metabolome coverage is in compromise with the sample type analyzed and the analytical methodology used (schemed in Figure 18). The scope of the analysis (untargeted or targeted) depends on the abundance of the metabolite in the sample and the sensitivity of the instrument to quantify the metabolite (see Figure 18). Metabolites' abundance depends principally on the matrix studied and the instruments' sensitivity for a metabolite (chromatography, fragmentation and ionization steps). Consequently, the combination of different types of sample treatments and analytical instruments can increase the metabolic coverage[189].

The metabolome covering of an untargeted approach focuses on large quantities of metabolites in a sample that can be detected by the instrumental technique. In contrast, the covering of a targeted approach centers with the sample and instrumental optimization for the detection of a specific metabolic pathway.

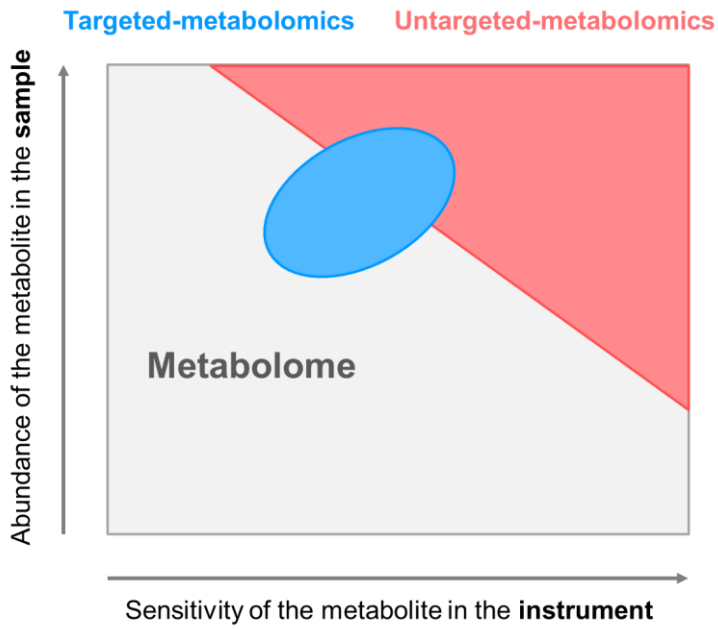


Figure 18. Schematic representation of a simulation of the whole metabolome, ordained in Y-axis metabolites by its abundance in the sample and in X-axis by its sensitivity in the instrument. The regions in color represents a possible metabolite covering by untargeted-metabolomics (in red) and by targeted-metabolomics (in blue).

The most common analytical techniques used in metabolomics studies are nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (MS)[180,191]. The MS-based techniques are usually in combination with a previous chromatographic separation coupled to the MS. The main advantages of the MS techniques in comparison with the NMR are the higher sensitivity with smaller sample volumes and relatively lower instruments costs associated. Moreover, the benefits of NMR techniques are the non-destructive sample preparation with no separation process[180].

f. Applications

Recently, metabolomics has become a potential tool for many scientific applications. A few examples of metabolomics applications are[180,189]:

a. To diagnose a pathology (metabolomics biomarkers).

b. To understand mechanism of diseases.

c. To monitor the effectiveness of a treatment (biomarkers of drug efficacy) and disease status (biomarkers of diseases).

d. To associate risk factors with certain pathologies.

e. To facilitate drug discovery and development (especially in drug safety by measuring acute preclinical drug toxicity).

f. To discriminate potential positives of drugs' consumption[192].

Furthermore, metabolomics has been also used as a tool to characterize related drug abuse out-comes, such as: drug addiction[193,194], drug acute intoxications[195], prolonged intoxications[196–199], drug-related diseases characterizations[179] or multi-dose drug administration[200]. These metabolomics studies have monitored biomarkers of the tricarboxylic acids, sugars and amino-acids that contributed notably to a better understanding of the drug-relation consequences. However, a difficult interpretation of the results was derived. Moreover, in some cases, the targeted metabolome selected is not directly linked to the drug's mechanism of action, providing an indirect measurement of the studied situation.

In the context of a growing challenge of the NPS evaluation, targeted metabolomics is proposed as potential tool to predict the *in vivo* drug pharmacology.

g. Metabolomics pathways

In this thesis, the targeted metabolomics pathways monitored were: monoamine neurotransmitters, amino acid neurotransmitters and related compounds and steroid hormones.

Monoamines pathways

Monoamine neurotransmitters are a group of molecules that contain one amino group connected to an aromatic ring by a carbon-carbon chain[157]. They are endogenously synthetized by the decarboxylation of the aromatic amino acids (phenylalanine, tyrosine and tryptophan). These neurotransmitters (serotonin, dopamine and noradrenaline) are involved in the regulation of cognitive process such as emotion or memory and related in the pathogenesis of several mental disorders[201]. The mechanism of action of several drugs of abuse is strongly and directly related to the synthesis, release, reuptake and metabolism of the monoamines neurotransmitters, being mainly all abuse drugs substrates of the monoamine transporters with different degrees of affinity[3,73].

In terms of synthesis, packaging, release and degradation, the monoamine neurotransmitters share similar metabolic pathways but differently distributed through the brain neurons. These biogenic amines are synthetized from the hydroxylation of essential amino acids and metabolized mainly by the mono-amine oxidase (MAO) and the catechol-O-methyl transferase (COMT) (see Figure 19). In detail, serotonin is formed by the dietary tryptophan while dopamine from dietary phenylalanine and tyrosine. Noradrenaline is formed by the hydroxylation of dopamine. The metabolism of monoamine neurotransmitters by MAO enzyme produces the metabolites: 5-Hydroxyindoleacetic (5-HIAA) (if the precursor is serotonin), DOPAL and posteriorly 3,4-Dihydroyxphenylacetic acid (Dopac) and homovanillic acid (HVA) (in the

case of dopamine). The action of the COMT produces normetanephrine (NM) and metanephrine (MM) (from noradrenaline) and 3-methoxy-tyramine (3-MT) (from dopamine) (see Figure 19)[202,203].

For the quantification of multiple monoamine neurotransmitters and related compounds, the use of mass spectrometry is preferred in order to achieve high sensitivity with a small amount of sample use[204]. There are several LC-MS/MS methods for the detection of Trp[205] and its metabolites as well as Tyr and its metabolic pathway[206]. The treatment applied for purifying the sample include solid-phase extracions (SPE) and liquid-liquid extractions (LLE)[157] or dilute and shoot strategies[204]. Furthermore, some methodologies have also quantified monoamines and metabolites by derivatizating these compounds with reagents such as Dansyl Chloride[207]. Due to the hydrophilicity and polarity of these compounds, monoamines are poorly retained in conventional reverse-phase columns[157] and they can be monitored either in positive or in negative mode (ESI) [ESI^+ protoning amino groups and ESI^- by desprotoning hydroxyl radicals][157].

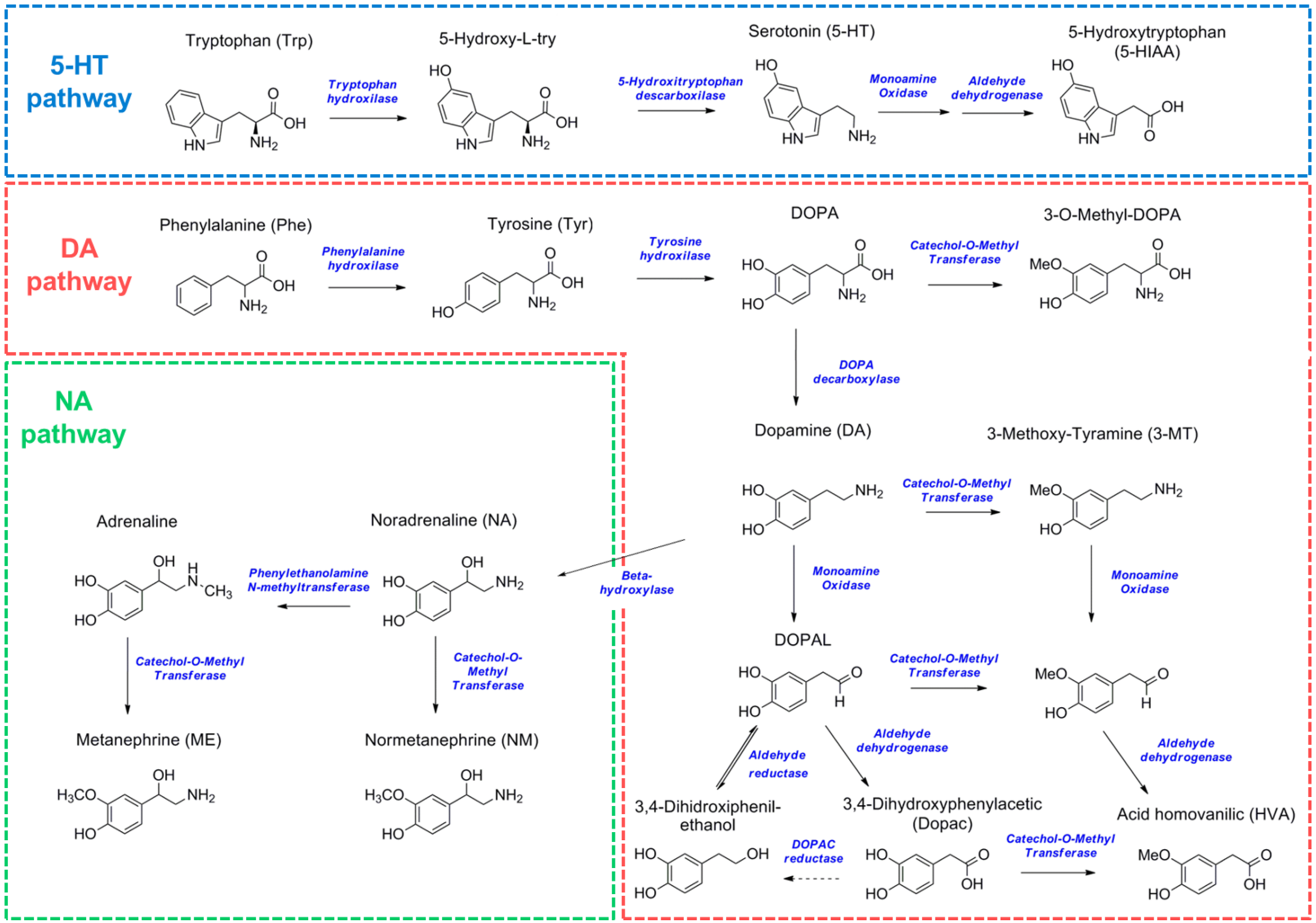


Figure 19- Metabolic pathway of monoamines neurotransmitters (precursors and metabolites).

Amino acids neurotransmitters

Other groups of neurotransmitters include amino acids (e.g. glutamic acid, glycine, γ-aminobutyric acid or glutamine), cholinergic (e.g. acetylcholine) and purines (e.g. adenosine)[208]. These diverse groups of neurotransmitters have in common their highly polar physicochemical characteristics. The long neutral amino acids (LNAA), which are brain neurotransmitters precursors, are the aromatic amino acids (Tyr, Phe and Trp) and the branched-chain amino acids (Leu, Ile and Val)[209]. These essentials amino acids (obtained from the diet) can modify their conversion to neurotransmitters. With regard to the acidic amino acids (glutamate, glutamine or GABA), they are neurotransmitters themselves, but their dietary intake does not have a direct access to brain tissue. Glutamate is the main excitatory neurotransmitter while GABA act as the principal inhibitor[210]. Another relevant neurotransmitter is acetylcholine, essential chemical implicated in the autonomic nervous system, in the neuromuscular junction and many other cell functions[211]. From the purines group, adenosine acts as a neuromodulator by playing an important role from the neuronal excitability[212]. Other compounds related to the amino acids neurotransmitters are carnitines (carnitine and acetylcarnitine) which are related to the body transport of fatty acids in the mitochondria[211]. Alterations of the concentrations of these compounds have been also related with the administration of several drugs of abuse[213,214]. In figure 20 a brief summary of the chemical structures and metabolic pathways of the mentioned compounds related to the amino acid neurotransmitters are shown.

For the quantification of amino acid neurotransmitters and related compounds, a number of methods for the analysis of polar neurotransmitters (e.g. glutamate or γ-aminobutyric acid) and related compounds in biological matrices have been developed using LC-MS/MS.

Typically amino acids and related compounds (such as carnitines) are monitored without any extraction step prior to the analysis. Furthermore they can be quantified with and without any derivatization step[157]. One of the challenge to quantify amino acids and related compounds is their high hydrophilicity and polarity, which causes low recoveries and poor retention times with in conventional reverse-phase columns (such as C_{18})[157,215].

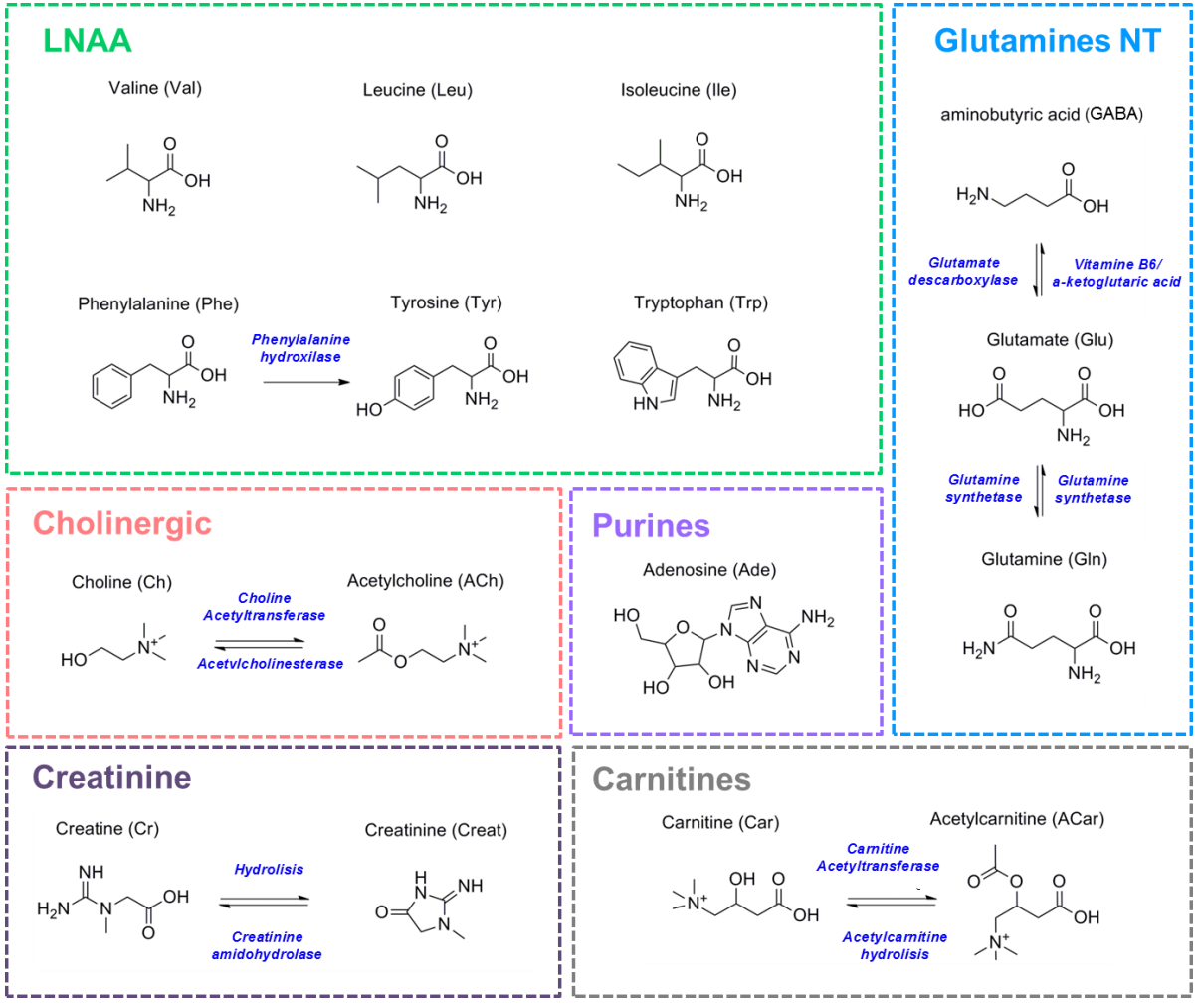


Figure 20. Chemical structures and metabolic pathways of amino acid neurotransmitters and related compounds (LNAA, glutamatergics, cholinergics, purines, creatinines and carnitines).

Steroid hormones

Steroid hormones are a group of cholesterol derived compounds secreted by the adrenal cortex, testes and ovaries and by the placenta during pregnancy. Steroid hormones are involved in development of sexual characteristics, immune functions and inflammation among many other pharmacological effects .They are classified into four groups, accordingly to their binding receptors: progestogens, corticoids (subdivided in glucocorticoids and mineralocorticoids), androgens and estrogens (see Figure 21). All groups of molecules have a cyclopentano-perhydrophenanthrene skeleton in common[157].

Cholesterol is the main precursor of the steroid hormones. It is firstly metabolized to pregnenolone and activated to progesterone (Prog), from the progestogens group. Progesterone is the precursor of 11-deoxy-corticosterone (DOC) and corticosterone (B), both with corticosteroid activity in rats. The inactive form of corticosterone is 11-dehydrocorticosterone (A). Through the metabolism of pregnenolone, androgens are formed. Androstenedione (AED) and testosterone (T) are responsible of the sexual hormones from the organism (see Figure 19).

Some drugs of abuse, are described to alter the hypothalamic–pituitary–adrenal (HPA) axis, increasing the release of some of the steroid hormones such as corticosterone in rats and cortisol in humans [199,216].

The quantification of the steroid hormones are typically performed by GC-MS after their hydrolysis and derivatization[217]. But, lately, the introduction of LC-MS/MS provided new advantages for the steroid hormones analysis, such as a shorter analysis time and a direct detection of conjugated steroids[157,217]. The steroid hormones have low polarity properties and consequently they are commonly excreted as phase II metabolites (such as glucuronides or sulphates). Traditionally, steroids are

released from its conjugated form by enzymatic hydrolysis. Enzymatic preparations (i.e. β-glucoronidase and/or sulfatase) are typically used. Then unconjugated steroids can be extracted from the matrix with LLE and SPE procedures[157,218]. These extraction steps are also useful for preconcentrating some of the low abundant steroids metabolites. For the sample collection, the presence of circadian rhythms for some of the steroids metabolites has to be minimized to have comparable results[157].

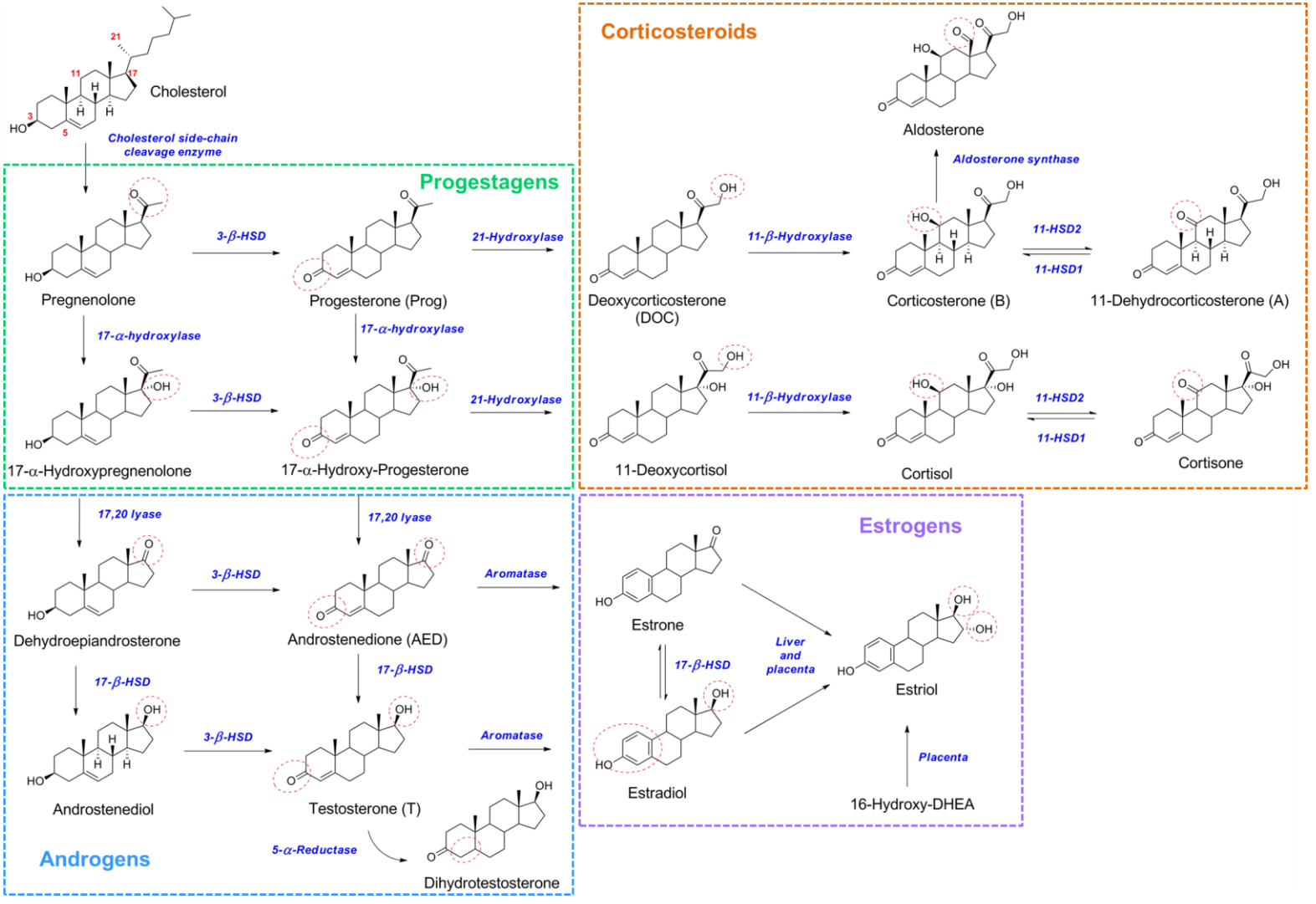


Figure 21. Metabolic pathway of steroid hormones of progestogens, corticosteroids, androgens and estrogens

2. Objectives and Hypothesis

The NPS phenomenon has become a global issue causing clinical consequences, delays on public health prevention and difficulties in legal enforcement. The aim of the presented thesis is to tackle the pharmacology evaluation of NPS by focusing on the human disposition of mephedrone, one of the most prevalent NPS and headline of the cathinones (in **Part I**) and by evaluating the potential of targeted metabolomics for the prediction of the NPS pharmacology (in **Part II**). Each approach has different hypothesis and objectives, explained below.

Part I

The initial **hypothesis** of Part I, was that a better understanding of mephedrone human pharmacology requires in depth studies on its metabolic fate. In vitro studies suggest that mephedrone metabolism would be regulated partially by polymorphic enzymes as CYP2D6 but little is known about its clinical relevance.

The general **aim** of Part I is to evaluate the human metabolic disposition of mephedrone. To achieve this main objective, 4 specific objectives were proposed:

a. To develop analytical strategies to monitor mephedrone and its metabolites in human matrices and in rodents brain.

b. To determine the pharmacokinetic parameters of mephedrone and its metabolites in human matrices under controlled conditions in a multi-dose regime.

c. To describe the pharmacokinetic/pharmacodynamics modeling evaluating the relationship between cardiovascular activity, serotonergic activity and subjective effects and mephedrone and its metabolites concentrations in a multi-dose regime.

d. To evaluate in humans the relevance of CYP2D6 polymorphism regulation of mephedrone disposition and its clinical implications.

Part II

The initial **hypothesis** of Part II, was that drugs of abuse and NPS would alter drug-specifically some targeted parts of the metabolome (monoamines pathway and steroids hormones) in the brain of rodents. Being capable to quantify these endogenous metabolites alterations after the acute drug administration, we expected to use these data as a tool to predict the pharmacological effect of novel substances using well characterized classical drugs as a reference.

The **aims** of Part II are to better understand the changes of a specific part of the metabolome underlying exposure to classical drugs and NPS and to develop a predictive pharmacology model using the specific-drug alterations of the targeted metabolome. To achieve these main objectives, 3 specific aims were proposed:

a. To develop and apply analytical methodologies to quantify endogenous metabolites following a targeted metabolomics approach.

b. To characterize the drug-specific alterations of the targeted metabolome induced by different classical drugs and NPS after its acute administration in rats.

c. To create a predictive model, capable to predict the pharmacological effect of NPS according to its targeted metabolomics profile.

3. Methods and Results

PART I

Human disposition of mephedrone

In order to characterize the pharmacokinetics of mephedrone in humans, our department performed a randomized, double-bind, crossover and placebo-controlled clinical trial at the Clinical Research Unit at IMIM with healthy volunteers. The biological samples obtained from this phase I clinical trial were the core study of the Part I of this thesis. In detail, the methodology used in each chapter follows as:

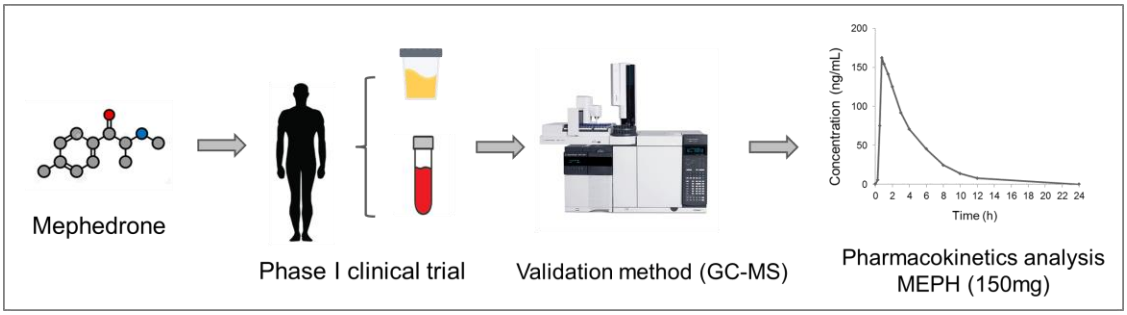
In **chapter 1**, a GC-MS methodological strategy was validated and experimental parameters of MEPH' pharmacokinetics were described in humans. The method was accurate and precise for MEPH quantification. However, due to the limitations of the GC-MS methodology to include the quantification of the main MEPH metabolites, a second analytical strategy was designed.

In **chapter 2**, a LC-MS/MS method (including 4 of the most relevant MEPH metabolites) was developed and validated in human matrices. Additionally, the methodology was also validated in rodent's brain to investigate the MEPH and metabolites permeability through the blood brain barrier (BBB). The experimental pharmacokinetic parameters for metabolites and MEPH were described in humans.

In **chapter 3**, the determination of MEPH and its metabolites (using the LC-MS/MS validated methodology from chapter 2) was performed in human samples, from volunteers who ingested different doses of MEPH (from 50 to 200 mg). Then, pharmacokinetics parameters were calculated. Moreover, the MEPH interaction with the serotoninergic system, its subjective and cardiovascular effects and its CYP interaction was also studied.

CHAPTER 1

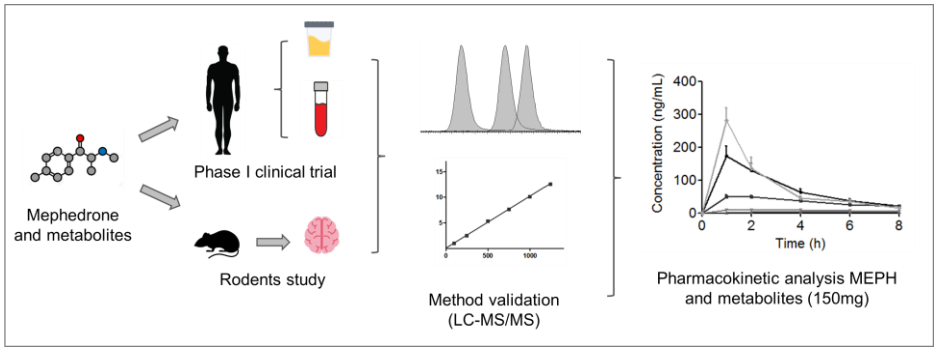
3.1. GC-MS quantification method for mephedrone in plasma and urine: application to human pharmacokinetics.



Olesti E, Pujadas M, Papaseit E, Pérez-Mañá C, Pozo ÓJ, Farré M, et al. GC-MS Quantification Method for Mephedrone in Plasma and Urine: Application to Human Pharmacokinetics. J Anal Toxicol. 2017 Mar 1;41(2):100–6. DOI: 10.1093/jat/bkw120

CHAPTER 2

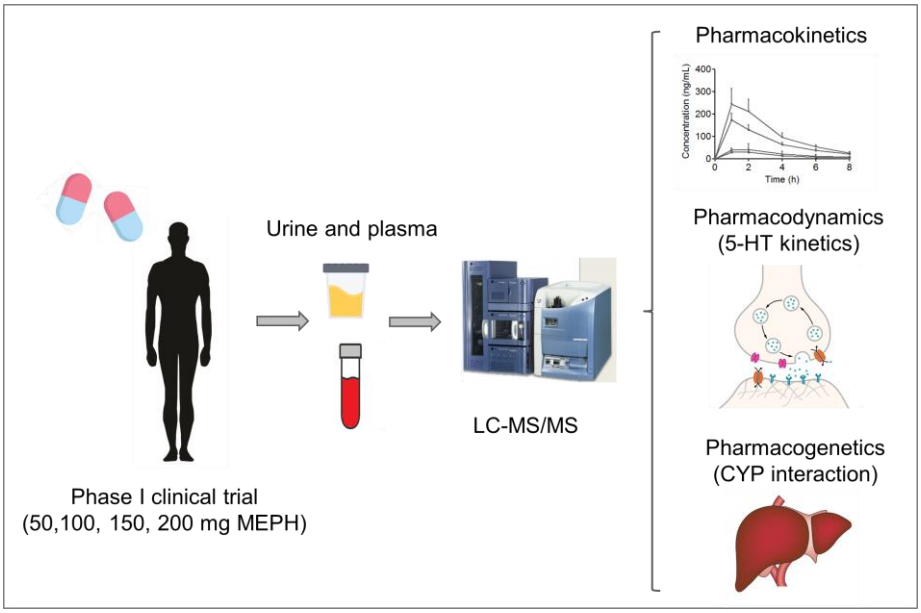
3.2. Pharmacokinetics of mephedrone and its metabolites in human by LC-MS/MS



Olesti E, Farré M, Papaseit E, Krotonoulas A, Pujadas M, de la Torre R, et al. Pharmacokinetics of Mephedrone and Its Metabolites in Human by LC-MS/MS. AAPS J. 2017 Nov 21;19(6):1767–78. DOI: 10.1208/s12248-017-0132-2

CHAPTER 3

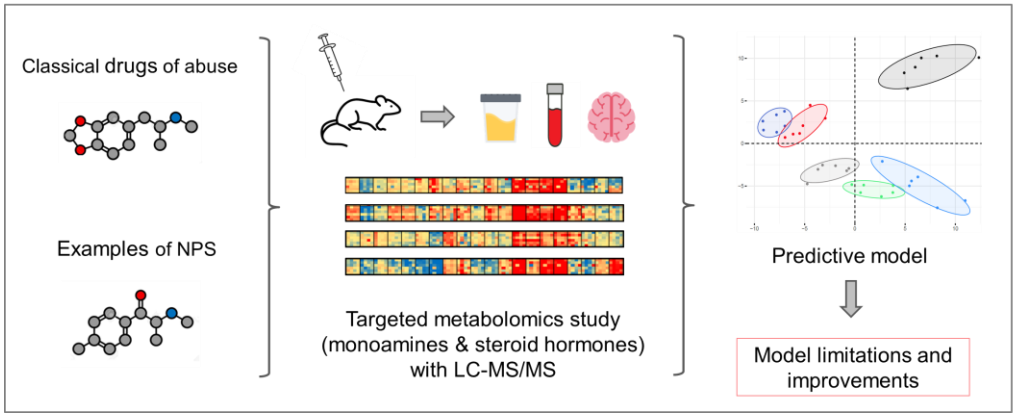
3.3. Dose-dependent pharmacology study of mephedrone and its metabolites: pharmacokinetics, serotoninergic effects and CYP interaction



Olesti E, Farré M, Carbó ML, Papaseit E, Perez-Mañá C, Torrens M, et al. Dose-Response Pharmacological Study of Mephedrone and Its Metabolites: Pharmacokinetics, Serotoninergic Effects, and Impact of CYP2D6 Genetic Variation. Clin Pharmacol Ther. 2019 Sep 8;106(3):596–604. DOI: 10.1002/cpt.1417

CHAPTER 4

3.4. Metabolomics predicts the pharmacological profile of new psychoactive substances



Olesti E, De Toma I, Ramaekers JG, Brunt TM, Carbó ML, Fernández-Avilés C, et al. Metabolomics predicts the pharmacological profile of new psychoactive substances. J Psychopharmacol. 2019 Mar 19;33(3):347–54. DOI: 10.1177/0269881118812103

4. General discussion

The tidal wave of new emerging substances has become a worrying issue world-wide[10]. This thesis aims to contribute to the pharmacological evaluation of these novel substances, by focusing on: the characterization of the human disposition of MEPH, one of the most problematic NPS, (part I) and on the evaluation of the potential of targeted metabolomics to predict the NPS' pharmacology profile (part II). To do so, several analytical approaches based on chromatography coupled to mass spectrometry have been developed and applied in order to shed light on the evaluation of NPS' pharmacology.

4.1. Mephedrone

In this thesis, as a result of the quantification of mephedrone and its metabolites in biological samples from a double-blind, placebo-controlled and randomized phase I clinical trial (Chapter 1 and Chapter 2), the human disposition of mephedrone was evaluated (Chapter 3). A brief summary of the main outputs obtained in this thesis from the human disposition study of MEPH are the followings (and summarized in Figure 54):

i. *Absorption*: In humans, the MEPH concentrations in biological matrices presented a large inter-individual variability, which could be explained by the fluctuating low bioavailability, an elevated hepatic first pass effect[20,149]. The absorption of MEPH was found to be erratic between and within individuals (Chapter 1 to 3). In a similar manner to other psychostimulant drugs (such as MDMA[289] or cocaine[290]), MEPH presented a rapid absorption (Tmax at 1.2h).

ii. *Distribution*: MEPH is rapidly and widely distributed throughout the organism. In Chapter 2, we have quantified MEPH concentrations in the rodent's brain tissue, which suggest that MEPH crosses the blood-brain-barrier. Interestingly, MEPH's active metabolite, NOR-

MEPH[148], nearly doubled the concentrations of MEPH in rodents' brain tissue (Chapter 2) but there was no correlation between the cardiovascular and subjective effects and the plasma of NOR-MEPH concentrations in humans.

iii. *Metabolism (CYP2D6)*: From urinary and plasmatic concentrations of MEPH and metabolites, we have reported different recoveries and kinetics of the synthetic cathinone and its metabolites according to the CYP2D6 phenotype of volunteers. Indeed, extensive CYP2D6 metabolizers had a lower exposure to MEPH in comparison with the intermediates metabolizers. In the absence of poor metabolizers among subjects included in clinical studies (this phenotype was an exclusion criteria) we can only speculate that these individuals will be more exposed to MEPH that might be associated to higher risks of clinical complications. The formation of the most abundant metabolites, nor-mephedrone and 4'-carboxy-mephedrone, was regulated by CYP2D6.

iv. *Excretion*: Regarding the MEPH excretion, all the studied metabolites were found in urine from 0 to 48h post drug administration, being COOH-MEPH the most abundant one.

v. *Cardiovascular and subjective effects and serotonin concentrations in blood*: The subjective and cardiovascular effects reported by volunteers and the 5-HT concentrations in blood correlated to MEPH concentrations in plasma. With regard to the metabolites, NOR-MEPH and DIHYDRO-MEPH were described to be neurochemically active[148], but only NOR-MEPH reached significant concentrations in rats' brain (Chapter 2) and no-correlation was found between any studied metabolite and any pharmacodynamics parameter (subjective, cardiovascular or 5-HT concentrations) effects in humans (Chapter 3).

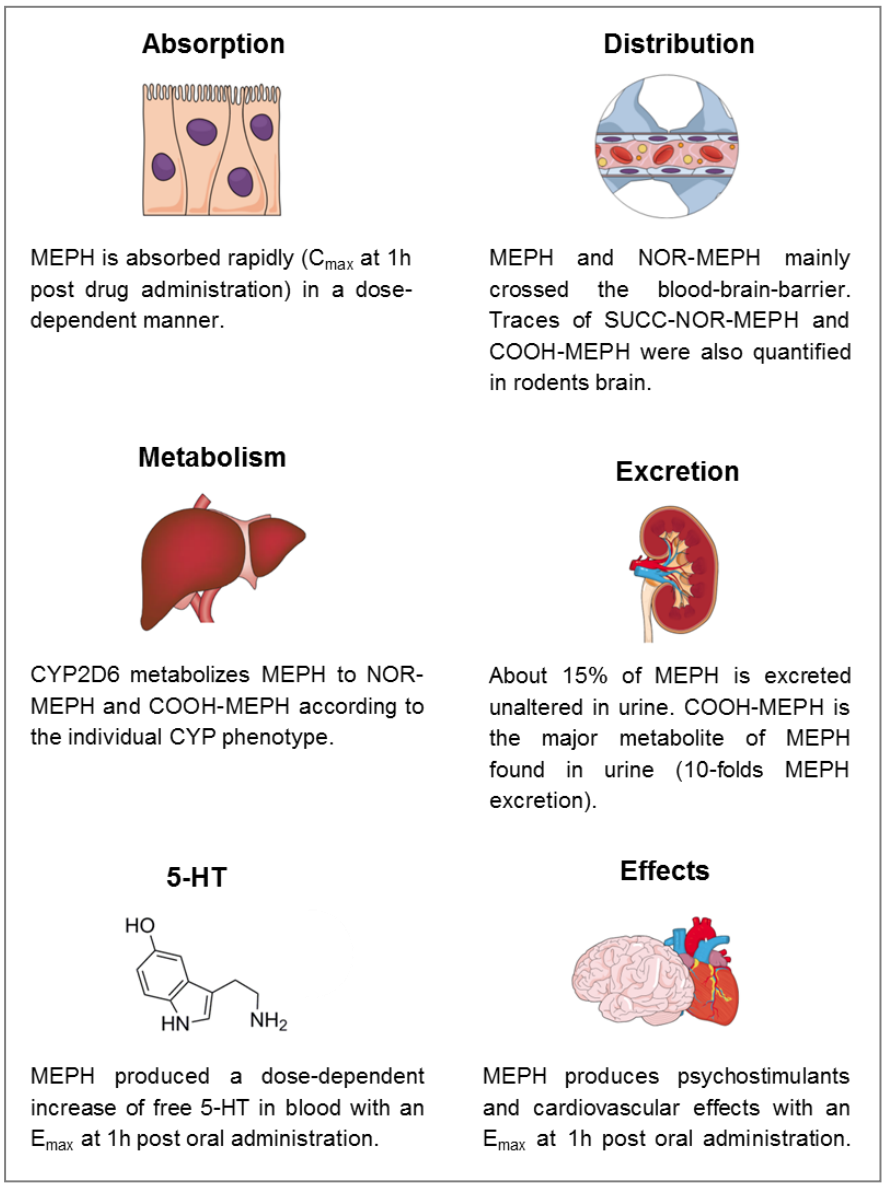


Figure 54. Schematic summary of the main outputs described in the thesis of the MEPH distribution in humans. The pictures were obtained from the webpage: www.mindthegraph.com.

Strengths and limitations

The strength of this work is the ability of combing metabolic data, with subjective and cardiovascular effects and biomarkers of the drug mechanisms of action (serotonergic activity). In addition the availability of subject's genotype and phenotype for CYP2D6 allowed to evaluate the contribution of this polymorphic enzyme in the regulation of MEPH metabolic disposition and to speculate on the clinical transnationality of the observations to clinical situations.

On the other hand, the report has some limitations as the low number of subjects per dose that participated in the trial, with the drawback that not all subjects ingested all doses. Although the contribution of CYP2D6 to MEPH metabolic disposition was further confirmed with an independent second set of subjects, the number of subjects participating in the study is low for genetic studies. In addition because poor metabolizers were not included in clinical trials for safety reasons, we can only speculate on the potential clinical implications of this phenotype. Another limitation is the fact that women were not include in clinical trials while there have been described that there are relevant differences in the drug's effects produced in both genders[291,292].

4.2. Analytical strategies

Small chemical molecules (either endogenous, such as dopamine and serotonin or exogenous, such as ecstasy or mephedrone) interact within organisms producing a broad range of effects. The importance of its quantification in biological samples relies in their relevance in pharmaco-toxicological studies and in targeted metabolomics approaches, among other disciplines[152]. Optimized chromatographic methods coupled to mass spectrometry techniques are necessary in order to satisfy the selectivity and sensitivity requirements to quantify different types of compounds (drugs, metabolites, neurotransmitters, etc.) in biological matrices[158]. The validation of bioanalytical methods are required in order to guarantee that the developed method is accurate, reproducible, robust and reliable for their diverse applications[156,221].

In the present thesis, we have validated 2 bioanalytical methods for the quantification of exogenous compounds (mephedrone and its metabolites, in Chapter 1 and 2 that were applied in Chapter 3) and 1 quantitative method for endogenous metabolites (amino acid neurotransmitters and related compounds, in Chapter 5). Furthermore, 2 additional methods were employed to quantify the targeted metabolome (monoamines neurotransmitters[204,207] and steroid hormones[218], in Chapter 4).

All analytes were quantified by using the selected reacting monitoring (SRM) mode of a liquid-chromatography coupled to a triple quadrupole mass analyzer, where both quadrupoles operated by filtering a selected *m/z*. Inherently, an increase of sensitivity, selectivity and specificity was achieved with SRM when compared to other scan modes (such as full scan or the other SIM mode used in the GC-MS method for MEPH[158]. The targeted analytes (MEPH and its metabolites, dansyl-monoamines, steroid hormones, polar NT and related compounds) presented a protonable

centre (basic) that favoured an efficient positive ionization (ESI+) in the interface, obtaining the molecular species of $[M+H]^+$, $[M+NH_4]^+$, $[M+H-H_2O]^+$, $[M+Na]^+$ and $[M]^+$. In all methods, at least two MS/MS transitions were monitoring in order to confirm the analyte identity (one for quantification and the other one for compound confirmation)[158]. Furthermore, suitable internal standards were selected when available in order to correct potential variations on the analytical procedure.

In the table 37, a brief summary of the experimental set-up of the analytical methodologies used and developed in the thesis is presented.

Method	Equipment	Column type	Derivatization	Sample Treatment	Targeted Matrices	Type of analytes	Analytes quantified	References
Mephedrone	GC-MS	Phenylmethylsiloxane capillary column (5%)	Yes (MSTFA)	PP and LLE	Human plasma and urine	Exogenous	1	Olesti E et al[223].
Mephedrone and metabolites	LC-MS/MS	ACQUITY UPLC BEH **C18** 1.7µm	No	PP and DS	Human plasma and urine and rats' brain areas	Exogenous	5	Olesti E et al[160].
Amino acid Neurotransmitters	LC-MS/MS	ACQUITY UPLC BEH **Amide** 1.7µm	No	PP and DS	Rats' plasma and brain areas	Endogenous	16	Olesti E et al. (submitted)
Steroid hormones	LC-MS/MS	ACQUITY UPLC BEH **C18** 1.7µm	No	PP and LLE	Rats' plasma and brain areas	Endogenous	6	Adapted from Marcos et al[218].
Monoamines	LC-MS/MS	ACQUITY UPLC BEH **C18** 1.7µm	Yes (Dansyl-Chloride)	PP and DS	Rats' urine and brain areas	Endogenous	12	Modified from Marcos et al[204] and Lu H et al[243].

Table 37. Summary of the principal characteristics of the analytical strategies used in this thesis. Brain areas include: PFC, prefrontal cortex; Cer, cerebellum; HC, hippocampus, St, striatum. Abbreviations: PP, Protein precipitation; DS, dilute and shoot and LLE, liquid-liquid extraction.

Methodological design

Each methodology was designed and optimized according to the targeted compounds, the biological matrix and the selected instrument. Therefore, different strategies were used depending on several factors including:

i. *Physicochemical properties of the analytes*: The physicochemical properties of the analytes were the main driving force to select part of the methodological design including sample treatment and chromatography. Thus, the low polarity of the steroid hormones advised to perform an extraction step in order to concentrate the metabolites whereas this step was not feasible for more polar analytes such as neurotransmitters. These properties were also critical in the selection of the chromatography. Thus, the quantification of derivatizated monoamine neurotransmitters and steroid hormones was achieved with a reversed-phase C_{18} column whereas the amino acids neurotransmitters required a HILIC amide column for its retention due their high hydrophilicity (difficult retention in C_{18}).

ii. *Abundance of the analyte in the biological matrix*: The analytical methodology must be able to quantify the expected amounts of the analyte in the selected matrix. In the case of endogenous compounds, these amounts can vary from the low ng/mg range of monoamine neurotransmitters in some brain areas to the µg/mL range of some amino acids in plasma. These differences on concentrations guided the sample treatment in some methods. As an example, whereas a 2-fold factor of pre-concentration (300 µL of the sample were evaporated and reconstituted in 150 µL) was needed for the quantification of monoamines, to quantify the amino acids neurotransmitters and related compounds a 20 fold dilution was required for their quantification.

iii. *Instrumental sensitivity*: Closely related with the physicochemical properties of the analyte and its abundance in the targeted matrix, the instrumental sensitivity for an analyte will define the methodological design. If the instrumental sensitivity for a specific analyte is too high for the amount present in the targeted matrix, a dilution strategy will be advised. That is the case of some polar neurotransmitters (Chapter 5). On the other hand, if the instrumental sensitivity was not enough, an additional strategy must be followed. In the case of monoamines neurotransmitters, a derivatization strategy (with Dansyl-Chloride) was required in order to improve the analyte ionization. After derivatization with Dansyl, a substantial gain of sensitivity was obtained. For instance, NA was poorly detected without derivatization in urine samples with S/N close to 3 (see figure 55a). The selected derivatization step improved substantially the sensitivity of the method. Dansyl-Chloride reacts with phenols and amines, forming mono-Dansyl, bis-Dansyl and tris-Dansyl species (e.g. Trp- Dansyl, 5-HT-bis-Dansyl or DA-tris-Dansyl). In the case of NA, the detection of the tris-dansyl derivative improved the sensitivity of the method more than one order of magnitude (Figure 55b) allowing for the determination of NA in all tested samples.

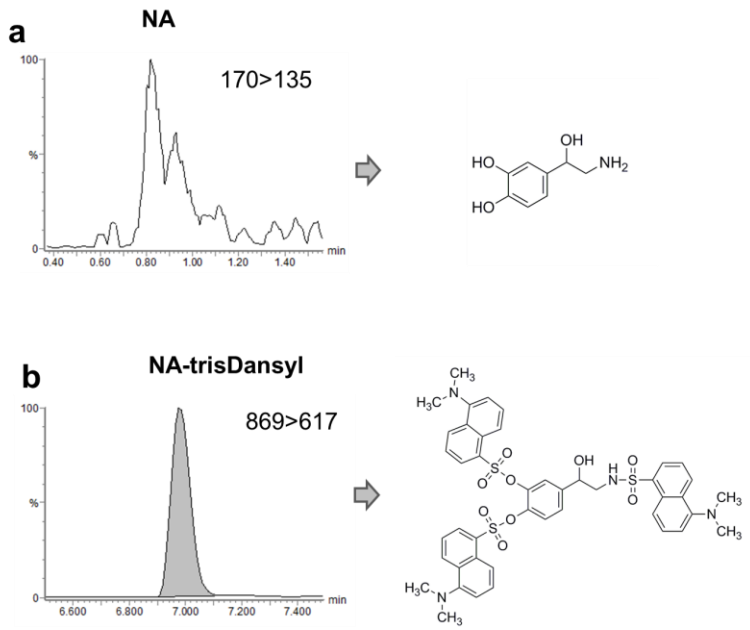


Figure 55- Chromatograms and chemical structures of a) norepinephrine and b) norepinephrine-trisDansyl in a real urine sample. The quantification transitions used were: 170>135 for NA and 869>617 for NA-trisDansyl.

iv. *Exogenous vs endogenous analytes*: The nature of the studied compounds (endogenous or exogenous) is also extremely relevant for the quantitative method development mainly for the selection of the validation strategy. For the validation of methods dealing with the quantification of exogenous compounds obtaining drug free-matrix for the validation procedure is not problematic and therefore, it is feasible to follow most of the guidelines for bioanalytical method validation based on spiking blank matrices[221,293]. In this thesis, the method validation for MEPH and metabolites was performed following the European guideline for bioanalytical method validation[221]. In contrast, endogenous compounds are inherently present in biological matrices, which complicate the validation of the method. As a consequence,

typical guidelines for bioanalytical method validation[221,293] cannot be strictly applied and, given the absence of specific guidelines for endogenous compounds, alternative approaches for the validation might be employed (such as, standard addition, the background subtraction, surrogate matrix and surrogate analyte method[156]). In the thesis the standard addition strategy[294] was applied for the quantitative validation of polar NTs and related compounds (in Chapter 5). With this validation procedure, we have proved that the "calculated" concentration (obtained with the solvent calculation curve) coincided with the "actual" concentration of the matrix (obtained with the addition standard in matrix). In this manner, the analysis of all the samples was performed by interpolating its values into the solvent calibration curve (identically as for monoamines neurotransmitters and steroids hormones, in Chapter 4).

v. *Selection of the instrument*: Besides the instrumental sensitivity, the inherent characteristics of the instrument selected for the determination also guide the methodological design. As an example, for the analysis of MEPH and metabolites, we developed two methodologies based on GC-MS[223] and LC-MS/MS[160] respectively. The pre-treatment procedure for both instruments differed substantially (schemed for urine analysis in Figure 56) since GC-MS required the MEPH derivatization for its detection (to make the analyte volatile and thermally stable for its retention through the column). Since the presence of water would hamper the derivatization procedure, an evaporation step (among previous cleaning-up steps) was needed for the quantification of MEPH by GC-MS. MEPH might be partially lost during evaporation and the use of its labelled isotope internal standard was required to correct for these losses. Since evaporation rates for MEPH and its metabolites are different (e.g. NOR-MEPH evaporated

faster than MEPH) the use of only on labeled internal standard was not enough for the proper correction of the metabolites. Therefore, the developed GC-MS method was able to quantify only the parent drug. The shift from GC-MS to LC-MS/MS permitted the inclusion of MEPH metabolites in a unique analysis. With the LC-MS/MS equipment, a rapid dilute and shoot strategy was validated, avoiding the evaporation loses of the analyte in urine. In plasma, a partial evaporation strategy minimized the differences on evaporation between MEPH and metabolites allowing for the use of the only available labeled compound (MEPH-d_3) as internal standard for all analytes. Additionally, due to the suitable chromatography and ionization of MEPH and its metabolites no derivatization step was needed.

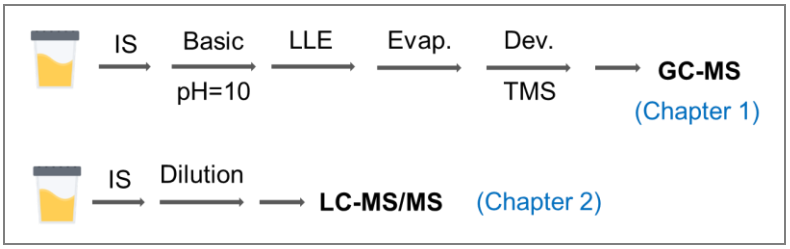


Figure 56- Schematic work-flow for the quantification of MEPH in human urine by GC-MS and by LC-MS/MS. Abbreviations: IS (internal standard), LLE (liquid-liquid extraction), Evap. (evaporation), Dev. (derivatization), TMS (derivative agent).

Another relevant difference between GC-MS and LC-MS/MS methodologies was the sensitivity reached by the instrument. With regard to the lowest concentration level at which the presence of our analyte can be detected (LOD), the LC-MS/MS methodology was 10 fold more sensitive than GC-MS [for LC-MS/MS (0.1ng/mL and 0.4 ng/mL), for GC-MS (1.1 ng/mL and 4.5 ng/mL) in urine and plasma

respectively]. Furthermore, the quantity of sample used for the GC-MS analysis was 50 to 20 folds (in urine and in plasma) higher than the needed for LC-MS/MS. Therefore, LC-MS/MS technique is more suitable than GC-MS for the MEPH and metabolites identification and quantification and it was the method used for pharmacological studies.

vi. *Matrix*: The selection of the matrices for the procedure mainly depends on the purpose of the study. In humans, mainly urine and plasma were used for the mephedrone and metabolites study, whereas in the targeted metabolomics study, the addition of rats' brain areas permitted an accurate drug-fingerprint characterization.

Matrix components co-eluting with the studied analyte (e.g. salts or phospholipids) can interfere in the compound ionization by producing the suppression or the enhancement of the analyte ionization[156,158]. Indeed, the presence of molecules in the matrix that compete for the same charges of the MS source produce that different analytes with the same concentration may produce different MS responses[156]. Therefore, without a proper correction, the matrix effect can bias the obtained concentration of the sample. In this thesis, all analytes (in all matrices) were quantified with an internal standard in order to overcome the matrix effect. Other strategies to minimize the matrix effect included the analytes purification or a clean-up sample treatment like the LLE performed for steroid hormones analysis.

Matrix interferences depend on the selected matrix and they guide to some extent the selection of the analytical strategy. Thus, the cleaning-up sample treatment in plasma and brain areas included a protein precipitation (PP) step. Different strategies to precipitate proteins were[295]: a) by changing the pH of the sample with the addition of acidic compounds (such as the perchloric acid, used in Chapter 1), b)

by decreasing the dielectric constant with the addition of an organic solvent (such as acetonitrile, used in Chapter 2, 4 and 5) and c) by increasing the ionic strength (not used in the thesis)[156]. In urine, this PP step is not required and a dilute-and-shoot approach (strategy used for MEPH and metabolites in Chapter 2, for monoamine neurotransmitters in Chapter 4 and for polar neurotransmitters and related compounds in Chapter 5) was used to minimize the matrix effect and to avoid low extraction recoveries[227]. However, the extraction steps were necessary in some cases for further cleaning up and concentrating the sample. For the quantification of MEPH (by GC-MS, Chapter 1) and steroid hormones (by LC-MS/MS, Chapter 4) liquid-liquid extraction step (LLE) was used to gain sensitivity[223] and to decrease matrix effect[218].

Overall, the analytical strategies developed and used in the presented thesis fit to the aimed purpose. Therefore, the developed methods can be considered as the ideal analytical tools for the evaluation of the NPS pharmacology.

4.3. Targeted metabolomics study

In order to perform a rapid pharmaco-toxicological evaluation of different NPS *in vivo*, a model based on targeted metabolomics was developed. With the selected analytical strategies (either developed or available), we have determined different metabolic pathways to be applied in targeted metabolomics approaches. Nowadays, the use of metabolomics strategies has been studied in different disciplines, such as: to characterize diseases behaviors[179] or to discover pathologies biomarkers[242]. For our model, we decided to select the accuracy and precision of the data obtained by targeted analysis than the wide coverage provided by untargeted approaches. We generated data for 34 metabolites in 6 different matrices at 2 different administration times. Each animal was defined by 298 data

points. This dataset was used for predicting the pharmacological profile of NPS. As far as we know, this is the first time that a targeted metabolomics study has been used as a tool to predict the pharmacological profile of novel drugs.

The preselection of the metabolic pathways is a prerequisite in targeted metabolomics. The classical drugs of abuse (and specially psychostimulants) are described to strongly interact with the monoamine neurotransmitters pathway[4]. Since NPS were designed to mimic the classical drugs, their mechanism of action is strongly similar to well-known drugs of abuse such as cocaine, ecstasy or THC[29,73,74]. For that reason, the monoamine neurotransmitters system (precursors, neurotransmitters and its metabolites) was firstly selected in order to obtain the maximal changes among psychostimulants. The alterations observed in monoamines, were specific of each studied drug and mainly expected (e.g. MDMA, described to produce serotoninergic effects, robustly depleted 5-HT and its metabolite, and COCA administration, described to block the DA uptake, strongly altered the DA pathway, see Chapter 4).

Moreover, all the assayed drugs were also described to interact with the steroid hormones pathway, producing stimulants and cardiovascular effects. Then, the inclusion of the steroid hormones pathway permitted a more detailed picture of the metabolome alterations after the administration of each drug of abuse (see Chapter 4). The steroids produced a high differentiation between drugs and vehicles as their alterations were significantly increased for all drugs at 1h post drug administration in comparison with the vehicle treatment. These alterations were in accordance with the scientific literature and they provided a predictive metabolic fingerprint for each assayed drug (classical; MDMA,

COCA, METH, HEROIN, THC) and NPS (MEPH, JWH-018) (see Chapter 4).

Using these two main pathways, the pharmacological effect of both MEPH and JWH-018 was successfully predicted. The rationale for choosing MEPH and JWH-018 as examples of NPS was their high consume and popularity[28], their extensive characterization in a scientific level[73,79,296] and their different drugs-type pharmacological effect[62,80,252]. In this way, either the administered dose or their pharmacological differences could be compared and supported by the literature.

The miss-prediction result obtained with the inclusion of two other synthetic cathinones (MDPV and α-PVP) revealed potential improvements of our metabolomics approach (additional information, Chapter 4). By adding a third metabolomics pathway (amino acids neurotransmitters and related compounds), a direct improvement of the targeted metabolomics prediction for psychostimulants was achieved. Indeed, both synthetic cathinones were highly affected by the targeted pathway and closely classified as COCA-like compound, in coherence with the literature[74]. Furthermore, the alterations were nearly identical between both analogues drugs (see Chapter 5).

Overall, our results confirm that the selection of the metabolic pathways is critical for targeted metabolomics approaches. Based on the metabolic pathways evaluated, the presented model was able to correctly predict the pharmacological effect of examples of NPS. For instance, the inclusion of other metabolic pathways (such as endocannabinoids[260]) could be required for the study of synthetic cannabinoids[297].

The use of large-scale biological screening methods has been devised as a possible approach to face the growing problem of NPS[10]. Thereby, the

use of *in silico* techniques that mimic the drug-receptor interaction[240,241] could be proposed as a tool to predict the NPS effect. However, according to experts in the pharmaceutical industry, nowadays it is still a challenge to provide a reliable guidance to predict the efficacy or toxicity of a compound when exposed to human population with *in silico* techniques[298]. And indeed, assays are even less reliable when predicting the compounds that interact with the Central Nervous System[298]. The predictions of the drugs-receptor interaction are valuable, but the interpretation of their biological relevance is still unclear[298]. In this context, we proposed the combination of experimental data (*in vivo*) and machine learning algorithms (*in silico*) as a strategy to predict the pharmacology of unknown drugs.

Regarding the selected matrices, the use of brain areas (PFC, Cer, HC and ST) was critical for the drug fingerprint characterization. The determination of the targeted pathways in different brain areas permitted a closer and accurate analysis of the direct drug effect. In contrast, the alterations described in plasma and urine were residual and not sufficient to independently characterize each assayed drug. These results would hamper the translation of such approach to humans.

Overall, our model demonstrated that with relatively short experimental analysis (quantification of the neuro-metabolomics markers), a predictive classification was achieved for known classical drugs and NPS. The presented predictive model is a preliminary model which has some strengths, potential improvements and inherent limitations.

Model strengths

The targeted metabolomics approach developed in this thesis has the following strength points:

i. *Combination of in vivo and in silico (learning algorithms analysis)*: The quantification of the selected part of the metabolome *in-vivo* permitted the biological characterization of each drug. In this way, the targeted metabolomics profile provides information about the effect of not only the parent drug but also the potential active metabolites of the administered drug (in case of pro-drugs). Moreover, the presence of toxic adducts or toxic reactions formed by the drugs could be indirectly detected by regarding to the metabolomics fingerprint alterations (e.g. the neurotoxicity of MDMA observed by a decrease in tryptophan hydroxylase activity[299]). Furthermore, the *in-vivo* study can provide first in-sights of the mechanism of action of the drug, its pharmacokinetic/toxicokinetic behavior and its active dose. With the *in vivo* data, the model constructed (*in silico*) permitted the prediction and drug-type classification of different NPS (synthetic cathinones and synthetic cannabinoids) pharmacological profile.

ii. *Robust and coherent neuro-metabolomics alterations*: Most of the neuro-metabolomics alterations described in the targeted metabolomics studies for the 3 metabolic pathways (monoamines and steroids in Chapter 4 and amino acid neurotransmitters and related compounds in Chapter 5) were homogeneous among the different individuals and among the different brain areas (see the heat map representation example in Figure 57 and in supplementary material of the thesis, Figures 60-77, where the individual data of each variable is placed in rows and each brain area in columns). In general, the increases or decreases of the metabolite trended to go into the same direction with

similar intensities. Therefore, the neuro-metabolomics fingerprints obtained can be considered as robust. Furthermore, the alterations produced by each drug were in accordance to the literature. The fingerprint was also coherent with the substance pharmacology and its mechanism of action. For instance, the MDMA depletion observed in Chapter 4 was strongly marked for 5-HT at 1h post-drug administration and consistently, its metabolite (5-HIAA) was then deeply decreased at 4h post MDMA administration (see figure 57).

iii. *Rapid pharmacological evaluation*: Another advantage of the proposed NPS evaluation strategy is the valuable information acquired within a short period of time at relatively low cost. Based on targeted metabolomics, the drug-type NPS classification could be predicted within a cost-effective short time period.

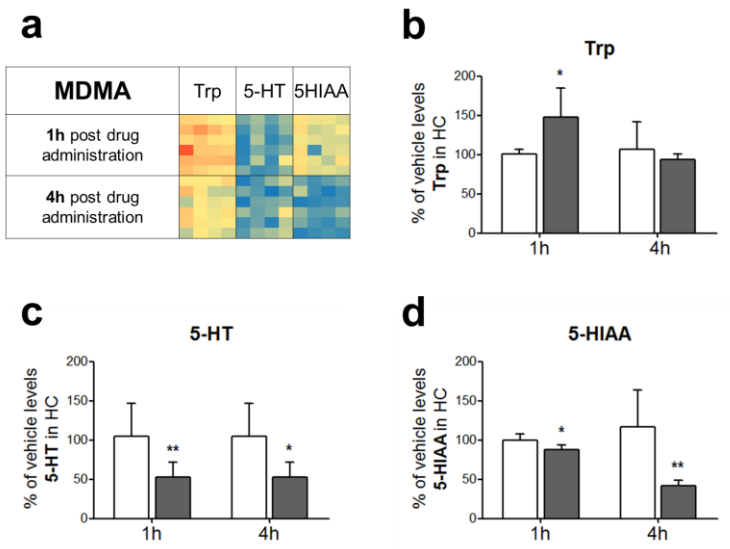


Figure 57- Partial heat map and graph bar of the alterations produced in the 5-HT pathway.

Potential improvements

The miss-prediction obtained with 2 metabolic pathways (monoamines and steroids) at 2 administration times (1-4h), revealed weak points of the constructed predictive model. These weak points are related with: a) the administered dose, b) the time of drug's administration and c) the targeted metabolic pathway. We hypothesized that the method might be improved by performing a pilot-dose-effect study for each drug, by increasing the time-of-drug administration points and by adding new metabolic pathways (additional information, Chapter 4). From these potential improvements we have successfully overcame the miss-prediction of two NPS by quantifying a new metabolic pathway (the amino acids neurotransmitters and related compounds; Chapter 5). In any case, each of these potential improvements would imply increases on the cost of the approach.

The model would benefit also from the addition of more reference substances in each drug-type classification group. In this way, each reference group would have wider range of compounds (e.g. serotoninergic drugs, dopaminergic drugs, etc.) and the probability to have overfitting would decrease.

Limitations

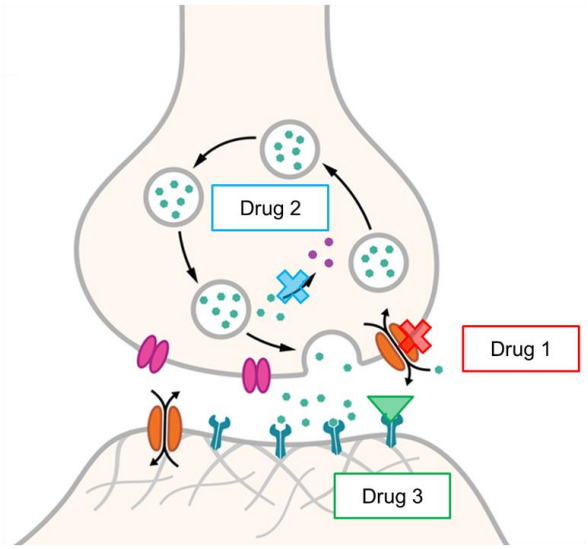
The targeted metabolomics model presented two inherent limitations of the study design: i) lack of a clear bridge between the rodent's described metabolomics and its translation into humans and ii) lack of complete picture of the drug effect.

i. *Translation into humans*: Due the complexity of the translation of research evidence from animals to humans[300], the targeted metabolomics fingerprint obtained for each drug in rats' matrices

(brain areas, plasma and urine) does not guarantee that these alterations are persisted on the complex human body. Although the alterations observed in rats' brain were in the same line that the reported effects in humans (e.g. MDMA was described to produce a serotoninergic effect in humans[301] and a grand affectation was observed in all rats' brain areas), there is not enough evidence for a direct translation from rodents to humans. Further experiments are required for that purpose.

ii. *Drug effect vs metabolomics*: Another limitation of the targeted metabolomics study is the fact that different metabolomics profiles could be obtained for a common pharmacological effect. In other words, drugs that globally produce a determinated NT related effect (e.g. serotoninergic or dopaminergic) could produce different metabolomics alterations due their different mechanism of action (see Figure 58 for a detailed example). In this situation, a possible bias with the drug-type classification could be made by classifying differently drugs with the same pharmacological effect. It is expected that this limitation might be minimized by the addition of a larger number of known drugs into the model.

a



b

Drug	Mechanism of action	Expected Effect	Expected metabolome	
			NT	Metabolite
Drug 1	Block the NT re-uptake	↑	↑	↑
Drug 2	Enzyme inhibition	↑	↑	↓
Drug 3	Interaction with the receptor	↑	↓	↓

Figure 58. Simulation of the mechanism of action of three hypothetic drugs that could globally produce a neurotransmitter related effect (expected effect). a) Schematic mechanism of action of the drugs b) Table of the different expected metabolomics alterations (neurotransmitter and metabolites) and the same expected effect of the 3 hypothesized drugs. Chemical synapsis draw was adapted from www.scistyle.com[113].

4.4. Future directions

Based on the strategies used in the thesis to evaluate the pharmacology of novel substances, future research studies can be drawn. In these studies, it would be of great interest to dig deeper into the disposition of MEPH and study other highly consumed and problematic toxic substances. Furthermore, the improvement of the metabolomics predictive model prototype would be required for its routinary implementation.

The suggested potential research lines for the human disposition approach developed in Part I, follows as:

a. To add and validate the quantification of other metabolites of MEPH with the LC-MS/MS methodology (such as 4'-Hydroxy-tolyl-MEPH).

b. To study the human disposition of MEPH after its ingestion by other routes of administration (such as insufflation).

c. To test the pharmacogenetics relation of MEPH and metabolites within a larger population. To investigate if there is a relation between the low CYP2D6 metabolizers and the patients with MEPH clinical intoxications.

d. To study the human disposition of other problematic and prevalent NPS (such as MDPV) by studying the pharmacokinetics, pharmacodynamics and pharmacogenetics under a Phase I Clinical trial.

e. To evaluate the potential of MEPH metabolites as biomarkers for the MEPH misuse to enhance athlete's performance.

The proposed potential future directions for the targeted metabolomics model prototype in Part II, follows as:

a. To enlarge the predictive model created by adding more reference drugs per each group.

b. To perform a dose-finding study in order to obtain the equivalence dose of the novel drug assayed with the doses used for the classical drugs of abuse.

c. To use the microdialysis technique to obtain more frequent snap-shots of the variations on the targeted metabolome among time.

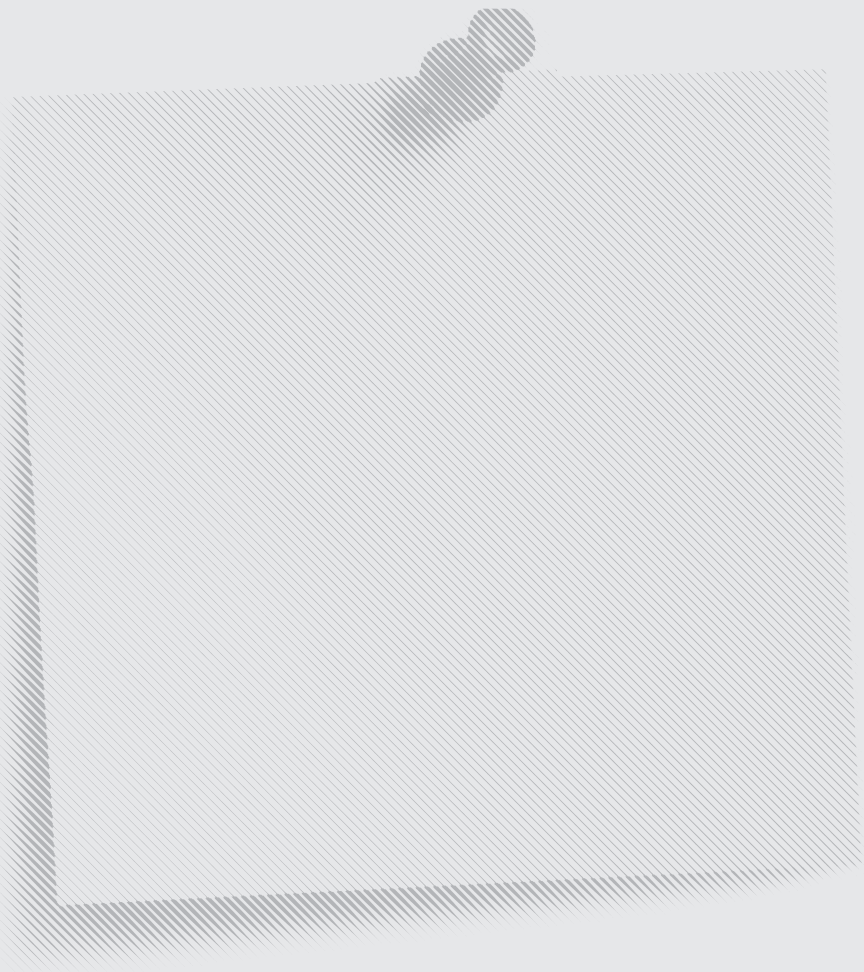
d. To develop novel targeted bioanalytical methods in different matrices in order to monitor a wider part of the metabolome (such as, estrogens, fatty acids, prostaglandins or leukotrienes among others).

e. To evaluate the potential of this approach in order to check the mechanism of action of drugs acting on the central nervous system (such as cognitive enhancer drugs).

f. To develop a new metabolomics predictive model with known commercial and therapeutic drugs in order to evaluate the adequacy as a drug candidate. The big-pharma industry could value the development of a targeted metabolomics approach for the facilitation of the evaluation of new candidate drugs.

g. To evaluate the translation of the model to humans. Since brain analysis is not feasible in humans, other biological matrices (such as hair or oral fluid among others) could be used in order to test potential alterations on the targeted metabolomics.

5. Conclusions



In summary, the work presented in the thesis has contributed to evaluate the pharmacology of novel psychoactive substances. Overall, the results obtained lead to the following conclusions:

Aim 1: Human disposition of mephedrone

1. The analysis of mephedrone and metabolites was achieved using gas chromatography–mass spectrometry and liquid chromatography coupled to tandem mass spectrometry. The later permitted an increase in sensitivity and in selectivity and also allowed the direct detection of mephedrone metabolites without a derivatization step.

2. The pharmacokinetics of mephedrone, nor-mephedrone, N-succinyl-nor-mephedrone, 1'-dihydro-mephedrone, and 4'-carboxy-mephedrone was described following analytical developments for the first time.

3. Mephedrone presents a rapid and erratic absorption (T_{max}= 1.2 h), with a low bioavailability and extensive hepatic metabolism. Is rapidly cleared (with a half-life of 2h). About 15% of mephedrone is excreted un-altered in urine and the most abundant excreted metabolite is 4'-Carboxy-mephedrone.

4. The administration of mephedrone induced a dose-dependent release of free serotonin concentrations in blood in parallel with an increase of the cardiovascular and subjective effects. There was a good correlation between these effects and mephedrone plasma concentrations.

5. For the first time we describe that the formation of the most abundant metabolites, nor-mephedrone and 4'-carboxy-mephedrone, was regulated by CYP2D6. Subjects with low CYP2D6 activity tend to accumulate mephedrone in the body which may explain some acute intoxications cases although this observation has to be balanced by the erratic oral absorption of the drug.

Aim 2: Prediction of the NPS pharmacology by targeted metabolomics

6. The use of liquid chromatography coupled to tandem mass spectrometry has allowed the validation of quantitative methods for the determination of exogenous (mephedrone and its metabolites) and endogenous compounds (amino acid neurotransmitters and related metabolites) in different biological matrices (from humans and from rodents). Furthermore, previous analytical methodologies developed in our laboratory with liquid chromatography coupled to tandem mass spectrometry have been used to quantify monoamine neurotransmitters and steroid hormones.

7. With regard to the targeted-metabolomics study, classical drugs of abuse (ecstasy, cocaine, methamphetamine, tetrahydrocannabinol and heroin) and examples of NPS (mephedrone, JWH-018, MDPV and α-PVP) were characterized by quantifying the neurochemical alterations of monoamines, steroid hormones, amino acid neurotransmitters and related compounds in brain areas, plasma and urine. Each drug of abuse had its specific metabolomics fingerprint in accordance to its pharmacological profile.

8. Based on the metabolomics fingerprints of classical drug, a predictive model using random forest algorithms was able to group the similarity of NPS with specific drug categories. In this manner, mephedrone was predicted to act as an ecstasy-like drug and JWH-018 as tetrahydrocannabinol-like drug. An improved analysis permitted the classification of MDPV and α-PVP as cocaine-like drugs.

In all, this thesis has contributed to i) study the human disposition of mephedrone ii) highlight the potential of using metabolomics in the search for evaluating the pharmacological profile of new psychoactive substances.

6. Bibliography

1. United Nations Office on Drugs and Crime. *World Drug Report*. (2018).

2. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report. 1–90 (2018).

3. Lüscher, C. & Ungless, M. a. The mechanistic classification of addictive drugs. *PLoS Med.* 3, e437 (2006).

4. Rothman, R. B. & Baumann, M. H. Monoamine transporters and psychostimulant drugs. *Eur. J. Pharmacol.* 479, 23–40 (2003).

5. Parolaro, D. & Vigano, D. Role of endocannabinoids in regulating drug dependence. *Neuropsychiatr. Dis. Treat.* 3, 711–721 (2007).

6. Vinson, G. P. & Brennan, C. H. Addiction and the adrenal cortex. *Endocr. Conncetions* 2, 1–14 (2013).

7. United Nations Office on Drugs and Crime. The International Drug Control Conventions. 1–160 (2013).

8. Brandt, S. D., King, L. A. & Evans-Brown, M. The new drug phenomenon. *Drug Test. Anal.* 6, 587–597 (2014).

9. United Nations Office on Drugs and Crime. *The challenge of new psychoactive substances*. (2013).

10. Madras, B. K. in *Neuropsychopharmacology of new psychoactive substances* 1–18 (2017).

11. Baumann, M. H., Glennon, R. A. & Wiley, J. L. *Neuropharmacology of New Psychoactive Substances (NPS)*. (Current Topics in Behavioral Neurosciences, 2017).

12. Gibbons, S. 'Legal Highs'–novel and emerging psychoactive drugs: a chemical overview for the toxicologist. *Clin. Toxicol.* 50, 15–24 (2012).

13. United Nations Office on Drugs and Crime. in 3–65 (2017).

14. Papaseit, E., Farré, M., Schifano, F. & Torrens, M. Emerging drugs in Europe. *Curr. Opin. Psychiatry* 27, 243–50 (2014).

15. Roman-Urrestarazu, A. & Corazza, O. *Novel Psychoactive Substances: Policy, Economics and Drug Regulation*. (2017).

16. Baumann, M. H. & Volkow, N. D. Abuse of New Psychoactive Substances: Threats and Solutions. *Neuropsychopharmacology* 41, 663–665 (2015).

17. European Monitoring Centre for Drugs and Drug Addiction. New psychoactive substances in Europe. Legislation and prosecution - current challenges and solutions. 1–25 (2016).

18. Simmler, L. D. *et al.* Pharmacological characterization of designer cathinones in vitro. *Br. J. Pharmacol.* 168, 458–470 (2013).

19. Martínez-Clemente, J., Escubedo, E., Pubill, D. & Camarasa, J. Interaction of mephedrone with dopamine and serotonin targets in rats. *Eur. Neuropsychopharmacol.* 22, 231–236 (2012).

20. Martínez-Clemente, J. *et al.* Mephedrone pharmacokinetics after intravenous and oral administration in rats: Relation to pharmacodynamics. *Psychopharmacology (Berl).* 229, 295–306 (2013).

21. Elmore, J. S. *et al.* Pharmacokinetic Profiles and Pharmacodynamic Effects for Methylone and Its Metabolites in Rats. *Neuropsychopharmacology* 42, 649–660 (2016).

22. Ellefsen, K. N. *et al.* Synthetic cathinone pharmacokinetics , analytical methods , and toxicological findings from human performance and postmortem cases. *Drug Metab. Rev.* 2532, (2016).

23. Webb, N. E. *et al.* Change in the new psychoactive substances associated with Emergency Department acute toxicity presentations associated with the introduction of the UK 2016 Psychoactive Substances Act Change in the new psychoactive substances associated with Emergency. *Clin. Toxicol.* 0, 1–6 (2018).

24. Hand, T. & Rishiraj, A. S. Seizures of Drugs in England and Wales 2008/09. *Home Office Statistical Bulletin* 0–19 (2009).

25. Home Office Crime and Policing Analysis Unit, 2016. Drug Misuse : Findings from the 2015 / 16 Crime Survey for England and Wales. 1–51 (2016).

26. Home Office Crime and Policing Analysis Unit, 2016. Drug Misuse : Findings from the 2016/17 Crime Survey for England and Wales. *2017* (2017).

27. Reuter, P. H. & Bryce, P. A. in *Novel Psychoactive Substances: Policy, Economics and Drug Regulation* (2017).

28. Baumann, M. H. *et al.* Bath Salts,Spice, and Related Designer Drugs: The Science Behind the Headlines. *J. Neurosci.* 34, 15150–15158 (2014).

29. Miliano, C. *et al.* Neuropharmacology of New psychoactive substances (NPS): Focus on the rewarding and reinforcing properties of cannabimimetics and amphetamine-like stimulants. *Front. Neurosci.* 10, 1–21 (2016).

30. Soria-Sanchez, L. M. New Psychoactive Substances: Risks and Challenges. *Toxicol. forensic Med.* 2, 37–38 (2016).

31. Helander, A. & Bäckberg, M. New Psychoactive Substances (NPS) – the Hydra monster of recreational drugs. *Clin. Toxicol.* 55, 1–3 (2016).

32. Helander, A., Bäckberg, M., Hultén, P., Al-Saffer, Y. & Beck, O. Detection of new psychoactive substance use among emergency room patients: Results from the Swedish STRIDA project. *Forensic Sci. Int.* 243, 23–29 (2014).

33. Wood, D. M. *et al.* Review of European-Drug Emergencies Network (Euro-DEN) training package for non-specialist workers to assess acute recreational drug and new psychoactive substance toxicity in night-time economy environments. *Drugs Educ. Prev. Policy* 1-5, 1465–3370 (2015).

34. United Nations Office on Drugs and Crime. in *World Drug Report* 1–60 (2017).

35. EMCDDA. www.emcdda.europa.eu. (2016).

36. Fattore, L. & Fratta, W. BeyondTHC : the new generation of cannabinoid designer drugs. 5, 1–12 (2011).

37. Brents, L. K. *et al.* Phase I Hydroxylated Metabolites of the K2 Synthetic Cannabinoid JWH-018 Retain In Vitro and In Vivo Cannabinoid 1 Receptor Affinity and Activity. *PLoS One* 6, 1–9 (2011).

38. Winstock, A. R. & Barratt, M. J. Synthetic cannabis: A comparison of patterns of use and effect profile with natural cannabis in a large global sample. *Drug Alcohol Depend.* 131, 106–111 (2013).

39. Simmler, L. D., Rickli, A., Hoener, M. C. & Liechti, M. E. Monoamine transporter and receptor in cathinones. *Neuro* 79, 152–160 (2013).

40. López-Arnau, R., Martínez-Clemente, J., Pubill, D., Escubedo, E. & Camarasa, J. Comparative neuropharmacology of three psychostimulant cathinone derivatives: Butylone, mephedrone and methylone. *Br. J. Pharmacol.* 167, 407–420 (2012).

41. Busardò, F. P., Kyriakou, C., Napoletano, S., Marinelli, E. & Zaami, S. Mephedrone related fatalities: A review. *Eur. Rev. Med. Pharmacol. Sci.* 19, 3777–3790 (2015).

42. Papaseit, E., Olesti, E., De La Torre, R., Torrens, M. & Farré, M. Mephedrone concentrations in cases of clinical intoxication. *Curr. Pharm. Des.* (2017).

43. Nelson, M. E., Bryant, S. M. & Aks, S. E. Emerging drugs of abuse. *Disease-a-Month* 60, 110–132 (2014).

44. Corazza, O. *et al.* Designer Drugs on the Internet : A Phenomenon Out-of-Control? The Emergence of Hallucinogenic Drug Bromo-Dragonfly. *Curr. Clin. Pharmacol.* 6, 125–129 (2011).

45. Hill, S. L. & Thomas, S. H. L. Clinical toxicology of newer recreational drugs. *Clin. Toxicol.* 49, 705–719 (2011).

46. Kersten, B. P. & Mclaughlin, M. E. Toxicology and Management of Novel Psychoactive Drugs. *J. Pharm. Pract.* 28, 50–65 (2015).

47. Karila, L., Billieux, J., Benyamina, A., Lançon, C. & Cottencin, O. The effects and risks associated to Mephedrone and Methylone in humans: A review of the preliminary evidences. *Brain Res. Bull.* (2016).

48. Schifano, F., Papanti, D. G., Orsolini, L. & Corkery, J. M. Novel Psychoactive Substances : the pharmacology of stimulants and hallucinogens. *Expert Rev. Clin. Pharmacol.* 2433, (2016).

49. Brown, D. T. *Cannabis*. (Harwood academic publishers, 1998).

50. European Drug Report. (2017).

51. Maldonado, R., Berrendero, F., Ozaita, A. & Robledo, P. Neurochemical basis of cannabis addiction. *Neuroscience* 181, 1–17 (2011).

52. Grotenhermen, F. Pharmacokinetics and Pharmacodynamics of Cannabinoids. *Clin. Pharmacokinet.* 42, 327–360 (2003).

53. Gaoni, Y. & Mechoulam, R. Isolation, Strucutre, and Partial Synthesis of an Active Constituent of Hashish. *J. Am. Chem. Soc.* 86, 1646–1647 (1964).

54. Seely, K. A., Lapoint, J., Moran, J. H. & Fattore, L. Spice drugs are more than harmless herbal blends : A review of the pharmacology and toxicology of synthetic cannabinoids. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 39, 234–243 (2012).

55. Theunissen, E. L. *et al.* Neurocognition and subjective experience following acute doses of the synthetic cannabinoid JWH-018 : A phase 1 , placebo-controlled , pilot study . *Br. J. Pharmacol.* (2017).

56. Armenian, P. *et al.* Intoxication from the novel synthetic cannabinoids AB-PINACA and ADB-PINACA: A case series and review of the literature. *Neuropharmacology* (2017).

57. Wiley, J. L., Marusich, J. A. & Thomas, B. F. in 231–248 (Current Topics in Behavioral Neurosciences, 2017).

58. Tai, S. & Fantegrossi, W. E. Pharmacological and Toxicological Effects of Synthetic Cannabinoids and Their Metabolites. *Curr. Top. Behav. Neurosci.* 32, 249–262 (2017).

59. Su, M. K., Seely, K. A., Moran, J. H. & Hoffman, R. S. Metabolism of Classical Cannabinoids and the Synthetic Cannabinoid JWH-018. *Clin. Pharmacol. Ther.* 97, 562–564 (2015).

60. Toennes, S. W. *et al.* Excretion of metabolites of the synthetic cannabinoid JWH-018 in urine after controlled inhalation. *J. Pharm. Biomed. Anal.* 150, 162–168 (2018).

61. Toennes, S. W. *et al.* Pharmacokinetic properties of the synthetic cannabinoid JWH-018 and of its metabolites in serum after inhalation. *J. Pharm. Biomed. Anal.* 140, 215–222 (2017).

62. Toennes, S. W., Geraths, A., Pogoda, W., Paulke, A. & Ramaekers, J. G. Pharmacokinetic properties of the synthetic cannabinoid JWH-018 in oral fluid after inhalation. *Drug Test. Anal.* 1–7 (2017).

63. Valente, M. J., Guedes de Pinho, P., de Lourdes Bastos, M., Carvalho, F. & Carvalho, M. Khat and synthetic cathinones : a review. *Arch. Toxicol.* 88, 15–45 (2014).

64. Al-motarreb, A., Baker, K. & Broadley, K. J. Khat: Pharmacological and Medical Aspects and its Social Use in Yemen. *Phyther. Res.* 16, 403–413 (2002).

65. South African National Biodiversity Institute, S. Catha Edulis; *2004*.

66. Kelly, J. P. Cathinone derivatives: A review of their chemistry, pharmacology and toxicology. *Drug Test. Anal.* 3, 439–453 (2011).

67. MNAR/11/1975, U. N. document. Etudes sur la composition chimique du Khat. (1975).

68. Toennes, S. W., Harder, S., Schramm, M., Niess, C. & Kauert, G. F. Pharmacokinetics of cathinone, cathine and norephedrine after the chewing of khat leaves. *Br. J. Clin. Pharmacol.* 56, 125–130 (2003).

69. UNODC. Laboratory and Scientific Section Portals (2018).

70. Baumann, M. H., Partilla, J. S. & Lehner, K. R. Psychoactive 'bath salts': Not so soothing. *Eur. J. Pharmacol.* 698, 1–5 (2013).

71. Prosser, J. M. & Nelson, L. S. The Toxicology of Bath Salts: A Review of Synthetic Cathinones. *J. Med. Toxicol.* 8, 33–42 (2012).

72. Karila, L., Megarbane, B., Cottencin, O. & Lejoyeux, M. Synthetic Cathinones: A New Public Health Problem. *Curr. Neuropharmacol.* 13, 12–20 (2015).

73. Baumann, M. H. *et al.* The designer methcathinone analogs, mephedrone and methylone, are substrates for monoamine transporters in brain tissue. *Neuropsychopharmacology* 37, 1192–203 (2012).

74. Baumann, M. H. *et al.* Powerful Cocaine-Like Action of 3,4-Methylenedioxypyrovalerone (MDPV), a Principal Constituent of Psychoactive 'Bath Salts' Products. *Neuropsychopharmacology* 4, 552–62 (2012).

75. Karila, L., Lafaye, G., Scocard, A., Cottencin, O. & Benyamina, A. Neuropharmacology MDPV and α-PVP use in humans: The twisted sisters. *Neuropharmacology* 1–8 (2017).

76. Glennon, R. A. & Young, R. Neurobiology of 3,4-methylenedioxypyrovalerone (MDPV) and α-pyrrolidinovalerophenone (α-PVP). *Brain Res. Bull.* 126, 111–126 (2016).

77. Kalix, P. Khat, an amphetamine-like stimulant. *J. Psychoactive Drugs* 26, 37–41 (2012).

78. Bonano, J. S., Glennon, R. A., De Felice, L. J., Banks, M. L. & Negus, S. S. Abuse-related and abuse-limiting effects of methcathinone and the synthetic 'bath salts' cathinone analogs methylenedioxypyrovalerone (MDPV), methylone and mephedrone on intracranial self-stimulation in rats. *Psychopharmacology (Berl).* Psychophar, 199–207 (2014).

79. World Health Organization. Mephedrone. (2014).

80. Papaseit, E. *et al.* Human Pharmacology of Mephedrone in Comparison to MDMA. *Neuropsychopharmacology* (2016).

81. Schifano, F. *et al.* Mephedrone (4-methylmethcathinone; 'Meow meow'): Chemical, pharmacological and clinical issues. *Psychopharmacology (Berl).* 214, 593–602 (2011).

82. Emcdda. Europol – EMCDDA Joint Report on a new psychoactive substance : 4-methylmethcathinone (mephedrone). 1–30 (2010).

83. Castrignanò, E. *et al.* A new approach towards biomarker selection in estimation of human exposure to chiral chemicals : a case study of mephedrone. 1–12 (2017).

84. Papaseit, E. *et al.* in *Current topics in behavioral neurosciences* (2016).

85. Pedersen, A. J., Reitzel, L. A., Johansen, S. S. & Linnet, K. In vitro metabolism studies on mephedrone and analysis of forensic cases. *Drug Test. Anal.* 5, 430–438 (2013).

86. Savona, E. U., Kleiman, M. A. R. & Calderoni, F. *Dual markets*. (2017).

87. Brunt, T. M., Poortman, a., Niesink, R. J. & van den Brink, W. Instability of the ecstasy market and a new kid on the block: mephedrone. *J. Psychopharmacol.* 25, 1543–1547 (2011).

88. Measham, F., Moore, K., Newcombe, R. & Welch, Z. Tweaking, bombing, dabbing and stockpiling: the emergence of mephedrone and the perversity of prohibition. *Drugs & Alcohol Today* 10, 14–21 (2010).

89. Claire, M., Hout, V. & Bingham, T. 'A Costly Turn On': Patterns of use and perceived consequences of mephedrone based head shop products amongst Irish injectors. *Int. J. Drug Policy* 23, 188–197 (2012).

90. Brunt, T. M., Niesink, R. J. M. & Brink, W. Van Den. Impact of a transient instability of the ecstasy market on health concerns and drug use patterns in The Netherlands. *Int. J. Drug Policy* 23, 134–140 (2012).

91. European Monitoring Centre for Drugs and Drug Addiction. *Report on the risk assessment of mephedrone in the framework of the Council Decision on new psychoactive substances*. (2011).

92. United Nations Office on Drugs and Crime. Single Convention on Narcotic Drugs, 1961. 1–44 (1961).

93. Advisory Council on the Misuse of Drugs, A. Consideration of the Novel Psychoactive Substances (‘Legal Highs’). (2011).

94. Dick, D. & Torrance, C. ‘MixMag Drugs Survey’,Mix Mag 225. 44–53 (2010).

95. Winstock, A. ‘The 2011 Mismag drugs survey’ Mixmag March. 46–59 (2011).

96. EMCDDA. Annual report 2011: the state of the drugs problem in Europe. Follow-up on mephedrone. (2011).

97. Hout, M. C. Van & Brennan, R. Curiosity killed M-Cat : A post-legislative study on mephedrone use in Ireland. *Drugs Educ. Prev. policy* 19, 156–162 (2012).

98. Measham, F., Wood, D. M., Dargan, P. I. & Moore, K. The rise in legal highs : prevalence and patterns in the use of illegal drugs and first- and second-generation ‘ legal highs ’ in South London gay dance clubs. *J. Subst. Use* 16, 263–272 (2011).

99. Hockenhull, J., Murphy, K. G. & Paterson, S. Mephedrone use is increasing in London. *Lancet* 387, 1719–1720 (2016).

100. Winstock, A., Mitcheson, L. & Marsden, J. Mephedrone : still available and twice the price. *Corresp. - Lancet* 376, 1 (2010).

101. Web, T. global web survey 2014 findings. *Last 12 month prevalence of top 20 drugs*. 1–19 (2014).

102. Wood, D. M. *et al.* The European Drug Emergencies Network (Euro-DEN). *Clin. Toxicol.* 52, 239–241 (2014).

103. Mccall, H., Adams, N. & Mason, D. What is chemsex and why does it matter ? 5790, 2–3 (2015).

104. Vardakou, I., Pistos, C. & Spiliopoulou, C. Drugs for youth via Internet and the example of mephedrone. *Toxicol. Lett.* 201, 191–195 (2011).

105. Winstock, A. *et al.* Mephedrone: use , subjective effects and health risks. *Addiction* 106, 1991–1996 (2011).

106. Jones, L., Reed, P. & Parrott, A. Mephedrone and 3,4-methylenedioxy-methamphetamine : Comparative psychobiological effects as reported by recreational polydrug users. *J. Psychopharmacol.* 12, 1313–1320 (2016).

107. Erowid. (2000). at www.erowid.org . Consulted February 2018.

108. Van Hout, M. C. An Internet Study of User's Cathinone 4-Methylethcathinone. *J. Psychoactive Drugs* 4, 37–41 (2014).

109. Winstock, A. R. *et al.* Mephedrone, new kid for the chop? *Addiction* 106, 154–161 (2011).

110. Wood, D. M., Hill, S. L., Thomas, S. H. L. & Dargan, P. I. Using poisons information service data to assess the acute harms associated with novel psychoactive substances. *Drug Test. Anal.* 6, 850–860 (2014).

111. de Sousa Fernandes Perna, E. B. *et al.* Neurocognitive performance following acute mephedrone administration, with and without alcohol. *J. Psychopharmacol.* 1–8 (2016).

112. Eshleman, A. J. *et al.* Substituted methcathinones differ in transporter and receptor interactions. *Biochem. Pharmacol.* 85, 1803–1815 (2013).

113. Thomas Splettstoesser. https://www.scistyle.com/. *2017* (2017).

114. Varner, K. J. *et al.* Comparison of the behavioral and cardiovascular effects of mephedrone with other drugs of abuse in rats. *Psychopharmacology (Berl).* 225, 675–685 (2013).

115. Freeman, T. P. *et al.* Cognitive and subjective effects of mephedrone and factors influencing use of a 'new legal high'. *Addiction* 107, 792–800 (2012).

116. Herzig, D. A., Brooks, R. & Mohr, C. Inferring about individual drug and schizotypy effects on cognitive functioning in polydrug using mephedrone users before and after clubbing. *Hum. Psychopharmacol.* 28, 168–182 (2013).

117. Kuypers, K. P. C. & Ramaekers, J. G. Transient memory impairment after acute dose of 75 mg 3.4-Methylene- dioxymethamphetamine. *Psychopharmacology (Berl).* 19, 633–639 (2005).

118. Kuypers, K. P. C. & Ramaekers, J. G. Acute dose of MDMA (75 mg) impairs spatial memory for location but leaves contextual processing of visuospatial information unaffected. *Psychopharmacology (Berl).* 189, 557–563 (2007).

119. Meneses, A. 5-HT systems : Emergent targets for memory formation and memory alterations 5-HT systems : emergent targets for memory formation and memory alterations. *Rev. Neurosci.* 24, 629–664 (2015).

120. Wood, D. M. *et al.* Case series of individuals with analytically confirmed acute mephedrone toxicity. 924–927 (2010).

121. Busardò, F., Kyriakou, C., Napoletano, S., Marinelli, E. & Zaami, S. Mephedrone related fatalities: a review. *Eur. Rev. Med. Pharmacol. Sci.* 19, 3777–3790 (2015).

122. Dargan, P. I., Sedefov, R., Gallegos, A. & Wood, D. M. The pharmacology and toxicology of the synthetic cathinone mephedrone (4-methylmethcathinone). *Drug Test. Anal.* (2011).

123. Corkery, J. M., Schifano, F. & Ghodse, A. H. in *Pharmacology* (ed. Galleli, L.) 355–380 (InTech - Open Access Publisher, 2012).

124. Maskell, P. D., Paoli, G. De, Seneviratne, C. & Pounder, D. J. Mephedrone (4-Methylmethcathinone) -Related Deaths. *J. Anal. Toxicol.* 35, 188–191 (2011).

125. Nicholson, P. J., Quinn, M. J. & Dodd, J. D. Headshop heartache : acute mephedrone ‘ meow ’ myocarditis. *Heart* 96, 2051–2053 (2010).

126. Wood, D. M. *et al.* Recreational Use of Mephedrone (4-Methylmethcathinone, 4-MMC) with Associated Sympathomimetic Toxicity. *J. Med. Toxicol.* 6, 327–330 (2010).

127. Wood, D. M., Greene, S. L. & Dargan, P. I. Clinical pattern of toxicity associated with the novel synthetic cathinone mephedrone. *Emerg. Med. J.* 28, 280–283 (2010).

128. James, D. *et al.* Clinical characteristics of mephedrone toxicity reported to the UK National Poisons Information Service. *Emerg. Med. J.* 28, 686–689 (2011).

129. Durham, M. Ivory wave: the next mephedrone? *Emerg. Med. J.* 28, 1059–1061 (2011).

130. Regan, L., Mitchelson, M. & Macdonald, C. Mephedrone toxicity in a Scottish emergency department. *Emerg. Med. J.* 28, 1055–1058 (2010).

131. Schifano, F., Corkery, Þ. J. & Ghodse, A. H. Suspected and Confirmed Fatalities Associated With in the United Kingdom. *J. Clin. Psychopharmacol.* 32, 710–714 (2012).

132. Sivagnanam, K., Chaudari, D., López, P., Sutherland, M. E. & Ramu, V. K. 'Bath salts' induced severe reversible cardiomyopathy. *Am. J. Case Rep*. 14, 288–291 (2013).

133. Barrio, P., Gaskell, M., Goti, J., Vilardell, S. & Fàbregas, J. M. Persistent psychotic symptoms after long-term heavy use of mephedrone: A two-case series Síntomas psicóticos persistentes después del uso abusivo prolongado de mefedrona: una serie de dos casos. *Addiciones* 28, 154–157 (2016).

134. Wood, D. M. & Dargan, P. I. Mephedrone (4- methylmethcathinone): What is new in our understanding of its use and toxicity. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 39, 227–233 (2012).

135. Vallersnes, O. M. *et al.* Psychosis associated with acute recreational drug toxicity : a European case series. *BMC Psychiatry* 16, 1–8 (2016).

136. European Monitoring Centre for Drugs and Drug Addiction. Hospital emergency presentations and acute drug toxicity in Europe. (2016). doi:10.2810/894142

137. England, D. of P. H. Adult substance misuse statistics from the National Drug Treatment Monitoring System (NDTMS). (2017).

138. Karila, L. & Reynaud, M. GHB and synthetic cathinones : clinical effects. *Drug Test. Anal.* 3, 552–559 (2010).

139. López-Arnau, R. *et al.* An integrated pharmacokinetic and pharmacodynamic study of a new drug of abuse, methylone, a synthetic cathinone sold as 'bath salts'. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* 45, 64–72 (2013).

140. Aarde, S. M. *et al.* Mephedrone (4-methylmethcathinone) supports intravenous self-administration in Sprague-Dawley and Wistar rats. *Addict. Biol.* 18, 786–799 (2013).

141. Wright, J. J. *et al.* Effect of Ambient Temperature on the Thermoregulatory and Locomotor Stimulant Effects of 4-Methylmethcathinone in Wistar and Sprague-Dawley Rats. *PLoS One* 7, (2012).

142. de la Torre, R. *et al.* Human Pharmacology of MDMA. *Ther. Drug Monit.* 26, 137–144 (2004).

143. de la Torre, R. *et al.* Pharmacology of MDMA in Humans. *Ann. New York Acad. Sci.* 914, 225–37 (2000).

144. Roden, D. M. & George, A. L. The genetic basis of variability in drug responses. 1, (2002).

145. Meyer, M. R., Wilhelm, J., Peters, F. T. & Maurer, H. H. Beta-keto amphetamines: Studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography - Mass spectrometry. *Anal. Bioanal. Chem.* 397, 1225–1233 (2010).

146. Khreit, O. I. G. *et al.* Elucidation of the Phase I and Phase II metabolic pathways of (±)-4'-methylmethcathinone (4-MMC) and (±)-4'-(trifluoromethyl)methcathinone (4-TFMMC) in rat liver hepatocytes using LC-MS and LC-MS2. *J. Pharm. Biomed. Anal.* 72, 177–185 (2013).

147. Pozo, Ó. J. *et al.* Mass Spectrometric Evaluation of Mephedrone In Vivo Human Metabolism : Identification of Phase I and Phase II Metabolites , Including a Novel Succinyl Conjugate s. *Drug Metab. Dispos.* 43, 248–257 (2014).

148. Mayer, F. P. *et al.* Phase I metabolites of mephedrone display biological activity as substrates at monoamine transporters. *Br. J. Pharmacol.* 2657–2668 (2016).

149. Pedersen, A. J., Reitzel, L. A. & Johansen, S. S. In vitro metabolism studies on mephedrone and analysis of forensic cases. (2012). doi:10.1002/dta.1369

150. Dictionary, C. E. Definition of analytical chemistry. (2018). at <https://www.collinsdictionary.com/dictionary/english/analytical-chemistry>. Consulted at June 2018.

151. Kellner, R., Mermet, J.-M., Otto, M., Valcárcel, M. & Widmer, H. M. *Analytical Chemistry: A Modern Approach to Analytical Science*. (2004).

152. Rodríguez-Morató, J., de la Torre, R. & Pozo, Ó. J. Potential of LC-MS/MS in Pharmacology and Toxicology: From Classical Applications to Personalized Medicine. *EC Pharmacol. Toxicol.* 2, 38–39 (2017).

153. Snyder, L. R., Kirkland, J. J. & Dolan, J. W. *Introduction to modern liquid chromatography*. (John Wiley & Sons, inc., 2010).

154. Niessen, W. M. A. *Liquid Chromatrography - Mass Spectrometry*. (CRC Press (Taylor & Francis Group), 2006).

155. Unger, K. K., Lamotte, S. & Machtejevas, E. in *Liquid Chromatography* 41–86 (Elsevier Inc., 2013). doi:10.1016/B978-0-12-415807-8.00003-1

156. Thakare, R., Chhonker, Y. S., Gautam, N., Alamoudi, J. A. & Alnouti, Y. Quantitative analysis of endogenous compounds. *J. Pharm. Biomed. Anal.* 128, 426–437 (2016).

157. Rodríguez-Morató, J., Pozo, Ó. J. & Marcos, J. Targeting human urinary metabolome by LC–MS/MS: a review. *Bioanalysis* (2018).

158. Hernández, F., Sancho, J. V. & Pozo, Ó. J. Critical review of the application of liquid chromatography/mass spectrometry to the determination of pesticide residues in biological samples. *Anal Bioanal Chem* 382, 934–946 (2005).

159. Gomis-Yagües, V. Espectrometría de masas. 1–43 (2008).

160. Olesti, E. *et al.* Pharmacokinetics of Mephedrone and Its Metabolites in Human by LC-MS / MS. 6, (2017).

161. Pozo, Ó. J. *et al.* Alternate steroid sulfatation pathways targeted by LC-MS/MS analysis of disulfates. Application to prenatal diagnosis of steroid synthesis disorders. *J Mol Endocrinol* 1–38 (2018).

162. Adaway, J. E. & Keevil, B. G. Therapeutic drug monitoring and LC–MS/MS. *J. Chromatogr. B* 883-884, 33–49 (2012).

163. Weinmann, W., Schaefer, P., Thierauf, A., Schreiber, A. & Wurst, F. M. Confirmatory Analysis of Ethylglucuronide in Urine by Liquid-Chromatography / Electrospray According to Forensic Guidelines. *J. Am. Soc. Mass Spectrom.* 15, 188–193 (2004).

164. Xiao, J. F., Zhou, B. & Ressom, H. W. Metabolite identification and quantitation in LC-MS/MS-based metabolomics. *Trends Anal. Chem.* 32, 1–14 (2012).

165. Ellefsen, K. N. *et al.* Validation of the only commercially available immunoassay for synthetic cathinones in urine : Randox Drugs of Abuse V Biochip Array Technology. *Drug Test. Anal.* 6, 728–738 (2014).

166. Ambach, L., Hernández Redondo, A., König, S. & Weinmann, W. Rapid and simple LC-MS/MS screening of 64 novel psychoactive substances using dried blood spots. *Drug Test. Anal.* 6, 367–375 (2014).

167. Mercolini, L., Protti, M., Catapano, M. C., Rudge, J. & Sberna, A. E. LC-MS/MS and volumetric absorptive microsampling for quantitative bioanalysis of cathinone analogues in dried urine, plasma and oral fluid samples. *J. Pharm. Biomed. Anal.* (2016).

168. Adamowicz, P. & Tokarczyk, B. Simple and rapid screening procedure for 143 new psychoactive substances by liquid chromatography-tandem mass spectrometry. *Drug Test. Anal.* 8, 652–667 (2016).

169. Sørensen, L. K. Determination of cathinones and related ephedrines in forensic whole-blood samples by liquid-chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 879, 727–736 (2011).

170. Hong, W. *et al.* Determination of Synthetic Cathinones in Urine Using Gas Chromatography – Mass Spectrometry Techniques. *J. Anal. Toxicol.* 1–5 (2015).

171. de Castro, A. *et al.* Liquid chromatography tandem mass spectrometry determination of selected synthetic cathinones and two piperazines in oral fluid. Cross reactivity study with an on-site immunoassay device. *J. Chromatogr. A* 1374, 93–101 (2014).

172. Strano-Rossi, S., Anzillotti, L., Castrignanò, E., Romolo, F. S. & Chiarotti, M. Ultra high performance liquid chromatography-electrospray ionization-tandem mass spectrometry screening method for direct analysis of designer drugs, 'spice' and stimulants in oral fluid. *J. Chromatogr. A* 1258, 37–42 (2012).

173. Kintz, P. Evidence of 2 Populations of Mephedrone Abusers by Hair Testing . Application to 4 Forensic Expertises. *Curr. Neu* 15, 658–662 (2017).

174. Lendoiro, E. *et al.* An LC-MS / MS methodological approach to the analysis of hair for amphetamine-type- stimulant (ATS) drugs , including selected synthetic cathinones and piperazines. *Drug Test. Anal.* 96–105 (2017).

175. Shah, S. a B. *et al.* Quantitative analysis of mephedrone using liquid chromatography tandem mass spectroscopy: Application to human hair. *J. Pharm. Biomed. Anal.* 61, 64–69 (2012).

176. Agency, W. anti-doping. WADA Technical Document – TD2018MRPL. 2–7 (2018).

177. Mazzoni, I., Barroso, O. & Rabin, O. in *Novel Psychoactive Substances* 43–56 (2017).

178. Horgan, R. P. & Kenny, L. C. 'Omic' technologies: proteomics and metabolomics. *Obstet. Gynaecol.* 13, 189–195 (2011).

179. Meinhardt, M. W. *et al.* The Neurometabolic Fingerprint of Excessive Alcohol Drinking. *Neuropsychopharmacology* 40, 1259–1268 (2015).

180. Wishart, D. S. Emerging applications of metabolomics in drug discovery and precision medicine. *Nat. Rev. Drug Discov.* 15, 473–484 (2016).

181. Patti, G. J., Yanes, O. & Siuzdak, G. Metabolomics-the apogee of the omics trilogy. *Nat. Rev. Mol. Cell Biol.* 13, 263–269 (2012).

182. Johnson, C. H., Ivanisevic, J. & Siuzdak, G. Metabolomics: beyond biomarkers and towards mechanisms. *Nat. Rev. Mol. Cell Biol.* 1–9 (2016).

183. Greef, J. Van Der, Wietmarschen, H. Van, Ommen, B. Van & Verheij, E. Looking back into the future: 30 years of metabolomics at TNO. *Mass Spectrom. Rev.* 32, 399–415 (2013).

184. Pauling, L., Robinson, A. B., Teranishit, R. O. Y. & Cary, P. Quantitative Analysis of Urine Vapor and Breath by Gas-Liquid Partition Chromatography. *Proc. Nat. Acad. Sci. USA* 68, 2374–2376 (1971).

185. Oliver, S. G., Winson, M. K., Kell, D. B. & Baganz, F. Systematic functional analysis of the yeast genome. *Trends Biotechnol.* 16, 373–378 (1998).

186. Lindon, J. C. *et al.* The Consortium for Metabonomic Toxicology (COMET): aims, activities and achievements. *Pharmacogenomics* 6, 691–699 (2005).

187. Wishart, D. S. *et al.* HMDB : the Human Metabolome Database. *Nucleic Acids Res.* 35, 521–526 (2018).

188. Wishart, D. S. *et al.* HMDB 4 .0: the human metabolome database for 2018. *Nucleic Acids Res.* 46, 608–617 (2018).

189. Wishart, D. S. Applications of Metabolomics in Drug Discovery and Development. *Drugs R D* 9, 307–322 (2008).

190. Hyötyläinen, T. Novel methodologies in metabolic profiling with a focus on molecular diagnostic applications. *Expert Rev. Mol. Diagn.* 12, 527–538 (2012).

191. Han, J., Datla, R., Chan, S. & Borchers, C. H. Mass spectrometry-based technologies for high-throughput metabolomics. *Bioanalysis* 1, 1665–1684 (2009).

192. Bijlsma, L., Gil-solsona, R., Hernández, F. & Sancho, J. V. What about the herb? A new metabolomics approach for synthetic cannabinoid drug testing. *Anal. Bioanal. Chem.* (2018).

193. Zaitsu, K. *et al.* Metabolic profiling of urine and blood plasma in rat models of drug addiction on the basis of morphine, methamphetamine, and cocaine-induced conditioned place preference. *Anal. Bioanal. Chem.* 406, 1339–1354 (2014).

194. Kaplan, K. A. *et al.* Neuronal metabolomics by ion mobility mass spectrometry: Cocaine effects on glucose and selected biogenic amine metabolites in the frontal cortex, striatum, and thalamus of the rat. *Anal. Bioanal. Chem.* 405, 1959–1968 (2013).

195. Zaitsu, K. *et al.* Metabolome disruption of the rat cerebrum induced by the acute toxic effects of the synthetic cannabinoid MAM-2201. *Life Sci.* 137, 49–55 (2015).

196. McClay, J. L. *et al.* Large-scale neurochemical metabolomics analysis identifies multiple compounds associated with methamphetamine exposure. *Metabolomics* 9, 392–402 (2013).

197. Wen, C. *et al.* Brain metabolomics in rats after administration of ketamine. *Biomed. Chromatogr.* 30, 81–84 (2016).

198. Zheng, T. *et al.* Metabolic phenotype of rats exposed to heroin and potential markers of heroin abuse. *Drug Alcohol Depend.* 127, 177–186 (2013).

199. Schaefer, T. L. *et al.* Short- and long-term effects of methamphetamine and 3,4-methylenedioxymethamphetamine on monoamine and corticosterone levels in the neonatal rat following multiple days of treatment. *J. Neurochem.* 104, 1674–1685 (2008).

200. Stone, D. M., Stahl, D. C., Hanson, G. R. & Gibb, J. W. The effects of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) on monoaminergic systems in the rat brain. *Eur. J. Pharmacol.* 128, 41–48 (1986).

201. Elhwuegi, A. S. Central monoamines and their role in major depression. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* 28, 435–451 (2004).

202. Eisenhofer, G. Catecholamine Metabolism: A Contemporary View with Implications for Physiology and Medicine. *Pharmacol. Rev.* 56, 331–349 (2004).

203. Keszthelyi, D., Troost, F. J. & Masclee, A. A. M. Understanding the role of tryptophan and serotonin metabolism in gastrointestinal function. *Neurogastroenterol. Motil.* 21, 1239–1249 (2009).

204. Marcos, J. *et al.* Targeting tryptophan and tyrosine metabolism by liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* 1434, 91–101 (2016).

205. González, R. R. *et al.* Development and validation of an ultra-high performance liquid chromatography – tandem mass-spectrometry (UHPLC – MS / MS) method for the simultaneous determination of neurotransmitters in rat brain samples. *J. Neurosci. Methods* 198, 187–194 (2011).

206. Lionetto, L., Lostia, A. M., Stigliano, A., Cardelli, P. & Simmaco, M. HPLC – mass spectrometry method for quantitative detection of neuroendocrine tumor markers: Vanillylmandelic acid , homovanillic acid and 5-hydroxyindoleacetic acid. *Clin. Chim. Acta* 398, 53–56 (2008).

207. Cai, H. L., Zhu, R. H. & Li, H. D. Determination of dansylated monoamine and amino acid neurotransmitters and their metabolites in human plasma by liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal Biochem* 396, 103–111 (2010).

208. Beckstead, R. M. The Life Cycle of Neurotransmitters. *A Surv. Med. Neurosci.* 32–44 (1996).

209. Fernstrom, J. I. Dietary amino acids and brain function. *J. Am. Diet. Assoc.* 94, 71–77 (1994).

210. Petroff, O. A. C. GABA and Glutamate in the Human Brain. *Neurosci.* 8, 562–573 (2002).

211. Simon P.. Neurotransmitters. *Advances in Pharmacology and Therapeutics*. (1979).

212. Sperlágh, B. & Vizi, E. S. The Role of Extracellular Adenosine in Chemical Neurotransmission in the Hippocampus and Basal Ganglia : Pharmacological and Clinical Aspects. *Curr. Top. Med. Chem.* 11, 1034–1046 (2011).

213. Jiao, D., Li, X. & Zhao, M. The role of the GABA system in amphetamine-type stimulant use disorders. *Front. Cell. Neurosci.* 9, 1–13 (2015).

214. Ballesteros-yáñez, I., Castillo, C. A., Merighi, S. & Gessi, S. The Role of Adenosine Receptors in Psychostimulant Addiction. *Front. Pharmacol.* 8, 1–18 (2018).

215. Chen, J. *et al.* Target-based metabolomics for the quantitative measurement of 37 pathway metabolites in rat brain and serum using hydrophilic interaction ultra-high-performance liquid chromatography – tandem mass spectrometry. *Anal Bioanal Chem* 408, 2527–2542 (2016).

216. Seibert, J. *et al.* Acute effects of 3,4-methylenedioxymethamphetamine and methylphenidate on circulating steroid levels in healthy subjects. *Neuroendocrinology* 100, 17–25 (2014).

217. Shackleton, C., Pozo, O. J. & Marcos, J. GC/MS in Recent Years Has Defined the Normal and Clinically Disordered Steroidome: Will It Soon Be Surpassed by LC/Tandem MS in This Role? *J. Endocr. Soc.* 2, 974–996 (2018).

218. Marcos, J. *et al.* Investigation of endogenous corticosteroids profiles in human urine based on liquid chromatography tandem mass spectrometry. *Anal. Chim. Acta* 812, 92–104 (2014).

219. Dargan, P. I., Sedefov, R., Gallegos, A. & Wood, D. M. The pharmacology and toxicology of the synthetic cathinone mephedrone (4-methylmethcathinone). *Drug Test. Anal.* 3, 454–463 (2011).

220. Torrance, H. & Cooper, G. The detection of mephedrone (4-methylmethcathinone) in 4 fatalities in Scotland. *Forensic Sci. Int.* 202, e62–e63 (2010).

221. European Medicines Agency. Guideline on bioanalytical method validation. *EMA Guidel.* 44, 1–23 (2012).

222. Pantano, F. *et al.* Neurotoxicity Induced by Mephedrone: An up-to-date Review. *Curr. Neuropharmacol.* 15, 738–749 (2017).

223. Olesti, E. *et al.* GC–MS Quantification Method for Mephedrone in Plasma and Urine: Application to Human Pharmacokinetics. *J. Anal. Toxicol.* 2–8 (2016).

224. Amaratunga, P., Lemberg, B. L. & Lemberg, D. Quantitative measurement of synthetic cathinones in oral fluid. *J. Anal. Toxicol.* 37, 622–628 (2013).

225. Linhart, I. *et al.* Metabolic profile of mephedrone: Identification of nor-mephedrone conjugates with dicarboxylic acids as a new type of xenobiotic phase II metabolites. *Toxicol. Lett.* 240, 114–121 (2016).

226. Cozzi, N. V., Sievert, M. K., Shulgin, A. T., Jacob, P. & Ruoho, A. E. Inhibition of plasma membrane monoamine transporters by β-ketoamphetamines. *Eur. J. Pharmacol.* 381, 63–69 (1999).

227. Deventer, K., Pozo, O. J., Verstraete, A. G. & Van Eenoo, P. Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology. *TrAC - Trends Anal. Chem.* 55, 1–13 (2014).

228. Cruickshank, C. C. & Dyer, K. R. A review of the clinical pharmacology of methamphetamine. *Addiction* 104, 1085–1099 (2009).

229. Wu, D., Victoria Otton, S., Inaba, T., Kalow, W. & Sellers, E. M. Interactions of amphetamine analogs with human liver CYP2D6. *Biochem. Pharmacol.* 53, 1605–1612 (1997).

230. Shimshoni, J. A. *et al.* 3-Methyl-methcathinone: Pharmacokinetic profile evaluation in pigs in relation to pharmacodynamics. *J. Psychopharmacol.* 29, 734–743 (2015).

231. Zhou, S. Polymorphism of Human Cytochrome P450 2D6 and Its Clinical Significance. *Clin Pharmacokinet* 48, 689–723 (2009).

232. De La Torre, R. *et al.* Non-linear pharmacokinetics of MDMA (’ecstasy') in humans. *Br. J. Clin. Pharmacol.* 49, 104–109 (2000).

233. Zolkowska, D., Rothman, R. B. & Baumann, M. H. Amphetamine Analogs Increase Plasma Serotonin : Implications for Cardiac and Pulmonary Disease. 318, 604–610 (2006).

234. Yubero-lahoz, S. *et al.* Effects of MDMA and related analogs on plasma 5-HT : Relevance to 5-HT transporters in blood and brain. *Eur. J. Pharmacol.* 674, 337–344 (2012).

235. Cuyàs, E. *et al.* Errors and reproducibility of DNA array-based detection of allelic variants in ADME genes: PHARMAchip. *Pharmacogenomics.* 11, 257–266 (2010).

236. Zhou, Y., Ingelman-Sundberg, M. & Lauschke, V. M. Worldwide Distribution of Cytochrome P450 Alleles: A Meta-analysis of Population-scale Sequencing Projects. *Clin. Pharmacol. Ther.* 102, 689–700 (2017).

237. Yubero-lahoz, S., Rodríguez, J., Faura, A. & Pascual, J. Determination of free serotonin and its metabolite 5-HIAA in blood human samples with consideration to pre-analytical factors. 1641–1646 (2014). doi:10.1002/bmc.3192

238. Schmid, Y. *et al.* CYP2D6 function moderates the pharmacokinetics and in a controlled study in healthy individuals. *Pharmacogenet. Genomics* 26, 397–401 (2016).

239. Fattore, L. Synthetic Cannabinoids — Further Evidence Supporting the Relationship Between Cannabinoids and Psychosis. *Biol. Psychiatry* 79, 539–548 (2016).

240. Strachan, R. T., Ferrara, G. & Roth, B. L. Screening the receptorome : an efficient approach for drug discovery and target validation. *Drug Discov. Today* 11, 708–716 (2006).

241. Kroeze, W. K. *et al.* PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nat. Struct. Mol. Biol.* 22, 362–369 (2015).

242. Näsström, E. *et al.* Salmonella Typhi and Salmonella Paratyphi A elaborate distinct systemic metabolite signatures during enteric fever. *Elife* 3, 1–19 (2014).

243. Lu, H. *et al.* Simultaneous quantification of neuroactive dopamine serotonin and kynurenine pathway metabolites in gender-specific youth urine by ultra performance liquid chromatography tandem high resolution mass spectrometry. *J. Pharm. Biomed. Anal.* 122, 42–51 (2016).

244. Bloomfield, M. A. P., Ashok, A. H., Volkow, N. D. & Howes, O. D. The effects of Δ9-tetrahydrocannabinol on the dopamine system. *Nature* 539, 369–377 (2016).

245. Breiman, L. Random forests. *Mach. Learn.* 45, 5–32 (2001).

246. Stone, D., Stahl, D., Hanson, G. & Gibb, J. The effects of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) on monoaminergic systems in the rat brain. *Eur. J. Pharmacol.* 128, 41–48 (1986).

247. Fleckenstein, A. E. *et al.* Methamphetamine-induced decrease in tryptophan hydroxylase activity : role of 5-hydroxytryptaminergic transporters. (1997).

248. Commission achieves EU-wide ban on ecstasy-like drug mephedrone. (2010).

249. Drug Enforcement Administration, D. 4-Methylmethcathinone (Mephedrone). 4 (2013).

250. Drug Enforcement Administration, D. JWH-018 [Synthetic Cannabinoid in Herbal Products]. 1 (2013).

251. Marshell, R. *et al.* In vivo effects of synthetic cannabinoids JWH-018 and JWH-073 and phytocannabinoid Δ 9 -THC in mice : Inhalation versus intraperitoneal injection. *Pharmacol. , Biochem. Behav.* 124, 40–47 (2014).

252. Atwood, B. K., Huffman, J., Straiker, A. & Mackie, K. JWH018, a common constituent of ' Spice ' herbal blends, is a potent and efficacious cannabinoid CB 1 receptor agonist. *Br. J. Pharmacol.* 585–593 (2010).

253. Drug Enforcement Administration, D. 3,4-Methylenedioxypyrovalerone (MDPV). 2012, 7183 (2013).

254. World Health Organization - Expert Committee on Drug Dependence. 3,4-Methylenedioxypyrovalerone (MDPV) - Critical Review Report. 1–37 (2014).

255. World Health Organization - Expert Committee on Drug Dependence. 1-Phenyl-2-(pyrrolidin-1-yl)pentan-1- one (α-PVP) -Critical Review Report. 16–20 (2015).

256. Novellas, J. *et al.* Concentrations of MDPV in rat striatum correlate with the psychostimulant effect. *J. os Psychopharmacol.* 11, 1209–1218 (2015).

257. Aarde, S. M., Creehan, K. M., Vandewater, S. A., Dickerson, T. J. & Taffe, M. A. In vivo potency and efficacy of the novel cathinone self-administration and locomotor stimulation in male rats. *Psychopharmacology (Berl).* 232, 3045–3055 (2015).

258. Baumann, M. H. *et al.* in *Neuropharmacology of New Psychoactive Substances (NPS)* 93–117 (Current Topics in Behavioral Neurosciences, 2016).

259. Wang, L., Pi, Z., Liu, S., Liu, Z. & Song, F. Targeted metabolome profiling by dual-probe microdialysis sampling and treatment using Gardenia jasminoides for rats with type 2 diabetes. *Sci. Rep.* 1–11 (2017).

260. Pastor, A., Farré, M., Fitó, M., Fernandez-Aranda, F. & de la Torre, R. Analysis of ECs and related compounds in plasma: artifactual isomerization and ex vivo enzymatic generation of 2-MGs. *J. Lipid Res.* 55, 966–977 (2014).

261. Imperato, A., C, M. & Gessa, G. L. Effects of cocaine and amphetamine on acetylcholine release in the hippocampus and caudate nucleus. 238, 377–381 (1993).

262. White, T. L. *et al.* Psychostimulant drug effects on glutamate , Glx , and creatine in the anterior cingulate cortex and subjective response in healthy humans. *Neuropsychopharmacology* 43, 1498–1509 (2018).

263. Gregg, R. A. *et al.* Synthetic cathinone MDPV downregulates glutamate transporter subtype I (GLT-1) and produces rewarding and locomotor-activating effects that are reduced by a GLT-1 activator. *Neuropharmacology* 108, 111–119 (2016).

264. Deutch, A. Y. in *Fundamental Neuroscience* (eds. Squire, L. R. et al.) 117–38 (2013).

265. Cuevas, J. in *xPharm: The comprehensive Pharmacology Reference* 1–7 (2007).

266. Parrot, S. *et al.* Capillary electrophoresis combined with microdialysis in the human spinal cord : A new tool for monitoring rapid peroperative changes in amino acid neurotransmitters within the dorsal horn. *Electrophoresis* 25, 1511–1517 (2004).

267. Yeon, J. *et al.* Profiling analysis of biogenic amines and their acidic metabolites in mouse brain tissue using gas chromatography – tandem mass spectrometry. *J. Chromatogr. B* 940, 94–103 (2013).

268. Zhao, L. *et al.* In vivo study on the neurotransmitters and their metabolites change in depressive disorder rat plasma by ultra high performance liquid chromatography coupled to tandem mass spectrometry. *J. Chromatogr. B* 988, 59–65 (2015).

269. Nurullah, S., Tague, S. E. & Lunte, C. Analysis of amino acid neurotransmitters from rat and mouse spinal cords by liquid chromatography with fluorescence detection. *J. Pharm. Biomed. Anal.* 107, 217–222 (2015).

270. Wojnicz, A. *et al.* Simultaneous determination of 8 neurotransmitters and their metabolite levels in rat brain using liquid chromatography in tandem with mass spectrometry : Application to the murine Nrf2 model of depression. *Clin. Chim. Acta* 453, 174–181 (2016).

271. Huang, F. *et al.* Simultaneous quantification of seven hippocampal neurotransmitters in depression mice by LC – MS / MS. *J. Neurosci. Methods* 229, 8–14 (2014).

272. Parrot, S., Neuzeret, P. & Denoroy, L. A rapid and sensitive method for the analysis of brain monoamine neurotransmitters using ultra-fast liquid chromatography coupled to electrochemical detection. *J. Chromatogr. B* 879, 3871–3878 (2011).

273. Cudjoe, E. & Pawliszyn, J. Optimization of solid phase microextraction coatings for liquid chromatography mass spectrometry determination of neurotransmitters. *J. Chromatogr. A* 1341, 1–7 (2014).

274. Konieczna, L., Roszkowska, A., Nied, M. & Tomasz, B. Hydrophilic interaction chromatography combined with dispersive liquid – liquid microextraction as a preconcentration tool for the simultaneous determination of the panel of underivatized neurotransmitters in human urine samples. *J. Chromatogr. A* 1431, 111–121 (2016).

275. Jandera, P. Stationary and mobile phases in hydrophilic interaction chromatography: a review. *Anal. Chim. Acta* 692, 1–25 (2011).

276. Zhao, X., Zhu, S., Yang, H., You, J. & Song, F. Simultaneous determination of amino acid and monoamine neurotransmitters in PC12 cells and rats models of Parkinson's disease using a sensitizing derivatization reagent by UHPLC–MS/MS. *J. Chromatogr. B* 995-996, 15–23 (2015).

277. Zhou, W. *et al.* A rapid and simple method for the simultaneous determination of four endogenous monoamine neurotransmitters in rat brain using hydrophilic interaction liquid chromatography coupled with atmospheric-pressure chemical ionization tandem mass spectrometry. *J. Chromatogr. B* 1002, 379–386 (2015).

278. Forgacsova, A., Galba, J., Garruto, R. M., Majerova, P. & Katina, S. A novel liquid chromatography/mass spectrometry method for determination of neurotransmitters in brain tissue: Application to human tauopathies. *J. Chromatogr. B* 1073, 154–162 (2018).

279. Bergh, M. S., Lise Bogen, I., Lundanes, E. & Leere Oiestad, A. M. Validated methods for determination of neurotransmitters and metabolites in rodent brain tissue and extracellular fluid by reversed phase UHPLC–MS/MS. *J. Chromatogr. B* 1028, 120–129 (2016).

280. Merbel, N. C. Van De. Quantitative determination of endogenous compounds in biological samples using chromatographic techniques. *Trends Anal. Chem.* 27, 924–933 (2008).

281. Perry, M., Li, Q. & Kennedy, R. T. Review of recent advances in analytical techniques for the determination of neurotransmitters. *Anal. Chim. Acta* 653, 1–22 (2009).

282. Dinis-oliveira, R. J. Metabolomics of drugs of abuse : a more realistic view of the toxicological complexity. *Bioanalysis* 6, 3155–3159 (2014).

283. Koob, G. F. Neurobiology of Addiction Toward the Development of New Therapies. *Ann. New York Acad. Sci.* 909, 170–185 (2000).

284. Yue, B. *et al.* Choline in Whole Blood and Plasma: Sample Preparation and Stability. *Clin. Chem.* 54, 590–593 (2008).

285. Coppola, M. & Mondola, R. 3,4-Methylenedioxypyrovalerone (MDPV): Chemestry, pharmacology and toxicology of a new designer drug of abuse marketed online. *Toxicol. Lett.* 208, 12–15 (2012).

286. Counselman, F. L., Mclaughlin, E. W., Kardon, E. M. & Bhambhani-bhavnani, A. S. Creatine Phosphokinase Elevation in Patients Presenting to the Emergency Department With Cocaine-Related Complaints. *Am. J. Emerg. Med.* 071, 221–223 (1997).

287. Welch, R. D., Tood, K. & Krause, G. S. Incidence of Cocaine-Associated Rhabdomyolysis. *Ann. Emerg. Med.* 155, 65–69 (1991).

288. Wilson, J. M., Carroll, M. E., Lac, S. T., DiStefano, L. M. & Kish, S. J. Choline acetyltransferase activity is reduced in rat nucelus accumbens after unlimited access to self-administration of cocaine. *Neurosci. Lett.* 180, 29–32 (1994).

289. Peiró, a. M. *et al.* Human pharmacology of 3,4-methylenedioxy-methamphetamine (MDMA, ecstasy) after repeated doses taken 2 h apart. *Psychopharmacology (Berl).* 225, 883–893 (2013).

290. Booze, R. M., Lehner, A. F., Wallace, D. R., Welch, M. A. & Mactutus, A. F. Dose-Response Cocaine Pharmacokinetics and Metabolite Profile Following Intravenous Administration and Arterial Sampling in Unanesthetized , Freely Moving Male Rats. *Neurotoxicol. Teratol.* 19, 7–15 (1997).

291. Pardo-Lozano, R. *et al.* Clinical Pharmacology of 3,4-Methylenedioxymethamphetamine (MDMA, '"Ecstasy"'): The Influence of Gender and Genetics. *PLoS One* 7, 1–10 (2012).

292. Brady, K. T. & Randall, C. L. Gender differences in substance use disorders. *Addict. Disord.* 22, 241–252 (1999).

293. Administration, F. and D. Analytical Procedures and Methods Validation for Drugs and Biologics. (2015).

294. Bader, M. A Systematic Approach to Standard Addition Methods in Instrumental Analysis. *J. Chem. Educ.* 57, 703–706 (1980).

295. Polson, C., Sarkar, P., Incledon, B., Raguvaran, V. & Grant, R. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography – tandem mass spectrometry. *J. Chromatogr. B* 785, 263–275 (2003).

296. Barbieri, M. *et al.* Neuropharmacology Synthetic cannabinoid JWH-018 and its halogenated derivatives JWH- 018-Cl and JWH-018-Br impair Novel Object Recognition in mice : Behavioral , electrophysiological and neurochemical evidence. *Neuropharmacology* 109, 254–269 (2016).

297. EMCDDA. Synthetic cannabinoids in Europe. (2017).

298. Leach, A. G. Predicting the activity and toxicity of new psychoactive substances : a pharmaceutical industry perspective. 739–745 (2014).

299. Perfetti, X. *et al.* Neurotoxic Thioether Adducts of 3, 4-Methylenedioxymethamphetamine Identified in Human Urine After Ecstasy Ingestion. *Drug Metab. Dispos.* 37, 1448–1455 (2009).

300. Hackam, D. G. Translation of Research Evidence From Animals to Humans. *JAMA* 296, 1731–1732 (2006).

301. Yubero-lahoz, S. *et al.* Effects of MDMA and related analogs on plasma 5-HT : Relevance to 5-HT transporters in blood and brain. *Eur. J. Pharmacol.* 674, 337–344 (2012).

302. Green, a R., Mechan, A. O., Elliott, J. M., Shea, E. O. & Colado, M. I. The Pharmacology and Clinical Pharmacology of 3 , 4-Methylenedioxymethamphetamine (MDMA , ‘ Ecstasy ’). *Pharmacol. Rev.* 55, 463–508 (2003).

303. Hotchkiss, J., Gibb, W. & Adair, J. Long-term effects of multiple doses of methamphetamine on tryptophan hydroxylase and tyrosine hydroxylase activity in rat brain. (1980).

304. Gribb, J. W. & Kogan, F. J. Influence of dopamine synthesis on methamphetamine-induced changes in striatal and adrenal tyrosine hydroxylase activity. *Arch. Pharmacol.* 7, 185–187 (1979).

305. Uhl, G. R., Hall, F. S. & Sora, I. Cocaine , reward , movement and monoamine transporters. 21–26 (2002).

306. Pettit, H. O., Pan, H.-T., Parsons, L. H. & Justice Jr., J. B. Extracellular Concentrations of Cocaine and Dopamine Are Enhanced During Chronic Cocaine Administration. *J. Neurochem.* 55, 798–804 (1990).

307. Negus, S. S. *et al.* Effect of antagonists selective for mu , delta and kappa receptors on the reinforcing effects of heroin in rats humans. *J. Pharmacol. Exp. Ther.* 265, 1245–1252 (1993).

308. Weil-Fugazza, J., Godefroy, F. & Besson, J.-M. Changes in brain and spinal tryptophan and 5-hydroxy-indoleacetic acid levels following acute morphine adminitration in normal and arthritic rats. *Brain Res.* 175, 291–301 (1979).

309. Wise, R. A., Leone, P., Rivest, R. & Leeb, K. Elevations of nucleus accumbens dopamine and DOPAC levels during intravenous heroin self-administration. *Synapse* 21, 140–148 (1995).

310. Baumann, M. H. *et al.* Methylenedioxypyrovalerone (MDPV), Its Metabolites , and Related Analogs. *Curr. Top. Behav. Neurosci.* (2016).

7. Supplementary Material

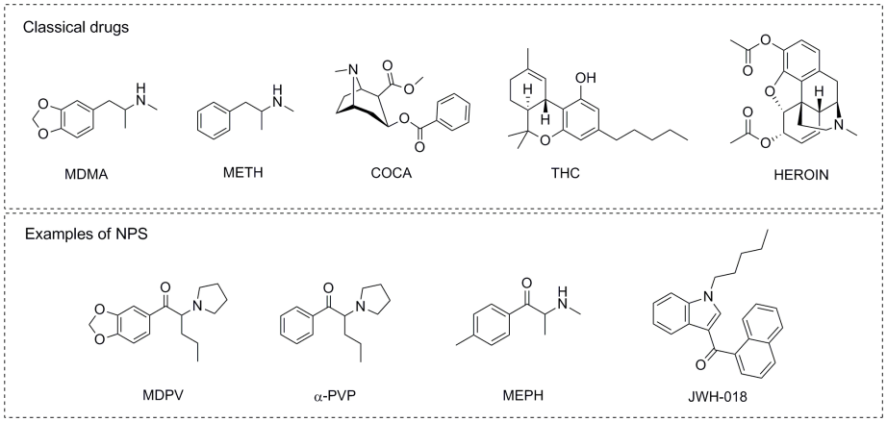


Figure 59. Chemical structures of the different drugs of abuse assayed in the metabolomics studies (part II) of this thesis. The classical drugs group included MDMA, METH, COCA, THC and HEROIN, whereas as examples on NPS we included MDPV, α-PVP, MEPH and JWH-018.

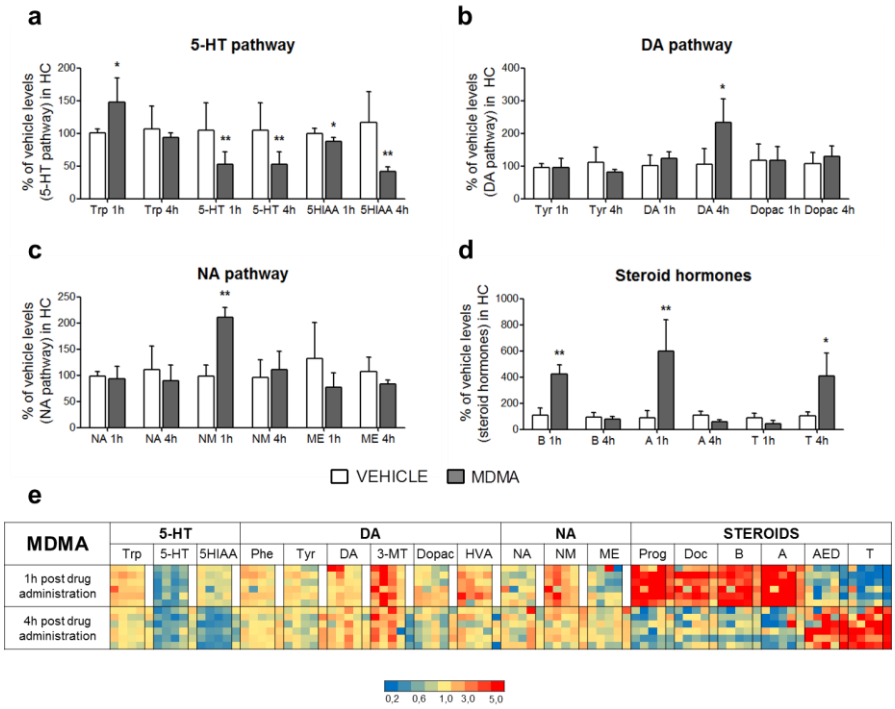


Figure 60. Detailed MDMA metabolomics fingerprint (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of MDMA in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after MDMA administration (1-4h).

MDMA administration (15 mg/kg) mainly altered the serotoninergic pathway in rat brain (Fig. 60 a). In HC, Trp concentrations at 1h post drug administration as compared to the saline vehicle were significantly increased ($p = 0.015$). We observed a sustained 5-HT depletion 1h and 4h post drug administration ($p = 0.002$ and $p = 0.026$) with a related decrease of its main metabolite, 5-HIAA ($p = 0.015$ and $p = 0.002$ at 1h and 4h post administration). The depletion of 5-HT was more pronounced 1h post administration whereas the drop of 5-HIAA was more marked at 4h post MDMA treatment. Those results were in concordance with the reduction of the tryptophan hydroxylase after the MDMA administration, described in literature[199,200,302]. Hippocampal DA concentrations at 4h treatment and NM at 1h were incremented in relation to the vehicle administration ($p = 0.026$ and $p = 0.002$ respectively) (Fig. 60 b-c). The steroid hormones (Fig. 60 d) were certainly altered in HC after the psychostimulant administration. Corticosteroids (such as B or A) were highly increased after 1h of MDMA administration and normalized at 4h post drug administration. On the other hand, androgens (like testosterone) were only boosted 4h post-drug administration ($p = 0.004$) with similar values to vehicle at 1h post administration. The homogeneous effect of the MDMA in the targeted metabolome showed similar tendencies between the individual animals (Fig. 60 e). The metabolome alterations described in hippocampus were extended also to the prefrontal cortex, the cerebellum and the striatum. Plasmatic steroids showed similar alterations to the ones observed in brain. Only slight changes were observed in the selected urinary metabolome.

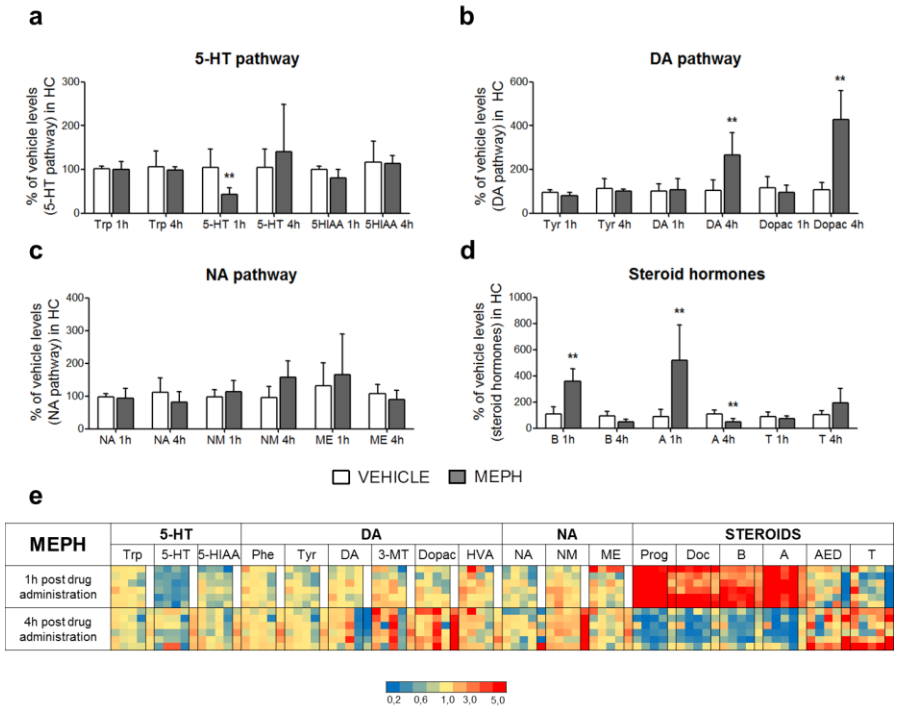


Figure 61. Detailed MEPH targeted metabolomics profile (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of MEPH in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after MEPH administration (1-4h).

MEPH administration (30 mg/kg) principally altered the 5-HT and DA pathway in the brain. As an example, in the hippocampus, MEPH decreased 5-HT concentrations 1h post drug administration (P = 0.02) (Fig. 61 a), similarly to the 5-HT depletion by MDMA (Fig 61 a). MEPH also altered the DA pathway in this brain area, increasing DA and Dopac (P = 0.004 and P = 0.002 respectively) at 4h post drug administration (Fig. 61 b). In the literature, MEPH is described to act as a substrate of DA and 5-HT receptors[18,19,73]. We did not find significant differences of hippocampal NA, NM and ME (Fig. 61 c). Regarding steroid hormones, corticosteroids were significantly increased (B, P = 0.002; and A, P = 0.002) at 1h post administration and A was decreased 4h post MEPH administration (A, P = 0.09) (Fig. 61 d). Overall, these changes of the metabolome in HC were extended in all brain areas (Fig. 61 e). Remarkable steroid hormones alterations were found in after MEPH administration. Thus, both progestogens and corticosteroids reached larger plasmatic concentrations 1h after administration. We also detected a substantial reduction in androgens. Plasmatic steroids tended to normalize 4h after MEPH administration. The most remarkable alterations found in urine were the decrease in DA levels and the increases in both Dopac and NM levels.

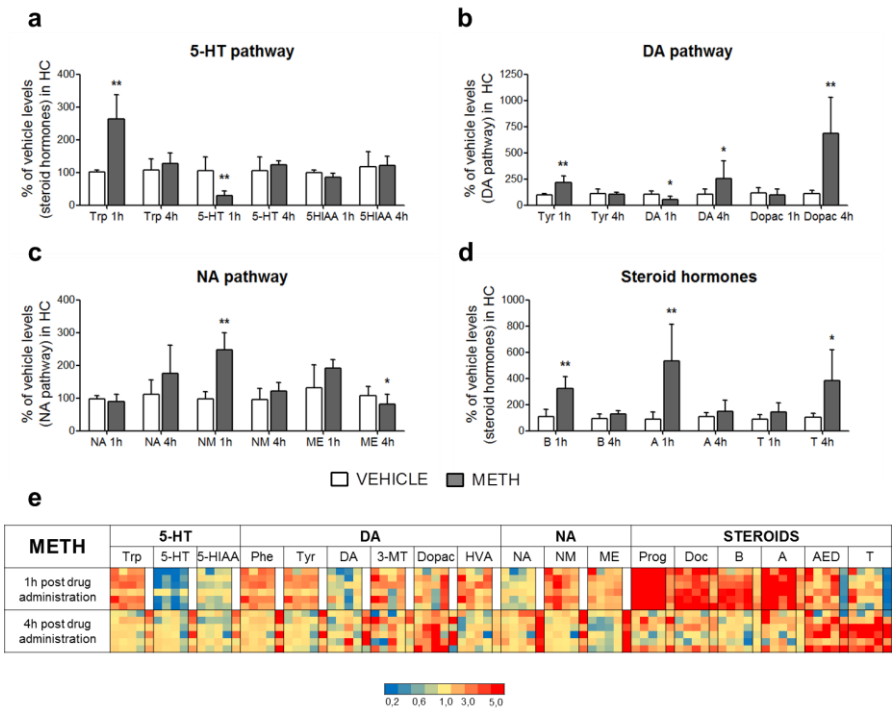


Figure 62. Detailed METH targeted metabolomics profile (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of METH in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after METH administration (1-4h).

METH administration (15 mg/kg) altered the targeted metabolomics profile of all the neurotransmitters and steroid hormones pathways when compared with the saline vehicle administration. Regarding the 5HT and the DA pathways, MEPH increased concentrations of the precursors at 1h post drug administration, (Trp, p = 0.002; Tyr, p = 0.004 in HC) and decreased concentrations of serotonin and dopamine (5-HT, p = 0.002; DA, p = 0.041 in HC) (Fig. 62 a-b). These results agree with the depletion of both tryptophan hydroxylase and tyrosine hydroxylase reported to be induced by METH[247,303,304]. At 4h post administration, DA and its metabolite Dopac were significantly increased (p = 0.015 and p = 0.002 respectively in HC) whereas not significant altered for 5HT and its main metabolite 5HIAA. In the NA pathway (see Fig. 62 c), NM increased at 1h post administration (p = 0.002 in HC) whereas ME decreased 4h post administration (p = 0.041 in HC). Regarding steroid hormones, corticosteroids rose (B and A p = 0.004 in HC) 1h post drug administration whereas testosterone increased 4h after METH administration (p = 0.015 in HC) (Fig. 62 d). The alterations were homogeneous in the selected brain areas and between individuals (Fig. 62 **e**). Plasmatic steroids behaved similarly to brain with the main exception of androgens which showed a marked depletion 1 h after METH administration. In urine, metabolites related to DA and NA notably incremented compared to the vehicle administration.

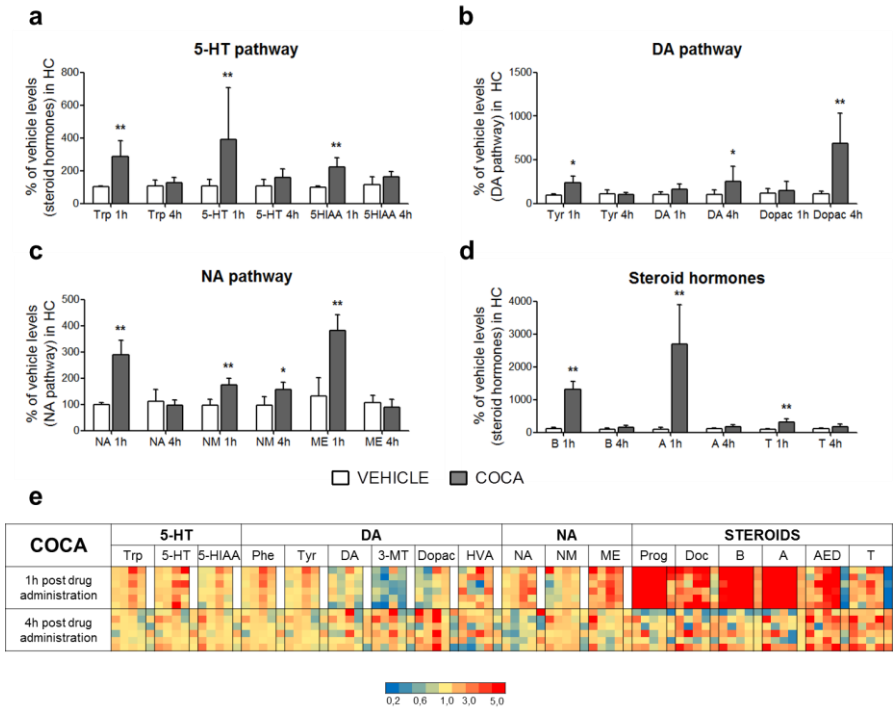


Figure 63. Detailed COCA targeted metabolomics profile (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of COCA in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after COCA administration (1-4h).

COCA administration (20 mg/kg) mainly rose metabolites and precursors in the monoamine neurotransmitters pathway (especially DA and NA) and in the steroid hormones in agreement with previous reports[305,306]. In the 5-HT pathway, we observed increased concentrations of hippocampal Trp, 5-HT and 5-HIAA at 1h post COCA administration ($p = 0.002$ in all cases) (Fig. 63 a). In the hippocampus, Tyr values were enhanced 1h after COCA administration ($p = 0.002$) compared to the vehicle condition, whereas DA and its metabolite, Dopac, increased 4h after COCA treatment ($p = 0.03$ and 0.04) (Fig. 60 b). In the NA pathway (Fig. 63 c), NA and its metabolites (NM and ME) were significantly incremented in the hippocampus ($p = 0.002$ for all compounds) with a sustained boost of NM ($p = 0.017$) at 4h post drug administration. Hippocampal corticosteroids and androgens markedly rose after 1h of the psychostimulant administration (p values for B, A and T of 0.002) (Fig. 63 d). Other brain areas behaved similarly for steroid hormones and DA pathway. Remarkably, the alterations observed in the hippocampus were more intense than the ones in other brain areas mainly in the 5HT pathway but also for NA pathway (Fig. 63 e). Plasmatic steroid hormones slightly increased at 1h after administration with the main exception of androgens. Their concentrations returned to basal levels 4h after administration. Changes in the urinary metabolome were almost undetectable (Fig. 63 e).

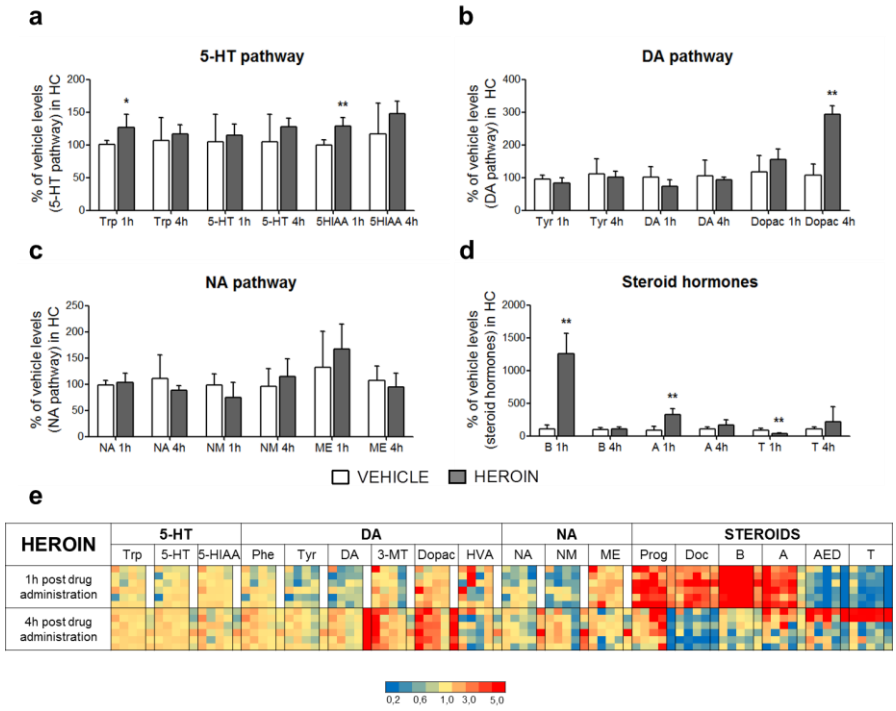


Figure 64. Detailed HEROIN targeted metabolomics profile (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of HEROIN in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after HEROIN administration (1-4h).

HEROIN administration (30 mg/kg) produced changes in comparison with the vehicle administration on the monoamine pathways and the steroid hormones even through the HEROIN principally interacts with the opioid receptors[307]. We found the significant increases of hippocampal Trp and 5-HIAA ($p = 0.015$ and $p = 0.002$ at 1h and 4h post administration respectively), already described in rats after morphine administration[308] (Fig. 64 a). In the DA pathway, hippocampal Dopac increased at 4h post administration in concordance with the literature[309] ($p = 0.04$, Fig. 64 b). We did not find significant changes on the NA metabolic pathway (Fig. 64 c). The selected corticosteroids (B and A), increased in the hippocampus at 1h post HEROIN administration ($p = 0.02$ and 0.04 respectively) whereas androgens (such as T) were significantly decreased ($p = 0.009$) also 1h post drug administration (Fig. 64 d). Results for steroid hormones, 5HT and NA pathways were consistent between all the brain areas studied. However, the alterations found in the dopaminergic pathway in the striatum followed a different trend when compared with the other areas. This difference was more notably observed at 4h (Fig. 64 e). Similar to the other drugs, the administration of HEROIN was followed by a rise in plasmatic corticosteroids and by a decrease of androgens. The urinary metabolome was characterized by an increment on DA and Dopac concentrations.

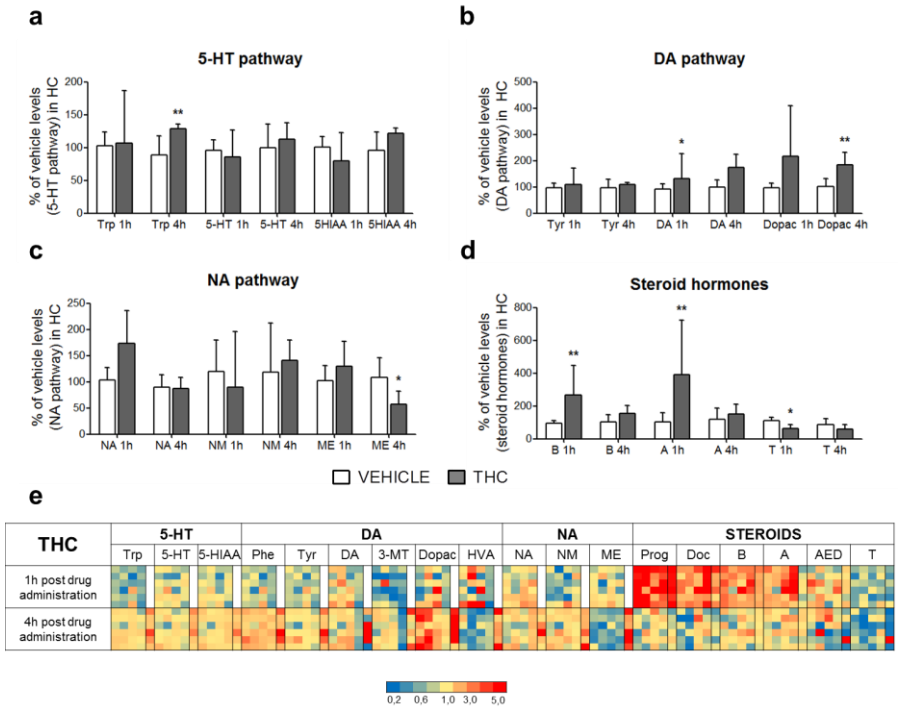


Figure 65. Detailed THC targeted metabolomics profile (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of THC in comparison with saline ethanol-vehicle administration (5% ethanol. 5% cremophor. 90% saline) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after THC administration (1-4h).

THC administration (15 mg/kg) changed the targeted metabolome profile of the rats. THC directly interacts with the cannabinoid receptors (mainly CB_1) and indirectly with the monoamine neurotransmitters and the steroid hormones[3,51]. In the 5-HT pathway in HC, only significant increases in the tryptophane at 1h post administration were observed ($p = 0.002$) (Fig. 65 a). Regarding the DA metabolism, hippocampal DA was risen 1h post administration ($p = 0.026$) whereas its metabolite Dopac was enhanced 4h after the THC administration ($p = 0.009$) (Fig. 65 b). In the NA pathway, significant alterations were observed in HC with a reduction of ME at 4h post drug administration ($p = 0.015$) (Fig. 65 c). Regarding steroid hormones, hippocampal corticosteroids incremented (B, $p = 0.002$; A, $p = 0.009$) and androgens decreased (T, $p = 0.026$) at 1h post drug administration (Fig. 65 d). Globally, THC produced similar changes in all tested individuals and brain areas with the main exception of DA metabolites (Fig. 65 e). Similar to other classical drugs, THC increased the plasmatic corticosteroids values (1h post drug administration) but the boost was less intense than the one observed for the psychostimulants tested (MDMA, COCA and METH). In urine, the most remarkable alteration was the increment in several metabolites belonging to the DA pathway.

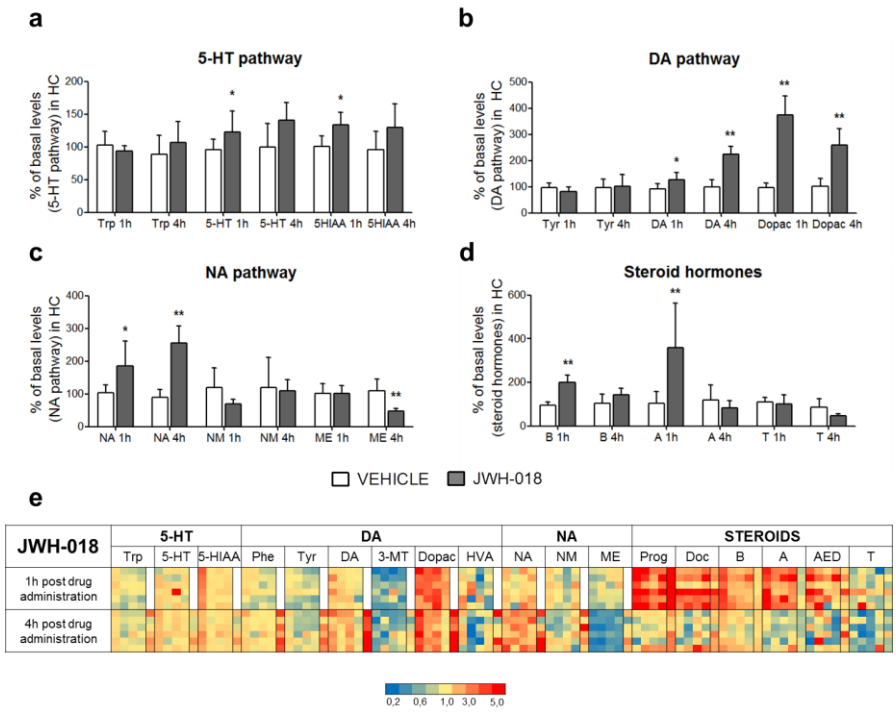


Figure 66. Detailed JWH-018 targeted metabolomics profile (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of JWH-018 in comparison with ethanol-vehicle administration (5% ethanol. 5% cremophor. 90% saline) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after JWH-018 administration (1-4h).

JWH-018 administration (10 mg/kg) altered the targeted metabolome profile of monoamines and steroid hormones. Regarding the 5-HT pathway, 5-HT and its principal metabolite (5-HIAA) increased 1h post drug administration ($p = 0.041$ and $p = 0.015$ respectively in HC) (Fig. 66 a). In the DA pathway, DA and its metabolite Dopac significantly increased ($p = 0.015$ and $p = 0.002$ for hippocampal DA at 1h and 4h treatment and $p = 0.002$ for hippocampal Dopac for 1h and 4h post drug administration) (Fig. 66 b). With regard to the NA metabolism, NA concentrations incremented 1h post administration ($p = 0.015$ in HC) and decreased (together with ME values) at 4h post JWH-018 administration ($p = 0.002$ for both metabolites in HC) (Fig. 66 c). Brain corticosteroids (B and A) significantly rose ($p = 0.002$ in both cases in HC) at 1h post drug administration and normalized 4h post JWH-018 administration (Fig. 66 d). Similar alterations were detected in all the selected brain regions (HC, PFC, Cer and St) (Fig. 66 e). Similarly to the other tested drugs, JWH-018 boosted its plasmatic steroid concentrations at 1h post administration, but with less intensity than the psychostimulant drugs. Finally, JWH-018 administration leads to an increase in several metabolites of the DA pathway in the urinary metabolome.

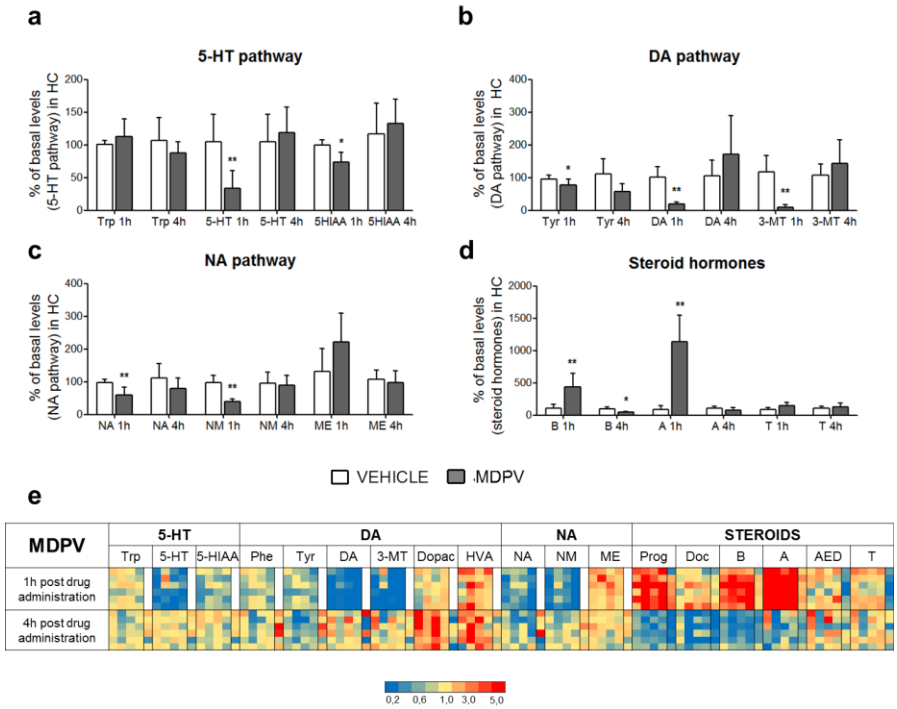


Figure 67. Detailed MDPV targeted metabolomics profile (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of MDPV in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after MDPV administration (1-4h).

MDPV administration (4 mg/kg) principally altered the principal monoamine neurotransmitters (5-HT and DA and NA). With regard to the 5-HT pathway, MDPV strongly decreased the 5-HT and its metabolite (5-HIAA) concentrations in HC and the other studied brain areas (p= 0.002 and p=0.015) at 1h post drug administration (see Fig. 67 a). MDPV was described to blockade the catecholamines transporters (specially DA and NA)[74,310] and we observed a 20% drop of the DA and 3-MT (metabolite of the COMT from DA) concentrations in comparison with the vehicle administration during the first hour of the drug administration in all brain areas (p = 0.002 for both compounds in HC) (see Fig. 67 b). Nevertheless, Dopac concentrations were boosted 4h post drug administration (p = 0.002). In the NA pathway, a marked depletion on NA and NM (metabolite of the COMT enzyme) values was observed for both compounds at 1h post MDPV administration (p =0.009 and p =0.002 in HC) (see Fig. 67 c) with residual alterations were observed 4h post drug administration. Regarding the steroid hormones profile, increases on the 1h post drug administration were found in Prog, B and A (p = 0.009, p = 0.002 and p = 0.002, respectively) (see Fig. 67 d). Then, at 4h post MDPV administrations a decrease on progestogens and corticoids was slightly observed. In plasma, a decrease in the androgens concentrations were observed at 1h post drug administration (p = 0.002 for AED and p = 0.041 for T) with a maintained drop of progestogens and corticoids (p = 0.02 for Prog, p = 0.002 for DOC, p = 0.004 for B and p = 0.004 for A) at 4h post MDPV treatment. In urine, no relevant alterations were observed after MDPV administration.

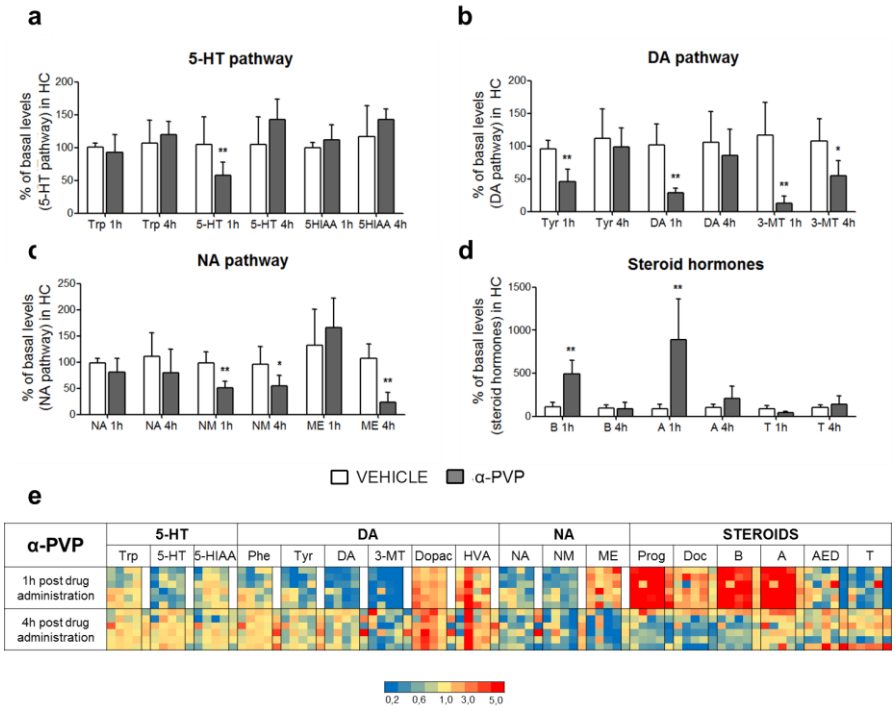


Figure 68. Detailed α-PVP targeted metabolomics profile (monoamines and steroid hormones pathway). a-d) Bars graph of the variations of precursors, neurotransmitters and metabolites of the serotonin, dopamine, noradrenaline and steroid hormones pathways after the administration of α-PVP in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after α-PVP administration (1-4h).

α-PVP administration (4 mg/kg), identically as MDPV, produced a profound alteration of the monoamine neurotransmitters. The concentrations of 5-HT (with respect to the vehicle administration) were deplected significantly at 1h post drug administration ($p = 0.002$ in HC) and returned to basal levels at 4h treatment (see figure 68 a). Homogeneously in all brain areas, DA and its metabolite 3-MT were decreased 1h post drug administration in HC ($p = 0.002$ in both cases). Tyr, precursor of DA was also hipocampusly deplected at 1h treatment ($p = 0.004$). Additionally, Dopac concentrations were slightly increased both at 1h and 4h post drug administration) (see figure 68 b). With regart to the NA pathway, the concentrations of NA where slighlty decreased and the values of its metabolite (NM) were significantly droped at 1h post drug administration ($p = 0.004$ in HC) (see figure 68 c). At 4h post α-PVP administration, ME values were reduced in comparison with the vehicle administration ($p = 0.002$). The steroid hormones were also altered after the administration of α-PVP, which significantly increased during the first hour the values of Prog ($p = 0.002$), B ($p = 0.004$) and A ($p = 0.002$) hippocampusly and in the other brain areas (see figure 68 d). At 4h these steroid hormones returned to basal levels. In plasma, the androgens were decreased ($p = 0.002$ for AED and T) at 1h post drug administration. In urine, no relevant changes were observed after the administration of α-PVP (see figure 68 e).

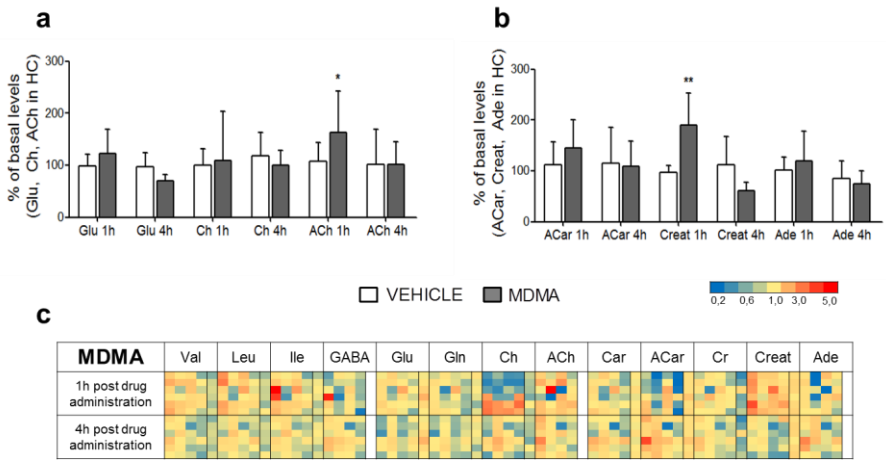


Figure 69. Detailed MDMA targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of MDMA in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. c) Heat map representation of all brain areas, urine and plasma after MDMA administration (1-4h).

MDMA administration (15 mg/kg) nearly unaltered the targeted amino acid neurotransmitters and related compounds pathway. Significant alterations were observed at ACh and Creat at 1h post drug administration ($p = 0.05$ and $p = 0.002$, respectively) (figure 69 a-b). ACar showed decreased concentrations in Cer and St brain areas, 1h post administration. With regard to the targeted metabolites at 4h post drug administration, no alterations in any brain area were observed (see figure 69 c).

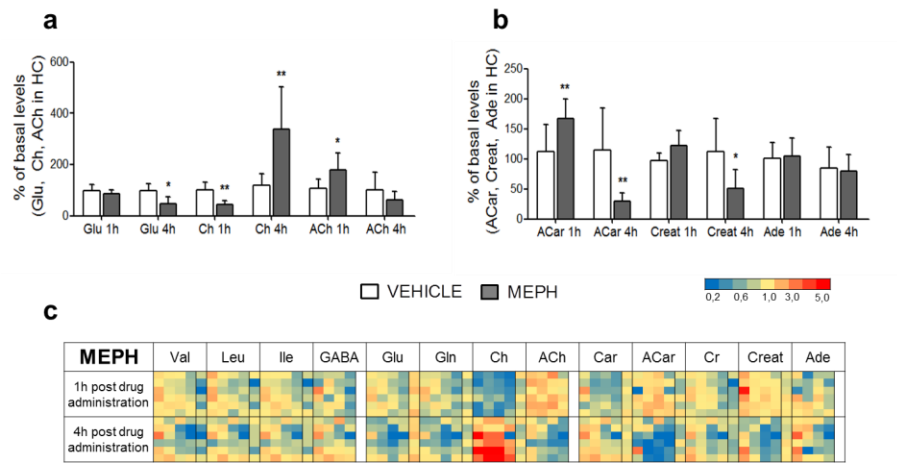


Figure 70. Detailed MEPH targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of MEPH in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. c) Heat map representation of all brain areas, urine and plasma after MEPH administration (1-4h).

MEPH administration (15 mg/kg) produced a deep depletion of Ch concentrations (in all brain areas) at 1h post administration ($p = 0.004$ with an increased recovery at 4h post treatment ($p = 0.009$) in HC. In reverse, ACar was boosted 1h post MEPH administration ($p = 0.002$), but significantly decreased at 4h post drug treatment ($p = 0.009$). Creat and Glu were also decreased at 4h post MEPH administration ($p = 0.015$ and $p = 0.026$, respectively, in HC) (figure 70 a-b). In coherence with the results in brain, plasma concentrations were increased 4h post drug administration ($p = 0.009$) (figure 70 c).

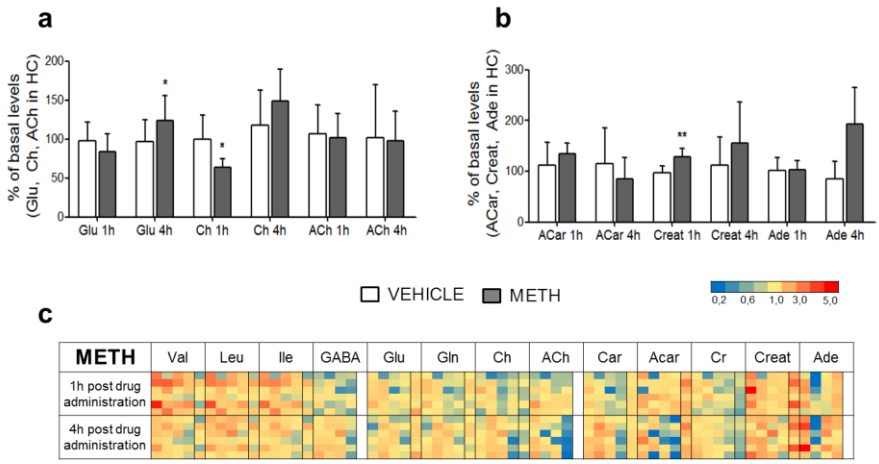


Figure 71. Detailed METH targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of METH in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. c) Heat map representation of all brain areas, urine and plasma after METH administration (1-4h).

METH administration (15 mg/kg) nearly unaffected the targeted amino acids and related compounds in all brain areas. Only slightly increases on Creat values in comparison with the vehicle administration were observed at 1h post drug administration ($p = 0.002$, in HC). Also, Ch concentrations were also decreased 1h post METH treatment ($p = 0.026$ in HC). At 4h post drug administrations, Glu was decreased significantly ($p = 0.041$) and it was increased at 1h post METH injection in plasma ($p = 0.004$) (figure 71 a-b). At the same time, carnitines and creatinines were also boosted in plasma 1h ($p = 0.002$ for Creat, $p = 0.002$ for Cr, $p = 0.002$ for Car and $p = 0.004$ for ACar) (figure 71 c).

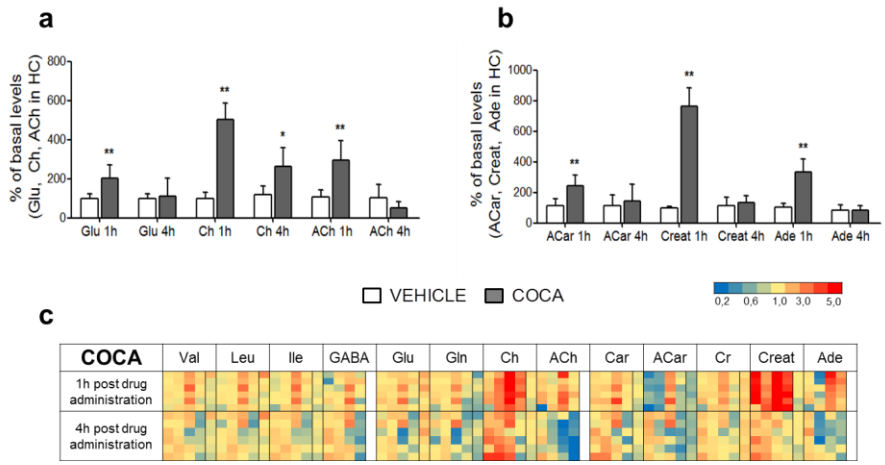


Figure 72. Detailed COCA targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of MDMA in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after MDMA administration (1-4h).

COCA administration (20 mg/kg) showed significant alterations in many targeted metabolites. The most relevant increases (around 6 to 8 folds the basal values) were observed during the 1st hour post drug administration in Ch and Creat concentrations ($p = 0.002$ for both analytes in HC). These increases was maintained for Ch at 4h post drug administration ($p = 0.026$ in HC). Additionally, increased concentrations of Glu, ACar, Ade and ACh were also observed at 1h post COCA administration ($p = 0.002$ for the first three compounds and $p = 0.009$ for ACh in HC) (see figure 72 a-b). In plasma, significant alterations were observed in Ch and in creatines ($p = 0.026$ for Ch, $p = 0.026$ for Cr and $p = 0.004$ for Creat) (figure 72 c).

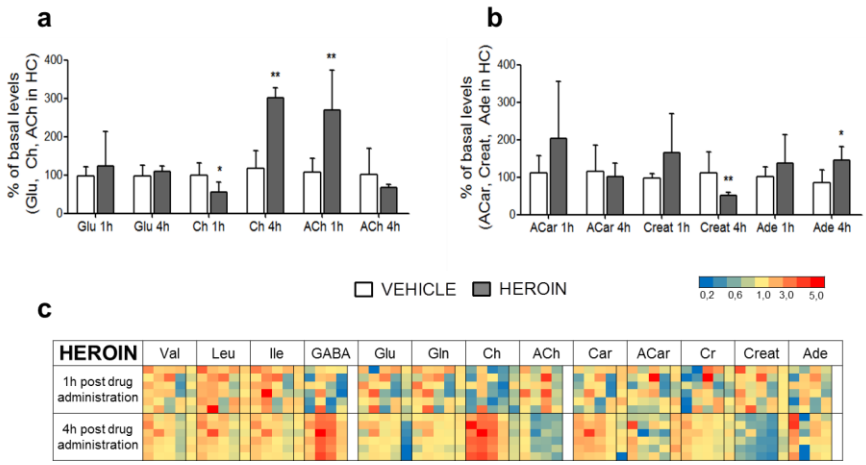


Figure 73. Detailed HEROIN targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of HEROIN in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after HEROIN administration (1-4h).

HEROIN administration (30 mg/kg) produced significant changes on the hippocampal cholinergic system at 1h post drug administration: a decrease on Ch ($p = 0.041$) and an increase on ACh ($p = 0.009$). Moreover, higher alterations were observed at 4h post drug administration, with a global increase of Ch and Car concentrations ($p = 0.002$ and $p = 0.009$, respectively in HC). Then, a decrease on Creat concentrations was observed at 4h post drug administration in comparison with the vehicle treatment ($p = 0.004$ in HC) (figure 73 a-b). With regard to the plasma values, Car concentrations were slightly higher that vehicle ($p = 0.026$) (figure 73 c).

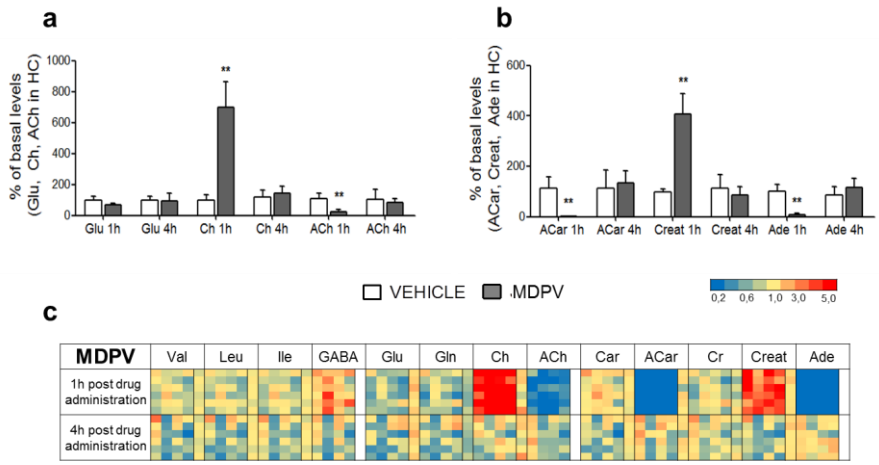


Figure 74. Detailed MDPV targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of MDPV in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after MDPV administration (1-4h).

MDPV administration (4 mg/kg) considerably altered 5 of the 13 targeted compounds at 1h post drug administration. Creat and Ch concentrations were 8 and 4 fold increased in comparison with vehicle administration whereas ACh, ACar and Ade were deeply decreased ($p = 0.002$, for all compounds in HC). These alterations were homogeneously extended in all brain areas and they were recovered at 4h post drug administration. Indeed, no alterations in comparison with the vehicle administration were seen in any amino acid neurotransmitters and related compounds at 4h post drug administration (see figure 74 a-b). In plasma, only a slightly decrease on the Creat concentrations was observed ($p = 0.041$) (figure 74 c).

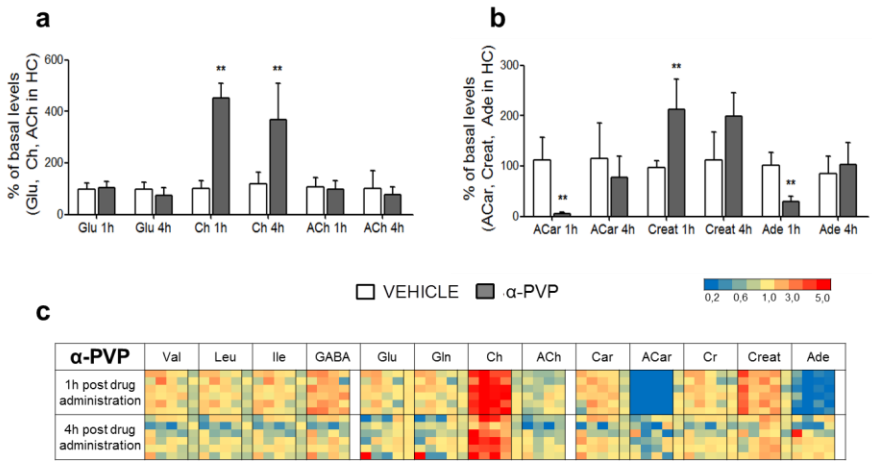


Figure 75. Detailed α-PVP targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of α-PVP in comparison with saline vehicle administration (0.9% NaCl) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after α-PVP administration (1-4h).

α-PVP administration (4 mg/kg) induced a 4-fold increase of Ch concentrations mantained at 1h and 4h post drug administration ($p = 0.002$ and $p = 0.009$, respectively in HC). Additionally, an increase of Creat values was observed at 1h post drug administration ($p = 0.002$ in HC) but it was recovered at 4h post α-PVP administration. With regard to the rest of metabolites, Ade and ACar were significantly deplected at the 1st hour treatment ($p = 0.002$ for both compounds in HC) (see figure 75 a-b). The variations produced in all metabolites were homogeneously extended in all brain areas. In coherence with the brain alterations, concentrations of Creat were boosted ($p = 0.002$) at 1h post drug administration (see figure 75).

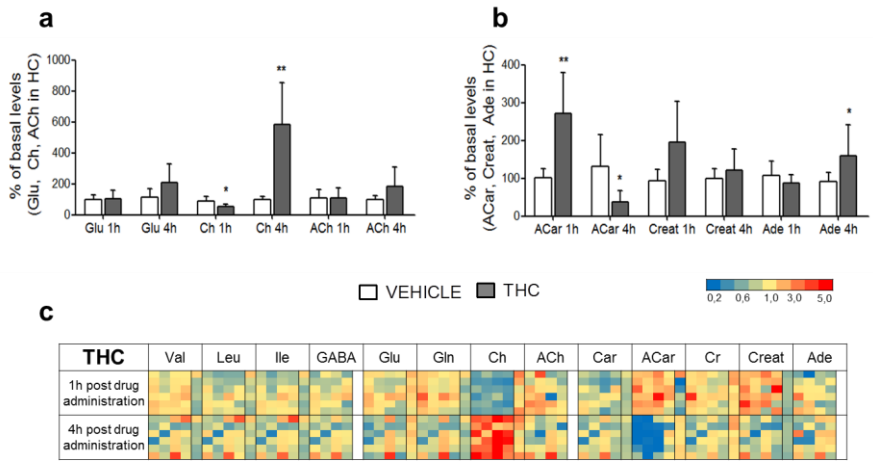


Figure 76. Detailed THC targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of THC in comparison with ethanol-vehicle administration (5% ethanol. 5% cremophor. 90% saline) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after THC administration (1-4h).

THC administration (15 mg/kg) induced a marked increase of ACar concentrations (in relation to the vehicle administration) at 1h post drug administration ($p = 0.002$) but with a significant decrease at 4h post drug treatment ($p = 0.015$). Reversely, Ch values were deplected 1h post THC administration ($p = 0.41$) and increased at 4h post drug treatment ($p = 0.002$). Moreover, Ade concentrations were increased 4h post drug administration ($p = 0.041$) (see figure 76 a-b). With regard to plasma concentrations, creatines were significantly altered (increased for Cr, $p = 0.41$ and decreased for Creat, $p = 0.002$) at 1h post drug administration (figure 76 c).

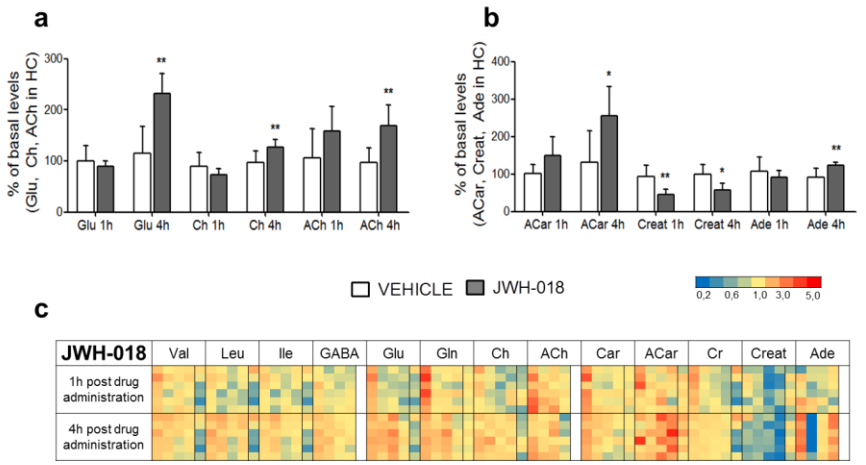


Figure 77. Detailed JWH-018 targeted metabolomics profile (amino acid neurotransmitters and related compounds). a-b) Bars graph of the variations of amino acid neurotransmitters (such as Glu, Ach and Ade) and related compounds (Ch, ACar and Creat) after the administration of JWH-018 in comparison with ethanol-vehicle administration (5% ethanol. 5% cremophor. 90% saline) in hippocampus. e) Heat map representation of all brain areas, urine and plasma after JWH-018 administration (1-4h).

JWH-018 administration (10 mg/kg) mainly decreased Creat in all brain areas in a maintained manner at 1h and 4h post drug administration ($p = 0.009$ and $p = 0.026$, respectively in HC). Furthermore, JWH-018 produced an small increase of the following metabolites: Glu, Gln, Ch, ACar, ACh ($p = 0.009$ for all of them) and Ade ($p = 0.002$) at 4h post drug administration (figure 77 a-b). Cocentrations of ACar were also increased in plasma at 4h treatment (0.026) (figure 77 c).

8. Annex

REVIEW

8.1. Mephedrone concentrations in cases of clinical intoxications

Papaseit E, Olesti E, de la Torre R, Torrens M, Farre M. Mephedrone Concentrations in Cases of Clinical Intoxication. Curr Pharm Des. 2018 Jan 22;23(36). DOI: 10.2174/1381612823666170704130213