APPLICATION OF ADVANCED MASS SPECTROMETRIC TECHNIQUES FOR THE IDENTIFICATION, METABOLIC CHARACTERISATION AND TOXICOLOGICAL ANALYSIS OF NEW PSYCHOACTIVE SUBSTANCES

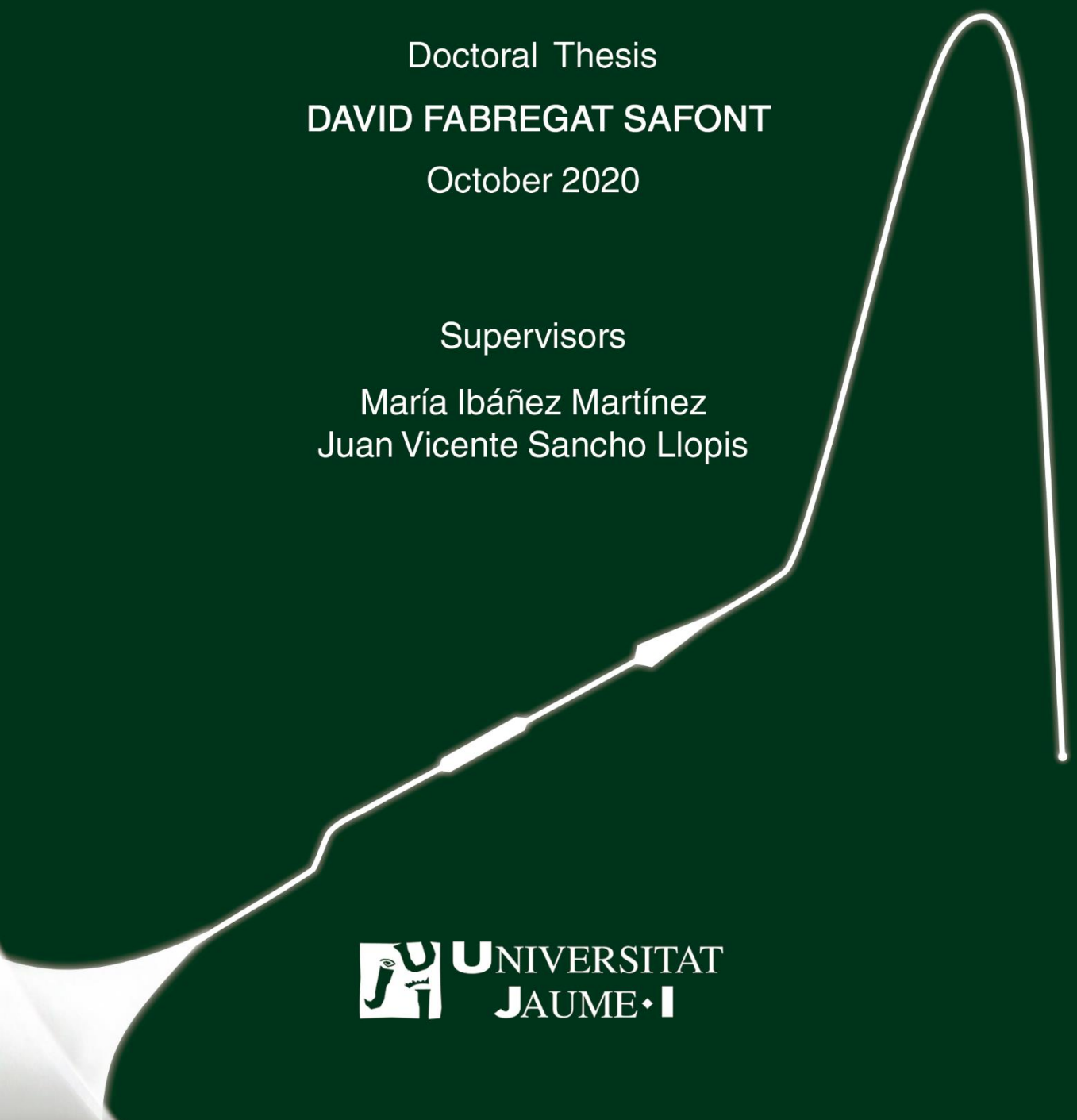
Doctoral Thesis

DAVID FABREGAT SAFONT

October 2020

Supervisors

María Ibáñez Martínez
Juan Vicente Sancho Llopis



UNIVERSITAT JAUME·I

Doctoral Programme in Science

Doctoral School of Universitat Jaume I

APPLICATION OF ADVANCED MASS SPECTROMETRIC TECHNIQUES FOR THE IDENTIFICATION, METABOLIC CHARACTERISATION AND TOXICOLOGICAL ANALYSIS OF NEW PSYCHOACTIVE SUBSTANCES

Thesis presented by David Fabregat Safont in fulfilment of the requirements for the degree of Doctor (PhD) from the Universitat Jaume I

DAVID|FABREGAT|SAFONT
Firmado digitalmente por DAVID|FABREGAT|SAFONT
Fecha: 2020.10.06 09:34:34 +02'00'

David Fabregat Safont

MARIA IBAÑEZ MARTINEZ - NIF:53220742F
Firmado digitalmente por MARIA IBAÑEZ MARTINEZ - NIF:53220742F
Fecha: 2020.10.06 08:49:26 +02'00'

Dr. María Ibáñez Martínez

JUAN VICENTE|SANCHO|LLOPIS
Firmado digitalmente por JUAN VICENTE|SANCHO|LLOPIS
Fecha: 2020.10.05 17:03:40 +02'00'

Dr. Juan V. Sancho Llopis

Castelló de la Plana, October 2020

This work responds to the commitment acquired with the *Ministerio de Educación, Cultura y Deporte* (MECD) in Spain, for granting David Fabregat Safont with a predoctoral fellowship belonging to the *Subprograma de Formación del Profesorado Universitario* (FPU-MECD), from 1st of October 2016 to 30th November 2020 (Grant FPU15/02033).

David Fabregat Safont has also received a grant for a research stay in the Forensic Medicine department of the University of Copenhagen (UCPH) in Denmark from 30th of April 2018 to 31st of July 2018, under the supervision of Prof. Dr. Med. Kristian Linnet. This stay was funded by the *Ministerio de Educación, Cultura y Deporte* (MECD) in Spain and belonging to the *Convocatoria de ayudas complementarias destinadas a beneficiarios del Subprograma de Formación del Profesorado Universitario* (Grant EST17/00024).

The works included in this thesis have been funded by the *Ministerio de Economía y Competitividad* in Spain (Projects CTQ2015-65603-P and BFU2016-77691-C2-1-P), NPS-Euronet co-funded by the European Commission (HOME/2014/JDRUG/AG/DRUG/7086), *Generalitat Valenciana* (PROMETEO/2014/023, PROMETEO/2016/076 and PROMETEO/2019/040), Universitat Jaume I (UJI-B2016-45 and UJI-B2018-19), Ghent University Research Council (Projects 01 N00814 and 01 J15517), and from *Fundación C.V. de investigación del Hospital Provincial de Castellón* (Project CAF 17-071).

Thesis by compendium of these publications:

I. Fabregat-Safont D, Fornís I, Ventura M, Gil C, Calzada N, Sancho JV, Hernández F, Ibáñez M. Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. *Drug Testing and Analysis*. 2017; 9:1073-1080. doi:10.1002/dta.2182. Impact Factor 2.993 (2017).

II. Fabregat-Safont D, Carbón X, Ventura M, Fornís I, Guillamón E, Sancho JV, Hernández F, Ibáñez M. Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-*N*-(2-(diethylamino)cyclohexyl)-*N*-methylbenzamide (U-49900). *Scientific Reports*. 2017; 7:6338. doi:10.1038/s41598-017-06778-9. Impact Factor 4.122 (2017).

III. Fabregat-Safont D, Carbón X, Gil C, Ventura M, Sancho JV, Hernández F, Ibáñez M. Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. *Forensic Toxicology*. 2018; 36:447-457. doi:10.1007/s11419-018-0422-0. Impact Factor 2.476 (2018).

IV. Fabregat-Safont D, Carbón X, Ventura M, Fornís I, Hernández F, Ibáñez M. Characterization of a recently detected halogenated aminorex derivative: *para*-fluoro-4-methylaminorex (4′F-4-MAR). *Scientific Reports*. 2019; 9:8314. doi:10.1038/s41598-019-44830-y. Impact Factor 3.998 (2019).

V. Fabregat-Safont D, Sancho JV, Hernández F, Ibáñez M. Rapid tentative identification of synthetic cathinones in seized products taking advantage of the full capabilities of triple quadrupole analyzer. *Forensic Toxicology*. 2019; 37:34-44. doi:10.1007/s11419-018-0432-y. Impact Factor 2.945 (2019).

VI. Fabregat-Safont D, Felis-Brittes D, Mata-Pesquera M, Sancho JV, Hernández F, Ibáñez M. Direct and Fast Screening of New Psychoactive Substances Using Medical Swabs and Atmospheric Solids Analysis Probe Triple Quadrupole with Data-Dependent Acquisition. *Journal of the American Society for Mass Spectrometry*. 2020; 31:1610-1614. https://doi.org/10.1021/jasms.0c00112. Impact Factor 3.255 (2019).

VII. Fabregat-Safont D, Mardal M, Noble C, Cannaert A, Stove CP, Sancho JV, Linnet K, Hernández F, Ibáñez M. Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. *Drug Testing and Analysis*. 2019; 11:1358-1368. doi:10.1002/dta.2659. Impact Factor 2.903 (2019).

VIII. Fabregat-Safont D, Mardal M, Sancho JV., Hernández F, Linnet K, Ibáñez M. Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation. *Journal of Pharmaceutical Analysis*. 2020; 10:147-156. doi:10.1016/j.jpha.2019.12.006. Impact Factor 2.673 (2019).

IX. Fabregat-Safont D, Barneo-Muñoz M, Martinez-Garcia F, Sancho JV, Hernández F, Ibáñez M. Proposal of 5-methoxy-*N*-methyl-*N*-isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. *Journal of Chromatography A*. 2017; 1508:95-105. doi:10.1016/j.chroma.2017.06.010. Impact Factor 3.716 (2017).

X. Fabregat-Safont D, Barneo-Muñoz M, Carbón X, Hernádez F, Martinez-Garcia F, Ventura M, Stove CP, Sancho JV, Ibáñez M. Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. *Accepted in Addiction Biology*. Impact Factor 4.121 (2019).

XI. Fabregat-Safont D, Ibáñez M, Baquero A, Sancho JV, Hernández F, Haro G. Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples. *Journal of Pharmaceutical and Biomedical Analysis*. 2020; 186:113298. doi:10.1016/j.jpba.2020.113298. Impact Factor 3.209 (2019).

XII. Fabregat-Safont D, Ripoll C, Orengo T, Sancho JV, Hernández F, Ibáñez M. Variation in the pattern of synthetic cannabinoid use by a female patient during 2018. *Adicciones*. 2020; 32:228-230. doi:10.20882/adicciones.1379. Impact Factor 3.167 (2019).

"*This thesis has been accepted by the co-authors of the publications listed above that have waved the right to present them as a part of another PhD thesis*"

This thesis has been developed and will be defended according to the requirements for obtaining the International PhD degree:

1. The present thesis has been written in English. The summary, objectives and conclusion sections have also been included in Valencian and Spanish.
2. The candidate has performed a research stay in the Department of Forensic Medicine of the University of Copenhagen from 30th of April 2018 to 31st of July 2018, under the supervision of Prof. Dr. Med. Kristian Linnet.
3. Previously to the defense, this work has been evaluated by two international independent reviewers directly related with the research field, Prof. Facundo Fernández (School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, USA) and Prof. Adrian Covaci (Department of Pharmaceutical Sciences, University of Antwerp, Antwerp, Belgium).
4. The thesis will be defended in Spanish and English, and the tribunal will be composed by at least one international expert from a non-Spanish university.

A la "uela"

“Use them with care, and use them with respect as to the transformations they can achieve, and you have an extraordinary research tool. Go banging about with a psychedelic drug for a Saturday night turn-on, and you can get into a really bad place, psychologically. Know what you're using, decide just why you're using it, and you can have a rich experience.”

Alexander Shulgin

PiHKAL: A Chemical Love Story (1991)

AGRAÏMENTS/ACKNOWLEDGEMENTS

Doncs ja està, tesi acabada. Es diu ràpid, però ací queda reflectida bona part de la meua vida dels darrers 4 anys. Però, aquest treball no és únicament meu. Aquest treball és el resultat de tots aquells que s'han esforçat en que aquesta recerca fóra possible, amb qui he col·laborat directament en els estudis que ací es recullen, i tots aquells que m'han recolzat fora de l'àmbit científic. Aquest treball és de tots nosaltres. I a tots vosaltres, moltes gràcies.

Aquesta tesi no s'haguera pogut realitzar, si no fóra pels meus directors de tesi María Ibáñez i Juanvi Sancho. A Maria, per guiar-me en el llarg camí que és una tesi, i ser tot un referent per a mi en l'àmbit científic. Moltes gràcies per recolzar totes aquelles "idees boges" que se'm passaven pel cap, i que van ser la llavor de molts dels treballs d'aquesta tesi. I com no, per acollir-me al teu despatx en tots aquells moments fluixos quan les coses no anaven com era d'esperar, tant en l'àmbit científic com personal. A Juanvi, moltes gràcies per totes les xarrades i raonaments sobre l'espectrometria de masses amb les que tant disfrute i aprenc una infinitat de coses. Per tindre sempre la porta del teu despatx oberta sempre que tenia algun dubte, fóra del tema que fóra, i dedicar-me uns minuts a discutir-ho. I com no, no podia deixar de passar l'oportunitat per agrair-te totes les xarrades durant el *coffee*, que anaven des de temes de ciclisme (i les corresponents sortides a seguir La Vuelta quan passava per Castelló), fins discussions en l'àmbit de la informàtica.

Me gustaria también agradecer a Félix Hernández, director del Institut Universitari de Plaguicides i Aigües, por aceptarme en su grupo de investigación y brindarme la oportunidad de realizar esta tesis doctoral. Dedicar també unes paraules a Clara Boix, amb qui vaig donar els meus primers passos en el món de la recerca mentre realitzava el Treball Final de Grau, i em va ajudar en eixos moments inicials quan tot et sembla extremadament complexe.

Per a fer una seguida “geogràfica” dels agraïments, voldria continuar agraïnt a tots els membres de l’Institut Universitari de Plaguicides i Aigües. Moltes gràcies a tots els que composeu aquest grup per haver-me ajudat, en major o menor grau, en la meua formació al llarg d’aquests quatre anys: Paco, Ximo, Toni, Roque, Elena, Carmen, Robert, Jose, Arantxa, Ana Maria, Noelia i Marina; i aquells que ja no estan al laboratori com Mercedes, Edu, Montse, Emma, Neus, Rubén i Carlos. I com no, he d’agrair a una de les persones més importants del IUPA, ja que sense ella el laboratori no podria funcionar com cal. Moltes gràcies a Vima pel seu bon humor que sempre ens treu un somriure, així com per l’interès que sempre mostra en tot allò que fem.

Si que m’agradaria dedicar algunes paraules amb més detall a la gent amb qui he estat amb major contacte, simplement perquè hem “patit” simultàniament (o seqüencialment) el que és realitzar una tesi, o estar dia a dia amb treballs experimentals. A Leticia i Alberto, companys de màster, de tesi, i de despatx (encara que no de forma simultània). A Eddie que, aunque nunca hayamos trabajado juntos, siempre te has interesado por mi investigación y hemos compartido largas charlas sobre nuestros ámbitos de estudio. A Ionut, qui sempre m’ha deixat “posar mà” i donar la meua opinió sobre qualsevol problema analític que tenia. A Borja, amb qui sempre és un plaer raonar de pràcticament qualsevol tema que se’m passava pel cap, i va fer que les xarrades de corredor foren simplement genials. A Tania, per tirar-me una maneta si necessitava qualsevol cosa de GC, i no sé si deuria donar-li les gràcies o no pel fet d’haver-me anomenat com a “Servei d’Informàtica del IUPA” i ser el seu assessor personal en temes tecnològics. A Jorge, compañero más allá del ecosistema del laboratorio, con quien he compartido incontables *craft beers* tanto en Castelló, como a lo largo de Bélgica y Dinamarca, así como el terrible calor del secarral de Villena. I finalment, moltes gràcies a Maria Mata, futura Graduada en Química, amb qui he treballat colze amb colze en alguns articles d’aquesta tesi, treballant sembre amb moltes ganes i il·lusió.

Encara sense sortir del laboratori, m'agradaria recordar també a tots aquells que han passat pel IUPA i amb qui he tingut l'oportunitat de treballar. A Mihaela Dalae, gracias por tu inmenso trabajo con el proyecto de los centros de menores, ya que no lo podríamos haber realizado sin ti. A Eulàlia Olesti i Óscar Pozo del IMIM, amb qui vaig tindre l'oportunitat de col·laborar en la realització d'estudis de metabolisme de NPS amb humans, i em van ensenyar molts aspectes del metabolisme que desconeixia. Y muy especialmente a Silvia Valverde que, aunque no participé directamente en el trabajo que realizó en el IUPA, me hizo recorrerme todas y cada una de las tiendas de Castelló buscando jalea real "fresca". Gracias también por todas esas tardes de cervezas en el Lupulove junto con Jorge, las cuales es obligado que se mantengan con cierta periodicidad.

M'agradaria continuar amb paraules per a tots aquells que han participat molt activament en els treballs que composen aquest mosaic anomenat tesi. En primer lloc, a Manoli Barneo, María José Sánchez i Ferran Martinez, de la Unitat Predepartamental de Medicina de l'UJI. Moltes gràcies per haver col·laborat amb la mateixa il·lusió que nosaltres en tots els estudis amb rates i ratolins, i per haver-nos ampliat un poc més els nostres escassos coneixements de medicina. Gran part d'aquesta tesi és gràcies a vosaltres.

Als companys d'Energy Control Iván Fornís, Cristina Gil, Xoán Carbón, i molt especialment a Mireia Ventura. El Capítol 1 no es podria haver realitzat si no fóra per vosaltres. Moltes gràcies per haver acceptat aquella proposta de col·laboració que us vam enviar fa ara 4 anys per a l'anàlisi de NPS, i per l'inesgotable esforç en treure endavant tots els treballs que hem tret junts.

A la gente con quien he trabajado en los estudios sobre la detección de cannabinoides sintéticos en consumidores. Gracias a Gonzalo Haro y Abel Baquero del Hospital Provincial de Castelló, y a Carmen Ripoll y Teresa Orengo de las UCAs de Valencia, por todo el esfuerzo e ilusión que pusieron y que permitió realizar esos trabajos.

A Eva Guillamón, por haberme ayudado con los datos de difracción de rayos X de monocristal, ya que sin ella no podríamos haber utilizado esta técnica para confirmar compuestos no reportados hasta la fecha. A Florenci González, per resoldre'm tots els dubtes que tenia en els primers anàlisis que vaig realitzar amb el RMN.

Special thanks to the people from the University of Copenhagen, where I spent 3 months of this thesis working with cell incubations and a Q Exactive. I would like to acknowledge Prof.Dr.Med. Kristian Linnet, responsible of the Forensic chemistry section of the Forensic Medicine laboratory of the University of Copenhagen, for accept my research stay. Thanks also to Dr. Petur Dalsgaard, who gave me lots of "tricks" and knowledge about the QTOF system and the UNIFI software. Thanks also to Kristian Brinch Mollerup and Anders Bork Davidsen for helping me with the QTOF and the Q Exactive, respectively. Muchas gracias también a la Dr. Carolina Noble, con quien compartí despacho en Copenhague y participó en uno de los trabajos de esta tesis. *Finally, thanks to Dr. Marie Mardal for leading me through all my research stay, helping me with the Q Exactive, FreeStyle and Compound Discoverer software, as well as recommend to me the nicest places in Copenhagen to have a beer.*

Thanks to Dr. Annelies Cannaert from University of Ghent (Belgium) for the collaboration in the SCRAs in vitro research, together with Carolina Noble and Prof.Dr. Christophe Stove (University of Ghent). And very special thanks to Prof.Dr. Christophe Stove, who participate also in the cathinone brain permeability study, providing an accurate revision and very interesting comments and suggestions, which significantly improved the manuscript.

I fins ací, l'àmbit científic. Però no perquè estiguen cap al final dels agraïments vol dir que no han col·laborat igual (o fins i tot més) per a que aquesta tesi fóra una realitat. Moltes gràcies als meus amics Pepe, Silvia, Adrián, Ignacio i Mar, per tots els moments que hem passat junts i que eren una de les meues principals fonts de recàrrega energètica després de cada setmana esgotadora al laboratori.

A les meues companyes del Grau en Química Silvia i Rosa, per compartir tots els moments complicats del llarg camí que són els estudis universitaris i que, una volta vàrem acabar eixa etapa, vam continuar l'amistat més enllà del Campus del Riu Sec. A Ester, qui va dissenyar la portada d'aquesta tesi en base a unes vagues idees sobre el *ion path* de un QTOF que li vaig dibuixar (prou malament) mentre preníem una cervesa.

Ja se que pot sonar a tòpic, però no puc deixar passar l'oportunitat d'agrair en aquesta tesi als meus pares, pel recolzament que sempre m'heu donat en totes i cadascuna de les decisions que he pres en la vida, foren encertades o no, així com per haver estat en tots i cadascun dels moments bons, roïns, i molt roïns de la meua vida. I supose que hauria de donar-vos gràcies per moltes més coses, però la quantitat de paraules que es poden escriure és finita.

Finalment, a una de les persones més importants per a mi. Moltes gràcies a la meua "auela". Qui va estar amb mi durant totes les meues etapes de la meua vida. Qui quan vaig iniciar el meu camí en la universitat, no va dubtar en comprar-me el millor portàtil que va trobar (i amb el qual encara he escrit la major part d'aquesta tesi durant la quarantena a causa de la pandèmia de COVID-19). Qui malauradament no va poder veure acabat aquest treball, però se que encara està amb mi quan estic escrivint aquestes paraules. Especialment a tu, dedique aquesta tesi.

A cadascú de vosaltres, moltes gràcies!

Summary

The search for novel psychotropic experiences, the desire of exploring the chemistry of synthetic drugs, and the need of skirting drug regulation and avoiding drug tests have promoted the rising of the so-called new psychoactive substances (NPS), especially in the last decade. Nowadays, the NPS consumption in Europe is a serious public health problem due to the unknown health effects for most of these substances, as well as the toxicity reported for some of them through reporting overdose and death cases. The fight against the NPS challenge must be performed in coordination with medical services, legal and social experts, and pharmacological, toxicological and forensic laboratories.

In this Doctoral Thesis, an in-depth investigation on NPS have been performed from the analytical chemistry point of view, a discipline present during toxicological and forensic analyses. The usefulness of cutting-edge mass spectrometry-based analytical chemistry for NPS research has been demonstrated through the different studies included hereafter.

The present work is structured in three blocks. The first is focused on the analysis of consumption products, including the analytical characterisation of different compounds, as well as the development of novel analytical strategies for their determination. The second includes the application of different *in vitro* and *in vivo* models for assessing the metabolism, potency and pharmacokinetics properties of different NPS. Finally, the third presents an investigation about the use of NPS among teenagers.

The first block is comprised by six research articles divided in two chapters. The first chapter presents the analysis of different legal highs purchased in a local smartshop, the analysis of research chemicals performed in collaboration with Energy Control, and the full analytical characterisation (as well as the investigation of the origin and reasons to be synthetized) of four novel NPS not previously reported. As no analytical reference standards were available by the time of performing these studies, compounds were unequivocally identified by

the combination of different and powerful analytical techniques: high-resolution mass spectrometry and nuclear magnetic resonance. Additionally, information about gas chromatography-mass spectrometry, Fourier-transformed infrared spectroscopy and, in some cases, single crystal X-ray diffraction, was provided. Concretely, a putative new phenethylamine, the opioid U-49900, the synthetic cathinone 5-PPDi (including a novel indanyl moiety), and the first halogenated aminorex derivative were reported.

The second chapter of this block includes two research articles, in which the full capabilities of the triple quadrupole mass analyser are explored for performing compound identification in research chemical and legal high samples, based on suspect screening strategies. The first work presents the application of the precursor ion scan and neutral loss scan modes for the tentative identification of synthetic cathinones, based on the common fragmentation pathways of these compounds. The second work shows the application of a modified atmospheric solids analysis probe for the direct analysis of NPS in legal highs and surfaces, using in this case a data-dependent acquisition mode and identifying the compounds based on their fragmentation spectra.

The second block of the thesis, formed by two chapters with two research articles each one, explores the applicability of different *in vitro* and *in vivo* models for assessing NPS pharmacology. The first chapter of this block, the fourth of this thesis, is focused on the *in vitro* approach, and includes a first research article about the study of the metabolic pathway of the synthetic cannabinoids 5F-APP-PICA and AMB-FUBINACA using pooled human hepatocytes and analysis by high-resolution mass spectrometry. Additionally, the potency of both compounds was evaluated by using an *in vitro* assay based on measuring their affinity for the specific receptors involved in its potency. The second study of this chapter shows the metabolic fate of four synthetic stimulants, including the synthetic cathinone 5-PPDi, following the same strategy described above: pooled human hepatocytes incubation and high-resolution mass spectrometry analysis.

The fifth chapter of the thesis, and second part of this block, assesses the pharmacology of NPS using *in vivo* approaches. The first research article is focused in the synthetic tryptamine 5-MeO-MiPT, identified in a legal high sample. Here, the metabolic fate and pharmacokinetics in blood and urine were explored using C57BL/J6 adult male mice. Moreover, the most suitable consumption biomarkers for this tryptamine were also proposed. The second research article of this chapter uses Sprague-Dawley adult female rats, for exploring the relationship between synthetic cathinone structure and their permeability through the blood-brain barrier. For that, a pre-target analytical methodology for the quantification of thirteen cathinones in cerebrum tissue was successfully validated and applied for their quantification in cerebrum samples from rats dosed with these compounds. A direct relationship between the moieties present in the cathinone and the permeability through the blood-brain barrier was observed, illustrating that cathinones follow a carrier-mediated process increased with the polarity of the compound.

The third block is formed by the sixth chapter, including two scientific articles. Both of them are based on the application of high-resolution mass spectrometry suspect screening strategies for the determination of synthetic cannabinoids consumption among teenagers. For this purpose, the information about the synthetic cannabinoids present in the herbal blends potentially consumed was used. The first research article is focused on the teenagers from five juvenile offenders' centres, and the results showed that some of these adolescents had been consuming herbal blends during their permits with stay at home for avoiding drug tests. The second article is the monitoring of a female teenager, who consumed synthetic cannabinoids, during 2018. In this case, a change on her consumption patterns was observed, which was afterwards related to the Spanish ban of the synthetic cannabinoid XLR-11 in July 2018.

All the information presented in this Doctoral Thesis will help forensic and toxicological laboratories working on NPS, providing useful information that includes analytical data for compound detection, novel instrumental methodologies, metabolic and pharmacokinetic information about the studied compounds, and showing the synthetic cannabinoids use among teenagers under control and/or treatment.

Resum

La cerca de noves experiències psicotròpiques, el desig d'explorar la química de les drogues sintètiques i la necessitat d'eludir la legislació sobre drogues i evitar així els controls, han promogut el sorgiment de les denominades noves substàncies psicoactives (NPS), especialment en l'última dècada. Hui dia, el consum de NPS a Europa és un greu problema de salut pública, pel fet que els efectes per a la salut de la majoria d'aquestes substàncies són encara desconeguts, així com la toxicitat que s'ha detectat per a algunes d'elles a través de diferents casos de sobredosis i morts. La lluita contra el problema de les NPS ha de realitzar-se en coordinació amb els serveis mèdics, els experts jurídics i socials, i els laboratoris farmacològics, toxicològics i forenses.

En aquesta Tesi Doctoral s'ha realitzat una investigació amb profunditat sobre les NPS des del punt de vista de la química analítica, disciplina present en les anàlisis toxicològiques i forenses. La utilitat de la química analítica especialitzada, basada en l'espectrometria de masses per a la investigació de les NPS, ha quedat demostrada a través dels diferents estudis que s'inclouen a continuació.

Aquest treball s'estructura en tres blocs. El primer se centra en l'anàlisi dels productes de consum, inclosa la caracterització analítica de diversos compostos, així com el desenvolupament de noves estratègies analítiques per a la seua determinació. El segon inclou l'aplicació de diferents models *in vitro* i *in vivo* per a avaluar el metabolisme, la potència i les propietats farmacocinètiques de diferents NPS. Finalment, el tercer presenta una investigació sobre l'ús de las NPS entre els adolescents.

El primer bloc està compost per sis articles d'investigació dividits en dos capítols. En el primer capítol, es presenta l'anàlisi de diferents *legal highs* adquirides en una *smartshop* local, l'anàlisi de *research chemicals* en col·laboració amb Energy Control, i la caracterització analítica completa (així com la investigació de l'origen i les raons per a ser sintetitzada) de quatre noves NPS no reportades

anteriorment. Com no es disposava de patrons de referència analítica en el moment de realitzar aquests estudis, els compostos es van identificar inequívocament mitjançant la combinació de diferents i potents tècniques analítiques: l'espectrometria de masses d'alta resolució i la ressonància magnètica nuclear. A més, es va proporcionar informació sobre les anàlisis mitjançant cromatografia de gasos-espectrometria de masses, espectroscòpia infraroja i, en alguns casos, difracció de raigs X de monocristall. Concretament, es van reportar una suposada nova fenetilamina, l'opiaci U-49900, la catinona sintètica 5-PPDi (que inclou el nou grup funcional indanil), i el primer derivat halogenat de l'aminorex.

El segon capítol d'aquest bloc inclou dos articles d'investigació, en els quals s'exploren totes les capacitats de l'analitzador de masses de triple quadrupol per a realitzar la identificació de compostos en mostres de *legal highs* i *research chemicals*, basant-se en estratègies de tipus *suspect screening*. El primer treball, presenta l'aplicació dels modes d'adquisició *precursor ion scan* i *neutral loss scan* per a la identificació temptativa de catinones sintètiques, basant-se en les rutes de fragmentació comunes d'aquests compostos. El segon treball, mostra l'aplicació de la sonda d'anàlisi de sòlids a pressió atmosfèrica, modificada per a l'anàlisi directa de NPS en *legal highs* i superfícies, utilitzant en aquest cas un mode d'adquisició de tipus *data-dependent* i identificant els compostos a partir dels seus espectres de fragmentació.

El segon bloc de la tesi, format per dos capítols amb dos articles d'investigació cadascun, explora l'aplicabilitat de diferents models *in vitro* i *in vivo* per a avaluar la farmacologia de les NPS. El primer capítol d'aquest bloc, el quart d'aquesta tesi, se centra en els models *in vitro*, i inclou un primer article d'investigació sobre l'estudi de la ruta metabòlica dels cannabinoides sintètics 5F-APP-PICA i AMB-FUBINACA, utilitzant incubació amb hepatòcits humans i anàlisis per espectrometria de masses d'alta resolució. A més, es va avaluar la potència de tots dos compostos mitjançant un assaig *in vitro* basat a mesurar l'afinitat d'aquests compostos pels receptors específics que intervenen en la seua potència.

El segon estudi d'aquest capítol mostra l'elucidació dels metabòlits de quatre estimulants sintètics, inclosa la catinona sintètica 5-PPDi, seguint la mateixa estratègia descrita anteriorment: incubació amb hepatòcits humans i anàlisis per espectrometria de masses d'alta resolució.

El cinqué capítol de la tesi, i la segona part d'aquest bloc, avalua la farmacologia de les NPS utilitzant models *in vivo*. El primer article d'investigació se centra en la triptamina sintètica 5-MeO-MiPT, identificada en una mostra de *legal high*. En aquest cas, es va estudiar el metabolisme i farmacocinètica en sang i orina usant ratolins C57BL/J6 mascles adults. A més, també es van proposar els biomarcadors de consum més adequats per a aquesta triptamina. El segon article d'investigació d'aquest capítol utilitza rates Sprague-Dawley femelles adultes, per a explorar la relació entre l'estructura de les catinones sintètiques i la seua permeabilitat a través de la barrera hematoencefàl·lica. Per a això, es va validar amb èxit una metodologia analítica de tipus *pre-target* per a la quantificació de tretze catinones en teixit cerebral, aplicant-se per a la quantificació d'aquests compostos en mostres de cervell de rates injectades amb aquestes catinones. Es va observar una relació directa entre els diferents grups funcionals de les catinones i la permeabilitat a través de la barrera hematoencefàl·lica, la qual cosa il·lustra que aquests compostos presenten un procés mediat per un portador transmembrana, l'afinitat pel qual augmenta amb la polaritat del compost.

El tercer bloc està format pel sisé capítol, que inclou dos articles científics. Tots dos es basen en l'aplicació d'estratègies de tipus *suspect screening* mitjançant espectrometria de masses d'alta resolució, per a la determinació del consum de cannabinoides sintètics entre adolescents. Per a això, es va utilitzar la informació sobre els cannabinoides sintètics presents en les *herbal blends* que segurament havien consumit els adolescents. El primer article d'investigació se centra en els adolescents de cinc centres de menors, i els resultats van mostrar que alguns d'aqueixos adolescents havien estat consumint *herbal blends* durant els seus permisos de cap de setmana per a anar a casa, a fi d'evitar que es detectara el consum substancies mitjançant les anàlisis de drogues rutinaris. El segon article

és el seguiment d'una adolescent que va consumir cannabinoides sintètics durant 2018. En aquest cas, es va observar un canvi en els seus patrons de consum, que posteriorment es va relacionar amb la prohibició a Espanya del cannabinoide sintètic XLR-11 al juliol de 2018.

Tota la informació presentada en aquesta Tesi Doctoral ajudarà als laboratoris forenses i toxicològics que treballen amb NPS, proporcionant informació útil que inclou dades analítiques per a la detecció de compostos no reportats anteriorment, noves metodologies instrumentals, informació metabòlica i farmacocinètica sobre els compostos estudiats, i mostrant l'ús dels cannabinoides sintètics entre els adolescents sota control i/o tractament.

Resumen

La búsqueda de nuevas experiencias psicotrópicas, el deseo de explorar la química de las drogas sintéticas y la necesidad de eludir la legislación sobre drogas y evitar así los controles, han promovido la aparición de las denominadas nuevas sustancias psicoactivas (NPS), especialmente en la última década. Hoy en día, el consumo de NPS en Europa es un grave problema de salud pública, debido a que los efectos para la salud de la mayoría de estas sustancias son aún desconocidos, así como la toxicidad que se ha detectado para algunas de ellas a través de diferentes casos de sobredosis y muertes. La lucha contra el problema de las NPS debe realizarse en coordinación con los servicios médicos, los expertos jurídicos y sociales, y los laboratorios farmacológicos, toxicológicos y forenses.

En esta Tesis Doctoral se ha realizado una investigación en profundidad sobre las NPS desde el punto de vista de la química analítica, disciplina presente en los análisis toxicológicos y forenses. La utilidad de la química analítica especializada, basada en la espectrometría de masas para la investigación de las NPS, ha quedado demostrada a través de los diferentes estudios que se incluyen a continuación.

El presente trabajo se estructura en tres bloques. El primero se centra en el análisis de los productos de consumo, incluida la caracterización analítica de diversos compuestos, así como el desarrollo de nuevas estrategias analíticas para su determinación. El segundo incluye la aplicación de diferentes modelos *in vitro* e *in vivo* para evaluar el metabolismo, la potencia y las propiedades farmacocinéticas de diferentes NPS. Por último, el tercero presenta una investigación sobre el uso de la NPS entre los adolescentes.

El primer bloque está compuesto por seis artículos de investigación divididos en dos capítulos. En el primer capítulo, se presenta el análisis de diferentes *legal highs* adquiridas en una *smartshop* local, el análisis de *research chemicals* en colaboración con Energy Control, y la caracterización analítica completa (así

como la investigación del origen y las razones para ser sintetizada) de cuatro nuevas NPS no reportadas anteriormente. Como no se disponía de patrones de referencia analítica en el momento de realizar estos estudios, los compuestos se identificaron inequívocamente mediante la combinación de diferentes y potentes técnicas analíticas: la espectrometría de masas de alta resolución y la resonancia magnética nuclear. Además, se proporcionó información sobre los análisis mediante cromatografía de gases-espectrometría de masas, espectroscopia infrarroja y, en algunos casos, difracción de rayos X de monocristal. Concretamente, se reportaron una supuesta nueva fenetilamina, el opiáceo U-49900, la catinona sintética 5-PPDi (que incluye el nuevo grupo funcional indanilo), y el primer derivado halogenado del aminorex.

El segundo capítulo de este bloque incluye dos artículos de investigación, en los que se exploran todas las capacidades del analizador de masas de triple cuadrupolo para realizar la identificación de compuestos en muestras de *legal highs* y *research chemicals*, basándose en estrategias de tipo *suspect screening*. El primer trabajo, presenta la aplicación de los modos de *adquisición precursor ion scan* y *neutral loss scan* para la identificación tentativa de catinonas sintéticas, basándose en las rutas de fragmentación comunes de estos compuestos. El segundo trabajo, muestra la aplicación de la sonda de análisis de sólidos a presión atmosférica, modificada para el análisis directo de NPS en *legal highs* y superficies, utilizando en este caso un modo de adquisición de tipo *data-dependent* e identificando los compuestos en base a sus espectros de fragmentación.

El segundo bloque de la tesis, formado por dos capítulos con dos artículos de investigación cada uno, explora la aplicabilidad de diferentes modelos *in vitro* e *in viv*o para evaluar la farmacología de las NPS. El primer capítulo de este bloque, el cuarto de esta tesis, se centra en los modelos *in vitro*, e incluye un primer artículo de investigación sobre el estudio de la ruta metabólica de los cannabinoides sintéticos 5F-APP-PICA y AMB-FUBINACA, utilizando incubación con hepatocitos humanos y análisis por espectrometría de masas de

alta resolución. Además, se evaluó la potencia de ambos compuestos mediante un ensayo *in vitro* basado en medir la afinidad de estos compuestos por los receptores específicos que intervienen en su potencia. El segundo estudio de este capítulo muestra la elucidación de los metabolitos de cuatro estimulantes sintéticos, incluida la catinona sintética 5-PPDi, siguiendo la misma estrategia descrita anteriormente: incubación con hepatocitos humanos y análisis por espectrometría de masas de alta resolución.

El quinto capítulo de la tesis, y la segunda parte de este bloque, evalúa la farmacología de las NPS utilizando modelos *in vivo*. El primer artículo de investigación se centra en la triptamina sintética 5-MeO-MiPT, identificada en una muestra de *legal high*. En este caso, se estudió el metabolismo y farmacocinética en sangre y orina usando ratones C57BL/J6 machos adultos. Además, también se propusieron los biomarcadores de consumo más adecuados para esta triptamina. El segundo artículo de investigación de este capítulo utiliza ratas Sprague-Dawley hembras adultas, para explorar la relación entre la estructura de las catinonas sintéticas y su permeabilidad a través de la barrera hematoencefálica. Para ello, se validó con éxito una metodología analítica de tipo *pre-target* para la cuantificación de trece catinonas en tejido cerebral, aplicándose para la cuantificación de estos compuestos en muestras de cerebro de ratas inyectadas con estas catinonas. Se observó una relación directa entre los diferentes grupos funcionales de las catinonas y la permeabilidad a través de la barrera hematoencefálica, lo que ilustra que estos compuestos presentan un proceso mediado por un portador transmembrana, cuya afinidad aumenta con la polaridad del compuesto.

El tercer bloque está formado por el sexto capítulo, que incluye dos artículos científicos. Ambos se basan en la aplicación de estrategias de tipo *suspect screening* mediante espectrometría de masas de alta resolución, para la determinación del consumo de cannabinoides sintéticos entre adolescentes. Para ello, se utilizó la información sobre los cannabinoides sintéticos presentes en las *herbal blends* que seguramente habían consumido los adolescentes. El primer

artículo de investigación se centra en los adolescentes de cinco centros de menores, y los resultados mostraron que algunos de esos adolescentes habían estado consumiendo *herbal blends* durante sus permisos de fin de semana para ir a casa, a fin de evitar que se detectara el consumo sustancias mediante los análisis de drogas rutinarios. El segundo artículo es el seguimiento de una adolescente que consumió cannabinoides sintéticos durante 2018. En este caso, se observó un cambio en sus patrones de consumo, que posteriormente se relacionó con la prohibición en España del cannabinoide sintético XLR-11 en julio de 2018.

Toda la información presentada en esta Tesis Doctoral ayudará a los laboratorios forenses y toxicológicos que trabajan con NPS, proporcionando información útil que incluye datos analíticos para la detección de compuestos no reportados anteriormente, nuevas metodologías instrumentales, información metabólica y farmacocinética sobre los compuestos estudiados, y mostrando el uso de los cannabinoides sintéticos entre los adolescentes bajo control y/o tratamiento.

CONTENTS

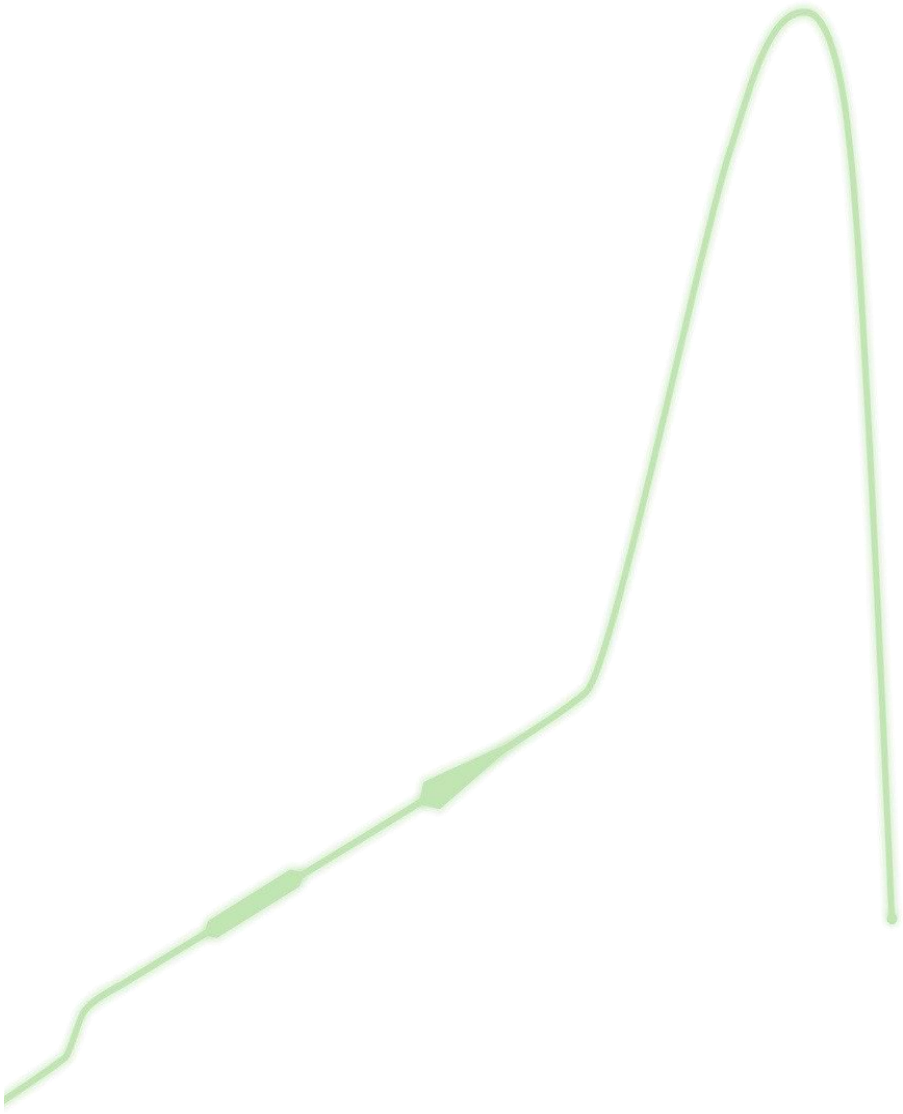
LIST OF ACRONYMS

$[M+H]^+$	Protonated molecule
$[M-H]^-$	Deprotonated molecule
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism, excretion
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
ASAP	Atmospheric solids analysis probe
BBB	Blood-brain barrier
CE	Collision energy
CID	Collision-induced dissociation
Da	Dalton
DDA	Data dependent acquisition
dd-MS^2	Data-dependent-MS-two, DDA acquisition mode for Thermo Sci. Q-Orbitrap instruments
DIA	Data independent acquisition
DOA	Drug of abuse
EI	Electron ionisation
EIC	Extracted ion chromatogram
EMCDDA	European Monitoring Centre for Drug and Drug Addiction
ESI	Electrospray ionisation
EWS	Early warning system
FIA	Flow-injection analysis
FTIR	Fourier-transformed infrared spectroscopy
FTMS	Fourier-transformed mass spectrum

FWHM	Resolution at full width at half maximum, used for HRMS instruments
GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
HCD	Higher-energy C-trap dissociation, used in Thermo Sci. instruments
HE	High energy function in the MS^E acquisition mode for Waters Corp. QTOF instruments
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
ICP	Inductively coupled plasma
IMS	Ion mobility spectrometry/separation
IMS-HRMS	High resolution mass spectrometry with ion mobility separation
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry
LE	Low energy function in the MS^E acquisition mode for Waters Corp. QTOF instruments
m/z	Mass to charge ratio
MeOH	Methanol
MOR	μ-opioid receptors
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^E	MS-to-the-E, DIA mode for Waters Corp. QTOF instruments
NPLC	Normal phase liquid chromatography
NPS	New psychoactive substance
nw-EIC	Narrow-window EIC

pHH	Primary human hepatocytes
pHLM	Pooled human liver microsomes
ppb	Part per billion
ppm	Part per million
ppt	Part per trillion
PRM	Parallel reaction monitoring, MS/MS acquisition mode for Thermo Sci. Q-Orbitrap instruments
pS9	Pooled human liver S9 fraction
Q	Quadrupole mass analyser
Q-HRMS	Hybrid quadrupole-high resolution mass spectrometry
QOrbitrap	Hybrid quadrupole-Orbitrap mass analyser
QqQ	Triple quadrupole mass analyser
QTOF	Hybrid quadrupole-time of flight mass analyser
RPLC	Reversed-phase liquid chromatography
SCRA	Synthetic cannabinoid receptor agonist
SFC	Supercritical-fluid chromatography
SRM	Single reaction monitoring
TOF	Time of flight mass analyser
UHPLC	Ultra-high-performance liquid chromatography
UNODC	United Nations Office on Drugs and Crime

OBJECTIVES



Objectives

The **main objective** of this Doctoral Thesis is to perform a comprehensive investigation on new psychoactive substances (NPS) by the use of advanced mass spectrometric techniques (MS) (including tandem low-resolution (MS/MS) and high-resolution (HRMS) mass analysers), as well as on the different analytical strategies to be applied, depending on the aim of the study. These strategies include compound characterisation, metabolite elucidation, development of quantification methodologies and application of screening analysis.

For a full investigation on NPS, the analytical pathway to follow should include the next steps: 1) to know what compounds are being consumed in Spain through the analysis of legal highs and research chemicals, 2) to develop analytical strategies for performing these analyses, 3) to perform metabolism and pharmacokinetics studies for some of these compounds, and 4) to apply the information obtained to urine analysis for determining NPS consumption.

Keeping in mind the main objective, the following **specific objectives** are stablished:

1) Analysis of legal highs and research chemicals, purchased or obtained from Spanish consumers, by combining different analytical techniques for compound identification when no analytical reference standard is available.
2) Full analytical characterisation of unknown/unreported NPS, as well as an in-depth investigation about their origin and reasons for synthesis.
3) Development of MS/MS analytical strategies for compound identification and suspect screening analysis of seizures, legal highs and research chemicals.
4) Evaluation of *in vitro* models for metabolite profiling and potency testing of selected compounds. Elucidation of the obtained metabolites based on HRMS data.

5) Application of *in vivo* models for metabolite profiling and pharmacokinetic studies of selected compounds. Development of a pre-target analytical strategy for the quantification of synthetic cathinones in cerebrum tissue.
6) Study on the prevalence of synthetic cannabinoids use among teenagers, by HRMS suspect screening analysis of urine samples, as well as of products potentially consumed.

Objectius

L'**objectiu principal** d'aquesta Tesi Doctoral és dur a terme una investigació exhaustiva sobre les noves substàncies psicoactives (NPS) mitjançant l'ús de tècniques avançades d'espectrometria de masses (MS) (utilitzant tant analitzadors en tàndem de baixa resolució (MS/MS) com analitzadors d'alta resolució (HRMS)), així com sobre les diferents estratègies analítiques a emprar en funció de l'objectiu de l'estudi. Aquestes estratègies inclouen la caracterització de compostos, l'elucidació de metabòlits, el desenvolupament de la metodologia de quantificació i l'aplicació d'anàlisi de tipus cribratge.

Per a una investigació completa sobre les NPS, la via analítica a seguir ha d'incloure els següents passos: 1) conéixer quins compostos s'estan consumint a Espanya a través de l'anàlisi de les *legal highs* i dels *research chemicals*, 2) desenvolupar estratègies analítiques per a realitzar aquestes anàlisis, 3) realitzar estudis de metabolisme i farmacocinètica per a alguns d'aquests compostos, i 4) aplicar la informació obtinguda a l'anàlisi d'orina per a determinar el consum de NPS.

Tenint en compte l'objectiu principal, s'estableixen els següents **objectius específics**:

1) L'anàlisi de *legal highs* i *research chemicals*, comprades o obtingudes de consumidors espanyols, mitjançant la combinació de diferents tècniques analítiques per a la identificació dels compostos quan no es disposa d'un patró analític de referència.
2) Caracterització analítica completa de les NPS desconegudes/no reportades, així com una investigació en profunditat sobre el seu origen i les raons de la seua síntesi.
3) Desenvolupament d'estratègies analítiques basades en MS/MS per a la identificació dels compostos i anàlisis de tipus *suspect screening* de confiscacions, *legal highs* i *research chemicals*.

4) Avaluació dels models *in vitro* per a realitzar estudis de metabolisme i assajos de potència de compostos seleccionats. Elucidació dels metabòlits obtinguts a partir de les dades de HRMS.
5) Aplicació de models *in vivo* per a realitzar estudis de metabolisme i estudis farmacocinètics de compostos seleccionats. Desenvolupament d'una estratègia analítica de tipus *pre-target* per a la quantificació de catinones sintètiques en el teixit cerebral.
6) Estudi de l'ús de cannabinoides sintètics entre els adolescents, mitjançant anàlisis de tipus *suspect screening* per HRMS de mostres d'orina, així com dels productes que se sospita que han consumit.

Objetivos

El **objetivo principal** de esta Tesis Doctoral es llevar a cabo una investigación exhaustiva sobre las nuevas sustancias psicoactivas (NPS) mediante el uso de técnicas avanzadas de espectrometría de masas (MS) (utilizando tanto analizadores en tándem de baja resolución (MS/MS) como analizadores de alta resolución (HRMS)), así como sobre las diferentes estrategias analíticas a emplear en función del objetivo del estudio. Estas estrategias incluyen la caracterización de compuestos, la elucidación de metabolitos, el desarrollo de la metodología de cuantificación y la aplicación de análisis de tipo *screening*.

Para una investigación completa sobre las NPS, la vía analítica a seguir debe incluir los siguientes pasos: 1) conocer qué compuestos se están consumiendo en España a través del análisis de las *legal highs* y de los *research chemicals*, 2) desarrollar estrategias analíticas para realizar estos análisis, 3) realizar estudios de metabolismo y farmacocinética para algunos de estos compuestos, y 4) aplicar la información obtenida al análisis de orina para determinar el consumo de NPS.

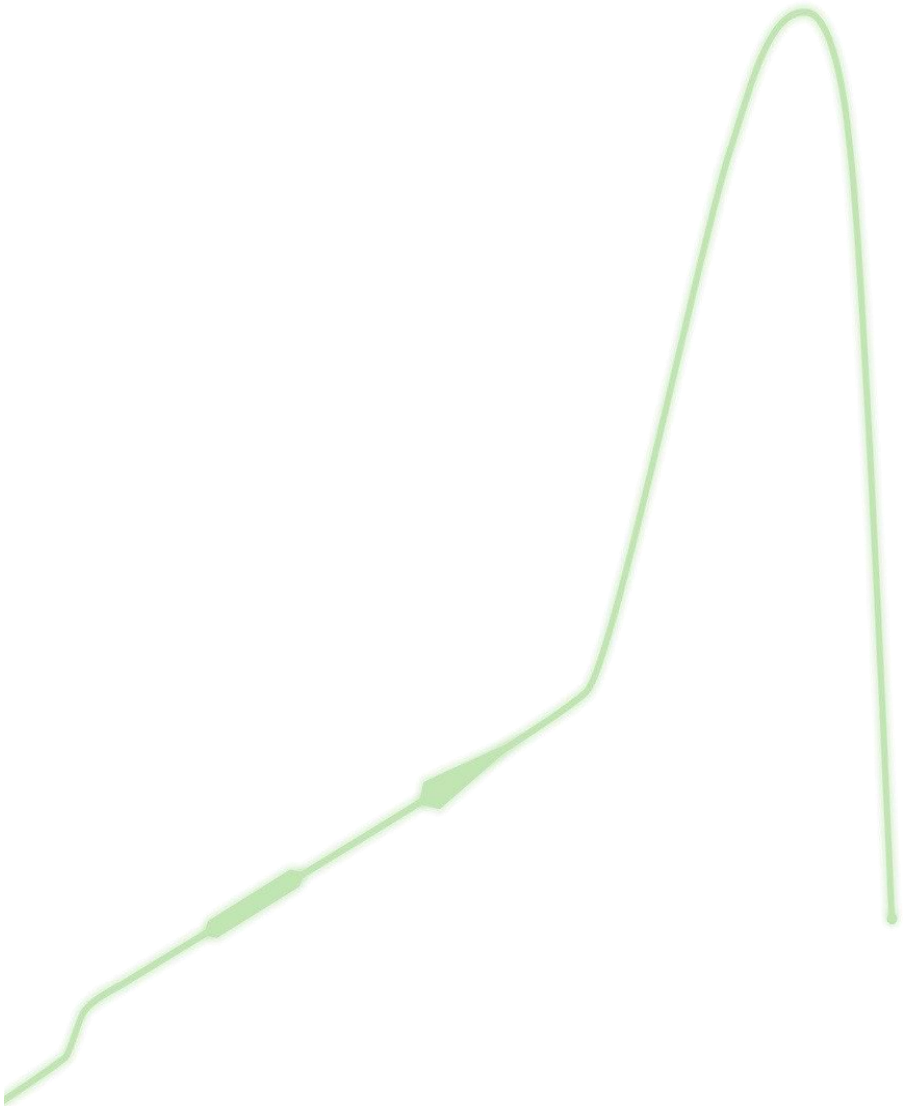
Teniendo en cuenta el objetivo principal, se establecen los siguientes **objetivos específicos**:

1) El análisis de *legal highs* y *research chemicals*, compradas u obtenidas de consumidores españoles, mediante la combinación de diferentes técnicas analíticas para la identificación de los compuestos cuando no se dispone de un patrón analítico de referencia.
2) Caracterización analítica completa de las NPS desconocidas/no reportadas, así como una investigación en profundidad sobre su origen y las razones de su síntesis.
3) Desarrollo de estrategias analíticas basadas en MS/MS para la identificación de los compuestos y análisis de tipo *suspect screening* de incautaciones, *legal highs* y *research chemicals*.

4) Evaluación de los modelos *in vitro* para realizar estudios de metabolismo y ensayos de potencia de compuestos seleccionados. Elucidación de los metabolitos obtenidos a partir de los datos de HRMS.
5) Aplicación de modelos *in vivo* para realizar estudios de metabolismo y estudios farmacocinéticos de compuestos seleccionados. Desarrollo de una estrategia analítica de tipo *pre-target* para la cuantificación de catinonas sintéticas en el tejido cerebral.
6) Estudio del uso de cannabinoides sintéticos entre los adolescentes, mediante análisis de tipo *suspect screening* por HRMS de muestras de orina, así como de los productos que se sospecha que han consumido.

CHAPTER 1

GENERAL INTRODUCTION



CHAPTER 1
GENERAL INTRODUCTION

1.1. A brief history of drugs origin

1.2. The current state of classical illicit drugs

1.3. New laws, new compounds: the psychoactive market change

1.4. Focusing on the new psychoactive substances of this thesis

- 1.4.1. Synthetic cannabinoids
- 1.4.2. Synthetic cathinones
- 1.4.3. Synthetic opioids
- 1.4.4. Tryptamines

1.5. Exploring the pharmacology of new psychoactive substances

- 1.5.1. *In vitro* approaches
- 1.5.2. *In vivo* approaches

1.6. Analytical methodologies for toxicological analysis

- 1.6.1. Chromatographic separation: Liquid chromatography
- 1.6.2. Mass spectrometry
  - 1.6.2.1. Atmospheric pressure ionisation
  - 1.6.2.2. Low-resolution mass analysers
  - 1.6.2.3. High-resolution mass analysers and hybrid instruments
- 1.6.3. Additional analytical techniques

1.7. Analytical strategies for NPS analysis in toxicological samples

- 1.7.1. Pre-target analysis
- 1.7.2. Wide-scope screening
- 1.7.3. Metabolite detection and elucidation

1.8. Literature

1.1. A brief history of drugs origin

Drugs.

We have been using drugs for many years. Maybe because they evade us from the reality. Perhaps due to the strange effects produced in our mind. Or we were deceived to use them by religious or social pressure. Anyway, we have been using drugs for many years, and for many reasons.

But, when did we start consuming drugs? The word "psychotropic" was originated in the Ancient Greece. It is made up of the terms *psycho* ("mind") and *tropos* (from *tropein* that means "to turn"), and it can be interpreted as "what turns the mind". One of the most ancient evidence of drug consumption was found in the archaeological site Shanidar IV (Iraq, dated around 48,000-46,000 BC), where some traces of pollen from different plants were found [1–3]. The *Ephedra altissima*, which has the alkaloid ephedrine that produces similar effects to amphetamine, was among the traces of pollen found in Shanidar IV [1–3].

There are also evidences of the opium use in Sumeria, Assyria, Ancient Egypt, Ancient Greece, Rome, China and India [4]. Opium is a dried latex from *Papaver somniferum* which contains the alkaloid morphine [4]. As an example, in the Odyssey (Homer, s. VIII BC) is described the use of a drug mixed with wine "to assuage suffering and to dispel anger, and to cause forgetfulness of all ills", when Helen tries to calm the men in the meeting arranged by Menelaus [5]. Parmenon of Byzantium (s. III BC) also wrote in an ancient iamb how the opium produces addiction [6]. Many years after, in the streets and brothels of the Roman Empire, the use of psychoactive substances was widely known and used, according to Lucius Mestrius Plutarchus (also known as Plutarch) who shown this fact in a verse of his *Moralia* [7,8]. In those years, health problems related to these substances were already known. Lucius Annaeus Seneca (Seneca the Young, or simply known as Seneca) described in his essay *De Providentia* (64 AD) how the consumption of certain substances could have adverse effects [9], called nowadays overdoses [8].

The medical use of these substances was transferred from Greek and Roman (for example, Plutarch and Aelius Galenus, better known as Galen of Pergamon) to Arabic authors [10]. There are evidences that the Arabic physician Yuhanna ibn Masawaih (777–857) prescribed the opium to relieve pain (for example, bladder stones and toothaches) and also to induce sleep [10]. By this time, physicians were also aware of the overdoses and how to treat them, as explained by Abbas al-Majusi in his *al-Malaki* [10].

The well-known cannabis, obtained from the *Cannabis sativa* plant, seems that was widely used in Ancient Egypt, as illustrated by several evidences available in literature [11]. According to this publication, the oldest example of medicinal cannabis in Ancient Egypt was found in *Papyrus Ramesseum III* (ca. 1,700 BC) for treating ocular problems [11,12]. This use can be comparable to the actual use of cannabis in glaucoma treatment or for anti-inflammatory purposes [11]. Another medical use was found in *Ebers Papyrus* (ca. 1,550 BC), were cannabis is used intra-vaginal, similarly to the application of cannabis as vaginal suppository in the 19th century for gynaecological disorders treatment [11]. Hopefully, these uses have been abandoned nowadays.

On the other side of the Atlantic Ocean, people had been using psychoactive substances many years before Christopher Columbus arrived to the West Indies. Pre-Columbian Mesoamerican cultures used a wide variety of plants and mushrooms that contain psychoactive compounds for their rituals and religious ceremonies, as they induce altered states of consciousness [13]. This fact is supported by the mushroom stones found in ritual contexts in Mesoamerica, dating from ca. 3,000 BC [13]. The rich variety of natural sources of hallucinogenic substances in this geographical region promoted the use of products producing psychoactive effects, such as the peyote cactus (*Lophophora williamsii*, containing the psychedelic alkaloid mescaline), hallucinogenic mushrooms (*Psilocybe* species, which have psilocybin and psilocin) and other psychedelic plants such as *Datura stramonium* and *Salvia divinorum* [13].

Other examples could be the "berserker" mood of the Vikings during the battle, produced by the consumption of the *Amanita muscaria* mushroom [14], or the tradition of chewing coca leaves (*Erythroxylum novogranatense* and *Erythroxylum coca*, which contains the well-known alkaloid cocaine) in South America [15]. According to J.W. Estes, the first report of cocaine to reach Europe could be around 1503, during the fourth voyage of exploration to the Americas [16]. From there, the cocaine dealing history is well-known, especially those related with Colombia [17]. It is impossible to include all the chapters of history related to drug use across the globe. But it is well-known the use of psychotropic substances over years and over the world.

Based on the premise of thousands of years of consumption, it is not surprising that, nowadays, and based on the information provided by the United Nations Office on Drugs and Crime (UNODC), around 271 million people aged between 15 and 64 (5.5% of the global population) have used drugs during 2017 [18]. If we focus in the European Union, around 96 million of people between 15 and 64 years (around 29% of adults) have used illicit drugs during their lives, according to the most recent report from the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) [19].

1.2. The current state of classical illicit drugs

Historically, cocaine, opium and cannabis seem to be the most commonly drugs used worldwide. This tendency on drug use has arrived until our days, and according to the EMCDDA, cannabis, cocaine, amphetamines and opioids are the most commonly used psychoactive substances in the European Union [19]. **Figure 1.1** shows the prevalence of these drugs in the European Union corresponding to the 2018 data.

The putative origin of psychoactive substances has been previously discussed, but now it is time to explore the prevalence, chemistry and pharmacology of the

illicit drugs or "drugs of abuse" (DOA), prior to deep into the novel psychotropic substances that are changing the actual market.

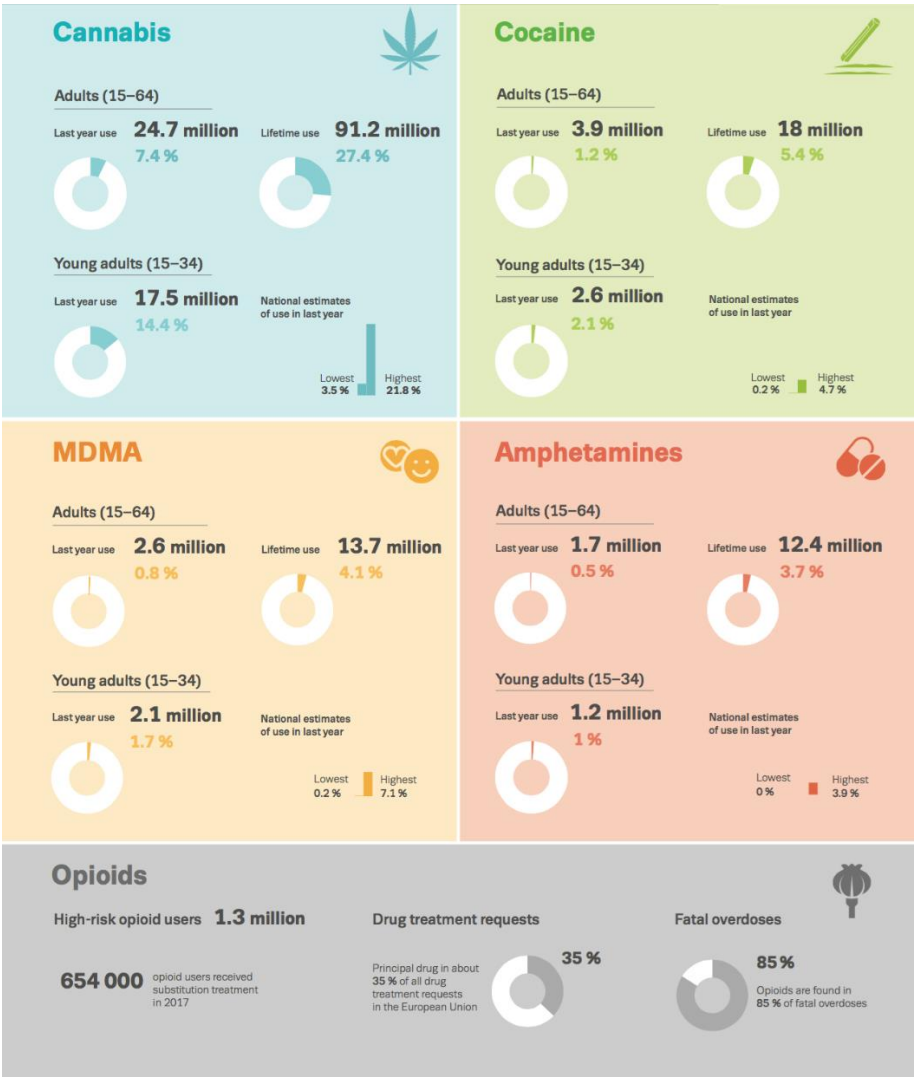


Figure 1.1. Estimation of drug use in the European Union according to the European Drug Report 2019 [19]. Adapted from the EMCCDA media library [CC BY-NC-ND 3.0].

Nowadays, the illicit drug most commonly used in Europe is cannabis, being consumed by around 14% of European in the 15-34 age group (young adults) last year (see **Figure 1.1**) [19]. Cannabis is generally smoked as herb (marijuana) or

resin (hashish) [19], and usually mixed with tobacco. Cannabis has more than one hundred of chemical compounds called cannabinoids, which most of them have been related with health benefits. For example, modulating physiological processes such as the appetite, pain-sensation, mood and fat/energy metabolism, among others [20]. This modulation is produced by the affinity of these compounds for the cannabinoids receptors of the endocannabinoid system [20]. The endocannabinoid receptors more related to the psychoactive effects of cannabis are CB_1 (central) and CB_2 (peripheral) receptors [21,22]. Regarding the cannabinoids present in cannabis, the most active compound, and major responsible of its psychotropic effects, is the Δ^9-tetrahydrocannabinol, better known as Δ^9-THC or simply THC [23,24]. The THC effects are well-known and have been widely studied: On the one hand, it produces the intensification of ordinary sensory experiences, time distortion, euphoria, relaxation and perceptual alterations [25]. Maybe, that is why many users affirm that "free-thinking, open people" use cannabis due to their triggered creativity [26]. On the other hand, cannabis produces also unpleasant effects such as anxiety, panic reactions, and affecting the short-term memory, motor skills and reaction time [25].

The second most consumed illicit drug is the stimulant cocaine, which has been consumed by 2.1% of European young adults during last year (**Figure 1.1**) [19]. Cocaine is an alkaloid obtained from the coca plant leaves after an extraction and purification process [27]. This illicit drug is commonly inhaled or "snorted" through the nose, being less common its intravenous injection [27]. Cocaine inhibits the dopamine uptake by the binding to the dopamine transporters in the striatum [28]. It produces a strong stimulation, euphoria and intense feeling of happiness, together with adverse effects such as agitation, delirium, psychosis, tachycardia and hypertension [29].

The 3,4-methylenedioxymethamphetamine (MDMA) is the third most frequently used illicit drug in the European Union, while the fourth are the amphetamines (**Figure 1.1**) [19]. In fact, MDMA belongs to the substituted amphetamine family, along with amphetamine and methamphetamine, among others.

Amphetamines are recreational drugs, usually presented as crystal and powder, which may be taken orally or snorted, while MDMA is usually consumed as tablets, known as ecstasy [19]. Similarly to cocaine, amphetamines, and specially MDMA, increase the levels of dopamine, norepinephrine and serotonin in brain, producing a stimulant and hallucinogenic effect [30,31]. Additionally, MDMA produces euphoria, arousal, enhanced mood, and heightened perceptions, together with other undesired effects such as nausea, headache, tachycardia, involuntary grinding of the teeth, and spasms of the jaw muscles [32].

Although opioids have been traditionally less used in Europe, ca. 1.3 million people in Europe are considered as high-risk opioid users [19]. As we already know, the first contact between people and opioids was through the consumption of opium, which contains the alkaloid morphine, widely used for the treatment of acute pain and chronic pain [33]. Nevertheless, the most known opioid is heroin (or diamorphine), a highly addictive recreational drug obtained from natural morphine [34]. Heroin is usually injected intravenously, snorted or smoked [35]. The psychoactive effects of this compound are produced by the binding to the μ-opioid receptors (MOR) in brain [34,36,37]. When MOR receptors are activated, the release of dopamine is increased, producing a sensation of pleasure [34,36,37]. So, the effects of using heroin is a feeling of pleasure or euphoria, together with nausea, itching, alteration of the mental functioning, and long term effects such as heart, liver, kidney and lungs problems, collapsed veins, insomnia, and severe mental disorders [35].

After reviewing the most commonly used illicit drugs (prevalence, pharmacological aspects and the effects produced), we are able to understand what is happening nowadays in the psychoactive market.

1.3. New laws, new compounds: the psychoactive market change

As we have seen above, Europe is mainly dominated by the cannabis and the stimulants market, including cocaine and amphetamines, being less important the use of opioids [19]. Nevertheless, the psychoactive substances market has changed in the last decade, maybe due to the toughening of illicit drug legislation, or perhaps by the desire to consume and explore novel types of drugs. This change has been promoted by the emergence of synthetic psychoactive compounds that produce, with greater or lesser intensity, similar effects to the classical illicit drugs.

These novel compounds have been called as "bath salts", "legal highs", "research chemicals", "spice"; but the UNODC uses the term "new psychoactive substances" or "NPS" for avoid confusion [38]. According to this organisation, an NPS is defined as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat" [38]. It is important to remark that the term "new" does not specify new inventions, as some of these compounds were synthetized decades ago, but refers to substances that have recently become available and used as psychoactive drug [38].

The origin of the NPS could be considered as "the revival of old forgotten medicines", as some of the first NPS identified were originally designed as pharmaceuticals. As examples, the synthetic opioids AH-7921, which was firstly synthetized in 1975 by Allen & Hanburys Ltd [39], or the U-47700 designed by The Upjohn Company in 1978 [40]. Their names are based on the pharmaceutical companies that developed them. Similarly, the well-known JWH synthetic cannabinoid series adopted the name of the researcher (John William Huffman) who synthetized them and explored the pharmacology of these compounds [41,42]. Many years later, these compounds were being consumed in streets, used as psychoactive drugs [43,44]. Nevertheless, the *guru* of the NPS is, without any doubt,

Alexander Shulgin, an American chemist who synthetized, and personally tested, more than 230 psychoactive compounds. His findings were published in two books, the *PiHKAL: A Chemical Love Story* ("Phenethylamines I Have Known And Loved", 1991) [45], and the *TIHKAL: The Continuation* ("Tryptamines I Have Known and Loved", 1997) [46], written with his wife Ann Shulgin. After the publication of these books, many of the NPS synthetized by the Shulgin started appearing in the drug market [47].

By the end of 2018, more than 730 different NPS were being monitored by the EMCDDA, 55 of which were detected for the first time in Europe in 2018 [19]. As it can be seen in **Figure 1.2**, the number of NPS notified by the EMCDDA for the first time in Europe increased until 2015. Nowadays, the number of notifications is around 1 novel compound per week.

But, why people are consuming/producing NPS? According to Reuter & Pardo [48], the existence of NPS can be justified based on four different premises:

1. Skirting drug regulation. As the NPS produce similar effects than the illicit drugs but they are not prohibited, these substances can be sold and consumed freely without legal consequences.
2. Avoid drug tests. As most of the existing NPS cannot be detected by the classical random drug tests, they are an adequate substitution to continue consuming psychoactive drugs and avoid detection.
3. Exploring new psychoactive experiences. Similarly to the Shulgin, some NPS users like trying different compounds, just for seeking new and attractive psychotropic trips.
4. Adulteration of classical drugs. Some illicit drugs producers adulterate those substances with NPS [19]. In some cases, NPS are sold as traditional drugs.

The NPS can be classified in different chemical families, being the most important ones the synthetic cannabimimetics (commonly known as synthetic cannabinoids), synthetic cathinones, phenethylamines, piperazines,

phencyclidines, tryptamines, benzofurans, synthetic opioids and benzodiazepines [49]. Based on the data reported by the EMCDDA, the most commonly seized substances in Europe are synthetic cannabinoids (51% of seizures in 2017) and synthetic cathinones (24% of seizures in 2017) [19]. Nevertheless, in the last years the synthetic opioids have been playing an important role, especially fentanyl derivatives, with 49 opioids detected in Europe since 2009 [19].

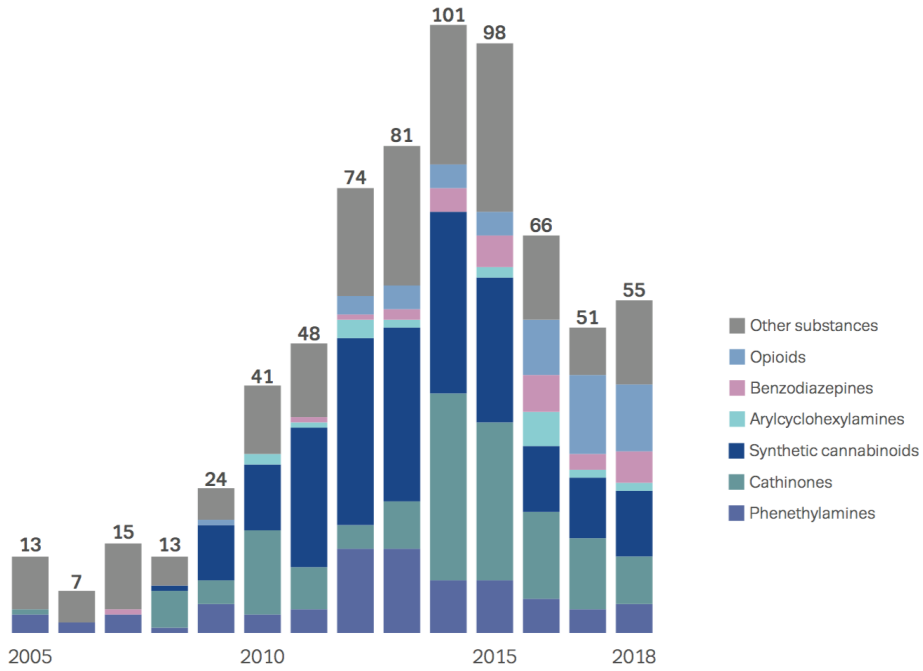


Figure 1.2. NPS notified for the first time to the EU Early Warning System since 2005 [19], classified based on their chemical class. Adapted from the EMCCDA media library [CC BY-NC-ND 3.0].

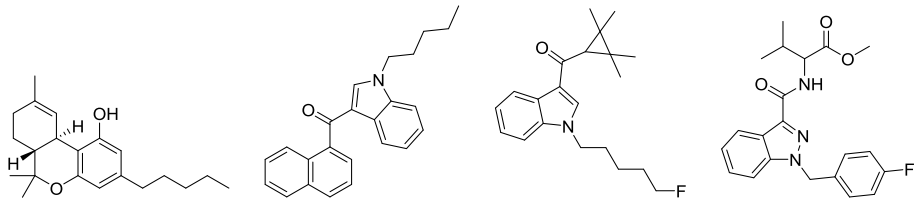
1.4. Focusing on the new psychoactive substances of this thesis

The studies included in the present thesis are mainly focused on the investigation of synthetic cannabinoids and synthetic cathinones, including additional studies related to a synthetic opioid and a tryptamine. So, it is mandatory to delve into these chemical families.

1.4.1. Synthetic cannabinoids

Synthetic cannabinoid receptor agonists (SCRA), commonly known as synthetic cannabinoids, are synthetic compounds that are agonist of the CB1 and CB2 receptors (similarly to THC, **Figure 1.3A**), and therefore, producing similar effects than cannabis [47]. First SCRAs subfamilies were developed for research and/or pharmaceutical purposes, being their name related to the origin of the substance, such as the already explained JWH (John W. Huffman, Clemenson University), HU (Raphael Mechoulam, Hebrew University), CP (Carl Pfizer, Pfizer Inc.) or AM series (Alexandros Makriyannis, University of Connecticut) [47]. Nowadays, SCRA represent the largest group of NPS monitored by the EMCDDA [19].

The most famous synthetic cannabinoid is, probably, the JWH-018 (**Figure 1.3B**), as it was the first SCRA identified in an herbal blend product in 2008, being rapidly identified as an agonist of the CB receptors [44,50]. Other JWH-SCRAs were identified in herbal blends [51], usually sold as a "synthetic marijuana", "spice", "incense" or just "collector's product, not for human consumption". Nevertheless, in 2012 started the disappearance of the JWH-type cannabinoids [52], being replaced by novel SCRAs such as XLR-11 (**Figure 1.3C**) and UR-144. Both compounds were commonly seized between 2012 and 2013, especially in the United States of America and Europe [47,53]. Comparably to the JWHs, XLR-11 and UR-144 started disappearing in 2013 after its ban in different countries, and again, novel SCRAs quickly replaced these compounds [47,53]. Since then, there has been a wide variety of cannabinoids available in the market (known as the third-generation SCRAs), as it can be exemplified after a quick search in the literature [47,54,55]. Some of them, such as the AMB-FUBINACA (**Figure 1.3D**) or the 5F-ADB (also known as 5F-MDMB-PINACA) have been related to different intoxication cases, and even deaths [56–60].



THC (**A**) JWH-018 (**B**) XLR-11 (**C**) AMB-FUBINACA (**D**)

Figure 1.3. Structures of THC (**A**) and three different SCRAs (**B**, **C** and **D**).

For some of this novel SCRAs, striking names were selected such as XLR-11, which is the name of the first liquid fuel rocket developed in the USA for use in aircraft. Another example is the case of the APINACA, known as AKB48, which is the name of a Japanese idol girl band. For the most recent SCRAs, their name comes from their chemical structure, following the system *LinkedGroup-TailCoreLinker*. For example, the SCRA APINACA corresponds to *N*-(1-**a**damantyl)-1-**p**entyl-1*H*-**in**d**a**zole-3-**c**arbox**a**mide. The EMCDDA has developed a web tool for naming synthetic cannabinoids based on their moieties, available on *http://www.emcdda.europa.eu/topics/pods/synthetic-cannabinoids*.

In this thesis, three different research articles focused on SCRAs are included. In one hand, **research article VII** explores the metabolic fate of two selected SCRAs, including the previously explained AMB-FUBINACA (**Figure 1.3D**), using pooled human hepatocytes, as well as their potency. On the other hand, two investigations on the consumption of SCRAs among teenagers are presented, one case on juvenile offenders' centres **(research article XI**), and the other on the consumption patterns of a SCRAs user in the city of Valencia (Spain) (**research article XII**). In both studies, the XLR-11 **(Figure 1.3C**) played an important role, and the 5F-ADB was also the focus of the second work.

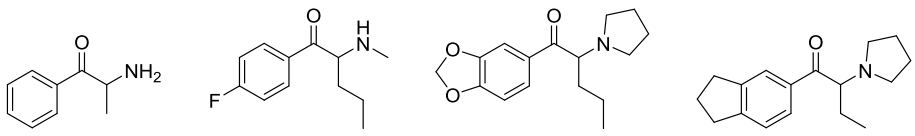
1.4.2. Synthetic cathinones

Synthetic cathinones are stimulants based on the structure of the natural alkaloid cathinone (**Figure 1.4A**) [61], present in the Catha edulis plant, commonly known as khat in the Middle East [62]. Similarly to amphetamines, these NPS produce

stimulant and euphoric effects by the alteration of the basal levels of dopamine, norepinephrine and serotonin receptors (5-HT) [47]. Nevertheless, high doses produce adverse effects such as psychosis, hallucinations, tachycardia and aggressive behaviours [63,64]. Synthetic cathinones are, indeed, the β-keto analogues of the amphetamines.

Cathinones are commonly found as powder or crystal, called "legal highs" or "bath salts", available on the Internet and smart-shops [65,66]. This chemical class of compounds represent nowadays the second largest group of NPS which is being monitored by the EMCDDA [19]. Starting with the structure of the cathinone (**Figure 1.4A**), different synthetic cathinones can be obtained by changing the three main moieties of the cathinone: N-functionalization, α carbon alkyl chain length, and aromatic ring substitution. Similarly to the SCRAs, the EMCDDA has a web tool for naming synthetic cathinones based on the previously indicated moieties, available on *http://www.emcdda.europa.eu/topics/pods/synthetic-cathinones-injection*.

The wide variety of combinations has promoted that, in the last years, tens of synthetic cathinones have been reported in literature [67–71]. The synthetic cathinones family includes halogenated cathinones such as 4-fluoro pentedrone (4F-pentedrone, **Figure 1.4B**), the famous 3',4'-methylenedioxypyrovalerone (MDPV, **Figure 1.4C**), and cathinones with novel aromatic functionalizations, such as the 3',4'-trimethylene-α-pyrrolidinobutiophenone (5-PPDi, **Figure 1.4D**). Nevertheless, as shown in **Figure 1.4**, the core of the cathinones remains unaltered, unlike SCRAs that present also differences in their core.



Cathinone (**A**) 4F-pentedrone (**B**) MDPV (**C**) 5-PPDi (**D**)

Figure 1.4. Structures of cathinone (**A**) and three different synthetic cathinones (**B**, **C** and **D**).

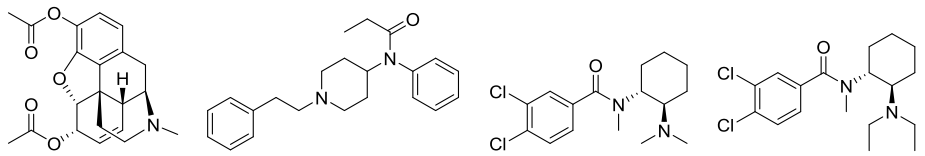
Four different research papers working with synthetic cathinones have been included in the present thesis. **Research article III** presents the identification and characterization of the novel cathinone 5-PPDi (**Figure 1.4D**), by the combination of different analytical techniques. Also, an approach for the rapid identification of these compounds in seized products has been developed in **research article V**. The metabolism of 5-PPDi using pooled human hepatocytes is presented in **research article VIII**. And finally, the blood-brain barrier (BBB) permeability of 13 synthetic cathinones has been studied using Sprague-Dawley rats (**research article X)**.

1.4.3. Synthetic opioids

The use of synthetic opioids has been historically related to medical purposes, as they present affinity for the MOR receptors similarly to morphine and thus, producing pain relief and anesthetic [33]. In fact, their properties have caused these compounds to be used as substances of abuse, for example, as a replacement of the heroin (**Figure 1.5A**). Likewise to this illicit drug, synthetic opioids are usually injected, smoked or taken orally. According to the EMCDDA, up to 49 new synthetic opioids have been detected in Europe since 2009, being 34 of them fentanyl derivatives [19].

Fentanyl (**Figure 1.5B**) is a potent synthetic opioid used for medical purposes, developed in 1963 by the Janssen Pharmaceutica [72,73]. Nowadays, fentanyl derivatives are the most used synthetic opioids worldwide [19,74–76], and also have been related to several intoxication and death cases [77–80]. Apart from fentanyl, other synthetic opioids have been widely used as substances of abuse. The most known cases are those related with the opioid U-47700 (**Figure 1.5C**). This compound, also developed by a pharmaceutical company [40], was almost forgotten until 2016, when the first death related to this compound was confirmed [81]. This year, the Drug Enforcement Administration of USA banned the U-47700, indicating that at least 46 overdoses deaths were produced by this opioid in 2016 [82]. In fact, a fast literature search illustrates the high number of intoxication cases

related to this compound [43,77,83]. Following the trend of the rest of NPS, when a compound is banned, different analogues appear on the street trying to replace it. In this case, different U-47700 analogues have been reported, including the U-49900 (**Figure 1.5D**) [47].



Heroin (**A**) Fentanyl (**B**) U-47700 (**C**) U-49900 (**D**)

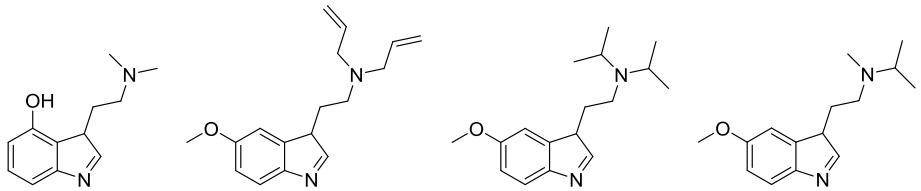
Figure 1.5. Structures of heroin (**A**) and three different synthetic opioids (**B**, **C** and **D**).

Research article II presents the first characterization and identification of the synthetic opioid U-49900 (**Figure 1.5D**) in a sample provided by an anonymous consumer. Additionally, its origin and rise were also explored, trying to understand why this compound appeared in the streets, based on previously reported opioids and changes in drug legislation.

1.4.4. Tryptamines

Tryptamines are included in the serotonergic psychedelics [46], together with lysergamides (such as the lysergic acid diethylamide or LSD) and phenethylamines [45]. Psychedelic drugs are those compounds that produce hallucinogenic effects by the activation of the serotonin 2A (5-HT2A) receptors [84,85]. These compounds are present in different *Psilocybe* genus mushrooms, commonly known as "magic mushrooms" [86]. The most commonly found tryptamine alkaloids present in these fungi are psilocin (4-OH-*N*,*N*-dimethyltryptamine, **Figure 1.6A**) and its phosphorylated analog psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine) [86]. Tryptamines are usually taken orally through a pill or capsule, among snorting, smoking or injecting intravenously [87,88].

Starting from the structure of natural tryptamines such as psilocin and DMT (*N*,*N*-dimethyltryptamine), different synthetic tryptamines have been produced, being the most important ones those synthetized and tested by Alexander Shulgin [46], such as the 5-MeOH-DALT (*N*,*N*-diallyl-5-methoxy tryptamine, **Figure 1.6B**). Some of these synthetic tryptamines have been related to overdose death cases, such as the 5-MeOH-DiPT or Foxy (5-methoxy-*N*,*N*-diisopropyltryptamine, **Figure 1.6C**) [89,90], and its analog 5-MeO-MiPT (5-methoxy-*N*-methyl-*N*-isopropyltryptamine, **Figure 1.6D**) [91,92].



Psilocin (**A**) 5-MeOH-DALT (**B**) 5-MeOH-DiPT (**C**) 5-MeOH-MiPT (**D**)

Figure 1.6. Structures of psilocin (**A**) and three different synthetic tryptamines (**B**, **C** and **D**).

The tryptamine 5-MeOH-MiPT (**Figure 1.6D**) is the focus of the **research article IX**. In this study, this tryptamine was identified in a pill from a smartshop. This finding, which demonstrated the consumption of this compound in Spain, was inciting the study of its metabolism and pharmacokinetics, using in this case C57BL/J6 mice and proposing also the most suitable consumption markers for this compound.

1.5. Exploring the pharmacology of new psychoactive substances

Once known what are the NPS and some of the most important families, this chapter will focus different pharmacological aspects such as pharmacokinetics and potency, among others.

One of the first steps when exploring the pharmacology of a certain NPS, is to know its metabolic fate, which is, the compounds (metabolites) produced after

the biotransformation of a substance [93–95]. The word "metabolism" comes from the Greek *metabole*, which means "change". So, this word is used for the identification of the transformation products of endogenous and exogenous compounds produced by biological processes, typically found in a living organism. The metabolic fate also includes the pharmacokinetics of the studied compound and its metabolites, which is the study of the movement of a certain compound in the body [96–98]. The knowledge of the metabolic fate of NPS is crucial for the establishment of the most suitable consumption biomarkers (the original compound or its metabolites) to be detected in toxicological analysis for determining the consumption of a certain NPS [47].

Once a xenobiotic compound gets into the organism, it is biotransformed in order to detoxify and excrete it by the combination of different enzymatic processes [99,100]. These biotransformations can be classified mainly into two groups:

- Phase I: Modification. The compound is usually oxidised, reduced, hydrolysed, and/or cycled, in order to introduce reactive polar moieties into the compound. Phase I metabolites are usually active compounds.
- Phase II: Conjugation. The activated phase I metabolites are conjugated with polar species such as glucuronic acid, sulphate, glutathione or glycine. Some functional groups, such as methyl or acetyl, can also be added. Conjugation occurs in active moieties such as carboxylic acid, hydroxyl, amino and sulfhydryl.

The elucidation of the metabolites produced after NPS consumption is very important as a certain metabolite could present also toxicity [101], or to be a psychopharmacological active compound [102]. The pharmacology of NPS also includes the study of the potency of the compounds, for example those used for determining the activity of the SCRAs [102–104].

The most realistic information in pharmacological studies is acquired from samples obtained from NPS users. Nevertheless, these samples are not easily accessible, and in some cases, especially those from intoxication cases, there is

no information about how long has it been since consumption. Thus, different approaches can be used, being the most important ones the *in vitro* and *in vivo* models. **Table 1.1** shows a comparison of the predictive value of different models for metabolism studies [105].

1.5.1. *In vitro* approaches

The *in vitro* approaches present a high versatility, as almost all the steps involved in the metabolism can be individually performed, from metabolite identification to potency testing.

Different models can be used for assessing NPS metabolism, such as pooled human liver microsomes (pHLM) [106], pooled human liver S9 fraction (pS9) [94], primary human hepatocytes (pHH) [107], or the use of single enzymes, such as recombinant P450 CYP enzymes [108]. The rat model can also be used for studying NPS metabolism, using rat microsomes and hepatocytes [109]. For drug metabolism, pHH are the most suitable cells due to the presence of enzymes and cofactors which allow obtaining phase I and phase II metabolites [110].

In vitro models can also be used for assessing prevalence of metabolites, estimation of the toxicity of NPS [47] (such as the nephrotoxicity of the SCRAs [111]), or the determination of the potency of NPS. On this way, the activity of SCRAs by the study of the affinity of these compounds to the CB1 and CB2 receptors has been recently reported in literature [102–104]. In these experiments, the activation of CB1 and CB2 receptors is monitored by the use of a G-protein coupled receptor (GPRC) activation assay [103].

The elucidation of NPS metabolites using an *in vitro* model has been explored in this thesis. Concretely, **research article VII** presents the elucidation of the metabolites of the SCRAs 5F-APP-PICA and AMB-FUBINACA after pHH incubation, including also the estimation of the potency of both compounds. The metabolism of three synthetic cathinones, including 5-PPDi, and one amphetamine, using pHH was also studied (**research article VIII**).

Table 1.1. Comparison of different models for metabolic studies.

Model	Physiological relevance	Compound throughput	Time needed	Cost	Comment
Human *in vivo*	Most	Lowest	Most	Most	Need regulatory approval, toxicology, formulation and bulk drug
Animal *in vivo*					Still considered best predictor, yet expensive and increasingly controversial
Isolated whole organ					Time-consuming, requires animal or human donor
Cellular					Generally considered reliable, *in vitro-in vivo* correlations are improving
Subcellular					Generally considered reliable, *in vitro-in vivo* correlations are improving
Isolated enzyme/receptor					Requires animal or human donor, enables higher throughput
Recombinant enzyme/receptor	Least	Highest	Least	Least	Now readily available, necessary for today's high throughput analysis

1.5.2. *In vivo* approaches

The use of animal *in vivo* approaches with living organisms presents a new dimension for pharmacological analysis. In this case, metabolites can be determined in different matrices such as blood, brain or urine, also allowing the study of the pharmacokinetic behaviour of the NPS and their metabolites. Moreover, the analysis of these matrices at different times after dosing allows the proposal of feasible consumption markers, including also the possibility of detecting long-term metabolites.

Different animal models can be used for the study of NPS metabolism, from zebrafish larvae [112] to pigs [113]. Nevertheless, the most used models are the rodents, mainly mice and rats, as can be illustrated by the numerous research articles published in this field [94,95,114,115]. With rodents, microdialysis can be used for obtaining extracellular liquid from the brain of conscious freely behaving rats [47]. This methodology is very useful for the determination of selected neurotransmitters, studying their pharmacokinetic at different times after drug administration [47].

Research article IX presents the metabolism and pharmacokinetic behaviour of the tryptamine 5-MeO-MiPT, using C57BL/J6 adult male mice. An additional application of in vivo models is presented in **research article X**, where the permeability of 13 synthetic cathinones through the BBB was studied using Sprague-Dawley adult female rats.

1.6. Analytical methodologies for toxicological analysis

NPS represent nowadays an important public health problem, as previously illustrated by the large number of NPS that are reported each year [19]. The constant on-going of NPS found in research chemicals and legal highs, creates a handicap for their detection, as novel compounds with unreported structures are continuously emerging. Moreover, these new drugs are being consumed and thus, their monitoring in forensic matrices must also be performed. But prior to the toxicological analysis of these samples, the pharmacological behaviour of these compounds, including metabolite identification and pharmacokinetics, should be performed in order to establish the most suitable consumption biomarkers. It can be noticed that the toxicological analysis of NPS presents an important scientific challenge, and here is where analytical chemistry plays a pivotal role.

Analytical chemistry can be defined as "the study and use of instruments and methods for the separation, identification and quantification of certain species in a certain sample", adapting the definition provided by Douglas A. Skoog in his *Fundamentals of Analytical Chemistry* [116]. This definition can be used for describing the studies included in this thesis: "the study and use of instruments and methods for the separation, identification, or quantification, of selected NPS and metabolites in forensic samples".

Within the wide variety of methodologies available in the (also wide) world of analytical chemistry, the hyphenated techniques, and especially chromatography coupled to mass spectrometry (MS), are probably the most powerful for toxicological analysis [47,117,118]. Although all the works presented in this thesis are based on the use of chromatography-MS, additional techniques such as nuclear magnetic resonance (NMR), were used in some cases for the unequivocal identification and characterisation of some NPS.

1.6.1. Chromatographic separation: Liquid chromatography

Chromatographic techniques include a wide variety of methods and instrumental techniques that allow the closely separation of the components of complex mixtures [119]. The chromatographic separations are carried out by the transport of the sample in a mobile phase through an immiscible stationary phase, being the compounds distributed between both phases in different degrees [119]. The distribution of the sample compounds between mobile and stationary phase depend on the partition coefficient of the compounds in both phases [119]. Compounds with low affinity for the stationary phase will elute early from the chromatographic column, while compounds with high affinity will elute later.

Mobile phase can be a gas (used in gas chromatography or GC), liquid (liquid chromatography or LC) or a supercritical fluid (supercritical-fluid chromatography or SFC) [119]; while the stationary phase is placed inside the chromatographic column, being solid particles (used in GC and LC), liquid adsorbed on a solid (GC and LC), organic species bonded to a solid surface (GC, LC and SFC) or even ion-exchange resins (LC) and polymeric solids (LC) [119].

For toxicological analysis and determination of NPS in different matrices, GC and LC have been widely used [47], both presenting advantages and drawbacks. GC-amenable compounds must be volatile and thermostable, and some of the NPS and most of their metabolites are not, needing a derivatization step [119]. Moreover, extracts to be analysed by GC must be water and salt-free, using a volatile organic solvent. Conversely, LC can be directly applied for the separation of NPS and metabolites in a wide variety of matrices (legal highs, herbal blends, urine, blood or brain), with minimal sample treatment procedure. For that, all the chromatographic separations included in this thesis were performed using LC systems, except for those studies regarding NPS characterization. In these cases, GC-MS using electron ionisation (EI) data were also included in order to provide a full characterisation of the compounds, and providing EI spectra that can be used in spectra libraries for compound identification.

LC, and more specifically High-Performance LC (HPLC), is probably the most widely used analytical separation technique due to its applicability to a wide range of compounds, such as proteins, hydrocarbons, carbohydrates, pesticides, steroids, metal-organic species, drugs [119] and NPS [47]. LC encompasses different separations based on the analyte retention mechanisms, being the most important the partition chromatography, adsorption chromatography, ion-exchange chromatography, and size exclusion chromatography [119]. The most used one in analytical chemistry is the partition chromatography, and most specifically, the bonded-phase chromatography [119]. In this case, an organic specie bonded to a solid surface, generally silica or polymeric particle bed, acts as a stationary phase [119].

The retention mechanism in partition LC is based on the analyte interaction between the mobile and stationary phases, usually due to Van der Waals interactions [119]. Depending on the polarity of the mobile phase, functionalisation of the stationary phase, and analytes to be separated, there are three different types of partition LC:

- Normal phase LC or NPLC. A highly polar stationary phase, usually silica, and a nonpolar mobile phase, such as hexane or *i*-propylether, are employed. The analyte elution order is from the nonpolar to the polar compounds, being therefore ideal for highly nonpolar analytes. Nowadays, it is scarcely used due to solvent incompatibilities with the MS systems [119].
- Reversed-phase LC or RPLC. It consists on a nonpolar stationary phase, usually a hydrocarbon (C_8 or C_{18}), and a polar mobile phase such as water combined with methanol or acetonitrile. The analyte elution order is from the polar to the nonpolar compounds, with a wide range of polarity. This is the most widely used due to the ionisation systems used for coupling LC to MS, together with the wide range of applications of this separation [119,120]. Different buffers can be added to the mobile phase to improve peak shape and ionisation efficiency in LC-MS couplings.

- Hydrophilic interaction LC or HILIC. In this case, a non-modified silica is used as stationary phase, and an aqueous-organic solvent (water and acetonitrile) as mobile. The elution order is from the nonpolar to the polar compounds, ideally used for highly polar analytes. In the last years, HILIC has become a very popular separation technique as it allows NPLC-type separations compatible to MS. One of the HILIC application fields is the metabolomics studies, for complementing RPLC [121].

Apart from the mobile and stationary phase, another important parameter that affect the chromatographic separation is the particle size and type of the analytical column. This parameter, together with the mobile phase flow, are key factors in the chromatographic performance as they affect the number of theoretical plates of the column, as evidenced in the Van Deemter equation [119]. Traditional HPLC columns present particle sizes between 3.5 and 5.0 µm, which allow a narrow range of optimal mobile phase flow. Nevertheless, in the last years a new LC separation has become extremely popular, the ultra-HPLC or UHPLC, which allows faster separations with increased chromatographic resolution by the use of columns packed with particles below 2 µm [122]. For this type of particles, the range of optimal solvent flow is wider than for HPLC particles, allowing higher flows without losing chromatographic efficiency and thus, having faster chromatographic separations. The main drawback of sub-2 µm particle size columns is the high back-pressure of the chromatographic system.

At this point, fused-core particle columns present an attractive alternative to the "classical" UHPLC columns. These particles have a solid core and a porous shell, usually functionalized with the same chemistries than porous sub-2 µm particle size columns [123]. The most commonly used are the 2.7 µm particle size columns, which present similar chromatographic performance (in terms of specificity, retention and selectivity) than sub-2 µm, but with substantial lower backpressures [124]. On this way, faster chromatographic separations without significant loss in efficiency and resolution can be achieved using 2.7 µm fused-core particle size columns [124].

In all the works presented in this thesis, UHPLC RPLC chromatographic separations have been used due to the wide range of NPS polarities, from the polar cathinones to the non-polar SCRAs. Nevertheless, different analytical columns have used, such as C_{18} 2.7 µm fused-core particle size column in **research articles I-IV**, **IX** and **XI-XII**, T3 1.6 µm fused-core particle size column in **research article X**, and HSS C_{18} porous 1.8 µm particle size column in **research articles VII-VIII**.

As information, the T3 column is a modified C_{18} functionalisation that increases the retention for polar compounds, while in the HSS (High Strength Silica) column the particle is a 100% silica particle, designed for high pressure separations.

1.6.2. Mass spectrometry

MS is an analytical methodology that determines the mass to charge (*m/z*) ratio of certain ions. An MS instrument can be considered, in a way, as a state-of-the-art analytical balance, as the mass (and charge) of a compound can be easily determined. The MS is a three-stage technique: the ions are produced in the ion source, separated by their *m/z* in the mass analyser, and detected in an ion detector.

Depending on the ion source, these ions can be atoms (for example, produced by an inductively coupled plasma or ICP), ionised molecules (those obtained in EI as $M^{+\cdot}$, where M represents a molecule) or (de)protonated molecules (obtained by atmospheric pressure ionisation (API), as $[M+H]^+$ or $[M-H]^-$). Additional ion sources can be used, such as matrix-assisted laser desorption/ionisation (MALDI), photoionisation, and different ambient ionisation sources [125], depending on the chemical properties of the analytes and the matrices.

Once the ions are produced, they are separated by their *m/z* in the mass analyser, which is the heart of a mass spectrometer [125]. Nowadays, mass analysers can be divided into two groups depending on their design and performance: low resolution (LR) and high-resolution (HR) mass analysers. But first, what is mass

resolution? The resolution of a mass analyser is its ability to separate two neighbouring ions that differ slightly in their mass [125]. LR mass analysers have usually unit resolution, in other words, can distinguish two ions separated 1 Dalton (Da). HR mass analysers (HRMS) have higher resolving power, and the mass difference that can detect depends on its resolution, but usually can differentiate two ions which masses differ few mDa.

After mass analyser, ions arrive to the detector, which provides information of the ion abundances by converting ion beams into an electric signal [125]. For ion detection, there are different available technologies such as electron multipliers, photomultipliers, multichannel plates, or multichannel arrays [125].

In the following sections, the different API sources and mass analysers used in this thesis are described.

1.6.2.1. Atmospheric pressure ionisation

The LC-MS coupling is based on API sources, which allow the ionisation of the compounds and evaporation of the mobile phase. In this way, API sources make compatible the coupling of a liquid phase technique (LC) and a gas phase (and high vacuum) technique (MS). Nowadays, the two most used API sources for LC separations are the electrospray ionisation (ESI) and the atmospheric pressure chemical ionisation (APCI).

Electrospray ionisation

The ESI is probably the most widely used interface for RPLC-MS coupling. Introduced by John B. Fenn [126,127], it is based on the application of a high electrical field to a solvent emerging from a capillary, producing small charged droplets thanks to the presence of a nebulizing gas. Once these droplets are completely evaporated, ions in gas phase are obtained [120]. In modern ESI sources, desolvation of charged droplets is pneumatically assisted using a heated gas, usually nitrogen, as it can be seen in **Figure 1.7**, where the scheme of a modern ESI source is shown.

The ESI source can be operated in positive ionisation mode, producing positive charges (protonated molecules, $[M+H]^+$), and in negative mode, producing negative charges (deprotonated molecules, $[M+H]^-$). Nevertheless, ESI can also produce adducts with sodium ($[M+Na]^+$), potassium ($[M+K]^+$) and ammonium ion ($[M+NH_4]^+$), as well as with chloride ($[M+Cl]^-$).

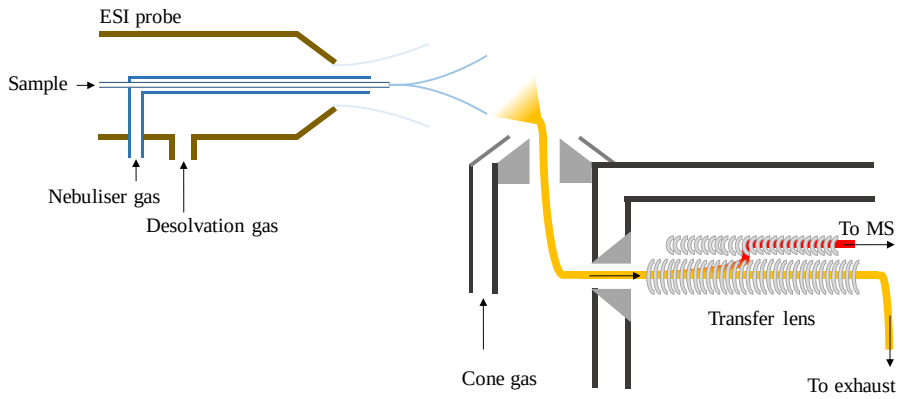


Figure 1.7. Scheme of an ESI source, based on a Waters Corp. Z-spray ionisation source.

The fundamentals of the ESI ionisation process have been widely studied and discussed [120]. When the solvent emerges from the capillary and the nebulization process is performed, small droplets with an excess of charge are produced. During the flight of these droplets, neutral solvent molecules from their surface are evaporated, reducing the size of the droplet and thus, increasing the charges at the droplet surface. When the charges are close enough, a Coulomb explosion is produced, disintegrating the droplet into smaller droplets. After several cycles, charged ions in gas phase will be produced, which can be mass analysed.

Nevertheless, ESI can be described as a mixed-mode ionisation, as different processes contribute to the ionisation of the analytes [120]. Two main processes are described to the formation of gas-phase analyte ions from microdroplets [125], briefly described hereunder.

- The *charge-residue model* of Dole [128,129] describes the ionisation of the compounds based on the pK_a of the analyte, following a soft desolvation process. After solvent evaporation and droplet disintegration, only one analyte ion is formed in each microdroplet as a solvated ion. After the complete evaporation of the solvent, the preformed analyte ion is released to the gas phase.
- The *ion-evaporation model* of Iribarne and Thomson [130,131] describes that gas-phase ions can be formed directly from the highly-charged microdroplets. It is based on the evidence that local field strength can be enough to allow the emission of preformed ions in solution to the gas phase.

Nevertheless, the chemical ionisation can be an additional process occurred in ESI [120]. In this case, chemical interactions can occur between gas-phase buffer ions coming from the mobile phase, and neutral analyte molecules. These reactions can happen either at the droplet surface or in gas phase after neutral molecules desolvation. These processes are the same than those occurring in APCI.

On this way, one of the benefits of ESI source is the improved ionisation efficiency for a wide range of molecules, such as small compounds like drugs and pesticides ionised by chemical ionisation processes, or peptides and proteins ionised by the charge-residue model [120]. On this sense, ESI can produce multiple-charged ions, which are extremely interesting for protein analysis. Nevertheless, the main drawback of the ESI is the typically high ion signal suppression produced during ionisation, due to the matrix compounds compete with the analyte molecules for the available charges. In some cases, the matrix effect can be also observed as a signal enhancement, as some compounds can affect the surface tension of the droplets, increasing ionisation efficiency.

The ESI has been used in almost all the studies of the present thesis, as is the most suitable ionisation technique for the determination of NPS (and metabolites)

using an LC separation. Different technologies have been used, depending on the mass analyser manufacturer: A Z-spray from Waters Corp. and a heated electrospray (HESI) from ThermoFisher Scientific.

Atmospheric pressure chemical ionisation

The APCI ionisation mechanism is based on solvent-mediated chemical ionisation by the reaction between solvent gas ions and analyte molecules, started by the electrons produced in a corona discharge pin [120]. **Figure 1.8** shows the scheme of an APCI source, where the corona discharge pin can be observed and placed after the APCI probe. First, the reagent gas (usually nitrogen, N_2) is ionised by its interaction with energetic electrons (e^-) produced in the corona pin, with a discharge current of few µA. Subsequently, these reagent gas ions react with the solvent molecules, generally water molecules (H_2O), ionising them. Finally, these ionised water molecules, together with the ionised nitrogen, can transfer the charge to the analyte molecules (M), ionising them and being mass analysed [120]. The APCI source can produce $[M+H]^+$ and/or molecular ions ($M^{+\cdot}$) depending on the analyte structure. According to Niessen, the ionisation reactions occurring in the APCI process are the following ones (the example shown corresponds to the positive ionisation mode) [120]:

$$N_2 + e^- \rightarrow N_2^{+\cdot} + 2e^-$$
$$N_2^{+\cdot} + H_2O \rightarrow N_2 + H_2O^{+\cdot}$$
$$H_2O^{+\cdot} + H_2O \rightarrow H_3O^+ + OH^-$$
$$H_3O^+ + M \rightarrow MH^+ + H_2O$$
$$H_2O^{+\cdot} + M \rightarrow M^{+\cdot} + H_2O$$

The charge transfer between nitrogen and water can be produced because the ionisation potential of water (12.6 eV) is lower than nitrogen (15.6 eV) [120]. In this case, ionisation occurs in gas phase. For that, LC eluate is rapidly evaporated in the heated fused-silica capillary of the APCI probe, usually at 600 ºC, as observed in **Figure 1.8**.

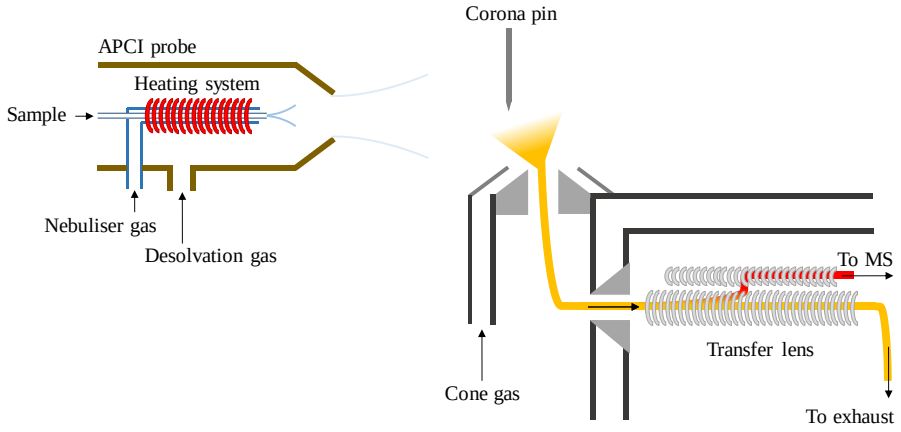


Figure 1.8. Scheme of an APCI source, based on a Waters Corp. Z-spray ionisation source.

A variation of the APCI source was developed for the direct analysis of solids and liquid samples, called atmospheric solids analysis probe or ASAP [132,133]. In this case, the APCI probe is replaced by a probe containing a glass capillary, where the solid/liquid sample is damped. The glass capillary is heated by the desolvation gas, producing the evaporation of the sample and the ionisation of the compounds in the corona pin. In **research article VI**, the ASAP source has been used for the suspect screening of NPS in consumption products and surfaces, using a triple quadrupole mass analyser.

1.6.2.2. Low-resolution mass analysers

As previously introduced, LR mass analysers are those able to distinguish between two ions which *m/z* differ on 1 Da, being the most common ones the quadrupole (Q) and the ion trap. In this thesis, the quadrupole has been indirectly used in all the studies presented in this work, used as tandem mass analyser or in a hybrid mass analyser.

Quadrupole mass analyser

A quadrupole is a mass analyser that consists of four parallel metal rods (electrodes). The ion separation is based on the application of direct-current (DC)

and radio-frequencies (RF) potentials to the electrodes, so that only one *m/z* has a stable trajectory in this high-frequency oscillating electric field [125]. The remaining *m/z* values present unstable vibrating trajectories, and they do not pass through the quadrupole rods. By changing DC and RF potentials, different *m/z* ions can be isolated.

The stable trajectories are obtained by the solution of the *Mathieu stability equation* (**Figure 1.9A** shows the stability regions in a linear ion trap [134], a mass analyser which working principles are similar to the Q), which describes the movement of an ion though the quadrupole based on different electrical and geometrical parameters [125]. This solution provides the two dimensionless parameters that stablish the stability regions (*x* and *y*) that will allow stable oscillations in the quadrupole for a certain *m/z* ion [134], as observed in **Figure 1.9A**. The inset shows the most important stability region where *x* and *y* stability bounds overlap, resulting in a small triangular stability region [134] (red triangle in **Figure 1.9A**) in *a* and *q* space. In terms of the *Mathieu stability equation*, ion stability in the Q is dependent on both *a* and *q* parameters, which are themselves dependent on the quadrupolar DC and main RF, respectively. The *m/z* range of stability is adjusted by modulating the electrical parameters, so that the triangular region of ion stability shrinks to include just the tip of the stability region for ion traps [134], as shown in **Figure 1.9B**. Further details about the *Mathieu stability equation* and the stability diagram for a quadrupole can be found in literature [125].

Quadrupoles can be operated in full-scan (or scan), scanning all the *m/z* values in a certain mass range and thus, obtaining a complete mass spectrum. Another working mode is the selected ion monitoring (SIM), where only specific *m/z* values are going to be isolated, obtaining an increment of the sensitivity and selectivity. Finally, a third working mode can be defined, when the quadrupole works only with RF potential. In this case, the quadrupole acts as an ion guide and thus, all the *m/z* values will cross the quadrupole. This application is used in hybrid Q-HRMS mass analysers working on full-spectrum acquisition mode.

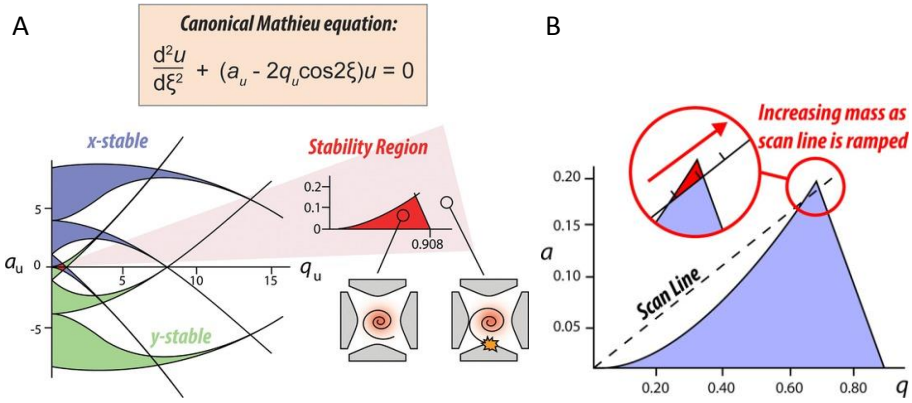


Figure 1.9. A Stability region of the linear ion trap, illustrating the stable solutions to *Mathieu's stability equations* (x and y). **B** Scanning and stability in the quadrupole. Figures adapted from J.P. Savaryn, et al. (2016) [134], with permission of the corresponding author Dr. N.L. Kelleher.

As commented above, quadrupoles present a 1 Da mass resolution, and a practical upper limit of *m/z* 4000 [125]. Nevertheless, these parameters can be improved by changing DC and RF standard parameters. The low cost of this mass analyser, together with its mechanical simplicity, high scan speeds, increased sensitivity and linear mass range are several of the reasons which explain why quadrupoles are so popular. Moreover, it can be coupled with ICP, GC, LC and other ion sources due to the quadrupole allows working at relatively high pressures in the ion source [125].

Triple quadrupole mass analyser

The triple quadrupole (QqQ) mass analyser is a tandem MS (MS/MS) instrument. MS/MS refers to the coupling of two stages of mass analysis, which can occur in time or space [125]. In MS/MS in space, two mass analysers are used for performing these two stages, while in MS/MS in time only one mass analyser is required, usually an ion trap. In fact, QqQ instrument is a MS/MS in space, as the two stages of mass analysis occur in different regions.

The working principle of an MS/MS system is based on the isolation of a specific target ion, produced in the ion source, in the first mass analyser (MS1). After this selection, the mass-selected ion is fragmented in the intermediate region, and these product ions are mass analysed in the second mass spectrometry stage (MS2) [125]. In the case of a generic QqQ instrument, the intermediate region is a collision cell, which produces ion fragmentation based on collision-induced dissociation (CID) [120].

The CID occurs in two-steps: a collision activation and a unimolecular dissociation [125]. The collision activation is achieved by the excitation through the collision of the fast-moving isolated ions in MS1 with an inert gas, usually nitrogen or argon [125]. In this step, ion translational energy is converted into ion internal energy, and obtaining a higher-energy state [120]. Due to this high energy, ion is decomposed into different product ions, in a process called fragmentation [125]. The fragmentation efficiency depends on the collision energy induced to the ions, obtaining more product ions with lower *m/z* values when using higher collision energies.

The QqQ instrument presents four different working modes, as each quadrupole can be operated in scan and SIM modes. **Table 1.2** shows a summary of the different working modes of a QqQ, as well as its application and information obtained [120,125].

Table 1.2. Working modes of a QqQ instrument.

Mode	MS1	MS2	Application
Product ion scan	SIM	Scan	Obtain the product ions of a certain precursor ion
Precursor ion scan	Scan	SIM	Monitor compounds that present the same product ion
Neutral loss scan	Scan	Scan	Monitor compounds that present the same mass difference between precursor and product ion (neutral loss)
Selected reaction monitoring	SIM	SIM	Monitor compounds with a specific CID reaction

The most commonly used working mode of the QqQ is the selected reaction monitoring (SRM). This mode is extremely used for target analysis, due to its high specificity, selectivity and sensitivity. The product ion scan is used for obtaining structural information of a specific ion, for example, for the elucidation of putative metabolites or transformation products. The precursor ion scan and neutral loss scan modes are used for the suspect analysis of compounds, for which some CID information is available. It is also used for detecting related compounds, as they usually present similar fragmentation. **Figure 1.10** shows the principles of the four explained working modes that can be used in QqQ instruments.

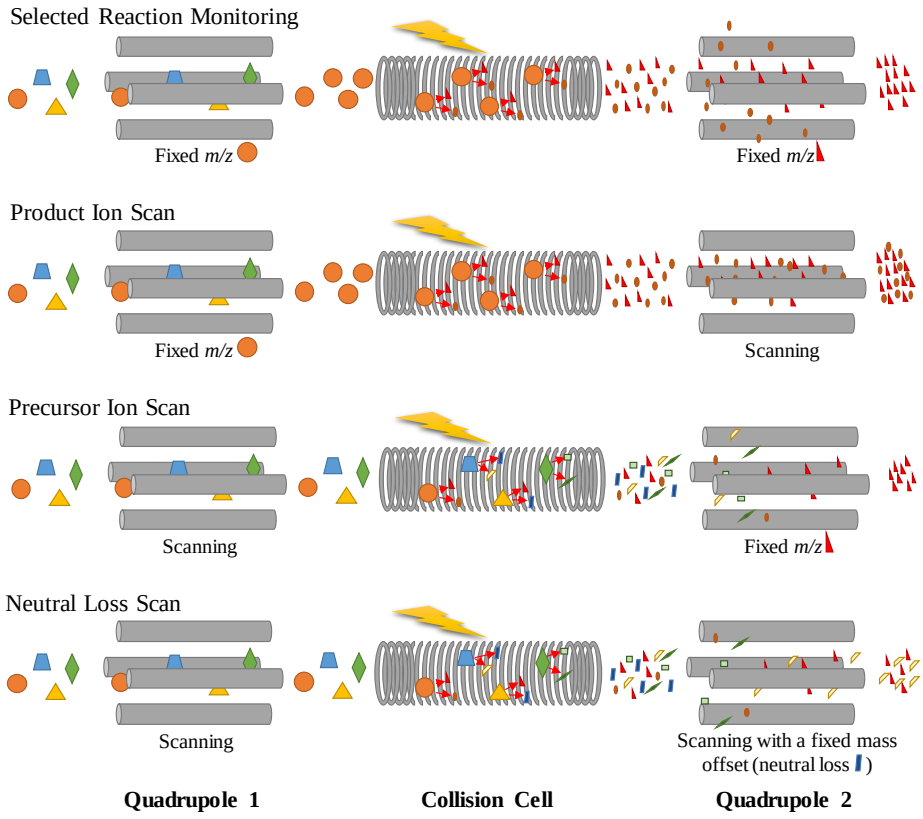


Figure 1.10. Scheme of the selected reaction monitoring, precursor ion scan and neutral loss scan working modes.

The QqQ has been used in three research articles of this thesis. In **research article V**, the precursor ion scan and neutral loss scan working modes were explored for the identification of synthetic cathinones. **Research article VI** presents the application of scan and product ion scan for a suspect screening of different NPS, and **research article X** uses the SRM for the quantification of 13 cathinones in rat brain samples.

1.6.2.3. High-resolution mass analysers and hybrid instruments

HRMS are probably the most versatile mass analysers for research purposes due to the information provided by these instruments. There are different mass analysers that allow the acquisition of data in full-spectrum and high-resolution, being the most commonly found in laboratories the time-of-flight (TOF) and the Orbitrap, together with high-complex instruments such as the magnetic sector and the Fourier-transform ion cyclotron resonance (FT-ICR) [125]. All these instruments acquire the full-spectrum of the sample without the preselection of the ions of interest, which allows the retrospective analysis of the samples.

Usually, HR mass analysers work with accurate masses, which is the experimentally determined *m/z* of an ion, with a certain mass accuracy and a certain mass error respect the exact mass of this ion [135]. Exact mass is the theoretical *m/z* value of an ion or molecule calculated based on a specific isotopic composition [135], and thus, the mass error is the difference between exact and accurate mass [135]. Mass error can be expressed as absolute (mDa) or relative values (part per million or ppm). For an accurate mass measurement, *m/z* axis must be properly calibrated with an appropriate periodicity and using a calibration solution, depending on the instrument [120]. An accurate mass measurement allows the determination of the elemental composition of an ion based on the measured *m/z*, being this ability one of the strengths of HRMS.

On this way, the chromatography-HRMS coupling allow data processing from different "points of view" thanks to the 3D data generated (chromatography/mass spectrometry/intensity). The narrow-window extracted ion chromatogram

(nw-EIC) shows the chromatographic behaviour of a selected accurate mass ion, while for a selected chromatographic peak, the accurate mass spectrum can be also obtained.

Additionally to the full-spectrum acquisition with high-resolution and accurate mass of the compounds present in a sample, hybrid instruments allow performing CID experiments. In this sense, the Q-HRMS hybrid mass analysers, equipped with a quadrupole, have become extremely popular due to the different working modes available. Apart from MS/MS experiments isolating a specific *m/z* ion in the quadrupole and measuring the accurate mass product ions in the HRMS analyser (product ion scan), two additional working modes are usually employed in Q-HRMS systems.

The most generic one is the data-independent acquisition (DIA). In this mode, information about the compounds present in the sample and their "product ions" are sequentially acquired in a single analysis. First, information about all the (de)protonated molecules is acquired in the HRMS, stablishing the Q in RF-only mode (or filtering a specific *m/z* range). After that, the CID is activated at a certain collision energy (or collision energy ramp), while the Q remains in RF-only (or filtering a certain *m/z* range). In this case, information about the CID product ions of the (de)protonated molecules previously acquired are detected, obtaining a fragmentation spectrum with high mass accuracy. DIA is the most appropriate working mode when no information is available about the compounds present in the sample, and is the most indicated for suspect and non-target analysis, including retrospective analysis. Examples of different DIA working modes are the MS^E from Waters Corp. (the Q is set as RF-only), and the SWATH from Sciex (the Q filters a certain *m/z* range).

The second working mode is the data-dependent acquisition (DDA). In this case, an automatic MS/MS acquisition filtering a specific *m/z* ion in the quadrupole is performed after a full-spectrum MS acquisition. Similarly to DIA, the first step of a DDA acquisition is performing a full-spectrum MS. Sequentially, MS/MS

are performed based on the ions observed in the full-scan: when the total-ion intensity or a selected-ion intensity (inclusion list) exceeds the stablished threshold, the MS/MS product spectrum of this ion will be acquired [125]. After performing automatic MS/MS for a certain number of *m/z* ions observed in the full-spectrum, another full-spectrum is performed and new MS/MS are automatically acquired. Additionally, an exclusion list can be used for avoiding automatic MS/MS from ions coming from the system. This working mode avoids the need of performing two consecutive injections for the identification of unknown components, as the information of the (de)protonated molecule (full-spectrum MS) and its product ion spectra (automatic MS/MS) are acquired in a single run [120]. DDA can also be run in QqQ instruments.

In this thesis, QTOF and QOrbitrap have been used in different studies, and the working principles of both instruments are described hereafter. Indeed, HRMS instruments have been widely used for the analysis of NPS and related compounds, such as the elucidation of the main metabolites of these substances, as well as for the screening of toxicological samples and unknown compound identification [47,65,117].

Hybrid Quadrupole-Time Of Flight mass analyser

The QTOF instrument is probably the most popular hybrid instrument [125]. But previously to explore this instrument, the main characteristics and working principles of a TOF mass analyser should be explained.

The TOF mass analyser is, in fact, one of the simplest mass analysers. The principle of ion separation is based on the velocities (v) differences [125]. The ions are pulsed and thus, they acquire a certain potential energy (E_p) based on the number of charges (z) and potential of the pulse (V). This Ep is converted into kinetic energy (E_k) due to the principle of energy conservation, and E_k depends on their mass (m) and velocity (v). So, the relationship between v, z, m and V can be stablished, as stated in *Eq*. 1 [120,125].

$$Eq.1:\ E_p = E_k \rightarrow z \cdot V = \frac{1}{2} \cdot m \cdot v^2 \rightarrow\ v = \sqrt{\frac{2 \cdot z \cdot V}{m}}$$

These ions are pushed into a field-free flight tube of length L, at the end of which is placed the detector, separating ions based on the acquired velocity. So, the time of flight of the ions through the tube can be determined (*Eq.* 2 [120,125]).

$$Eq.2:\ \ t = \frac{L}{v} = L\sqrt{\frac{m}{2 \cdot z \cdot V}}$$

Therefore, the arrival times (time of flight or t_{flight}) of these ions provides information about its m/z ratio, as it can be seen in *Eq.* 3 [120].

$$Eq.3:\ \ t_{flight}^2 = \frac{m \cdot L^2}{2 \cdot z \cdot V} = {}^{m}/_{z} \cdot \frac{L^2}{2 \cdot V}$$

As it can be deduced from Eq. 3, the t_{flight} depends only on the *m/z* ratio of the ions, as the remaining parameters are constant in the TOF instrument. Nevertheless, TOF instruments require a calibration, which periodicity depends on the manufacturer, for obtaining the mathematical function that link *m/z* ratio and t_{flight}. On the one hand, these working principles make the TOF analyser an instrument with a high ion transmission, fast spectrum acquisition and an unlimited mass range [120]. On the other hand, the two main limitations are that TOF resolution is restricted to the length of the flight tube, and ions with the same m/z can acquire different kinetics energies in the acceleration, giving different arrival times [120].

The increment of the tube length and suppression of these kinetic energy differences can be solved with the use of reflectrons. A reflectron is an energy-correcting device that minimizes the effects of kinetic energy spreads [125]. This ion mirror consists of a ring electrodes with a progressively increasing repelling potential. **Figure 1.11** shows the scheme of a QTOF instrument, placing the reflectron at the end of the flight tube and acting as an ion mirror.

When ions enter in this device, they are slowed down by the repelling electric field until they stop, to be reaccelerated again reversing their direction. On this way, ions with an excess of kinetic energy arrive earlier to the reflectron but also penetrate more in the device, while ions with the same *m/z* but lower energy penetrate less. In other words, the extra kinetic energy, and thus velocity, is "neutralized" by the reflectron, and ions with exactly the same *m/z* arrive at the same time to the detector [125]. Moreover, the presence of a reflectron or additional ion mirrors increase the length of the flight tube, increasing therefore the instrument resolution [125]. Up to two different configurations are usually used by TOF manufacturers. The TOF in "V" geometry presents a one-stage reflectron (as illustrated in **Figure 1.11**), while TOF in "W" geometry presents a two-stage reflectron, with an ion mirror between pusher and detector. Examples of TOF and QTOF instruments operated in "V" geometry are the benchtop QTOF instruments from Waters Corporation, Agilent Technologies and Bruker. Instruments with "W" geometry are the Synapt G2 series and Synapt XS from Waters Corp, which can be also operated in "V" geometry.

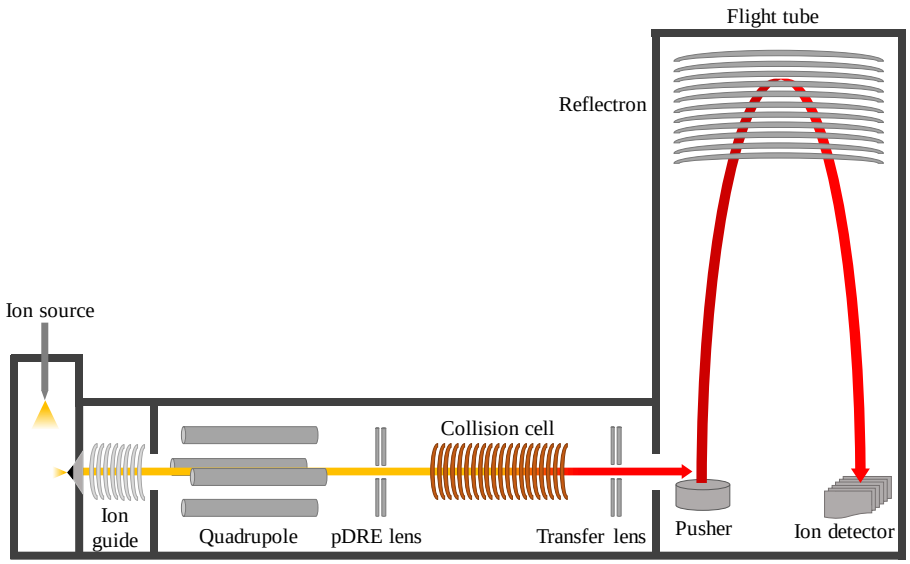


Figure 1.11. Hybrid QTOF mass spectrometer scheme, based on a Xevo G2 QTOF from Waters Corp.

Almost all the instruments designed for LC present orthogonal acceleration (oa) [120]. It can be seen in **Figure 1.11** that ions enter into the flight tube perpendicularly to the flight path, and they are subsequently pushed to the reflectron. This configuration enables that oa-TOF instruments to be used with continuous-beam ion sources, for example UHPLC-API [125]. This setup minimizes the spatial and energy spreads of ions entering into the flight tube by minimizing the turnaround effect [125].

An upgrade of the TOF analyser is the hybrid QTOF, equipped with a quadrupole and a collision cell [125], as it can be observed in **Figure 1.11**. The Q can be operated as a mass-resolving or RF-only quadrupole, acquiring data in MS/MS or DIA, respectively. The QTOF can also be operated in DDA acquisition mode, switching the quadrupole from RF-only to mass-resolving for automatic MS/MS acquisition. The collision cell is based on CID fragmentation, using argon (the most usual) or nitrogen.

In this work, the QTOF system used has been a Xevo G2 QTOF from Waters Corp., a benchtop instrument with a "V" geometry and a resolution of ~20,000 at FWHM for *m/z* 556, operated mainly in DIA acquisition mode, and MS/MS when necessary. Nevertheless, DIA acquisition when using Waters Corp. instruments is called MS^E acquisition mode, based on the working principle of this acquisition. When using MS^E, two different functions are sequentially acquired. In the low energy function (LE), the Q acts as RF-only, and thus, the full-spectrum MS spectrum of the (de)protonated molecules (and adducts, if exists) present in the sample is acquired. In the high energy function (HE), the Q remains in RF-only, but the collision cell applies a collision energy (typically a ramp), acquiring the accurate-mass fragment ions of the (de)protonated molecules observed in the LE. The resolution of the TOF analyser is the same for LE and HE functions. On this way, sequential information of intact molecules and their fragments is acquired, being a true DIA acquisition mode in which all the information of the sample is gathered in a single injection.

The MS^E acquisition mode allows performing post-target, suspect and non-target screening, as well as retrospective analysis. This working mode was used in all the research articles using the Xevo G2 QTOF instrument.

In spite of the complete information obtained by MSE, MS/MS spectra should be acquired for an unequivocal assignment of the fragments coming from a certain precursor ion, as under MS^E mode all the ions present in that moment in the collision cell are fragmented. For example, in metabolite elucidation studies, MS/MS at different collision energies for all the metabolites detected and elucidated using MS^E data are highly recommended for an accurate structure elucidation.

Hybrid Quadrupole-Orbitrap mass analyser

Since its commercial availability in the 2000 decade, the Orbitrap and hybrid Orbitrap mass analysers have become a very popular HRMS instrument [136]. Designed by Alexander Makarov [137], this HRMS mass analyser rapidly showed its potential when equipped with ESI sources [138]. An Orbitrap is an orbital trapping system based on an axial spindle-like central electrode and a coaxial barrel-like outer electrode [125], as it can be observed in **Figure 1.12**. The ions trapped in the central electrode present a rotation around it and a harmonic oscillation along it. Similar to a FT-ICR mass analyser, the frequency of harmonic oscillations of trapped ions are related to its *m/z* value, obtained by measuring the induced current of these ions by the outer electrode [125]. The measured ion frequencies are then converted to mass spectra using a Fourier-transformed [125].

The working principle of the Orbitrap mass analyser enables the design of high-field Orbitrap instruments, with enhanced mass resolution. Nowadays, there are Orbitrap-based instruments, such as the Orbitrap Fusion Lumos from Thermo Sci., that allows the full-spectrum acquisition at 500,000 FWHM (*m/z* 200), and up to 1,000,000 FWHM in ultra-HR mode [139].

The potential distribution inside the trap, created by the electrostatic field between both electrodes, is defined in *Eq.* 4 [125].

$$Eq.4:\ U(r,z) = \frac{k}{2}\left(z^2 - \frac{r^2}{2}\right) + \frac{k}{2}\cdot R_m^2 \cdot Ln\left(\frac{r}{R_m}\right) + C$$

where *r* and *z* are cylindrical coordinates being *z=0* the plane of symmetry of the field, *k* the field curvature, R_m the radius and *C* a constant. The trapped ions describe a spiral which is the combination of rotation and oscillations through the central electrode. So, three different frequencies are obtained from these oscillations: frequency of radial oscillation (ω_r), frequency of rotation (ω_φ), and frequency of axial oscillation (ω) [125]. Nevertheless, only the axial oscillation (*Eq.* 5, where *z* indicates the charge of the ion) can be used for determining *m/z* values, as this parameter is completely independent of the energy and distribution of the ions [125].

$$Eq.5:\ \omega = \sqrt{\frac{z}{m\cdot k}} \rightarrow {}^{m}/_{z} = \frac{1}{k\cdot\omega^2}$$

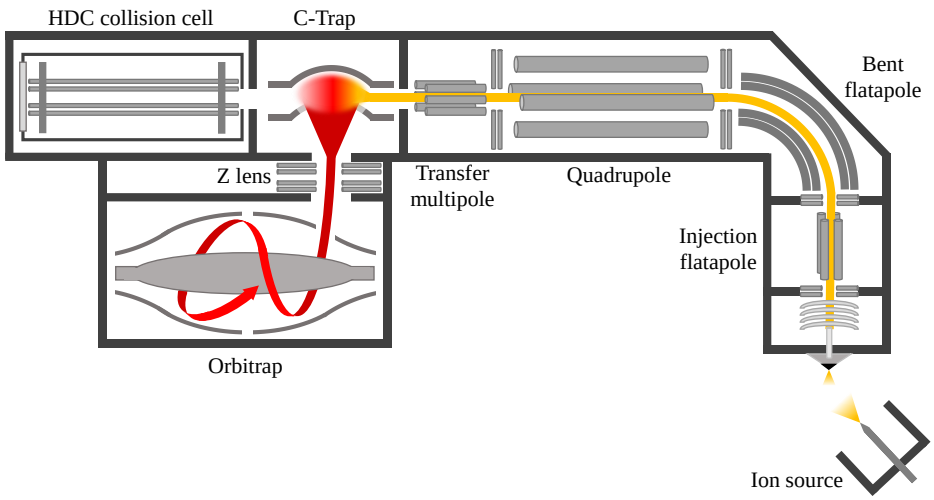


Figure 1.12. Hybrid QOrbitrap mass spectrometer scheme, based on a Q Exactive instrument from Thermo Scientific.

Ions are introduced into the Orbitrap mass analyser through a narrow ion-injection channel perpendicularly to the central electrode. Nevertheless, the ion beams must present narrow spatial and temporal distributions in order to assure stability and coherence of trapped ions during frequency measurements [140]. This efficient ion introduction is obtained by the use of the C-trap (**Figure 1.12**), which is a curved RF-only quadrupole ion trap between ion optics (and Q if present) and the Orbitrap [140]. The C-trap injects ions coming from ion source into the Orbitrap or into the HCD cell (Higher-energy C-trap dissociation, if present), to be injected into the Orbitrap after fragmentation [140]. The C-trap is based on the accumulation and stabilisation of ions by the application of RF, which are injected into the Orbitrap after ion cooling down.

The HDC cell is based on a CID variation that uses higher RF in order to retain product ions in the C-trap. When ions coming from the ion source are trapped in the C-trap, they can be injected into the HDC cell and thus, fragmented. After that, product ions return to the C-trap, are cooled down and injected into the Orbitrap. One of the drawbacks of using ion traps between the collision cell and mass analyser, is the formation of adducts between product ions and neutral molecules, such as water molecules [141].

Hybrid instruments based on the Orbitrap have been developed, using linear ion trap (LTQ-Orbitrap) and quadrupole (QOrbitrap). A Q Exactive from Thermo Scientific equipped with a hybrid QOrbitrap mass analyser has been used in two studies included in this thesis (**research articles VII** and **VIII**). As MS/MS instrument, DIA, DDA and product ion scan can be used. Nevertheless, only the two last working modes have been used in this thesis. For the DDA acquisition (called in this case, dd-MS^2), a full-spectrum acquisition has been performed using a mass resolution of 70,000 FWHM at *m/z* 200 (called FTMS), while automatic MS/MS have been performed at 35,000 FWHM and at 25 eV collision energy. Additionally, product ion scan (called parallel-reaction monitoring or PRM) has also been used for favouring compound elucidation, stablishing the Q

at 1 Da isolation window, the Orbitrap resolution at 17,500 FWHM and acquiring data at three different collision energies.

The DDA is highly useful when the composition of the sample is slightly known, as for example, *in vitro* incubations with pHH, in order to avoid automatic MS/MS acquisitions due to major compounds of the sample without analytical interest. Moreover, the DDA provides also a "pure" product ion scan for those ions of interest and thus, it is not mandatory to perform PRM experiments.

1.6.3. Additional analytical techniques

There are additional analytical techniques used for routine toxicological analysis such as GC-MS and Fourier-transformed infrared spectroscopy (FTIR). Both techniques have demonstrated their potential for the determination of NPS, especially GC-MS when using EI, due to the availability of spectral libraries that could be used for compound identification [142]. Regarding FTIR, this analytical technique can also be used for a rapid identification of NPS in consumption products, as it provides a fast analysis, and a library of IR spectra can be constructed for compound identification [143]. GC-MS and FTIR have been used in research articles included in **Chapter 2**, where different NPS have been identified and fully characterised. This type of information is highly useful for forensic laboratories, typically equipped with these techniques, as it can be used for the detection and identification of the reported NPS.

The main handicap during novel NPS identification is the lack of analytical reference standards, needed for an unequivocal identification of a certain compound, when using LC-MS or GC-MS. For those compounds, nuclear magnetic resonance (NMR) spectroscopy, together with the information obtained by HRMS, has demonstrated to be extremely useful for the final identification of the compounds [54,68,144,145]. Different experiments can be performed by NRM, being those used in this thesis, the following ones [146]:

- ^{1}H NMR. Used for detecting hydrogen-1 (^{1}H) nuclei. Provides information about the chemical surroundings of each ^{1}H nuclei (chemical shift, δ), as well as the relative number of ^{1}H present in the molecule (integration) and their interaction (coupling) with neighbouring ^{1}H nuclei (multiplicity).
- ^{13}C NMR. Used for detecting carbon-13 (^{13}C) nuclei. Provides information about the chemical surroundings of each ^{13}C nuclei (δ).
- COSY. Homonuclear correlation spectroscopy, it is a bidimensional NMR experiment (2D NMR) that determines the coupling between neighbouring nuclei. It is commonly used for determining the coupling between ^{1}H nuclei.
- HSQC. Heteronuclear single-quantum coherence/correlation. It is a 2D NMR experiment that stablishes the relations between ^{1}H nuclei and ^{13}C (or nitrogen-15, ^{15}N) nuclei at 1 bond distance.

The NMR has been widely used in **research articles I-IV** included in **Chapter 2**. Although the combination of HRMS and NMR provides enough information for an unequivocal compound identification, there are additional techniques that allow the direct elucidation of the structure of a compound. In this sense, single-crystal X-ray diffraction allows the identification of the position of atoms, and how are they bonded, into a molecule [147]. X-ray diffraction data determine the chemical bond lengths and angles, as well as the atoms size, measured as the diffraction of an X-ray radiation through the crystal. The main inconvenience of this technique is the need of a well-formed crystal of the pure compound, in order to acquire high-quality data and perform structure elucidation. This technique has used in **research articles I** and **II** for the unequivocal elucidation of two non-reported NPS.

1.7. Analytical strategies for NPS analysis in toxicological samples

The previously explained analytical techniques open the possibility to the development of different analytical strategies for the determination of NPS and related products in different matrices, as well as any ionisable compound in any matrix of interest. So, the knowledge of the fundamentals of each mass spectrometry technique, especially MS/MS and HRMS, is basic for understanding the different strategies that could be used.

Hereunder the main strategies used in this thesis are briefly described.

1.7.1. Pre-target analysis

In the pre-target analysis, the compounds of interest must be defined previously to the MS analysis. In this case, as the compounds to be determined are known and reference standards are available, instrumental conditions can be optimised for each analyte, in order to maximize sensitivity, selectivity and perform an accurate quantification of these compounds.

Usually, pre-target analysis is used for trace-level quantification purposes, using chromatographic techniques coupled to MS/MS instruments, commonly QqQ mass analysers working in SRM acquisition mode [148–150]. Nevertheless, pre-target analysis can also be performed by hybrid Q-HRMS instruments, working in MS/MS acquisition mode. The availability of reference standards allows the optimisation of all the steps of the analytical methodology, but the main limitation is the selection of the compounds: only those considered previously to the analysis will be detected.

This strategy has used in the **research article X** for the quantification of 13 synthetic cathinones in rat brain samples by UHPLC-MS/MS using QqQ mass analyser.

1.7.2. Wide-scope screening

On the other side, the wide-scope screening allows the detection of almost any ionisable compound in the sample. In this case, the compounds of interest are not (or are not necessarily) defined before MS analysis. In this way, HRMS are the most suitable instruments for this type of analysis, but they can also be performed using MS or MS/MS instruments working in scan mode. Although, a "pseudo" wide-scope screening can be performed using QqQ instruments working in precursor/neutral loss ion scan, as the compounds of interest are not previously defined, but some information are available before analysis.

Nevertheless, wide-scope screening is usually performed by chromatographic techniques coupled to HRMS, working in DIA or DDA acquisition modes [65,151]. These working modes allow the acquisition of all the analytical information available in the sample, so retrospective analysis can also be performed. The main drawback of this methodology is the huge amount of analytical data generated, being most of them irrelevant for the initial purpose of the analysis. So, different approaches can be stablished for data processing.

Post-target screening

A list of compounds of interest is searched in the data generated, filtering the results based on chromatographic retention time, accurate mass and product ions detection. For this processing, analytical reference standards of all the compounds of interest are needed, as retention time is dependent of the chromatographic separation used [151].

An example of post-target screening can be found in **research article IX**, where the tryptamine 5-MeO-MiPT was tentatively identified in a pill sample, and unequivocally identified after reference standard purchase.

Suspect screening

Similar to the post-target analysis, a list of compounds of interest is searched, but in this case, only information about accurate mass is available as it can be theoretically determined for the selected analytes. Information about product ions can also be included, based on information available in literature and spectral databases [151].

In this thesis, a suspect screening strategy has been used for the determination of SCRAs in herbal blend products and their major metabolites in real urine samples (**research articles XI** and **XII**).

Non-target screening

This is, without any doubt, the most complex data processing procedure. In this case, compounds are automatically detected based on chromatographic and spectral information using spectral deconvolution algorithms. Once the compounds are detected, the identification is performed based on the accurate-mass product ion spectra observed, by comparison with online spectral databases or by elucidation of compound if it is an unreported molecule or no MS information is available [151]. Nevertheless, is almost impossible and impractical the elucidation of all the detected compounds during HRMS DIA/DDA analysis. The selection of the compounds of interest (prioritization) can be performed based on peak intensity, isotope pattern, statistical analysis (for example, omics approaches [139]), or toxicological aspects.

In the **research article I**, a putative NPS found in a research chemical sample was elucidated following a non-target screening strategy.

1.7.3. Metabolite detection and elucidation

Apart from compound detection and identification after sample analysis, the wide-scope strategies can also be applied for the detection and elucidation of related compounds. In toxicological analysis, one of the most interesting fields

of work is the elucidation of potential NPS metabolites that could be used as consumption biomarkers, as explained in the pharmacology section of this thesis.

Here, only information about the precursor compound is available, while the potential metabolites must be detected and elucidated based on the analytical information available. For this purpose, the use of HRMS DIA/DDA acquisition is extremely useful due to the accurate-mass acquisition that allows elemental composition calculation, used for metabolite identification [101,152–155]. Nevertheless, the use of MS/MS, and specially QqQ, can also be used for metabolite identification using precursor ion scan and neutral loss scan [156–160].

Different approaches based on HRMS DIA/DDA data can be used for metabolite detection and elucidation, being almost all of them used in combination with the other approaches.

Binary comparison

For this non-target screening approach data processing, a control sample (control) must be analysed together with the samples of interest (analyte) in order to search for those compounds that are only present in the sample of interest [101]. For example, a urine sample obtained from an NPS consumer volunteer before administration must be analysed together with the urine sample obtained after a certain time after consumption [101]. In this case, compounds present in control and analyte samples are automatically compared using processing software, based on chromatographic retention time, accurate-mass and product ions (depending on the software). In this way, endogenous compounds present in urine are discarded, and only those compounds uniquely present in analyte samples are considered as potential metabolites, elucidated by another strategy explained down below.

This approach can be used for a wide variety of matrices, provided that a control sample is available. It can also be used for the detection of transformation products obtained after degradation processes, or for the non-target detection of compounds in environmental samples.

Expected biotransformations

This suspect screening approach consists on the search for plausible metabolites based on a list of potential biotransformations that could occur on the compound [101,155].

This list of potential biotransformations include phase I transformations (oxidations, alkyl cleavage, dehalogenations, among others) and phase II conjugations (glucuronide, sulphate, acetyl, etc.). With the aim of not searching for illogical metabolites, the structure of the compound and metabolising system must be carefully evaluated for the selection of the transformations. For example, it is not logical to include an oxidative dechlorination biotransformation if the compound of interest does not present a chlorine atom.

The biotransformed compound is searched in the HRMS data based on its exact mass. Depending on the software, these potential metabolites can be detected by obtaining the nw-EIC of the expected biotransformations, or by the comparison between the accurate-mass of the detected peak (after a peak picking process) and the putative biotransformations that could occur.

This approach is highly useful when combined with the binary comparison approach, as only those compounds present in the analyte sample are considered after the biotransformation search. Nevertheless, this approach only considers the accurate-mass of the (de)protonated molecule, and no information about the position in which this biotransformation has occurred is provided. For this, the fragmentation spectra must be evaluated.

Common fragment and neutral loss search

This approach is based on the search for compounds present in the analyte sample which produce the same product ions or neutral losses than the compound of interest. So, it can be considered as a suspect screening strategy. It is expected that the metabolites (and transformation products) present similar mass spectrometric behaviour than the compound under study [155].

The concept of this approach is very easy: when the nw-EIC of the product ions from compound of interest are obtained, different chromatographic peaks are observed in the chromatogram. These peaks are compounds that present the same product ion, and potential metabolites. After that, the elemental composition of the precursor ion of this fragment can be determined based on its accurate-mass and thus, the biotransformation that had occurred.

Additionally, with software which is able to perform peak picking, the common fragment search can be complemented with the neutral loss search, as all the product ions from all the detected peaks in the sample have been processed.

The mass shift of some fragments and/or neutral losses between metabolite and parent can helps for determine the exact position where the biotransformation has occurred. This strategy can be complemented with binary comparison and expected biotransformations approaches.

Isotope pattern search

Another suspect screening strategy is based on the search for a specific isotope pattern, especially for halogenated compounds. This approach can only be used when the software performs automatic peak picking, as the spectrum of each compound in the sample is going to be evaluated.

It is based on the selection of those compounds that present a characteristic isotope pattern, typically used when the compound of interest has chlorine and/or bromine atoms. The halogenated compounds are xenobiotic and therefore must be related to the compound of interest, being potential metabolites.

This approach is extremely useful and complementary to the expected biotransformation approach if no control sample is available, especially for the detection of unexpected metabolites. It is possible that some metabolites can be produced by reactions would not considered in the expected biotransformation list and, therefore, they would not be detected applying this approach.

Mass defect filtering

The mass defect filtering (also known as fractional mass filtering) is based on the premise that narrow ranges of changes in the mass defect dimension can be applied regardless of the molecular formula, fragmentation, or isotope pattern of metabolites [161].

The key of this suspect screening approach is that the mass defect of phase I and phase II metabolite ions is usually enclosed in a 50 mDa mass window [161]. For example, a hydroxylation generates a mass defect variation of -5 mDa, a demethylation of -23 mDa, while the glucuronide conjugation generates a mass defect variation of +32 mDa [161]. Nevertheless, it should be taken into account that some biotransformations can present a mass defect variation higher that 50 mDa, such as the glutathione conjugation, that generates a +68 mDa change in the mass defect [161]. Additionally, metabolites that had experimented two biotransformations can present a mass defect variation higher than 50 mDa.

So, after a peak picking process, the software can consider only potential metabolites those compounds that present a mass defect variation lower that, for example, 50 mDa. In this way, most of the compounds present in the sample are discarded, and a shorter list of potential metabolites is obtained. This approach is an alternative to the binary comparison, as no control sample is needed. Additionally, it can be complemented with common fragmentation pathway for metabolite elucidation.

In silico prediction

In the last years, different software able predict the metabolites produced from a specific compound using a specific metabolic system have developed. The software proposes a list of potential metabolites that could be generated, and a suspect screening strategy is used for searching these compounds in the HRMS data [162].

In this Doctoral Thesis, a thorough investigation on NPS has been carried out, using advanced MS techniques. For this purpose, different methodologies have been developed and applied for the detection, identification and characterisation of these substances and their metabolites in toxicological analysis.

In **Chapter 2**, four different unreported NPS are identified and characterised by the combination of HRMS and NMR. The results of the research chemicals and legal highs analysis performed during the last four years are also presented. **Research article I** shows the elucidation of a putative new phenethylamine, while **research article II** presents the identification and characterisation of the novel synthetic opioid U-49900. Additionally, two synthetic stimulants are characterised in **research articles III**, focused on the cathinone 5-PPDi, and **IV**, which illustrates the detection of a novel halogenated aminorex derivative.

Chapter 3 presents the development of two analytical strategies for the identification of NPS in consumption products, based on the use of triple quadruple instruments. In **research article V**, a methodology for the identification of unknown synthetic cathinones in research chemical samples was developed, based on the application of precursor ion and neutral loss scan working modes. **Research article VI** presents an approach for the suspect screening of different NPS in consumption products and surface analysis, based on the ASAP source and DDA acquisition mode.

Regarding pharmacological aspects of the NPS, the *in vitro* model for the study of metabolite profiling and potency tested is explored in **Chapter 4**. **Research article VII** is focused on the use of pHH for metabolite identification of two SCRA, as well as on the application of an *in vitro* assay for assessing the potency of both compounds. The use of pHH for metabolite profiling was also used in **research article VIII**, in which the metabolic fate of four synthetic stimulants, included the cathinone 5-PPDi, is investigated.

Chapter 5 explores some pharmacological aspects of the NPS, but in this case using *in vivo* models. The metabolic fate and pharmacokinetics, as well as the proposal of urinary biomarkers, for the synthetic tryptamine 5-MeO-MiPT in mice is presented in **research article IX**. The pharmacokinetic of NPS is also the focus of **research article X**, in which the relationship between synthetic cathinone structures and their permeability through the blood-brain barrier is assessed in rats, using previously validated methodology for the quantification of these compounds in cerebrum tissue.

Finally, **Chapter 6** shows the application of HRMS suspect screening strategies for the investigation of SCRA consumption among teenagers. **Research article XI** is focused on the use of these substances in teenagers interned in juvenile offenders' centres. A specific case report is presented in **research article XII**, about the monitoring of the consumption patterns of a teenager under treatment in the city of Valencia (Spain).

The studies included in this thesis, all of them based on advanced MS techniques, have been developed in order to:

1. provide analytical information about emerging NPS,
2. provide novel analytical strategies for their identification,
3. increase the knowledge about the pharmacology of these compounds,
4. propose consumption biomarkers for some NPS, and finally,
5. show the use of these compounds by a population group as delicate as teenagers.

1.8. Literature

1. Leroi-Gourham, A. Shanidar et ses fleurs. Páleorient 24, 79–88 (1999).

2. Leroi-Gourham, A. Rites et langage á Shanidar? Bull. la Société Préhistorique Française 27, 291–293 (2000).

3. Lietava, J. Medicinal plants in a Middle Paleolithic grave Shanidar IV? J. Ethnopharmacol. 35, 263–266 (1992).

4. Schiff, P. L. Opium and Its Alkaloids. Am. J. Pharm. Educ. 66, 186–194 (2002).

5. Hume, E. H. A Note on Narcotics in Ancient Greece and in Ancient China. Bull. N. Y. Acad. Med. 10, 618–622 (1934).

6. Becerra Romero, D. La adormidera en el Mediterráneo oriental: planta sagrada, planta profana. Habis 7–16 (2006).

7. Plutach. Moralia.

8. Becerra Romero, D. Las formas habituales de consumir drogas en la Antigüedad a partir de la obra de Porfirio 'De abstinentia'. Faventia 28, 67–78 (2006).

9. Seneca the Young. De Providentia.

10. Hamarneh, S. Pharmacy in medieval Islam and the history of drug addiction. Med. Hist. 16, 226–237 (1972).

11. Russo, E. B. History of Cannabis and Its Preparations in Saga, Science, and Sobriquet. Chem. Biodivers. 4, 1614–1648 (2007).

12. Manniche, L. An ancient Egyptian herbal. (University of Texas, 1989).

13. Carod-Artal, F. J. Alucinógenos en las culturas precolombinas mesoamericanas. Neurología 30, 42–49 (2015).

14. Fabing, H. D. On Going Berserk: A Neurochemical Inquiry. Sci. Mon. 83, 232–237 (1956).

15. Allen, C. J. To be Quechua: The Symbolism of Coca Chewing in Highland Peru. Am. Ethnol. 8, 157–171 (1981).

16. Estes, J. W. The European reception of the first drugs from the New World. Pharm. Hist. 37, 3–23 (1995).

17. Lee, R. W. Colombia's cocaine syndicates. Crime, Law Soc. Chang. 16, 3–39 (1991).

18. United Nations Office on Drugs and Crime. World Drug Report 2019. (2019).

19. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2019. (2019).

20. Andre, C. M., Hausman, J.-F. & Guerriero, G. Cannabis sativa: The Plant of the Thousand and One Molecules. Front. Plant Sci. 7, 1–17 (2016).

21. Pertwee, R. G. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol. Ther. 74, 129–180 (1997).

22. Pertwee, R. G. The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ9-tetrahydrocannabinol, cannabidiol and Δ9-tetrahydrocannabivarin. Br. J. Pharmacol. 199–215 (2008).

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

24. Mechoulam, R. Marihuana Chemistry. Science (80-.). 168, 1159–1165 (1970).

25. Hall, W. & Solowij, N. Adverse effects of cannabis. Lancet 352, 1611–1616 (1998).

26. Sandberg, S. Cannabis culture: A stable subculture in a changing world. Criminol. Crim. Justice 13, 63–79 (2013).

27. National Institute on Drug Abuse. Cocaine: 1977. NIDA Res. Monogr. (1977). doi:10.1007/978-1-4684-6033-9_2

28. Ritz, M., Lamb, R., Goldberg & Kuhar, M. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science (80-.). 237, 1219–1223 (1987).

29. Zimmerman, J. L. Cocaine Intoxication. Crit. Care Clin. 28, 517–526 (2012).

30. National Institute on Drug Abuse. Drug facts: 3,4-methylenedioxy-methamphetamine (MDMA) (Ecstasy/Molly). DrugFacts. Natl. Inst. Drug Abus. 1–4 (2018).

31. Freye, E. Pharmacological Effects of MDMA in Man. in Pharmacology and Abuse of Cocaine, Amphetamines, Ecstasy and Related Designer Drugs 151–160 (Springer Netherlands, 2009). doi:10.1007/978-90-481-2448-0_24

32. Meyer, J. 3,4-methylenedioxymethamphetamine (MDMA): current perspectives. Subst. Abuse Rehabil. 83 (2013). doi:10.2147/SAR.S37258

33. Sawynok, J. The therapeutic use of heroin: A review of the pharmacological literature. Can. J. Physiol. Pharmacol. 64, 1–6 (1986).

34. National Institute on Drug Abuse. Research Report Series: Heroin. NIDA Res. Rep. Ser. (2014).

35. National Institute on Drug Abuse. Drug facts: Heroin. DrugFacts. Natl. Inst. Drug Abus. 1–8 (2019).

36. Johnson, S. & North, R. Opioids excite dopamine neurons by hyperpolarization of local interneurons. J. Neurosci. 12, 483–488 (1992).

37. Goldstein, A. Heroin Addiction: Neurobiology, Pharmacology, and Policy. J. Psychoactive Drugs 23, 123–133 (1991).

38. United Nations Office on Drugs and Crime (UNODC). What are NPS? (2019). Available at: https://www.unodc.org/LSS/Page/NPS. (Accessed: 30th October 2019)

39. Harper, N. J. & Veitch, G. B. A. 1-(3,4-dichlororbenzamidomethyl)-cyclohexyldimethylamine. Patent No. US3975443A. Allen & Hanburys Limited, London, England. (1976).

40. Szmuszkovicz, J. Analgesic N-(2-aminocycloaliphatic)benzamides. Patent No. US4098904A. The Upjohn Company, Michigan, USA. (1978).

41. Aung, M. M. et al. Influence of the N-1 alkyl chain length of cannabimimetic indoles upon CB1 and CB2 receptor binding. Drug Alcohol Depend. 60, 133–140 (2000).

42. Huffman, J. W. et al. Structure–activity relationships for 1-alkyl-3-(1-naphthoyl)indoles at the cannabinoid CB1 and CB2 receptors: steric and electronic effects of naphthoyl substituents. New highly selective CB2 receptor agonists. Bioorg. Med. Chem. 13, 89–112 (2005).

43. Schneir, A., Metushi, I. G., Sloane, C., Benaron, D. J. & Fitzgerald, R. L. Near death from a novel synthetic opioid labeled U-47700: emergence of a new opioid class. Clin. Toxicol. 3650, 1–4 (2016).

44. 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. 160, 585–593 (2010).

45. Shulgin, A. & Shulgin, A. PIHKAL: A Chemical Love Story. (Transform Press, 1991).

46. Shulgin, A. & Shulgin, A. TIHKAL: The Continuation. (Transform Press, 1997).

47. Maurer, H. H. & Brandt, S. D. New Psychoactive Substances. Pharmacology, Clinical, Forensic and Analytical Toxicology. Handbook of Experimental Pharmacology 252, (Springer International Publishing, 2018).

48. Reuter, P. H. & Pardo, B. A. New Psychoactive Substances: The Regulatory Experience and Assessment of Options. in Novel Psychoactive Substances 155–177 (Springer International Publishing, 2017). doi:10.1007/978-3-319-60600-2_12

49. Zawilska, J. B. & Wojcieszak, J. Novel Psychoactive Substances: Classification and General Information. in Synthetic Cathinones 11–24 (Springer International Publishing, 2018). doi:10.1007/978-3-319-78707-7_2

50. Auwärter, V. et al. 'Spice' and other herbal blends: harmless incense or cannabinoid designer drugs? J. Mass Spectrom. 44, 832–837 (2009).

51. Ibáñez, M. et al. Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends. J. Mass Spectrom. 48, 685–694 (2013).

52. Kikura-Hanajiri, R., Kawamura, N. U. M. & Goda, Y. Changes in the prevalence of new psychoactive substances before and after the introduction of the generic scheduling of synthetic cannabinoids in Japan. Drug Test. Anal. 6, 832–839 (2014).

53. Bijlsma, L. et al. Mass spectrometric identification and structural analysis of the third-generation synthetic cannabinoids on the UK market since the 2013 legislative ban. Forensic Toxicol. 35, 376–388 (2017).

54. Liu, C., Jia, W., Hua, Z. & Qian, Z. Identification and analytical characterization of six synthetic cannabinoids NNL-3, 5F-NPB-22-7 N , 5F-AKB-48-7 N , 5F-EDMB-PINACA, EMB-FUBINACA, and EG-018. Drug Test. Anal. (2017). doi:10.1002/dta.2160

55. Qian, Z., Jia, W., Li, T., Hua, Z. & Liu, C. Identification and analytical characterization of four synthetic cannabinoids ADB-BICA, NNL-1, NNL-2, and PPA(N)-2201. Drug Test. Anal. 9, 51–60 (2017).

56. Adams, A. J. et al. "Zombie" Outbreak Caused by the Synthetic Cannabinoid AMB-FUBINACA in New York. N. Engl. J. Med. 376, 235–242 (2017).

57. Gatch, M. B. & Forster, M. J. Cannabinoid-like effects of five novel carboxamide synthetic cannabinoids. Neurotoxicology 70, 72–79 (2019).

58. Barceló, B. et al. Acute intoxication caused by synthetic cannabinoids 5F-ADB and MMB-2201: A case series. Forensic Sci. Int. 273, e10–e14 (2017).

59. Kusano, M. et al. Fatal intoxication by 5F–ADB and diphenidine: Detection, quantification, and investigation of their main metabolic pathways in humans by LC/MS/MS and LC/Q-TOFMS. Drug Test. Anal. 10, 284–293 (2018).

60. Tournebize, J., Gibaja, V. & Kahn, J.-P. Acute effects of synthetic cannabinoids: Update 2015. Subst. Abus. 7077, 1–23 (2016).

61. 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).

62. 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).

63. Banks, M. L., Worst, T. J., Rusyniak, D. E. & Sprague, J. E. Synthetic Cathinones ("Bath Salts"). J. Emerg. Med. 46, 632–642 (2014).

64. Karch, S. Cathinone Neurotoxicity ("The "3Ms"). Curr. Neuropharmacol. 13, 21–25 (2015).

65. Ibañez, M. et al. Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. TrAC - Trends Anal. Chem. 57, 107–117 (2014).

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

67. Taschwer, M., Weiß, J. A., Kunert, O. & Schmid, M. G. Analysis and characterization of the novel psychoactive drug 4-chloromethcathinone (clephedrone). Forensic Sci. Int. 244, e56–e59 (2014).

68. Majchrzak, M., Rojkiewicz, M., Celiński, R., Kuś, P. & Sajewicz, M. Identification and characterization of new designer drug 4-fluoro-PV9 and α-PHP in the seized materials. Forensic Toxicol. 34, 115–124 (2016).

69. Qian, Z., Jia, W., Li, T., Liu, C. & Hua, Z. Identification and analytical characterization of four synthetic cathinone derivatives iso-4-BMC, β -TH-naphyrone, mexedrone, and 4-MDMC. Drug Test. Anal. 9, 274–281 (2017).

70. Apirakkan, O. et al. Analytical characterization of three cathinone derivatives, 4-MPD, 4F-PHP and bk-EPDP, purchased as bulk powder from online vendors. Drug Test. Anal. 1–7 (2017). doi:10.1002/dta.2218

71. Doi, T. et al. Identification and characterization of a-PVT, a-PBT, and their bromothienyl analogs found in illicit drug products. Forensic Toxicol. 34, 76–93 (2016).

72. Janssen, P. A., Niemegeers, C. J. & Dony, J. G. The Inhibitory Effect of Fentanyl and Other Morphine-Like Analgesics on the Warm Water Induced Tail Withdrawl Reflex in Rats. Arzneimittelforschung 13, 502–507 (1963).

73. Stanley, T. H. The history and development of the fentanyl series. J. Pain Symptom Manage. 7, S3–S7 (1992).

74. Quintana, P. et al. The hidden web and the fentanyl problem: Detection of ocfentanil as an adulterant in heroin. Int. J. Drug Policy 40, 78–83 (2017).

75. Centers for Disease Control and Prevention. Increases in Fentanyl Drug Confiscations and Fentanyl-related Overdose Fatalities. Accessed 2017-02-23. emergency.cdc.gov (2015). Available at: https://emergency.cdc.gov/han/han00384.asp. (Accessed: 23rd February 2017)

76. Armenian, P. et al. Fentanyl and a Novel Synthetic Opioid U-47700 Masquerading as Street "Norco" in Central California: A Case Report. Ann. Emerg. Med. 69, 87–90 (2017).

77. Coopman, V., Blanckaert, P., Van Parys, G., Van Calenbergh, S. & Cordonnier, J. A case of acute intoxication due to combined use of fentanyl and 3,4-dichloro-N-[2-(dimethylamino)cyclohexyl]-N-methylbenzamide (U-47700). Forensic Sci. Int. 266, 68–72 (2016).

78. Centers for Disease Control and Prevention. Increases in Fentanyl-Related Overdose Deaths — Florida and Ohio, 2013–2015. Accessed 2017-02-23. cdc.gov (2016). Available at: Increases in Fentanyl-Related Overdose Deaths — Florida and Ohio, 2013–2015. (Accessed: 23rd February 2017)

79. Lozier, M. J. et al. Acetyl Fentanyl, a Novel Fentanyl Analog, Causes 14 Overdose Deaths in Rhode Island, March–May 2013. J. Med. Toxicol. 11, 208–217 (2015).

80. Helander, A., Bäckberg, M. & Beck, O. Intoxications involving the fentanyl analogs acetylfentanyl, 4-methoxybutyrfentanyl and furanylfentanyl: results from the Swedish STRIDA project. Clin. Toxicol. 54, 324–332 (2016).

81. Elliott, S. P., Brandt, S. D. & Smith, C. The first reported fatality associated with the synthetic opioid 3,4-dichloro- N -[2-(dimethylamino)cyclohexyl]- N -methylbenzamide (U-47700) and implications for forensic analysis. Drug Test. Anal. 8, 875–879 (2016).

82. Department of Justice. Drug Enforcement Administration (DEA). Schedules of Controlled Substances: Temporary Placement of U-47700 Into Schedule I. Federal Register 81 (173) 61636–61638 (2016). Available at: https://www.deadiversion.usdoj.gov/fed_regs/rules/2016/fr1114.htm.

83. Domanski, K. et al. Two cases of intoxication with new synthetic opioid, U-47700. Clin. Toxicol. 3650, 1–5 (2016).

84. Kometer, M., Schmidt, A., Jancke, L. & Vollenweider, F. X. Activation of Serotonin 2A Receptors Underlies the Psilocybin-Induced Effects on Oscillations, N170 Visual-Evoked Potentials, and Visual Hallucinations. J. Neurosci. 33, 10544–10551 (2013).

85. Kraehenmann, R. et al. Dreamlike effects of LSD on waking imagery in humans depend on serotonin 2A receptor activation. Psychopharmacology (Berl). 234, 2031–2046 (2017).

86. Kuhn, C. Buzzed: the straight facts about the most used and abused drugs from alcohol to ecstasy. (W.W. Norton, 2003).

87. Tittarelli, R., Mannocchi, G., Pantano, F. & Romolo, F. Recreational Use, Analysis and Toxicity of Tryptamines. Curr. Neuropharmacol. 13, 26–46 (2015).

88. Araújo, A. M., Carvalho, F., Bastos, M. de L., Guedes de Pinho, P. & Carvalho, M. The hallucinogenic world of tryptamines: an updated review. Arch. Toxicol. 89, 1151–1173 (2015).

89. Smolinske, S. C., Rastogi, R. & Schenkel, S. Foxy methoxy: a new drug of abuse. J. Med. Toxicol. 1, 22–5 (2005).

90. Tanaka, E., Kamata, T., Katagi, M., Tsuchihashi, H. & Honda, K. A fatal poisoning with 5-methoxy-N,N-diisopropyltryptamine, Foxy. Forensic Sci. Int. 163, 152–154 (2006).

91. Shimizu, E. et al. Combined intoxication with methylone and 5-MeO-MIPT. Prog. Neuro-Psychopharmacology Biol. Psychiatry 31, 288–291 (2007).

92. Nagai, F., Nonaka, R. & Satoh Hisashi Kamimura, K. The effects of non-medically used psychoactive drugs on monoamine neurotransmission in rat brain. Eur. J. Pharmacol. 559, 132–137 (2007).

93. Caspar, A. T., Brandt, S. D., Stoever, A. E., Meyer, M. R. & Maurer, H. H. Metabolic fate and detectability of the new psychoactive substances 2-(4-bromo-2,5-dimethoxyphenyl)- N- [(2-methoxyphenyl)methyl]ethanamine (25B-NBOMe) and 2-(4-chloro-2,5-dimethoxyphenyl)- N- [(2-methoxyphenyl)methyl]ethanamine (25C-NBOMe) in human and r. J. Pharm. Biomed. Anal. 134, 158–169 (2017).

94. Richter, L. H. J., Maurer, H. H. & Meyer, M. R. Metabolic fate of the new synthetic cannabinoid 7'N-5F-ADB in rat, human, and pooled human S9 studied by means of hyphenated high-resolution mass spectrometry. Drug Test. Anal. 11, 305–317 (2019).

95. Welter, J., Brandt, S. D., Kavanagh, P., Meyer, M. R. & Maurer, H. H. Metabolic fate, mass spectral fragmentation, detectability, and differentiation in urine of the benzofuran designer drugs 6-APB and 6-MAPB in comparison to their 5-isomers using GC-MS and LC-(HR)-MSn techniques. Anal. Bioanal. Chem. 407, 3457–3470 (2015).

96. 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).

97. Pedersen, A. J., Petersen, T. H. & Linnet, K. In vitro metabolism and pharmacokinetic studies on methylone. Drug Metab. Dispos. 41, 1247–1255 (2013).

98. Kevin, R. C. et al. In vitro and in vivo pharmacokinetics and metabolism of synthetic cannabinoids CUMYL-PICA and 5F-CUMYL-PICA. Forensic Toxicol. 35, 333–347 (2017).

99. Liston, H. L., Markowitz, J. S. & DeVane, C. L. Drug Glucuronidation in Clinical Psychopharmacology. J. Clin. Psychopharmacol. 21, 500–515 (2001).

100. Jakobyz, W. B. & Ziegler, D. M. The Enzymes of Detoxication. J. Biol. Chem. 265, 20715–20718 (1990).

101. Pozo, O. 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. Drug Metab. Dispos. 43, 248–257 (2014).

102. Noble, C., Cannaert, A., Linnet, K. & Stove, C. P. Application of an activity-based receptor bioassay to investigate the in vitro activity of selected indole- and indazole-3-carboxamide-based synthetic cannabinoids at CB1 and CB2 receptors. Drug Test. Anal. 1–11 (2018). doi:10.1002/dta.2517

103. Cannaert, A., Storme, J., Franz, F., Auwärter, V. & Stove, C. P. Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. Anal. Chem. 88, 11476–11485 (2016).

104. Cannaert, A., Franz, F., Auwärter, V. & Stove, C. P. Activity-Based Detection of Consumption of Synthetic Cannabinoids in Authentic Urine Samples Using a Stable Cannabinoid Reporter System. Anal. Chem. 89, 9527–9536 (2017).

105. Korfmacher, W. A. Using Mass Spectrometry for Drug Metabolism Studies. (CRC Press, 2004).

106. Holm, N. B., Pedersen, A. J., Dalsgaard, P. W. & Linnet, K. Metabolites of 5F-AKB-48, a synthetic cannabinoid receptor agonist, identified in human urine and liver microsomal preparations using liquid chromatography high-resolution mass spectrometry. Drug Test. Anal. 7, 199–206 (2015).

107. Carlier, J., Diao, X., Wohlfarth, A., Scheidweiler, K. & Huestis, M. A. In Vitro Metabolite Profiling of ADB-FUBINACA, A New Synthetic Cannabinoid. Curr. Neuropharmacol. 15, 682–691 (2017).

108. Wrighton, S. A., Ring, B. J. & Vandenbranden, M. The Use of In Vitro Metabolism Techniques in the Planning and Interpretation of Drug Safety Studies. Toxicol. Pathol. 23, 199–208 (1995).

109. Mardal, M., Annaert, P., Noble, C., Oorts, M. & Linnet, K. Metabolism of the synthetic cannabinoid 5F-PY-PICA by human and rat hepatocytes and identification of biliary analytical targets by directional efflux in sandwich-cultured rat hepatocytes using UHPLC-HR-MS/MS. J. Pharm. Biomed. Anal. 149, 296–307 (2018).

110. Manier, S. K., Richter, L. H. J., Schäper, J., Maurer, H. H. & Meyer, M. R. Different in vitro and in vivo tools for elucidating the human metabolism of alpha-cathinone-derived drugs of abuse. Drug Test. Anal. 10, 1119–1130 (2018).

111. Silva, J. P., Araújo, A. M., de Pinho, P. G., Carmo, H. & Carvalho, F. Synthetic Cannabinoids JWH-122 and THJ-2201 Disrupt Endocannabinoid-Regulated Mitochondrial Function and Activate Apoptotic Pathways as a Primary Mechanism of In Vitro Nephrotoxicity at In Vivo Relevant Concentrations. Toxicol. Sci. 169, 422–435 (2019).

112. Richter, L. H. J. et al. Tools for studying the metabolism of new psychoactive substances for toxicological screening purposes – A comparative study using pooled human liver S9, HepaRG cells, and zebrafish larvae. Toxicol. Lett. 305, 73–80 (2019).

113. Schaefer, N. et al. Metabolic patterns of JWH-210, RCS-4, and THC in pig urine elucidated using LC-HR-MS/MS: Do they reflect patterns in humans? Drug Test. Anal. 9, 613–625 (2017).

114. Savchuk, S. et al. In vivo metabolism of the new synthetic cannabinoid APINAC in rats by GC–MS and LC–QTOF-MS. Forensic Toxicol. 35, 359–368 (2017).

115. Nordmeier, F., Richter, L. H. J., Schmidt, P. H., Schaefer, N. & Meyer, M. R. Studies on the in vitro and in vivo metabolism of the synthetic opioids U-51754, U-47931E, and methoxyacetylfentanyl using hyphenated high-resolution mass spectrometry. Sci. Rep. 9, 13774 (2019).

116. Skoog, D. A., West, D. M., Holler, F. J. & Crouch, S. R. Fundamentals of Analytical Chemistry, 9th edition. (Brooks Cole/Cengage Learning, 2014).

117. Pasin, D., Cawley, A., Bidny, S. & Fu, S. Current applications of high-resolution mass spectrometry for the analysis of new psychoactive substances: a critical review. Anal. Bioanal. Chem. 409, 5821–5836 (2017).

118. Maurer, H. H. & Meyer, M. R. High-resolution mass spectrometry in toxicology: current status and future perspectives. Arch. Toxicol. 90, 2161–2172 (2016).

119. Skoog, D. A., Holler, F. J. & Nieman, T. A. Principles of instrumental analysis, 5th edition. (Harcourt College Publishers, 1998).

120. Niessen, W. M. A. Liquid Chromatography-Mass Spectrometry, 3rd edition. (CRC Press, 2006).

121. Tang, D.-Q., Zou, L., Yin, X.-X. & Ong, C. N. HILIC-MS for metabolomics: An attractive and complementary approach to RPLC-MS. Mass Spectrom. Rev. 35, 574–600 (2016).

122. Tiller, P. R., Yu, S., Castro-Perez, J., Fillgrove, K. L. & Baillie, T. A. High-throughput, accurate mass liquid chromatography/tandem mass spectrometry on a quadrupole time-of-flight system as a ‘first-line’ approach for metabolite identification studies. Rapid Commun. Mass Spectrom. 22, 1053–1061 (2008).

123. Salisbury, J. J. Fused-core particles: A practical alternative to sub-2 micron particles. J. Chromatogr. Sci. 46, 883–886 (2008).

124. Abrahim, A. et al. Practical comparison of 2.7μm fused-core silica particles and porous sub-2μm particles for fast separations in pharmaceutical process development. J. Pharm. Biomed. Anal. 51, 131–137 (2010).

125. Dass, C. Fundamentals of contemporary mass spectrometry. (John Wiley & Sons, 2007).

126. Yamashita, M. & Fenn, J. B. Electrospray ion source. Another variation on the free-jet theme. J. Phys. Chem. 88, 4451–4459 (1984).

127. Whitehouse, C. M., Dreyer, R. N., Yamashita, M. & Fenn, J. B. Electrospray interface for liquid chromatographs and mass spectrometers. Anal. Chem. 57, 675–679 (1985).

128. Dole, M. et al. Molecular Beams of Macroions. J. Chem. Phys. 49, 2240–2249 (1968).

129. Gieniec, J. et al. Electrospray mass spectroscopy of macromolecules: Application of an ion-drift spectrometer. Biol. Mass Spectrom. 11, 259–268 (1984).

130. Iribarne, J. V. & Thomson, B. A. On the evaporation of small ions from charged droplets. J. Chem. Phys. 64, 2287 (1976).

131. Thomson, B. A. & Iribarne, J. V. Field induced ion evaporation from liquid surfaces at atmospheric pressure. J. Chem. Phys. 71, 4451–4463 (1979).

132. Tose, L. V, Murgu, M., Vaz, B. G. & Romão, W. Application of Atmospheric Solids Analysis Probe Mass Spectrometry (ASAP-MS) in Petroleomics: Analysis of Condensed Aromatics Standards, Crude Oil, and Paraffinic Fraction. J. Am. Soc. Mass Spectrom. 28, 2401–2407 (2017).

133. Zydel, F. et al. Rapid screening of chemical warfare nerve agent metabolites in urine by atmospheric solids analysis probe-mass spectroscopy (ASAP-MS). Drug Test. Anal. 4, 308–311 (2012).

134. Savaryn, J. P., Toby, T. K. & Kelleher, N. L. A researcher's guide to mass spectrometry-based proteomics. Proteomics 16, 2435–2443 (2016).

135. Murray, K. K. et al. Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013). Pure Appl. Chem. 85, 1515–1609 (2013).

136. Makarov, A. Orbitrap journey: taming the ion rings. Nat. Commun. 10, 3743 (2019).

137. Makarov, A. Electrostatic Axially Harmonic Orbital Trapping: A High-Performance Technique of Mass Analysis. Anal. Chem. 72, 1156–1162 (2000).

138. Hu, Q. et al. The Orbitrap: a new mass spectrometer. J. Mass Spectrom. 40, 430–443 (2005).

139. Fenaille, F., Barbier Saint-Hilaire, P., Rousseau, K. & Junot, C. Data acquisition workflows in liquid chromatography coupled to high resolution mass spectrometry-based metabolomics: Where do we stand? J. Chromatogr. A 1526, 1–12 (2017).

140. Perry, R. H., Cooks, R. G. & Noll, R. J. Orbitrap mass spectrometry: Instrumentation, ion motion and applications. Mass Spectrom. Rev. 27, 661–699 (2008).

141. Alechaga, É., Moyano, E. & Galceran, M. T. Ion-molecule adduct formation in tandem mass spectrometry. Anal. Bioanal. Chem. 408, 1269–1277 (2016).

142. Strano Rossi, S. et al. An analytical approach to the forensic identification of different classes of new psychoactive substances (NPSs) in seized materials. Rapid Commun. Mass Spectrom. 28, 1904–1916 (2014).

143. Coelho Neto, J. Rapid detection of NBOME's and other NPS on blotter papers by direct ATR-FTIR spectrometry. Forensic Sci. Int. 252, 87–92 (2015).

144. Casale, J. F. & Hays, P. a. The Characterization of α-Pyrrolidinopentiophenone. Microgram J. 9, 33–38 (2012).

145. Gambaro, V. et al. Identification and characterization of a new designer drug thiothinone in seized products. Forensic Toxicol. 34, 174–178 (2016).

146. Ernst, R. R., Bodenhausen, G. & Wokaun, A. Principles of nuclear magnetic resonance in one and two dimensions. Magnetic Resonance in Medicine (Oxford University Press, 1988). doi:10.1002/mrm.1910070215

147. Nycz, J. E., Pazdziorek, T., Malecki, G. & Szala, M. Identification and derivatization of selected cathinones by spectroscopic studies. Forensic Sci. Int. 266, 416–426 (2016).

148. Borova, V. L., Gago-Ferrero, P., Pistos, C. & Thomaidis, N. S. Multi-residue determination of 10 selected new psychoactive substances in wastewater samples by liquid chromatography–tandem mass spectrometry. Talanta 144, 592–603 (2015).

149. Vircks, K. E. & Mulligan, C. C. Rapid screening of synthetic cathinones as trace residues and in authentic seizures using a portable mass spectrometer equipped with desorption electrospray ionization. Rapid Commun. Mass Spectrom. 26, 2665–2672 (2012).

150. Aldlgan, A. A. & Torrance., H. J. Bioanalytical methods for the determination of synthetic cannabinoids and metabolites in biological specimens. TrAC Trends Anal. Chem. 80, 444–457 (2016).

151. Hernández, F., Ibáñez, M., Bade, R., Bijlsma, L. & Sancho, J. V. Investigation of pharmaceuticals and illicit drugs in waters by liquid chromatography-high-resolution mass spectrometry. TrAC Trends Anal. Chem. 63, 140–157 (2014).

152. Gandhi, A. S. et al. First Characterization of AKB-48 Metabolism, a Novel Synthetic Cannabinoid, Using Human Hepatocytes and High-Resolution Mass Spectrometry. AAPS J. 15, 1091–8 (2013).

153. Mardal, M. et al. Metabolism of the synthetic cannabinoids AMB-CHMICA and 5C-AKB48 in pooled human hepatocytes and rat hepatocytes analyzed by UHPLC-(IMS)-HR-MS E. J. Chromatogr. B 1083, 189–197 (2018).

154. Castaneto, M. S. et al. Identification of AB-FUBINACA metabolites in human hepatocytes and urine using high-resolution mass spectrometry. Forensic Toxicol. 33, 295–310 (2015).

155. Ibáñez, M. et al. Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry. Anal. Bioanal. Chem. 408, 151–164 (2016).

156. Pozo, O. J., Ventura, R., Monfort, N., Segura, J. & Delbeke, F. T. Evaluation of different scan methods for the urinary detection of corticosteroid metabolites by liquid chromatography tandem mass spectrometry. J. Mass Spectrom. 44, 929–944 (2009).

157. Matabosch, X. et al. Detection and characterization of prednisolone metabolites in human urine by LC-MS/MS. J. Mass Spectrom. 50, 633–642 (2015).

158. McLeod, M. D. et al. Constant Ion Loss Method for the Untargeted Detection of Bis-sulfate Metabolites. Anal. Chem. 89, 1602–1609 (2017).

159. Matabosch, X. et al. Detection and characterization of triamcinolone acetonide metabolites in human urine by liquid chromatography/tandem mass spectrometry after intramuscular administration. Rapid Commun. Mass Spectrom. 28, 1829–1839 (2014).

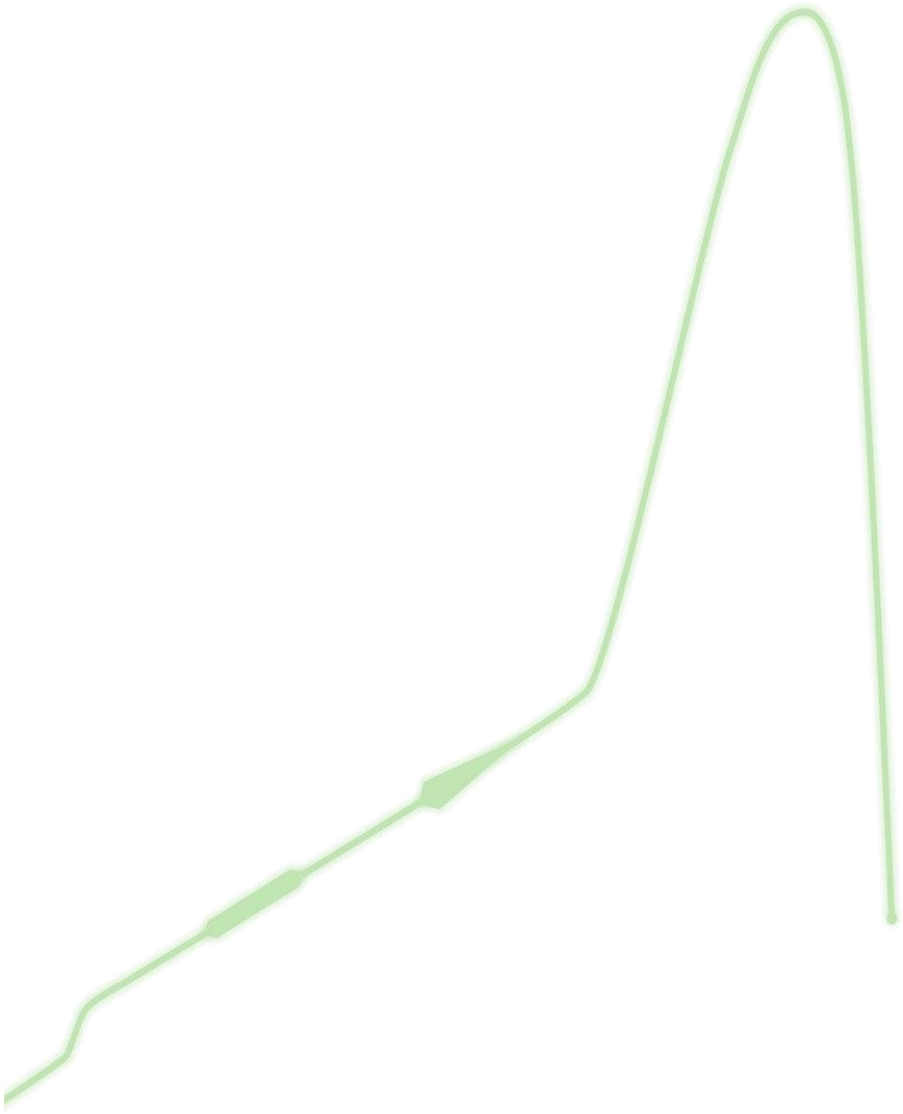
160. Matabosch, X. et al. Detection and characterization of betamethasone metabolites in human urine by LC-MS/MS. Drug Test. Anal. 7, 663–672 (2015).

161. Zhang, H., Zhang, D., Ray, K. & Zhu, M. Mass defect filter technique and its applications to drug metabolite identification by high-resolution mass spectrometry. J. Mass Spectrom. 44, 999–1016 (2009).

162. Bijlsma, L., Berntssen, M. H. G. & Merel, S. A Refined Nontarget Workflow for the Investigation of Metabolites through the Prioritization by in Silico Prediction Tools. Anal. Chem. 91, 6321–6328 (2019).

CHAPTER 2

IDENTIFICATION OF NPS IN LEGAL HIGHS AND RESEARCH CHEMICALS



CHAPTER 2
IDENTIFICATION OF NPS IN LEGAL HIGHS AND RESEARCH CHEMICALS

2.1. Introduction

2.2. Research article I

> "Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain".
> *Drug Testing and Analysis, 2017; 9:1073.*

2.3. Research article II

> "Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900)".
> *Scientific Reports, 2017; 7:6338.*

2.4. Research article III

> "Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance".
> *Forensic Toxicology, 2018; 36:447.*

2.5. Research article IV

> "Characterization of a recently detected halogenated aminorex derivative: *para*-fluoro-4-methylaminorex (4′F-4-MAR)".
> *Scientific Reports, 2019; 9:8314.*

2.6. Discussion of the results obtained

2.7. Literature

2.1. Introduction

As it has been explained in the general introduction, the psychoactive substances market is in a continuous change. Approximately, novel NPS are reported to the EMCCDA at a rate one per week [1], so we can imagine the numerous NPS that can be found nowadays in the streets. This on-going change poses a challenge for the medical services that handle intoxication cases, politicians that try to legislate these compounds, and forensic and toxicological laboratories that analyse seizures and potential consumption products. The monitoring performed by analytical labs provide information about the compounds that are currently being consumed, as well as the detection of novel molecules not previously reported.

Different European projects, based on the analysis of consumption products for obtaining information about consumption patterns, have been developed in the last years. One of the most known projects is the Trans-European Drug Information project (TEDI) [2]. The TEDI project is a collaborative network of different European drug checking services that share the data obtained from the analysis of products, as well as their expertise in drug analysis, with the European monitoring system [2]. Although the TEDI project is focused on the monitoring of illicit drugs and their adulterants for harm reduction purposes, it is also working on reporting NPS across Europe, for evaluating the changes in the drug market tendencies [3]. Additionally, the TEDI project has also published a document with the main guidelines for drug checking using analytical techniques such as GC-MS, HPLC and NMR, if available [4].

Another important project for drug analysis is the European project RESPONSE, coordinated by the police service from Slovenia [5]. This project is focused on the analysis and characterisation of NPS in order to share analytical data that allow their identification by other forensic laboratories. The RESPONSE project has, by the date of writing this part of the thesis, analytical information about 755 compounds, including NPS, potential psychoactive compounds, or precursor compounds commonly used for the synthesis of NPS [6]. For almost all the

compounds included in the RESPONSE database, information about GC-EI-MS, FTIR and HPLC-ESI-TOF is provided, as well as 1H NMR for some compounds [6]. In the project webpage, there is also available a FTIR database that can be downloaded and used for compound identification by other analytical laboratories [5].

Regarding databases, different European forensic laboratories, coordinated by the University of Copenhagen, have developed a HRMS database (HighResNPS.com) with information of more than 1500 compounds. This suspect list can be downloaded and used in different HRMS systems and different data processing software [7]. The available accurate-mass fragmentation data is obtained from different sources, including the analysis of analytical reference standards, data from the EMCDDA, analysis of seized compounds, or reports from the RESPONSE project [7]. Another interesting database is the Cayman Spectral Database, which includes the GC-EI-MS spectra of hundreds of Cayman Chemical's emerging forensic drug standards [8]. This database can be downloaded as NIST format (National Institute of Standards and Technology), which is the most commonly used format for EI spectral databases, as well as Agilent MSD ChemStation format [8].

As explained in the general introduction, the most commonly used analytical techniques for drug testing in routine analysis are GC-MS and FTIR, and in some cases, LC-MS. Although the EI and FTIR databases allow the tentative identification of the compounds present in a consumption product based on experimental spectra, the analytical reference standards are mandatory for the unequivocal identification of their identity. Moreover, the high fragmentation obtained by EI can produce misidentifications, especially for similar compounds such as cathinones that differ in one moiety of the molecule. This fact poses a handicap for the identification of unreported compounds when using GC-MS, as the information about the molecular ion can be not available due to the EI fragmentation.

For that, LC-HRMS presents an alternative for the analysis of NPS in consumption products, as accurate-mass information about the (de)protonated molecule can be obtained thanks to the soft ionisation produced by ESI sources [9]. Moreover, the use of hybrid Q-HRMS instruments allow the acquisition of accurate-mass product ions, allowing the elucidation of unknown compounds and a more confident tentative identification for those compounds without analytical reference standard available. In spite of the high-quality information provided by these systems, the analytical reference standard is needed for a definite compound identification based on chromatographic retention time and spectral information.

At this point, the use of additional analytical techniques that provide structural information, is crucial for the final identification of NPS without the need of analytical reference standards. NMR is, probably, one of the most powerful techniques for obtaining structural information of organic compounds. The combination of HRMS and NMR for compound identification has proven its potential [10], and this approach has been widely used for the characterisation of novel NPS [11–13]. The limitation for using NMR is the purity of the compound, as this technique detects all the organic molecules present in the sample. It is suitable for the analysis of research chemicals, as they can be directly dissolved in deuterated solvents, but it is not adequate for the analysis of legal highs, such as pills and herbal blends, because potential compounds from the pill excipients and herbs can also be extracted together with the active compound.

Despite all the information provided by HRMS and NMR, in some specific cases, it could not be enough for an unequivocal identification. In these cases, the use of single-crystal X-ray diffraction provides accurate information about the compound structure, as well as the conformation of the molecule [14]. This technique has been used for the elucidation of some NPS such as synthetic cathinones [15,16].

During the development of the studies included in the present thesis, a collaboration with Energy Control (Barcelona, Spain), an NGO included in the TEDI project, was stablished for the analysis of some research chemicals. Energy Control usually performs the identification of NPS by GC-MS, and in some cases by LC-MS for confirmation. For problematic identifications, a small amount of sample was sent to our lab and analysed by UHPLC-HRMS (Xevo G2 QTOF, Waters) and NMR (Bruker Ascend 400MHz, Bruker Daltonics). Within this collaboration, more than 100 research chemical samples were analysed, performing the tentative identification based on the accurate-mass fragmentation observed in HRMS analyses, and confirming the structure by ^{1}H and ^{13}C NMR.

In some special cases, unreported compounds, and even completely unknown molecules, were found. Here, analysis by FTIR and single-crystal X-ray diffraction was also performed in order to obtain a complete characterisation of the compound. **Figure 2.1** shows the analytical workflow followed for the identification of NPS in research chemical samples.

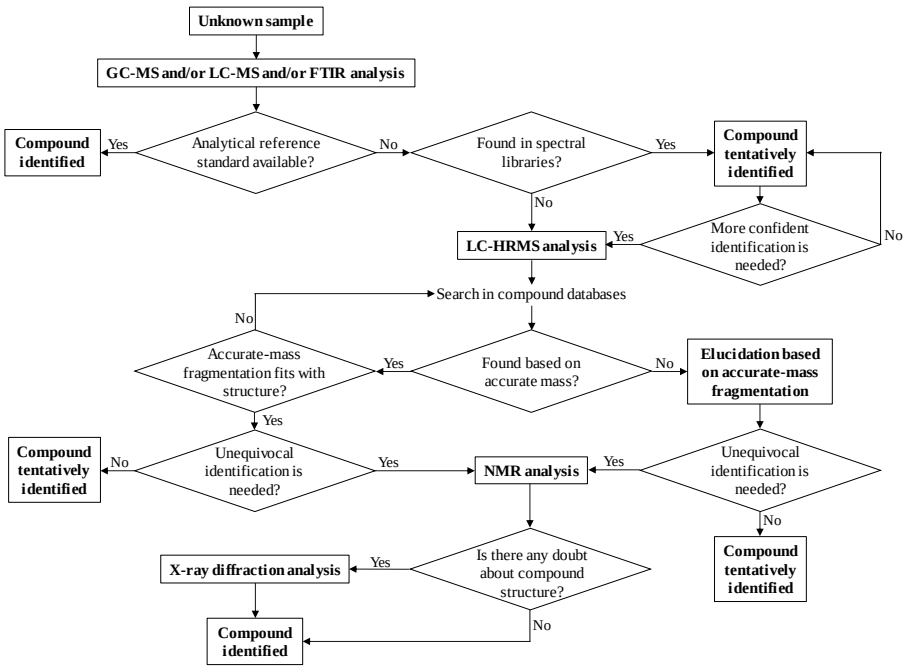


Figure 2.1. Analytical workflow for the analysis of research chemical samples in the collaborative work with Energy Control.

In this chapter, the main results of the collaborative work with Energy Control are presented and discussed, especially those works related with unknown or unreported novel compounds that were fully-characterised by the combination of different analytical techniques. The information presented in this chapter illustrates, on the one side, the benefits of combining different analytical techniques for compound identification and, on the other side, the continuous emergence of novel NPS derivatives that try to bypass the ban on certain compounds.

Research article I presents the complete elucidation of an unknown compound detected in a research chemical. The research article describes step-by-step the data interpretation for the elucidation of the unknown structure. **Research article II** shows the first characterisation of the novel synthetic opioid U-49900, found after the prohibition of the well-known U-47700. In this case, the origin, availability and psychoactive effects of this compound were also investigated through the reports of different drug users. In the **research article III**, the analytical characterisation of a novel synthetic cathinone containing an uncommon indanyl group was performed, trying also to find and discuss the reasons to synthetize this cathinone known as 5-PPDi. Finally, the **research article IV** presents the characterisation of a halogenated aminorex derivative, the 4'F-4-MAR. The identification of this aminorex derivative suggests that maybe these compounds could shortly appear again in the streets to replace the already banned cathinones 4-methylaminorex (or 4-MAR) and some derivatives.

Additionally, information about all the identified compounds during the collaboration with Energy Control, as well as those detected during the analysis of the legal highs sold in a local smartshop, is also included and discussed.

2.2. Research article I

Correspondence case reports

Drug Testing and Analysis

Received: 10 November 2016 Revised: 27 February 2017 Accepted: 28 February 2017 Published online in Wiley Online Library: 17 April 2017

(www.drugtestinganalysis.com) DOI 10.1002/dta.2182

Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain

D. Fabregat-Safont,[a] I. Fornís,[b] M. Ventura,[b] C. Gil,[b] N. Calzada,[b] J.V. Sancho,[a] F. Hernández[a] and M. Ibáñez[a]*

Introduction

The term 'new psychoactive substances' (NPS) refers to synthetically changed natural compounds or newly designed compounds intended to elicit a psychotropic response such as stimulation, hallucination or sedation.[1] According to the European Monitoring Centre for Drugs and Drug Addiction of the European Union, more than 560 NPS are currently being monitored, and 98 of these substance were reported for the first time in 2015.[2,3] Despite that, there are still many NPS that are not being monitored, and therefore their health effects are not yet studied. Hence, many cases of intoxication and death related to NPS have been reported in the past few years, highlighting the public health risks of these substances.[4] One of the challenges of monitoring NPS is the continuous structural evolution to evade regulation, so when one substance is banned or controlled, several new compounds replace it in the market.

NPS are usually found with various appearances, like incenses, bath salts, herbal blends or party pills, sold through an unregulated market, different websites or smart-shops.[4,5] These products are typically known as 'legal highs'. Synthetic cannabinoids, synthetic cathinones and amphetamines constitute the largest groups of NPS, although opioids, tryptamines, benzodiazepines, piperazines and phenethylamines are also common.[2,3] Synthetic cathinones and amphetamines are sold as replacements for stimulants, and have been reported in pills, crystal and sniff powder.[6] Synthetic cannabinoids are commonly sold as herbal blends or spices, replacing cannabis.[7]

Forensic laboratories, universities, research institutes, public health centres and law enforcement agencies play an important role in monitoring these types of substances, which are also encountered in customs seizures and medical emergencies.[8] Various analytical approaches have been reported for the analysis of NPS in legal highs samples. Gas chromatography coupled to mass spectrometry (GC–MS) using electron ionization (EI) is a fast and reliable technique for the identification of these compounds based on the use of spectral libraries.[9] However, fragmentation spectra of novel NPS may not be available. Moreover, some of these compounds are non-volatile and thermolabile, requiring additional derivatization steps in GC–MS analysis. Liquid chromatography (LC) coupled to high-resolution MS (HRMS) has proven to be a powerful technique for the screening of NPS in legal highs samples.[4,10–12] Moreover, HRMS allows screening analyses without reference standards being available (tentative identification).[11,13,14] HRMS/MS also has potential application in the compound structural elucidation of unknown substances by using accurate-mass fragmentation data.[15,16]

As complementary tools to HRMS instruments, additional spectroscopic techniques can be used for the unequivocal characterization of NPS. Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy are useful for a fast evaluation of molecules, identifying functional groups without destroying the sample and with reasonable running costs.[10] Nuclear magnetic resonance (NMR) spectroscopy has proved to be one of the most powerful techniques for the structural elucidation and characterization of organic molecules, and has been applied to the identification and characterization of synthetic cannabinoids[17] and synthetic cathinones.[18] X-ray crystallography, especially single-crystal X-ray diffraction, allows an unequivocal characterization of the structure of a molecule, but only if the compound can be formed as X-ray-suitable crystals. Thus, the combination of spectroscopic and mass spectrometric techniques provides a versatile workflow for structural elucidation, characterization and identification of unknown substances.[19]

The aim of the work reported here was the determination of the main compound present in a crystal sample from an anonymous Spanish consumer. The structure of a new designer drug derivate was elucidated after an exhaustive analysis using various analytical techniques. Characterization of the molecule was performed by GC–MS, LC-HRMS using hybrid quadrupole time-of-flight (QTOF) mass analyser, NMR and single-crystal X-ray diffraction. Additionally, melting point determination, FTIR and ultraviolet

* *Correspondence to: M. Ibáñez, Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12071 Castellón, Spain. E-mail: ibanezm@uji.es*

a *Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12071 Castellón, Spain*

b *Energy Control (Asociación Bienestar y Desarrollo), c/ Independencia 384, 08041 Barcelona, Spain*

Drug testing and Analysis, 2017; 9:1073

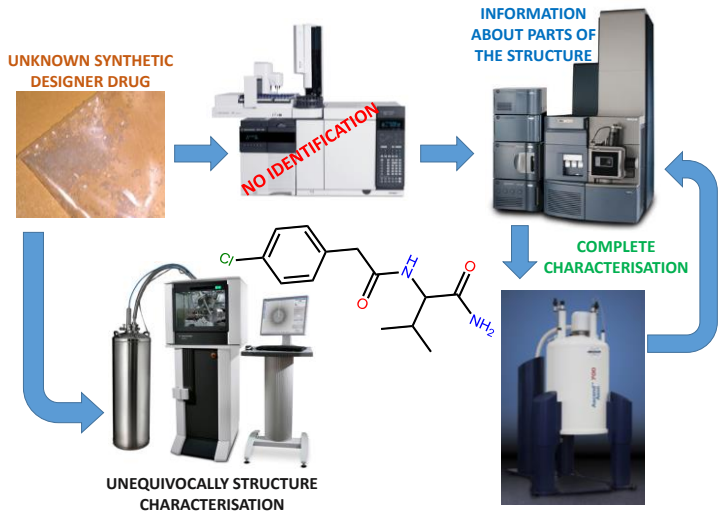
Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain

D. Fabregat-Safont [a], I. Fornís [b], M. Ventura [b], C. Gil [b], N. Calzada [b], J.V. Sancho [a], F. Hernández [a] and M. Ibáñez [a]*

[a] Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12071 Castellón, Spain.

[b] Energy Control (Asociación Bienestar y Desarrollo), c/ Independencia 384, 08041 Barcelona, Spain.

Graphical abstract



Keywords New psychoactive substances; 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide; High resolution mass spectrometry; Nuclear magnetic resonance spectroscopy; Single-crystal X-ray diffraction.

Introduction

The term "new psychoactive substances" (NPS) refers to synthetically-changed natural compounds or newly designed compounds intended to elicit a psychotropic response such as stimulation, hallucination or sedation [1]. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) of the European Union, more than 560 NPS are currently being monitored, and 98 of these substances were reported for the first time in 2015 [2,3]. Despite that, there are still many NPS that are not being monitored, and therefore their health effects are not yet studied. Hence, many cases of intoxication and death related to NPS have been reported in the past few years, highlighting the public health risks of these substances [4]. One of the challenges of monitoring NPS is the continuous structural evolution to evade regulation, so when one substance is banned or controlled, several new compounds replace it in the market.

NPS are usually found with different appearances, like incenses, bath salts, herbal blends or party pills, sold through an unregulated market, different websites or smart-shops [4,5]. These products are typically known as "legal highs". Synthetic cannabinoids, synthetic cathinones and amphetamines constitute the largest groups of NPS, although opioids, tryptamines, benzodiazepines, piperazines and phenethylamines are also common [2,3]. Synthetic cathinones and amphetamines are sold as replacement for stimulants, and have been reported in pills, crystal and sniff powder [6]. Synthetic cannabinoids are commonly sold as herbal blends or spices, replacing cannabis [7].

Forensic laboratories, universities, research institutes, public health centres and law enforcement agencies play an important role for monitoring this type of substances, which are also encountered in customs seizures and medical emergencies [8]. Different analytical approaches have been reported for the analysis of NPS in legal highs samples. Gas chromatography coupled to mass spectrometry (GC-MS) using electron ionization (EI) is a fast and reliable

technique for the identification of these compounds based on the use of spectral libraries [9]. However, fragmentation spectra of novel NPS may not be available. Moreover, some of these compounds are non-volatile and thermolabile, requiring additional derivatization steps in GC-MS analysis. Liquid chromatography (LC) coupled to high resolution MS (HRMS), has proven to be a powerful technique for the screening of NPS in legal highs samples [4,10–12]. Moreover, HRMS allows screening analyses without reference standards being available (tentative identification) [11,13,14]. HRMS/MS also has potential application in the compound structural elucidation of unknown substances by using the accurate-mass fragmentation data [15,16].

As complementary tools to HRMS instruments, additional spectroscopic techniques can be used for the unequivocal characterization of NPS. Fourier transform infrared (FTIR) spectroscopy or Raman spectroscopy are useful for a fast evaluation of the molecule, identifying functional groups without destroying the sample and with reasonable running costs [10]. Nuclear magnetic resonance (NMR) spectroscopy has proved to be one of the most powerful techniques for the structure elucidation and characterization of organic molecules, and has been applied to the identification and characterization of synthetic cannabinoids [17] and synthetic cathinones [18]. X-ray crystallography, especially single-crystal X-ray diffraction, allows an unequivocally characterization of the structure of the molecule, but only if the compound can be formed as X-ray suitable crystals. Thus, the combination of spectroscopic and mass spectrometric techniques provides a versatile workflow for structural elucidation, characterization and identification of unknown substances [19].

The aim of this work was the determination of the main compound present in a crystal sample from an anonymous Spanish consumer. The structure of a new designer drug derivate was elucidated after an exhaustive analysis using different analytical techniques. Characterization of the molecule was performed by GC-MS, LC-HRMS using hybrid quadrupole time-of-flight (QTOF) mass analyser, NMR and single-crystal X-ray diffraction crystallography. Additionally, melting

point determination, FTIR and ultraviolet (UV) analyses were performed. Current information reported in this paper will be useful for other laboratories in order to monitor the presence of this novel NPS or other related compounds in seizures and biological samples.

Experimental

Legal high sample

The sample, consisting of small crystals, was submitted by an anonymous consumer to Energy Control for analysis. According to the information provided by the consumer, it was purchased from the Internet, but no information was received about the consumption or psychoactive effects of this product. Energy Control is a project based on risks reduction, belonging to the Spanish non-governmental organization Asociación Bienestar y Desarrollo. Energy Control offers an anonymous drug testing service. Consumers can bring their samples to one of the 4 Energy Control headquarters (located in Madrid, Catalonia, Balearic Islands and Andalucia), send them by mail, or submit during outreach work in nightlife settings, such as music festivals, clubs, or underground raves. This service allows the monitoring of illegal drug markets, and the detection of trends in substances and their use, making this information available to all stakeholders. Additionally, analyses of both national and international samples received contribute to understanding of what is happening at the street level.

Reagents and chemicals

For GC-MS analysis, HPLC-grade methanol (MeOH) was purchased from Panreac (Panreac, Barcelona, Spain). For LC-HRMS analysis, HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), acetone, formic acid (HCOOH) and sodium hydroxide (NaOH) were purchased from Scharlau (Scharlab, Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA,

USA). Leucine enkephalin was acquired from Sigma-Aldrich (St. Louis, MO, USA). For NMR analysis, deuterated dimethyl sulfoxide (DMSO-d_6), deuterated chloroform ($CDCl_3$) and deuterated water (D_2O) were purchased from Sigma-Aldrich.

Sample treatment

For GC-MS analysis, 10 mg of sample were extracted with 10 mL of MeOH assisted with sonication during 15 min, due to the compound was soluble in MeOH. Finally, the extract was centrifuged to remove insoluble material and afterwards directly injected into GC-MS system.

For LC-HRMS analysis, 25 mg of sample were weighted in 2 mL propylene tubes and extracted with 1 mL of acetone in an ultrasonic bath for 15 min. Acetone has been successfully used for extracting NPS from legal highs samples [11,14]. After centrifugation at 12000 rpm during 12 min, the supernatant was 100-fold diluted with HPLC-grade water, and 10 µL of the extract were injected in the LC-HRMS system, using MS^E acquisition mode (see Instrumentation section for detailed information about this acquisition mode).

For NMR analysis, 10 mg of sample were extracted in 0.6 mL of DMSO-d_6. An additional sample treatment was performed by addition of D_2O to the DMSO-d_6 extract, in order to avoid amide signals in the ^1H NMR experiment. Additionally, 10 mg of sample were treated by 0.6 mL of $CDCl_3$ in order to study diastereotopic hydrogens.

For single-crystal X-ray diffraction, an adequate needle-shaped crystal was selected from the sample.

For FTIR analysis, sample was prepared with 5% of the unknown compound and 95% of potassium bromide, homogenized in an agate mortar and compressed under a pressure of 5000 kg/cm^2.

UV analysis was performed using the extract prepared for LC-HRMS analysis.

Instrumentation

For GC-MS analysis, an Agilent 7890B gas chromatograph was coupled to a 5977A quadrupole mass spectrometer detector (Agilent; Santa Clara, CA, USA). The gas chromatograph was fitted with a G4513A auto-sampler injector. Insert liners packed with silanized glasswool were used, and the injector and the interface were operated at 280 ºC. 1 µL of sample was injected in split mode, with a split ratio 1:10, into a 30 m 0.25 mm i.d., 0.25 µm film thickness 5% phenylmethylsilicone column (HP-5MS, Agilent Technologies). Helium was used as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially maintained at 90 ºC for 2 min and programmed to reach 320 ºC at 20 ºC per min. It was finally maintained at 320 ºC for 9.5 min (total run time was 21.5 min). The mass spectrometer was operated in electron ionization (EI) mode at 70 eV. MS system worked in SCAN acquisition mode, acquiring from *m/z* 40 to 400 Da. In order to identify the unknown compound, fragmentation spectrum was compared with four spectral libraries: the Searchable Mass Spectral Library NIST/EPA/NIH Mass Spectral Library, Data Version: NIST 14; Searchable Mass Spectral Library Version 2.3 (http://www.swgdrug.org/ms.htm), Searchable Mass Spectral Library Cayman Spectral Library (CSL) (https://www.caymanchem.com/app/template/SpectralLibrary.vm) and Energy Control's internal mass spectral library. Analytical data were acquired and processed using MassHunter B.06.00 (Agilent) operation software.

LC-HRMS analysis was performed using an ACQUITY UPLC ultra-high performance liquid chromatography (UHPLC) system (Waters, Mildford, MA, USA) coupled to a XEVO G2 QTOF hybrid quadrupole time-of-flight (QTOF) mass spectrometer (Waters Micromass, Manchester, UK) with an orthogonal Z-spray electrospray ionization (ESI) interface operating in positive ionization mode. The chromatographic separation was performed using a Cortecs C18 (Waters) 2.7 µm particle size analytical column 100 x 2.1 mm at a flow rate of 0.3 mL/min. The column temperature was set to 40 ºC. The mobile phases used were H2O with 0.01% HCOOH (A) and MeOH with 0.01% HCOOH (B). The

mobile phase gradient was performed as follows: 10% of B at 0 min, 90% of B at 14 min linearly increased, 90% of B at 16 min, and finally 10% B at 18 min in order to return to initial conditions. The injection volume was 10 µL. Nitrogen (Praxair, Valencia, Spain) was used as desolvation and nebulizing gas. The desolvation gas flow was set at 1000 L/h. The TOF resolution was ~20000 at FWHM at *m/z* 556. The range acquired by the MS system was from *m/z* 50 to 1000. A capillary voltage of 0.7 kV and a cone voltage of 10 V were used during all the chromatographic run. Argon 99.995% (Praxair, Valencia, Spain) was used as a collision gas. The interface temperature was set to 650 ºC and the source temperature to 120 ºC. For MS^E experiments, two acquisition functions with different collision energy were created. The low energy function (LE) used a collision energy of 4 eV in order to obtain information about the protonated molecule and adducts (if present), while the high energy function (HE) applied a collision energy ramp from 15 to 40 eV, in order to promote fragmentation of the compounds. For further details, please see the references [14]. Calibration of the mass-axis was performed daily from *m/z* 50 to 1000 Da using a 1:1 mixture of 0.05 M NaOH:5% HCOOH, diluted 1:25 with ACN:H2O 80:20 mixture. For accurate mass measurement, a 2 µg/mL leucine enkephalin solution in ACN:H2O 50:50 with 0.1% HCOOH was used as lock-mass, pumped at a flow rate of 20 µL/min. The leucine enkephalin protonated molecule (*m/z* 556.2771) was used for recalibrating the mass axis and ensure an accurate mass during all the chromatographic run. MS data were acquired in centroid mode using MassLynx data station operation software, version 4.1 (Waters).

NMR analyses were performed using a Varian VNMRS 500 MHz spectrometer at 303 K using DMSO-d_6 (Varian Medical Systems, Palo Alto, CA, USA). The residual solvents signals at δ = 2.50 ppm for ^{1}H (DMSO) and at δ = 39.51 ppm for ^{13}C (DMSO-d_6) were used as internal references. Characterization of the compound was performed using 5 experiments: ^{1}H NMR, ^{13}C NMR, correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC). NMR experiment data were

collected using the Varian Vnmr 2.2c software (Varian Medical Systems, Palo Alto, CA, USA).

For single-crystal X-ray diffraction crystallography, an Agilent SuperNova diffractometer (Agilent Technologies) was used. The diffractometer was equipped with an Atlas CCD detector (Agilent Technologies), and CuKα radiation (λ = 1.54184 Å) was used. Sample was kept at 199.95 K during data collection. Experimental data was acquired using the SHELXS-2013 software (Yale University, New Haven, CT, USA), using the OLEX software package (Olex AS, Trondheim, Norway).

For FTIR analysis, a Jasco FT/IR-6200 FTIR spectrometer (Jasco Inc., Easton, MD, USA) was used. Data acquisition was performed at 23 ºC between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1} and performing 32 acquisitions.

UV analysis was performed using a Waters Alliance 2795 LC system (Waters, Milford, MA, USA) equipped with a Waters 2998 Photodiode Array Detector (Waters). The LC separation was performed using an Atlantis C18 column (5µm, 2.1 x 50 mm; Waters) at a flow rate of 0.2 mL/min. The isocratic mobile phase used was a mixture of H2O:ACN 70:30. The injection volume was 20 µL. UV acquisition was performed between 200 and 500 nm.

Melting point range was determined using a Techne Stuart digital melting point apparatus (Stuart, Stone, Staffordshire, UK) using open capillary tubes

Results and discussion

The complete analysis of the experimental data obtained by LC-HRMS and NMR could be found in Supplementary Information (Experimental data analysis).

Gas chromatography-mass spectrometry

Initially, the sample was analysed by GC-MS. The Total Ion Chromatogram (TIC) showed an intense and single peak at 7.20 min, showing that the sample

contained a highly pure compound. The application of different spectral libraries did not retrieve results. At this point, the interpretation of fragmentation in EI spectrum was performed. The EI spectrum of the chromatographic peak at 7.20 min showed four intense m/z ions (**Figure 1**). Ions at *m/z* 224 and 125 presented the characteristic $^{35}Cl/^{37}Cl$ isotopic pattern, which indicates the presence of a chlorine atom in the structure. Additional fragment ions were observed at *m/z* 44, 72 and 89, the fragment ion at *m/z* 72 being the base peak. Ions above *m/z* 224 were also observed. However, it was impossible to determine if these ions came or not from the compound due to their low intensity. Ion at *m/z* 224 could correspond to the molecular ion of the compound; however, due to the high fragmentation obtained under EI, this could not be confirmed. Thus, additional analytical techniques were necessary for the elucidation of the structure.

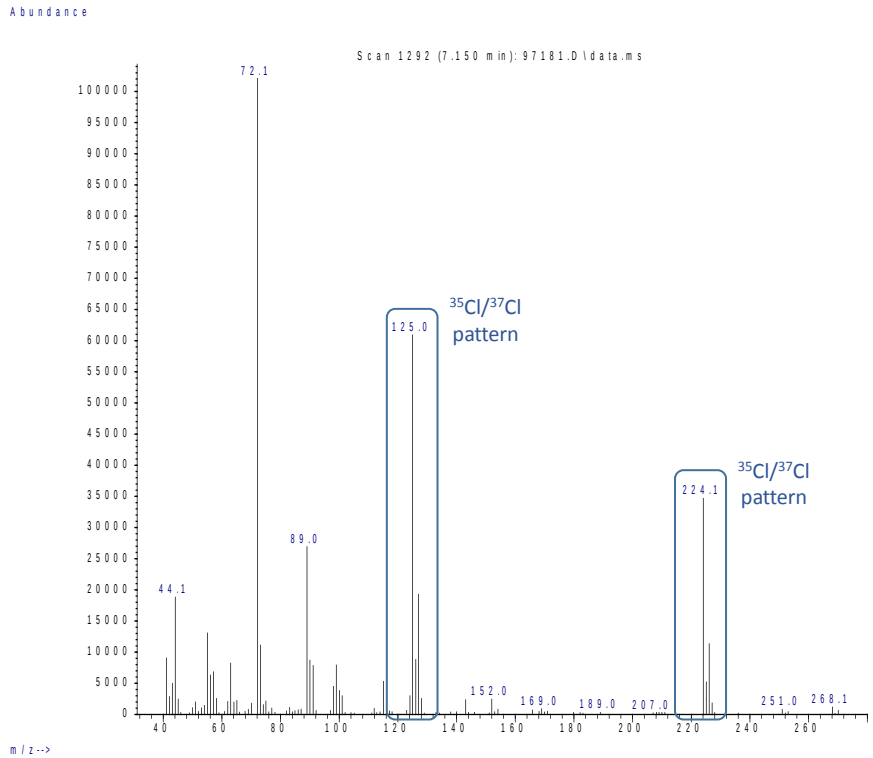


Figure 1. EI mass spectrum of the unknown compound.

Liquid chromatography-high resolution mass spectrometry

Figure 2 (left) shows the LE and HE spectra of the chromatographic peak at 8.17 min, corresponding to the unknown compound. Three ions were observed in the LE function at *m/z* 224.0835, 252.0786 and 269.1053. The use of a soft ionization technique showed that ions at *m/z* 224 and *m/z* 252 were in-source fragments, demonstrating that the protonated molecule was the ion at *m/z* 269. The use of UHPLC provides narrow chromatographic peaks, which are very useful for determining if a fragment ion comes from a selected protonated molecule (**Figure 2**, right). The elemental composition of the $[M+H]^+$ ion was calculated to be $C_{13}H_{18}ClN_2O_2^+$, with a mass error of -1.5 ppm (*m/z* 269.1053). Other feasible molecular formulas were rejected due to the associated high mass error. The elemental composition of the two in-source fragments were also obtained ($C_{13}H_{15}ClNO_2^+$ for the fragment at *m/z* 252.0786, and $C_{12}H_{15}ClNO^+$ for *m/z* 224.0835), corresponding to the loss of an ammonia molecule, followed by carbon monoxide loss. These fragments might indicate the presence of a terminal amide group loss. The characteristic $^{35}Cl/^{37}Cl$ isotopic pattern was also observed for the three ions.

Regarding HE spectrum, three collision-induced fragments were observed at *m/z* 125.0151, 89.0386 and 72.0803. After calculating their elemental composition, the presence of a chlorophenyl group bonded to a methylene group, known as chlorotropylium (fragment at *m/z* 125.0151, $C_7H_6Cl^+$) was supposed. The fragment at *m/z* 89.0386 ($C_7H_5^+$) would therefore correspond to the loss of HCl molecule from the chlorotropylium ion (*m/z* 125). Finally, the fragment at *m/z* 72.0803 corresponded to an amine functionalized with four carbon atoms ($C_4H_{10}N^+$).

In order to enhance the confidence on the results obtained, MS/MS experiments at 10, 30 and 40 eV collision energy were additionally performed, showing the same fragmentation as the observed after performing MS^E acquisition (**Figure S1**).

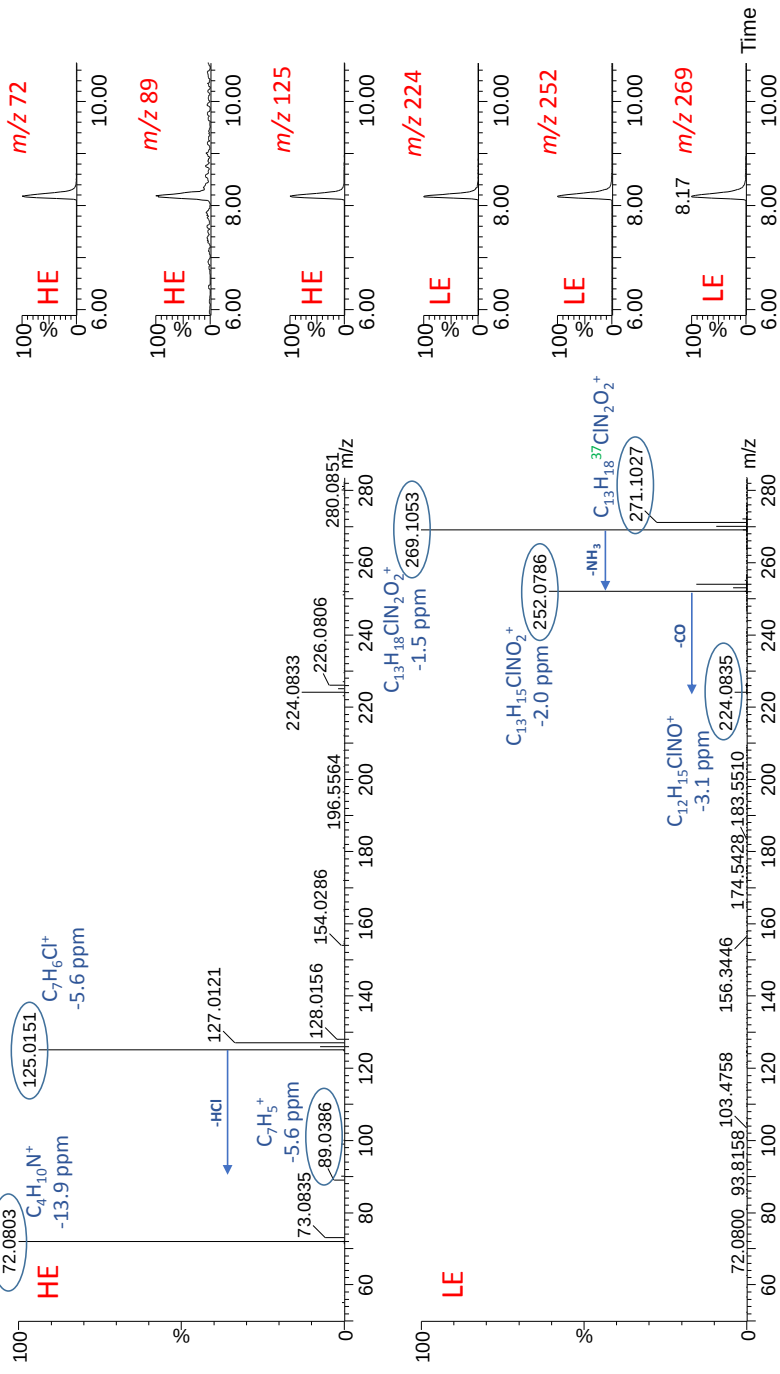


Figure 2. MS^E spectra of the unknown compound. **Right** Extracted ion chromatograms (EIC) (0.02 Da mass window) for protonated molecule and fragment ions in LE and HE functions. **Left** LE (bottom) and HE (top) spectra of the unknown compound.

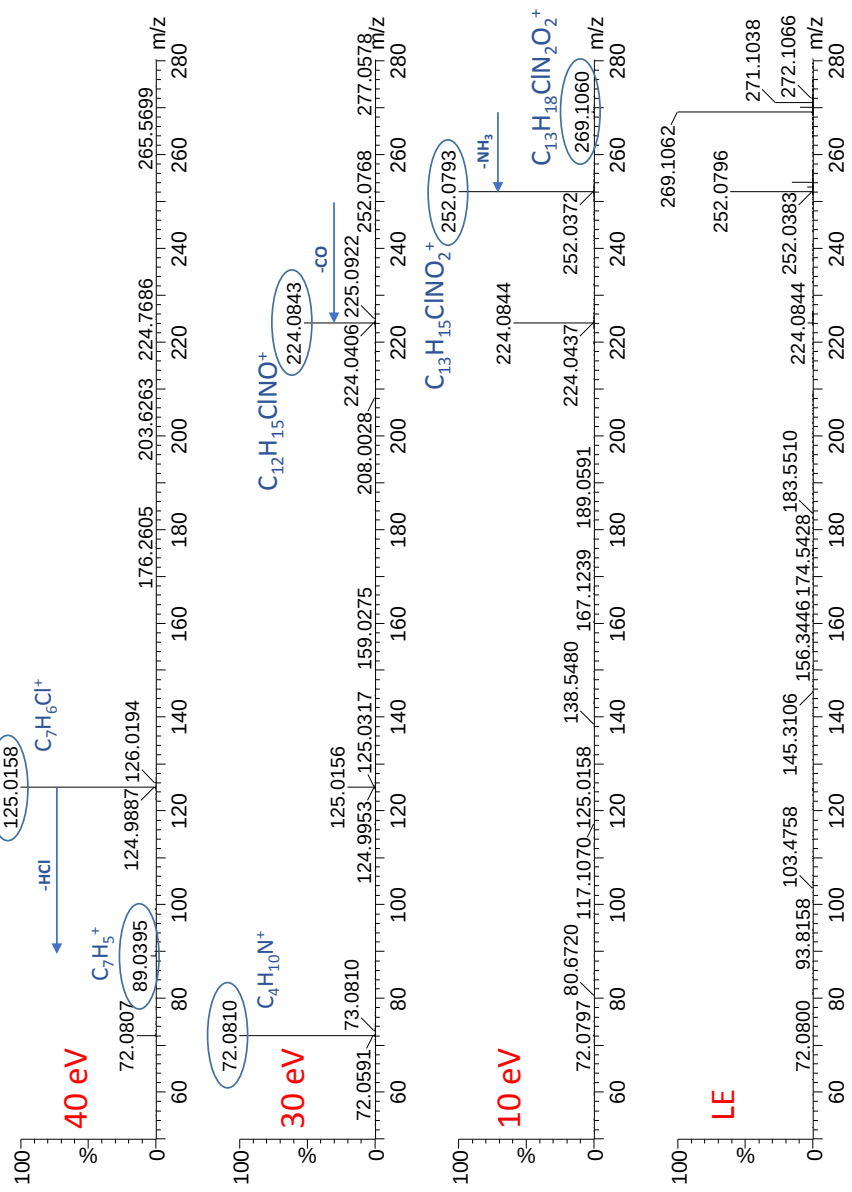


Figure S1. MS/MS (CID) spectra of the unknown compound at 10, 30 and 40 eV.

LC-HRMS data analysis determined the presence of a terminal amide and a chlorotropylium group. Nevertheless, additional spectroscopic techniques were required for the complete identification and characterization of the molecule.

Nuclear magnetic resonance

Different NMR experiments were carried out. First, ^{1}H NMR spectrum (500 MHz, DMSO-d_6) was performed. Three broad signals corresponding to amine protons (related to terminal amide group predicted during LC-HRMS analysis) were observed. In order to remove these signals and obtain a cleaner spectrum, deuterated water was added to the extract and ^{1}H NMR spectrum (500 MHz, DMSO-d_6 and D_2O) was acquired again (**Figure S2** top). The addition of D_2O removed amide and amine ^{1}H NMR signals produced by proton transfer between amine/amide hydrogens and solvent deuterium. The ^{1}H NMR signal analysis determined the presence of a methyl group bonded to a CH group, and the presence of aromatic hydrogens. Considering the fragment at *m/z* 125, corresponding to a chlorotropylium fragment, substituents of the phenyl ring could be a chlorine atom and a methylene group. The other ^{1}H NMR signals could not be assigned until the complete elucidation of the molecule.

The ^{1}H NMR signal interpretation was performed as follows. A doublet at δ = 0.77 ppm indicated the presence of a methyl group in the molecule bonded to a CH group. Another representative signal was at δ = 7.25 ppm, corresponding to aromatic hydrogens. The presence of the pair of doublets indicated that the benzene ring was para-substituted. Considering the presence of a chlorotropylium moiety, these substituents could be a chlorine atom and a methylene group. Other unassigned signals were a multiplet at δ = 1.92 ppm, a double doublet at δ = 3.45 ppm and a doublet at δ = 3.97 ppm.

Secondly, ^{13}C NMR spectrum (500 MHz, DMSO-d_6 and D2O) was acquired to estimate the functional groups of the molecule based on the type of carbon atom (**Figure S2** bottom). Two methyl groups were identified, related to the methyl ^{1}H NMR signals. Three CH_2 or CH groups were also identified, and based on the chemical shift, one of them could be bonded to an oxygen or nitrogen atom. These three CH2/CH groups corresponded to the three unassigned signals in ^{1}H NMR spectrum. Aromatic carbon signals were also identified according to the four

aromatic signals obtained. Two of these carbons would be functionalized according to the chemical shift observed in ^{13}C NMR, indicating the presence of a quaternary assignment. These aromatic signals were in accordance with the aromatic hydrogens detected in ^{1}H NMR spectrum and the chlorotropylium fragment (*m/z* 125) observed during LC-HRMS analysis. The last two additional signals corresponded to amide or ester carbons. As previously commented, LC-HRMS analysis determined the presence of a terminal amide group. Considering that, the molecular formula indicated the presence of two oxygen atoms and two nitrogen atoms, and based on the ^{13}C NMR spectrum, the presence of a second amide group in the molecule was undeniable.

^{13}C NMR signal interpretation was performed as follows. Signals at δ = 22.87 and 24.15 ppm corresponded to methyl groups, which could be related to the methyl doublet signal in ^{1}H NMR spectrum at δ = 0.77 ppm. Signals at δ = 35.1 and 46.32 ppm corresponded to CH_2/CH groups, while signal at δ = 63.09 ppm could be a methylene group bonded to an oxygen or nitrogen atom, based on its chemical shift. These CH_2/CH groups could be related to the three unassigned signals in ^{1}H-NMR spectrum. Signals around δ = 135 ppm corresponded to aromatic carbons: four non-functionalised (δ = 133.36 and 135.98 ppm), a methylene functionalised carbon (δ = 136.52 ppm) and a chlorine functionalised carbon (δ = 140.01 ppm). These aromatic signals corresponded to the aromatic hydrogens detected in ^{1}H NMR spectrum and the chlorotropylium ion (*m/z* 125). The last two signals at δ = 176.20 and 179.05 ppm corresponded to amide or ester carbons. As previously commented, LC-HRMS analysis determined the presence of a terminal amide group. So, it was undeniable the presence of a second amide group in the molecule.

The methylene bond between the chlorophenyl and the amide group presented a double doublet signal as seen before, as these hydrogens were diastereotopic. Diastereotopic hydrogens are produced by different surrounding electron density for each hydrogen atom bonded to the same carbon.

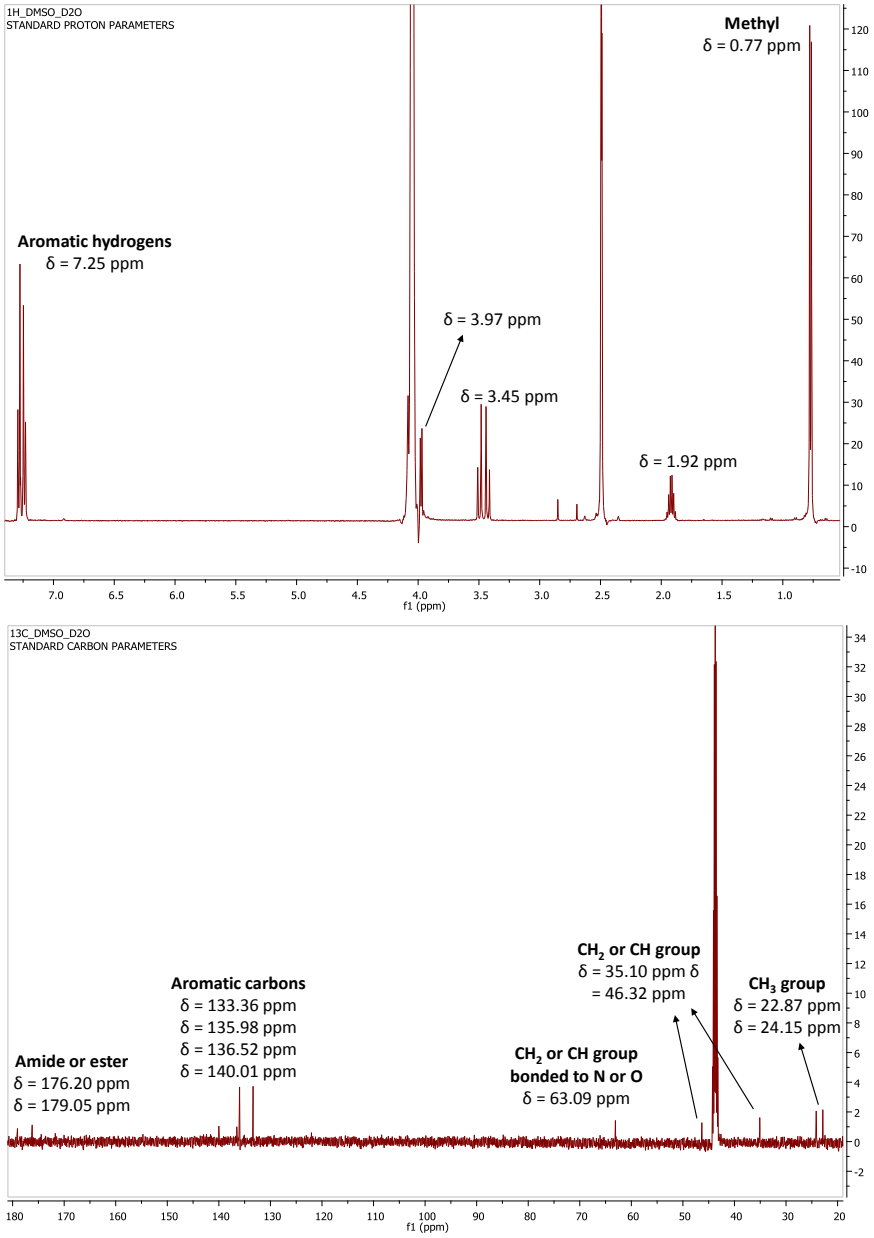


Figure S2. NMR spectra of the unknown substance. **Top** ^{1}H NMR spectrum. **Bottom** ^{13}C NMR spectrum.

For this compound, the difference in the surrounding electron density might be produced by the formation of intramolecular hydrogen bonds, which would block the free-rotation of the methylene bond. While rotation is blocked, each hydrogen

could be near to different parts of the molecule. Thus, the presence of an electronegative group near one of the hydrogens would affect its surrounding electron density. This difference causes that these hydrogens produce different resonances in ^{1}H NMR and so, they are unequivalent for NMR analysis and an interaction between them is produced, giving a double of doublets. In some occasions, the solvent could affect diastereotopic hydrogens [29]. In order to evaluate the solvent effect, an additional ^{1}H NMR spectrum (500 MHz, $CDCl_3$) was acquired, using deuterated dichloromethane. **Figure S3** shows ^{1}H NMR signals for the methylene bond hydrogens using DMSO-*d*$_6$ (**bottom**) and using $CDCl_3$ (**top**). It can be observed that deuterated chloroform produced lower diastereotopicity than deuterated dimethyl sulfoxide, and NMR signals in $CDCl_3$ did not show a clear double doublet, looking more as a doublet. No further information was found to explain this behaviour, but it is possible that the presence of a sulfoxide group could promote the formation of hydrogen bonds between the molecules, blocking the free-rotation of the methylene bond.

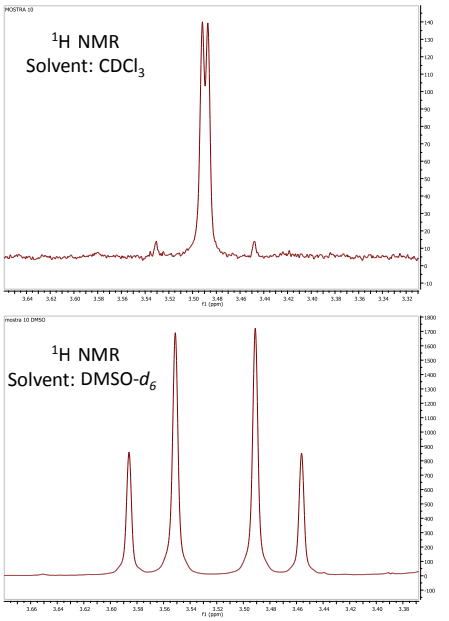


Figure S3. ^{1}H NMR signal for diastereotopic hydrogens in C7. **Top** Signal obtained when using $CDCl_3$ for dissolving the sample. **Bottom** Signal obtained when using DMSO-*d*$_6$ for dissolving the sample.

^{1}H NMR and ^{13}C NMR experiments did not allow the complete characterization of the molecule, so additional NMR experiments were performed. COSY is a two-dimensional NMR spectrum (2D NMR) that allows the determination of ^{1}H correlations present in the compound (which is a homonuclear through-bond correlation method). COSY can be complemented with HSQC experiments (heteronuclear through-bond correlation method), which match ^{1}H NMR and ^{13}C NMR signals, differentiating CH_3/CH groups (in red and yellow spots) from CH_2 groups (blue spots). The combination of COSY and HSQC (both spectra were acquired at 500 MHz, DMSO-d_6 and D_2O) provides enough information for the tentative identification of this compound [19]. **Figure 3** shows COSY and HSQC for the sample, identifying NMR signals based on the structure of the compound. COSY (**Figure 3**, top) shows the correlation between the hydrogens of the molecule, while HSQC (**Figure 3**, bottom) indicates which proton corresponds to each carbon signal. The correlation between both methyl groups (C1 and C2, doublet at δ = 0.77 ppm in ^{1}H NMR) and two CH groups (C3 and C4, multiplet at δ = 1.92 ppm and doublet at δ = 3.97 ppm in ^{1}H NMR, respectively), suggested the presence of an isopropyl group bonded to another CH, according to C4 ^{1}H NMR signal multiplicity (Figure 3, top). The chemical shift of C4 (δ = 63.09 ppm in ^{13}C NMR, **Figure 3**, bottom) indicated that this carbon was bonded to a nitrogen or oxygen atom. Regarding LC-HRMS analysis, the fragment at *m/z* 72 corresponded to an amine bonded to four carbon atoms. This would be in concordance with the structure composed of a CH group (C4) bonded to a nitrogen atom and an isopropyl group (C1, C2 and C3), predicted in NMR experiments. The combination of the information obtained using NMR and LC-HRMS could establish that the fragment at *m/z* 72 corresponded to a 2-methylpropanamine group, which in combination with fragments at *m/z* 72, 224, 252 and 269) (see **Figure 2**) and the presence of two amide groups determined by NMR (see **Figure 3**), suggested that the molecule presented an *N*-isobutylacetamide group.

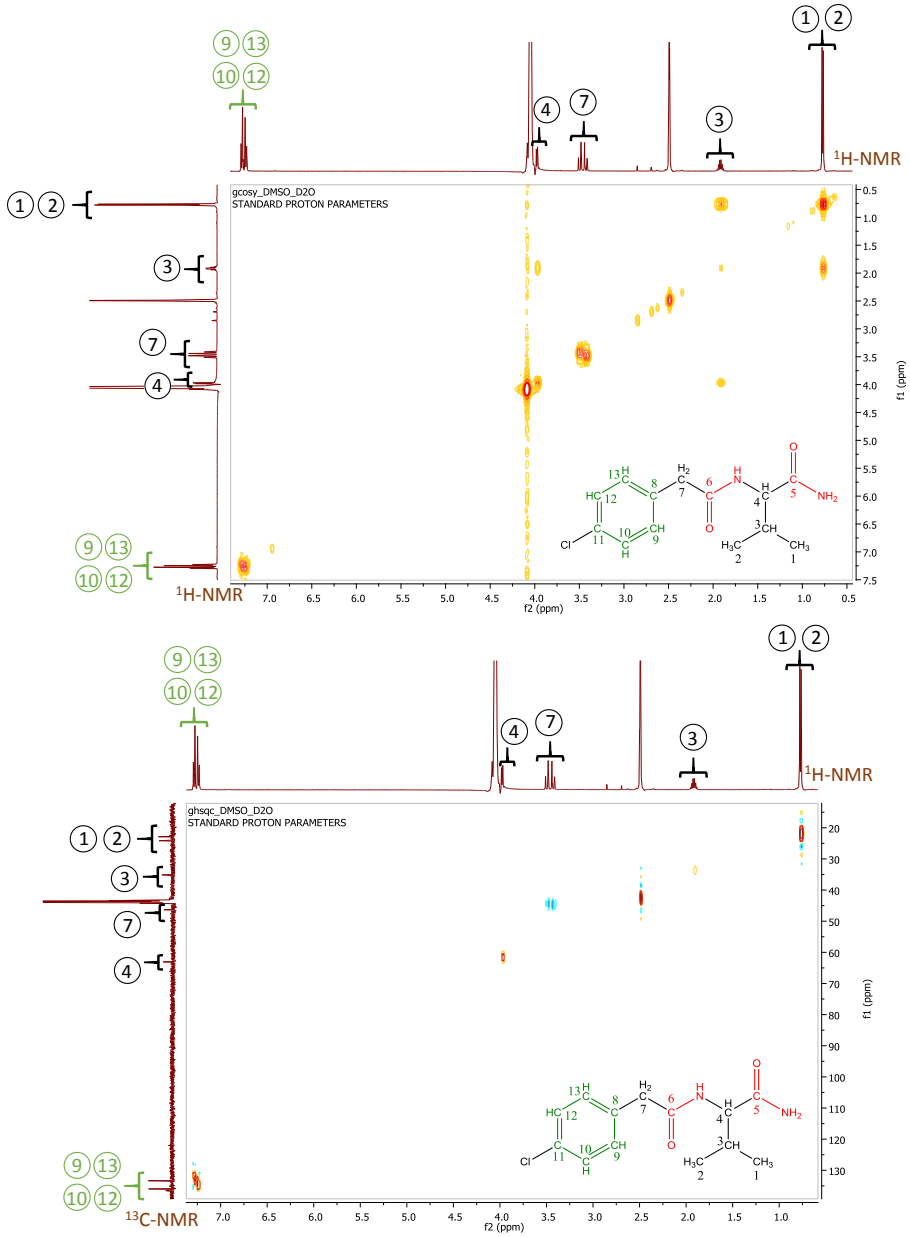


Figure 3. 2D NMR spectra of the unknown substance. **Top** COSY spectrum, showing the correlation between hydrogens of the molecule. **Bottom** HSQC spectrum, linking ^{1}H and ^{13}C NMR signals. CH_3 and CH groups appear as red and yellow spots, and CH_2 groups as blue spots.

The double of doublets at δ = 3.45 ppm in ^{1}H NMR corresponded to a methylene bond between the amide and the chlorophenyl group, because no proton correlations were observed in COSY spectrum. The multiplicity of this signal indicates that the two hydrogens in the CH_2 group were diastereotopic, therefore these hydrogens had different surrounding electron density. Finally, the other substituent of the CH group linked to the amide, was bonded to the carbonyl of the terminal amide group present in the molecule (regarding that the presence of this group was predicted during LC-HRMS analysis).

After this thorough interpretation of both 2D NMR spectra, the compound could be identified as 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, a putative new psychoactive substance.

After compound identification, NMR signals were assigned. ^{1}H and ^{13}C NMR signal assignment can be found in **Table S1**. In order to enhance the confidence in the structure, HMBC (a heteronuclear through-bond correlation method) 2D NMR experiment was performed. In this experiment, correlations over 2-4 bonds were observed, confirming the identity of the compound. HMBC spectrum can be found in Supplementary Information (**Figure S4**).

The characterized compound has a valinamide group (*N*-(1-carbamoyl-2-methylprop-1-yl) group), present in some third-generation synthetic cannabinoids such as 5F-AB-PINACA, AB-CHMFUPPYCA, AB-CHMINACA, AB-FUBINACA or 5F-AB-FUPPYCA [20–22]. However, the valinamide group can be found in vast diversity of compounds. Therefore, the presence of this moiety in the compound under discussion is not enough to assume similarity with synthetic cannabinoids as no information about its psychoactive effects is available. Once identified, the fragmentation pathway of the new compound obtained by LC-HRMS was proposed (**Figure 4**).

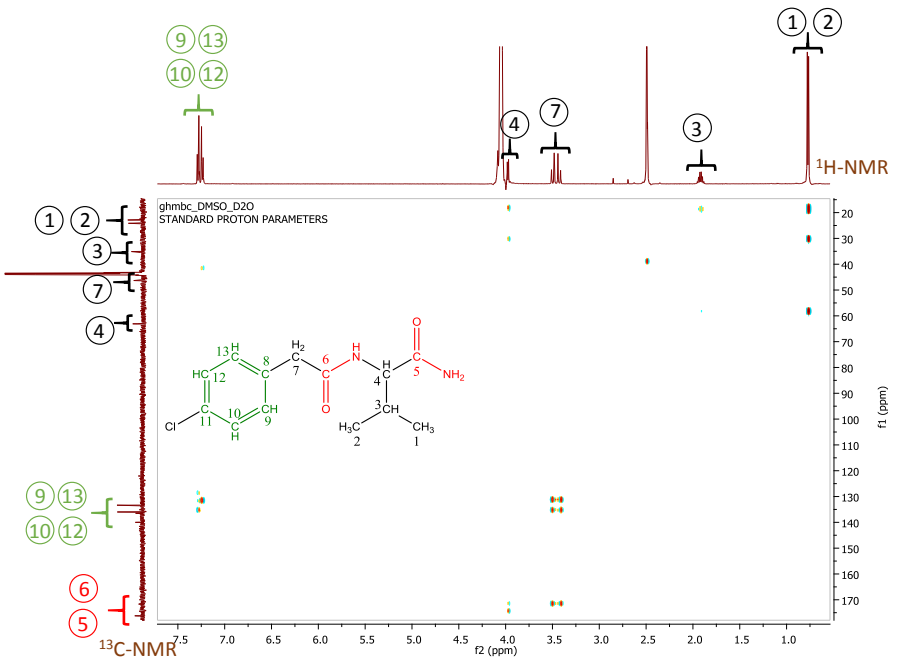


Figure S4. HMBC spectrum of the unknown compound. Correlations over 2-4 bonds are showed between ^{1}H and ^{13}C NMR signals.

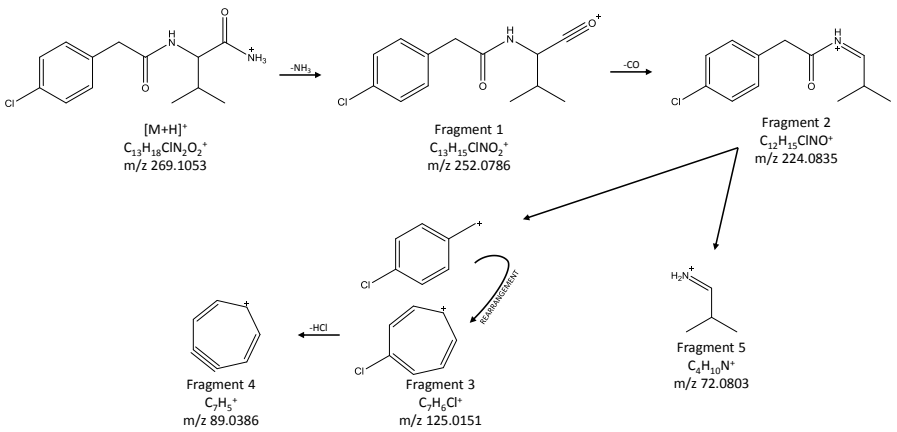
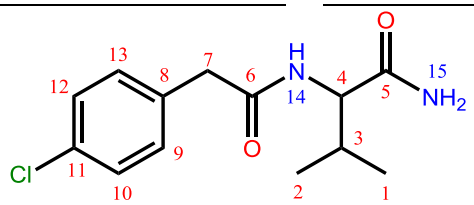


Figure 4. Proposed collision induced dissociation (CID) fragmentation pathway for the 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide.

Table S1. ^{1}H and ^{13}C NMR signal assignment.

1H NMR signal assignment			**13C NMR signal assignment**	
Number of H atom	**δ (ppm)**	**Multiplicity**	**Number of C atom**	**δ (ppm)**
1	0.77	Doublet	1	22.87
2	0.77	Doublet	2	24.15
3	1.92	Multiplet	3	35.10
4	3.97	Doublet	4	63.09
5	-	-	5	176.20
6	-	-	6	179.05
7	3.45	Double doublet	7	46.32
8	-	-	8	136.52
9	7.24	Doublet	9	133.36
10	7.28	Doublet	10	135.98
11	-	-	11	140.01
12	7.28	Doublet	12	135.98
13	7.24	Doublet	13	133.36



Single-crystal X-ray diffraction crystallography

In order to unequivocally confirm the structure of the compound, single-crystal X-ray diffraction analysis was also performed. Using OLEX2 software package [23], the structure was solved by using Superflip structure solution program [24] for charge-flipping methods and refined by the full-matrix least-squares method using ShelXL refinement package [25], applying multi-scan method to perform absorption corrections. **Table 1** lists collected data and refinement parameters.

Table 1. Crystallographic data for the characterized compound.

Parameter	**Data**
Empirical formula	$C_{13}H_{17}ClN_2O_2$
Formula weight	268.74
Temperature (K)	199.95(10)
Crystal system	Monoclinic
Space group	$P2_1$
Unit cell dimensions	
a, Å	4.81884(8)
b, Å	22.1502(2)
c, Å	6.83798(9)
α ≡	90.00
β ≡	108.6266(15)
γ ≡	90.00
Volume ($Å^3$)	691.643(16)
Z	2
ρ_{calc} (mg/mm^3)	1.290
Absorption coefficient μ (mm^{-1})	2.421
F(000)	284.0
Crystal size (mm^3)	0.368 x 0.146 x 0.083
2Θ range for data collection (º)	7.98 to 133.18
Index ranges	$-5 \leq h \leq 5$
	$-26 \leq k \leq 26$
	$-8 \leq l \leq 8$
Reflections collected	12265
Independent reflections	2448 [R(int) = 0.0199]
Absorption correction	Multi-scan
Refinement method	Full-matrix least-squares on F^2
Data/restrains/parameters	2448/1/170
Goodness of fit on F^2	1.070

Table 1. Crystallographic data for the characterized compound (continuation).

Final R indices [I > 2σ(I)]	R_1 = 0.0318
	wR_2 = 0.0804
R indices (all data)	R_1 = 0.0320
	wR_2 = 0.0807
Largest difference in peak/hole ($e \cdot A^{-3}$)	0.35/-0.44
Flack parameter	0.003(14)

Crystals suitable for single-crystal X-ray diffraction experiments were selected directly from the sample (**Figure 5**). The compound structure was refined in the monoclinic space group P21, with the following unit cell dimensions: a = 4.81884(8) Å, b = 22.1502(2) Å, c = 6.83798(9) Å, α = γ = 90.00º and β = 108.6266(15)º. **Figure 6** shows the structure predicted after performing the refinement. The flack parameter was 0.003, which indicates that this compound was crystallized with a high-enantiomeric purity of one of the plausible isomers. Thus, the unknown compound was unequivocally confirmed to be 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide that, to our knowledge, has not been registered in the IUPAC and CAS database.

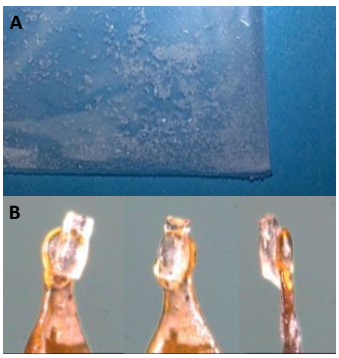


Figure 5. A Bulk sample containing the new characterized compound. Small needle-shaped crystals can be observed. **B** Single crystal selected from sample observed through X-ray diffractometer.

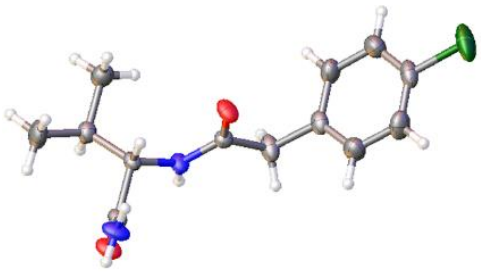


Figure 6. Predicted structure after processing single-crystal X-ray diffraction data. Grey spheres represent carbon atoms; white spheres, hydrogen atoms; red spheres, oxygen atoms; blue spheres, nitrogen atoms; and the green sphere represents the chlorine atom.

In order to facilitate data sharing, X-ray diffraction data and structure refinement were checked and included in the Cambridge Crystallographic Data Centre (CCDC). Supplementary crystallographic data for this compound can be found in the CCDC 1522776 file free of charge available at CCDC webpage, www.ccdc.cam.ac.uk/data_request/cif.

Additional characterization

In order to complete the characterization of the compound, the melting point range was determined and stablished between 223 and 225 ºC. FTIR and UV absorption spectra and instrumental conditions can also be found in **Figures S5** and **S6**, respectively.

Conclusions

In this work, a putative new psychoactive substance identified as 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide ($C_{13}H_{17}ClN_2O_2$) was characterized. The complete identification of the compound required the combination of different analytical techniques, such as GC-MS, LC-HRMS, NMR and X-ray crystallography. The fragmentation pathway of this compound in LC-HRMS has also been proposed, in order to make easier the future identification of related compounds by common fragmentation analysis. Unfortunately, the psychoactive effects and toxicity have not been evaluated yet. The strategy applied in this work has proven to be a powerful workflow for the identification and characterization of novel NPS. The information obtained about this new compound will be useful for forensic laboratories, toxicological studies or to enhance the Early Warning Systems.

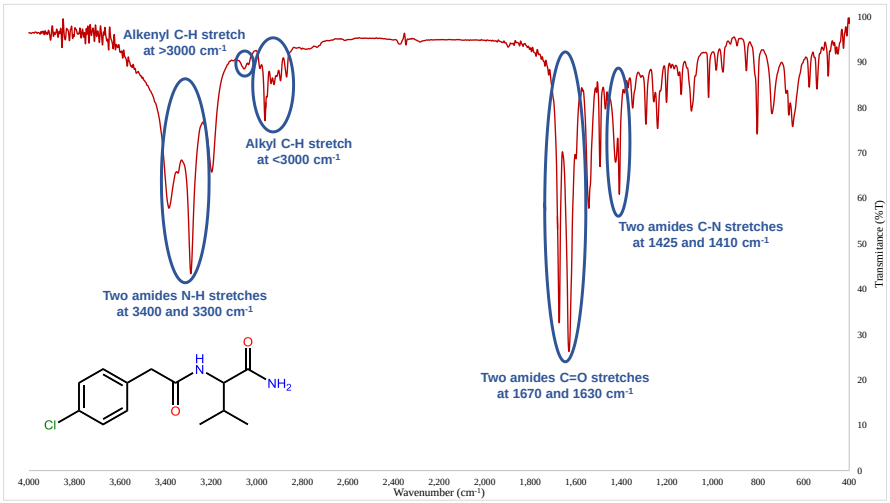


Figure S5. FTIR spectrum of the characterized compound. Carbonyl bands of amide groups are observed at 1600-1700 cm^{-1}. Aromatic bands are observed up to 3000 cm^{-1}. Amine bands of amide groups are observed around 3300 cm^{-1}.

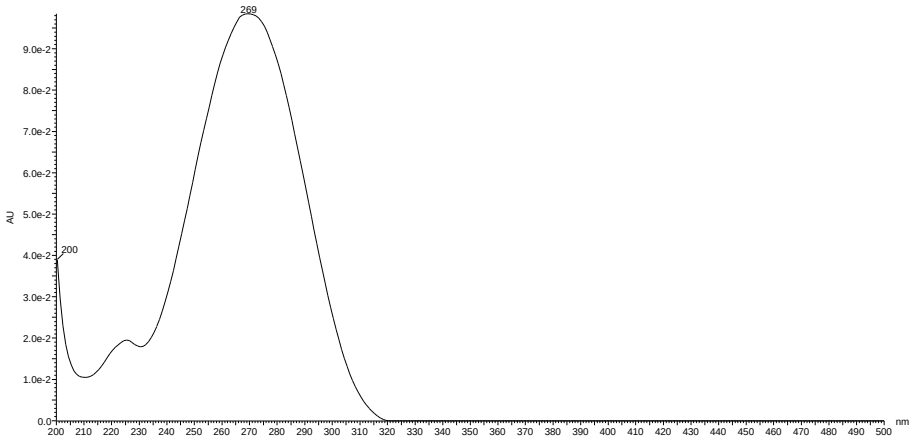


Figure S6. UV spectrum of the characterized compound.

Acknowledgements

The authors acknowledge financial support from Generalitat Valenciana (Group of Excellence Prometeo II 2014/023 and from the Ministerio de Economía y Competitividad in Spain (Project: CTQ2015-65603-P). The authors also acknowledge NPS-Euronet (HOME/2014/JDRUG/AG/DRUG/7086), co-funded by the European Union. This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein. The authors are very grateful to the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I (UJI) for the use of NMR, X-ray crystallography and FTIR. They also wish to thank Cristian Vicent (SCIC), Gabriel Peris (SCIC), José Pedra (SCIC) and Florenci V. González (UJI) for skillful technical assistance and useful comments. David Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (Grant FPU15/02033). Energy Control acknowledges the grants from Subdirecció General de Drogodependències, Departament de Salut, Generalitat de Catalunya and Plan Nacional sobre Drogas.

References

[1] J. B. Zawilska. "Legal Highs" – An Emerging Epidemic of Novel Psychoactive Substances, in Int. Rev. Neurobiol. - Neuropsychiatr. Complicat. Stimul. Abus., 2015, 273.

[2] European Monitoring Centre for Drugs and Drug Addiction. New psychoactive substances in Europe. An update from the EU Early Warning System. EMCDDA–Europol Jt. Publ., 2015, 12.

[3] European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2016. EMCDDA–Europol Jt. Publ., 2016, 80.

[4] D. Favretto, J. P. Pascali, F. Tagliaro. New challenges and innovation in forensic toxicology: Focus on the "New Psychoactive Substances." J. Chromatogr. A, 2013, 1287, 84.

[5] D. González, M. Ventura, F. Caudevilla, M. Torrens, M. Farre. Consumption of new psychoactive substances in a Spanish sample of research chemical users. Hum. Psychopharmacol Clin Exp, 2013, 28, 332.

[6] S. Gwak, L. E. Arroyo-Mora, J. R. Almirall. Qualitative analysis of seized synthetic cannabinoids and synthetic cathinones by gas chromatography triple quadrupole tandem mass spectrometry. Drug Test. Anal., 2015, 7, 121.

[7] L. Fattore, W. Fratta. Beyond THC: the new generation of cannabinoid designer drugs. Front. Behav. Neurosci., 2011, 5, 1.

[8] S. Strano Rossi, S. Odoardi, A. Gregori, G. Peluso, L. Ripani, G. Ortar, G. Serpelloni, F. S. Romolo. An analytical approach to the forensic identification of different classes of new psychoactive substances (NPSs) in seized materials. Rapid Commun. Mass Spectrom., 2014, 28, 1904.

[9] A. Thaxton, T. S. Belal, F. Smith, J. DeRuiter, K. M. Abdel-Hay, C. R. Clark. GC-MS studies on the six naphthoyl-substituted 1-n-pentyl-indoles: JWH-018 and five regioisomeric equivalents. Forensic Sci. Int., 2015, 252, 107.

[10] J. Lobo Vicente, H. Chassaigne, M. V. Holland, F. Reniero, K. Kolář, S. Tirendi, I. Vandecasteele, I. Vinckier, C. Guillou. Systematic analytical characterization of new psychoactive substances: A case study. Forensic Sci. Int., 2016, 265, 107.

[11] M. Ibañez, J. V. Sancho, L. Bijlsma, A. L. N. Van Nuijs, A. Covaci, F. Hernandez. Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. TrAC - Trends Anal. Chem., 2014, 57, 107.

[12] I. Marginean, W. F. Rowe, I. S. Lurie. The role of ultra high performance liquid chromatography with time of flight detection for the identification of synthetic cannabinoids in seized drugs. Forensic Sci. Int., 2015, 249, 83.

[13] F. Hernández, L. Bijlsma, J. V Sancho, R. Díaz, M. Ibáñez. Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry. Anal. Chim. Acta, 2011, 684, 87.

[14] M. Ibañez, L. Bijlsma, A. L. N. Van Nuijs, J. V. Sancho, G. Haro, A. Covaci, F. Hernandez. Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends. J. Mass Spectrom., 2013, 48, 685.

[15] F. Y. Lai, C. Erratico, J. Kinyua, J. F. Mueller, A. Covaci, A. L. N. van Nuijs. Liquid chromatography-quadrupole time-of-flight mass spectrometry for screening in vitro drug metabolites in humans: Investigation on seven phenethylamine-based designer drugs. J. Pharm. Biomed. Anal., 2015, 114, 355.

[16] L. Bijlsma, C. Boix, W. M. a Niessen, M. Ibáñez, J. V Sancho, F. Hernández. Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment. Sci. Total Environ., 2013, 443, 200.

[17] N. Uchiyama, K. Asakawa, R. Kikura-Hanajiri, T. Tsutsumi, T. Hakamatsuka. A new pyrazole-carboxamide type synthetic cannabinoid AB-CHFUPYCA [N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1H-pyrazole-5-carboxamide] identified in illegal products. Forensic Toxicol., 2015, 33, 367.

[18] T. Doi, B. Akiko Asada, B. Akihiro Takeda, B. Takaomi Tagami, M. Katagi, B. Shuntaro Matsuta, B. Hiroe Kamata, B. Masami Kawaguchi, Y. Satsuki, B. Yoshiyuki Sawabe, B. Hirotaka Obana. Identification and characterization of a-PVT, a-PBT, and their bromothienyl analogs found in illicit drug products. Forensic Toxicol., 2016, 34, 76.

[19] L. Bijlsma, B. Miserez, M. Ibáñez, C. Vicent, E. Guillamón, J. Ramsey, F. Hernández. Identification and characterization of a novel cathinone derivative 1-(2,3-dihydro-1H-inden-5-yl)-2-phenyl-2-(pyrrolidin-1-yl)- ethanone seized by customs in Jersey. Forensic Sci. Int., 2016, 144.

[20] G. McLaughlin, N. Morris, P. V. Kavanagh, J. D. Power, B. Twamley, J. O'Brien, B. Talbot, G. Dowling, S. D. Brandt. The synthesis and characterization of the "research chemical" N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1H-pyrazole-5-carboxamide (3,5-AB-CHMFUPPYCA) and differentiation from its 5,3-regioisomer. Drug Test. Anal., 2015, DOI 10.1002/dta.1864.

[21] M. H. Chen, A. Dip, M. Ahmed, M. L. Tan, J. P. Walterscheid, H. Sun, B. Teng, A. Mozayani. Detection and Characterization of the Effect of AB-FUBINACA and Its Metabolites in a Rat Model Michael. J. Cell. Biochem., 2016, 117, 1033.

[22] N. Uchiyama, Y. Shimokawa, R. Kikura-Hanajiri, Y. Demizu, Y. Goda, T. Hakamatsuka. A synthetic cannabinoid FDU-NNEI, two 2H-indazole isomers of synthetic cannabinoids AB-CHMINACA and NNEI indazole analog (MN-18), a phenethylamine derivative N–OH-EDMA, and a cathinone derivative dimethoxy-α-PHP, newly identified in illegal products. Forensic Toxicol., 2015, 33, 244.

[23] O. V. Dolomanov, L. J. Bourhis, R. J. Gildea, J. A. K. Howard, H. Puschmann. OLEX2: A complete structure solution, refinement and analysis program. J. Appl. Crystallogr., 2009, 42, 339.

[24] L. Palatinus, G. Chapuis. SUPERFLIP - A computer program for the solution of crystal structures by charge flipping in arbitrary dimensions. J. Appl. Crystallogr., 2007, 40, 786.

[25] G. M. Sheldrick. A short history of SHELX. Acta Crystallogr. Sect. A Found. Crystallogr., 2007, 64, 112.

2.3. Research article II

www.nature.com/scientificreports

OPEN

Received: 16 March 2017
Accepted: 16 June 2017
Published online: 24 July 2017

Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900)

D. Fabregat-Safont[1], X. Carbón[2], M. Ventura[2], I. Fornís[2], E. Guillamón[3], J. V. Sancho[1], F. Hernández[1] & M. Ibáñez[1]

New psychoactive substances have been rapidly growing in popularity in the drug market as non-illegal drugs. In the last few years, an increment has been reported on the use of synthetic alternatives to heroin, the synthetic opioids. Based on the information provided by the European Monitoring Centre for Drug and Drug Addiction, these synthetic opioids have been related to overdoses and deaths in Europe and North America. One of these opioids is the U-47700. A few months ago, U-47700 was scheduled in the U.S. and other countries, and other opioid derivatives have been appearing in order to replace it. One of these compounds is U-49900, an analog of U-47700. A white powder sample was obtained from an anonymous user in Spain. After an accurate characterization by gas chromatography-mass spectrometry, ultra-high performance liquid chromatography-high resolution mass spectrometry, nuclear magnetic resonance and single-crystal X-ray diffraction; and complemented by Fourier-transformed infrared spectroscopy, ultraviolet and circular dichroism spectrophotometry, the drug sample was unequivocally identified as U-49900. The information provided will be useful for the Early Warning System and forensic laboratories for future identifications of the U-49900, as well as in tentative identifications of other related opioids.

In recent years, new psychoactive substances (NPS) have rapidly emerged in the drug market as a "legal" alternative to controlled drugs[1,2]. The term "NPS" includes different compound families, such as synthetic cannabinoids, cathinones and opioids. In the last decade, an increase in the use of synthetic opioids as substance of abuse has been reported[3], with the potential to pose serious risks to public health and safety. According to the EMCDDA *European Drug Report 2016*, synthetic opioids have been involved in overdose drug related deaths in some parts of Europe and North America, these compounds having been found in 82% of fatal overdoses[4]. For years, the diversity of available synthetic opioids in the grey market has been limited. Fentanyl analogues are limited by their high potency, making them unwieldy and dangerous to handle by users without proper safety procedures; thus, opioids such as MT-45 have been demonstrated to cause dangerous side effects[5]. Around 2012, a new class of synthetic opioids emerged. AH-7921 (Fig. 1), an opioid around 0.8 times as potent as morphine, developed at Allen & Hanburys Ltd in 1975[6], resurfaced on internet stores sold under the guise of being a research chemical "not for human consumption". Effects described by users were similar to those of classical opioids: euphoria, sedation, pinned pupils, etc.[7], and it saw relatively little popularity until its ban in 2013. Shortly after, an analogue of AH-7921 appeared on the market, U-47700 (Fig. 1), developed by Upjohn in 1978[8]. In the Upjohn patent,

[1]Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain. [2]Energy Control (Asociación Bienestar y Desarrollo), c/Independencia 384, 08041, Barcelona, Spain. [3]Departament de Química Física I Analítica, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain. Correspondence and requests for materials should be addressed to M.I. (email: ibanezm@uji.es)

Scientific Reports, 2017; 7:6638

Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-*N*-(2-(diethylamino)cyclohexyl)-*N*-methylbenzamide (U-49900)

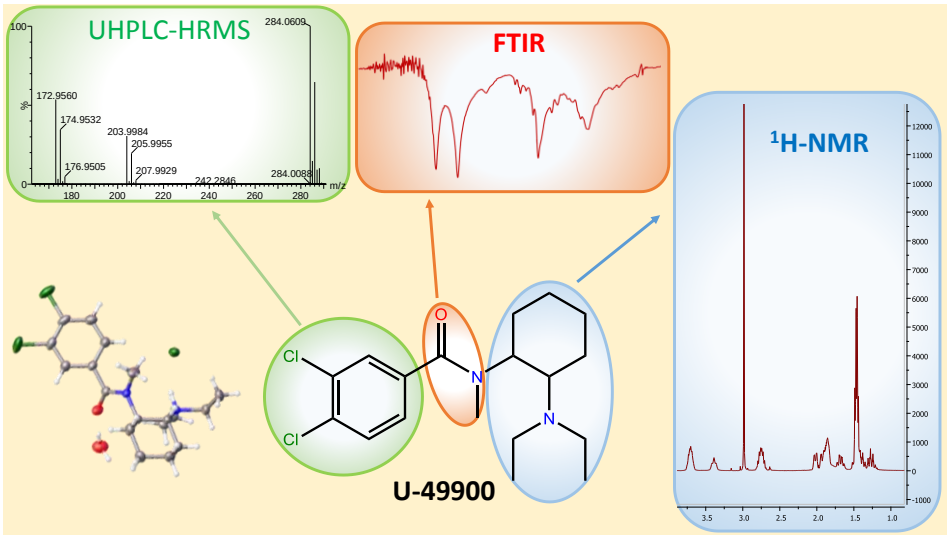
D. Fabregat-Safont [1], X. Carbón [2], M. Ventura [2], I. Fornís [2], E. Guillamón [3], J.V. Sancho [1], F. Hernández [1] & M. Ibáñez [1*]

[1] Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12071 Castellón, Spain.

[2] Energy Control (Asociación Bienestar y Desarrollo), c/ Independencia 384, 08041 Barcelona, Spain.

[3] Departament de Química Física i Analítica, University Jaume I, Avda Sos Baynat s/n, 12071 Castellón, Spain.

Graphical abstract



Abstract

New psychoactive substances have been rapidly growing in popularity in the drug market as non-illegal drugs. In the last few years, an increment has been reported on the use of synthetic alternatives to heroin, the synthetic opioids. Based on the information provided by the European Monitoring Centre for Drug and Drug Addiction, these synthetic opioids have been related to overdoses and deaths in Europe and North America. One of these opioids is the U-47700. A few months ago, U-47700 was scheduled in the U.S. and other countries, and other opioid derivatives have been appearing in order to replace it. One of these compounds is U-49900, an analog of U-47700. A white powder sample was obtained from an anonymous user in Spain. After an accurate characterization by gas chromatography-mass spectrometry, ultra-high performance liquid chromatography-high resolution mass spectrometry, nuclear magnetic resonance and single-crystal X-ray diffraction; and complemented by Fourier-transformed infrared spectroscopy, ultraviolet and circular dichroism spectrophotometry, the drug sample was unequivocally identified as U-49900. The information provided will be useful for the Early Warning System and forensic laboratories for future identifications of the U-49900, as well as in tentative identifications of other related opioids.

Keywords New psychoactive substances; Opioids; U-49900; 3,4-dichloro-*N*-(2-(diethylamino)cyclohexyl)-*N*-methylbenzamide; High resolution mass spectrometry; Nuclear magnetic resonance spectroscopy.

In recent years, new psychoactive substances (NPS) have rapidly emerged in the drug market as a "legal" alternative to controlled drugs [1,2]. The term "NPS" includes different compound families, such as synthetic cannabinoids, cathinones and opioids. In the last decade, an increase in the use of synthetic opioids as substance of abuse has been reported [3], with the potential to pose serious risks to public health and safety. According to the EMCDDA *European Drug Report 2016*, synthetic opioids have been involved in overdose drug related deaths in some parts of Europe and North America, having been found in 82% of fatal overdoses [4]. For years, the diversity of available synthetic opioids in the grey market has been limited. Fentanyl analogues are limited by their high potency, making them unwieldy and dangerous to handle by users without proper safety procedures; thus, opioids such as MT-45 have been demonstrated to cause dangerous side effects [5]. Around 2012, a new class of synthetic opioids emerged. AH-7921 (**Figure 1**), an opioid around 0.8 times as potent as morphine, developed at Allen & Hanburys Ltd in 1975 [6], resurfaced on internet stores sold under the guise of being a research chemical "not for human consumption". Effects described by users were similar to those of classical opioids: euphoria, sedation, pinned pupils, etc. [7], and it saw relatively little popularity until its ban in 2013. Shortly after, an analogue of AH-7921 appeared on the market, U-47700 (**Figure 1**), developed by Upjohn in 1978 [8]. In the Upjohn patent, U-47700 is the compound most selective for the mu opioid receptor among all studied. It's theorized to have a potency of around 7.5 times that of morphine, and binds to the mu opioid receptor (MOR) with a Ki (±SEM) of 0.91 nM (±0.11), to kappa opioid receptor (KOR) at 110 nM (±11), and poorly so to the delta opioid receptor (DOR) at 480 nM (±110). In comparison, morphine has Ki (±SEM) values at the MOR, KOR, and DOR of 0.213 (±0.019), 27.9 (±2.7), and 111 (±14) nM, respectively.

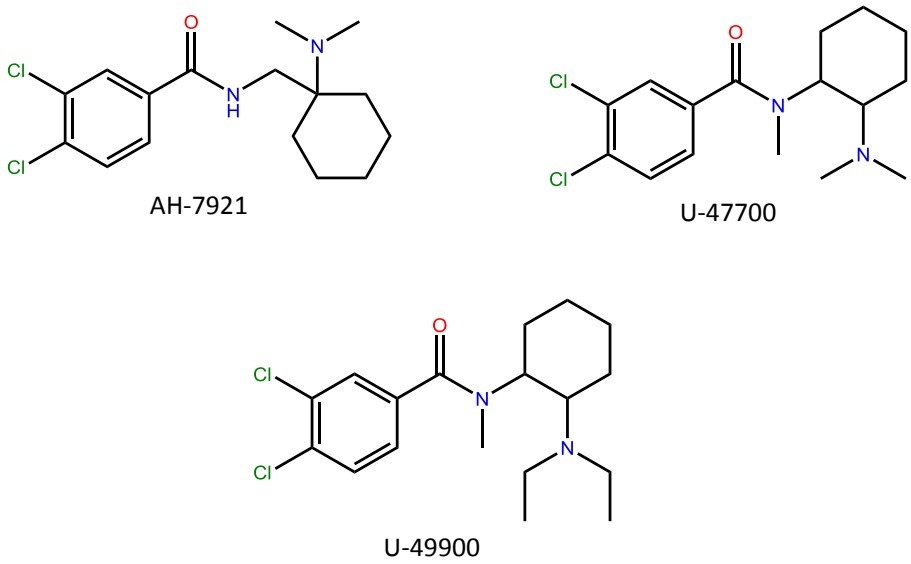


Figure 1. Chemical structure for AH-7921, U-47700 and expected structure for U-49900.

U-47700's presents a certain set of characteristics that make it particularly attractive to users, that until its appearance no other single substance had had. It is described by users as being more euphoric than fentanyl analogues, more potent than AH-7921, cheap, and widely available (at the time of writing this article) [9]. It is no surprising that it saw a surge in popularity, and has been the cause of at least 46 confirmed fatalities, as well as 88 reports from forensic laboratories of U-47700 submissions in the U.S. alone [10], as well as several fatalities in the rest of the globe [11–16]. With a common dose being six to eight milligrams, the cost per dose is no more than 0.32 USD [17]. As a response to its growing popularity, the DEA placed U-47700 in emergency scheduling along with furanyl-fentanyl in November 11th, 2016 [10].

The NPS market is quick to adapt however, and shortly before U-47700 was scheduled, U-51754, another synthetic opioid from the same Upjohn patent surfaced on the market. This substance is not as selective for KOR, and users describe it as being more dysphoric and dissociating than U-47700 [18]. The most recent compound to surface, and a potential replacement for U-47700,

is U-49900. Although both substance names bear a striking similarity, U-49900 does not appear on the same Upjohn patent as U-47700 or U-51754, and is in fact a completely novel substance that has not been described in literature until now.

In this work, a sample (labelled as U-49900) was obtained by Energy Control from an anonymous Spanish user. After an exhaustive analysis by gas chromatography coupled to single-quadrupole mass spectrometry (GC-MS), ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS), nuclear magnetic resonance (NMR) and single-crystal X-ray diffraction, the compound was unequivocally characterized and thus, identified. Additionally, Fourier-transformed infrared (FTIR) spectroscopy, ultraviolet (UV) analysis, circular dichroism (CD) and melting point range determination were performed in order to obtain a fully-characterization of the compound. This methodology has proved to be powerful for the identification of NPS, even for unknown substances [19]. The compound was confirmed to be the diethyl analogue of U-47700. We present the detailed analytical characterization of 3,4-dichloro-*N*-(2-(diethylamino)cyclohexyl)-*N*-methylbenzamide, henceforth referred to as U-49900 (**Figure 1**). Available information about its effects, price, availability, etc. has also been included.

Results

Analytical characterization of U-49900

Gas chromatography-quadrupole mass spectrometry

Firstly, the sample was analysed by GC-MS. A unique peak at 7.77 min was observed in the Total Ion Chromatogram (TIC), demonstrating the high purity of the sample (**Figure 2**, top). The EI spectrum of this peak showed a high fragmentation, being the base peak the ion at *m/z* 112 (**Figure 2**, bottom). This peak did not show the expected isotopic pattern produced by the putative presence of two chlorine atoms in the molecule. Additional fragment ions with

an important intensity were observed at *m/z* 56, 84, 126, 144, 154 and 173. In this case, the ion at 173 presented the characteristic isotopic pattern corresponding to the presence of two chlorine atoms. Ions above *m/z* 200 were also observed, but with a lower intensity. Ions at *m/z* 284, 327 and 356 also presented the two-chlorine pattern. The molecular ion expected at *m/z* 356 was observed, but with a very low intensity, due to the high fragmentation obtained by EI. Despite all the information provided, this technique did not allow the unequivocal characterization of the molecule and thus, additional techniques were used for the confirmation of the compound.

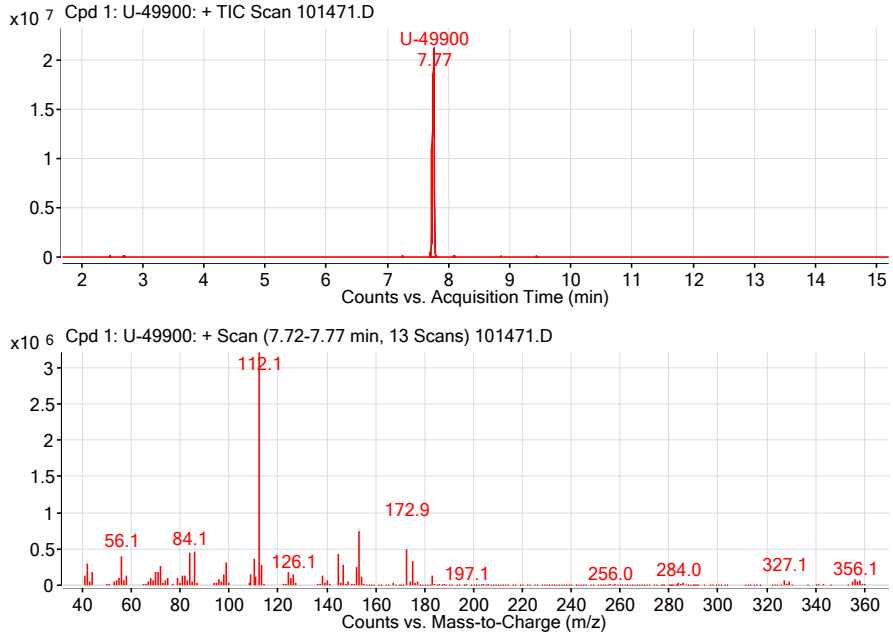


Figure 2. GC-EI-MS analysis of the sample containing U-49900. Top: TIC of the sample analysis. Bottom: EI spectrum for the chromatographic peak at 7.77 min.

Liquid chromatography-high resolution mass spectrometry

UHPLC-HRMS analysis was performed in order to tentatively identify the compound based on its fragmentation. The soft ionization interface used allows the acquisition of the protonated molecule in the LE spectrum. In **Figure 3** (left)

the LE spectrum of the chromatographic peak at 6.72 min is showed. A unique peak at *m/z* 357.1500 was observed, corresponding the protonated molecule ($[M+H]^+$) of the compound. The accurate-mass acquisition allowed the determination of the elemental composition of this *m/z* peak, being calculated as $C_{18}H_{27}Cl_2N_2O^+$ with a mass error of 0.0 ppm. Other possible elemental compositions were neglected due to the high mass error obtained. The determined elemental composition fitted with the theoretical elemental composition of U-49900. The isotopic pattern of the $[M+H]^+$ showed an important peak at *m/z* 359, corresponding to the presence of two chlorine atoms in the molecule. The ratio between both peaks (65% approximately) was the expected for the ratio $^{35}Cl_2/^{35}Cl^{37}Cl$. Similarly, the ratio between $[M+H]^+$ and the peak at *m/z* 361 corresponding to the ratio between $^{35}Cl_2/^{37}Cl_2$ was also observed. No significant fragment ions were observed in the LE function.

The HE spectrum showed four important collision-induced fragment (CID) ions at *m/z* 284.0609, 203.9984, 172.9560 and 81.0707 (**Figure 3**, left). In order to evaluate if this fragment ions came from the protonated molecule, extracted ion chromatograms (EIC) were generated (**Figure 3**, right). Ions at *m/z* 284, 204 and 173 showed the isotopic pattern corresponding to the presence of two chlorine atoms. Based on the expected structure of U-49900, these three ions would correspond to fragments which contain the dichlorophenyl group. This assumption was confirmed once their elemental composition was determined. Fragment at *m/z* 284 ($C_{14}H_{16}Cl_2NO^+$, 0.0 ppm) corresponds to the diethylamino loss; fragment at *m/z* 204 ($C_8H_8Cl_2NO^+$, 0.5 ppm) corresponds to the cyclohexyl loss from fragment at *m/z* 284; and fragment at *m/z* 173 ($C_7H_3Cl_2O^+$, -0.6 ppm) would be the methylamine loss of fragment 204. The other important fragment, at *m/z* 81 ($C_6H_9^+$, 3.7 ppm), corresponds to the cyclohexyl ring, whose formation was already observed in the fragment at *m/z* 204 (a cyclohexyl ring loss from fragment *m/z* 284). All these fragments have been previously described in literature for the U-47700, and it was expected that some of them could also be observed for the U-49900 [12].

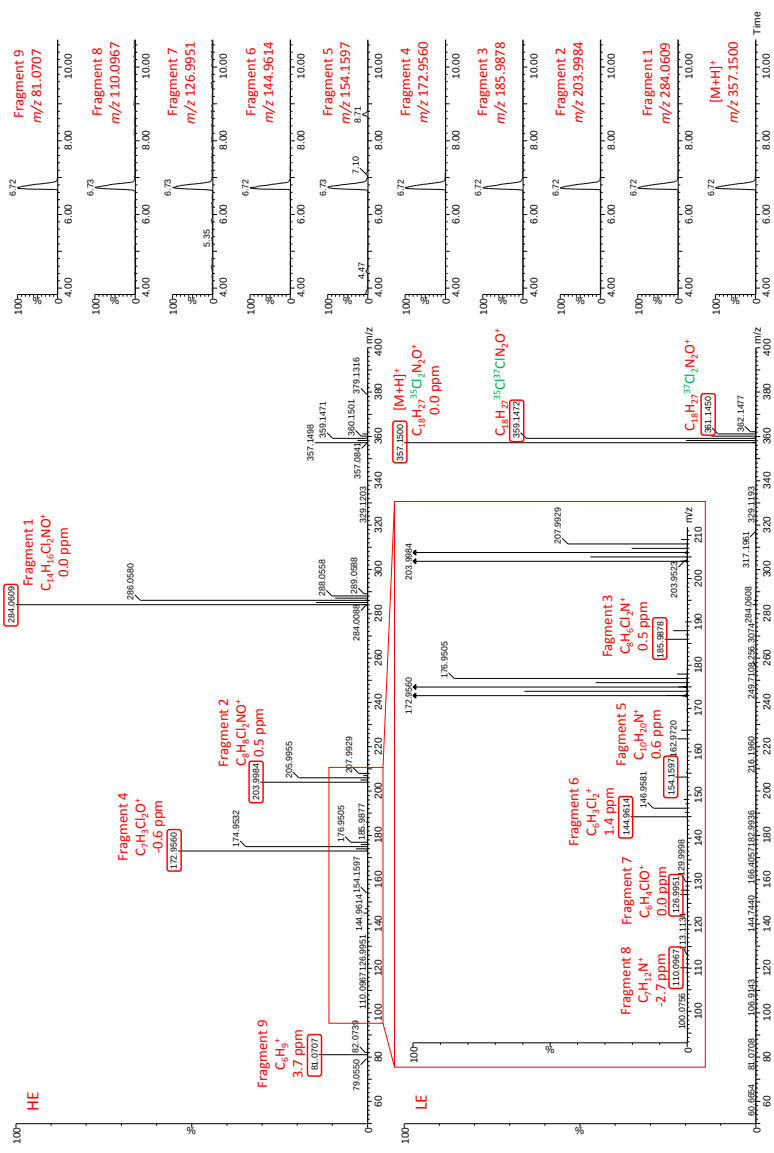


Figure 3. UHPLC-HRMS MS^E spectra of the U-49900. **Right** Extracted ion chromatograms (EIC) (0.02 Da mass window) for protonated molecule in LE function, and fragment ions in HE function. **Left** LE (bottom) and HE (top) spectra of U-49900

An accurate examination of the HE allowed the detection of five additional fragment ions, not previously observed due to their low intensity. The ions at *m/z* 185.9878 and 144.9614 also presented the isotopic pattern corresponding to the presence of two chlorine atoms. Fragment at *m/z* 186 ($C_8H_6Cl_2N^+$, 0.5 ppm) corresponds to the water loss from fragment at *m/z* 204. Thus, fragment at *m/z* 204 would present two different sequential fragmentations: a water loss (fragment at *m/z* 186) and a methylamine loss (*m/z* 173). The remaining

dichlorinated fragment at *m/z* 145 ($C_6H_3Cl_2^+$, 1.4 ppm) corresponds to the dichlorophenyl group. This fragment could be produced from fragment *m/z* 173 by a carbon monoxide loss, or from fragment *m/z* 186 by an azirine loss. Both fragments (at *m/z* 186 and 145) have also been described for the U-47700 [12].

Three minor fragments were observed at *m/z* 154.1597, 126.9951 and 110.0967. Fragments at *m/z* 154 ($C_{10}H_{20}N^+$, 0.6 ppm) and *m/z* 110 ($C_7H_{12}N^+$, -2.7 ppm) correspond to the diethylcyclohexylamine group (which would come from $[M+H]^+$) and the methylcyclohexylamine group (which would come from fragment at *m/z* 284 by C-N amide bond fragmentation), respectively. Additionally, both fragments would be the precursors of fragment at *m/z* 81 (the cyclohexyl group), previously described. Regarding fragment at *m/z* 127 ($C_6H_4ClO^+$, 0.0 ppm), this fragment would correspond to a 2-chlorophenol group, but this structure is not present in the supposed structure of U-49900. This fragment seems to be produced by an adduct formation with a neutral molecule present in the collision cell. The formation of fragment-adducts with small molecules in the collision cell has been recently described in literature [20]. According to literature, water adducts are the most commonly observed, due to water traces coming from the same instrument or CID gas impurities [20]. Based on this study, the fragment at *m/z* 127 would come from the dichlorophenyl fragment (*m/z* 145), experiencing a neutral HCl loss followed by a water addition, and subsequent formation of the 2-chlorophenol fragment. In this case, no information related with the U-47700 fragmentation was found in literature for fragments at *m/z* 154 (on the basis of the structure, this ion could not be present in U-47700), 127 and 110.

Finally, MS/MS experiments were performed in order to increase the confidence of the CID fragments acquired (**Figure S1**).

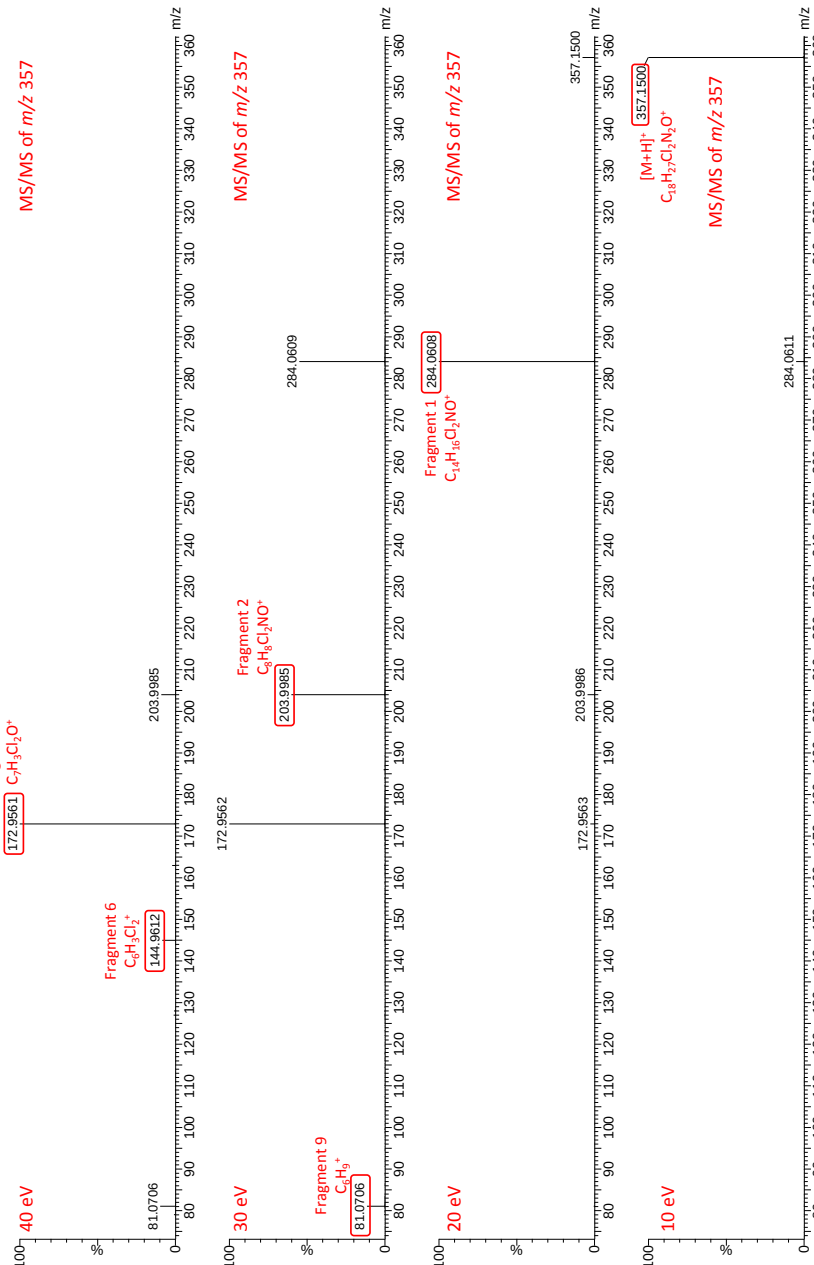


Figure S1. MS/MS spectra of U-49900 at 10, 20, 30 and 40 eV collision energy.

Once the compound was tentatively identified based on its accurate-mass fragmentation, a plausible fragmentation pathway was proposed (**Figure 4**). Nevertheless, a tentative identification was not enough to characterize the compound, thus additional spectroscopic techniques were applied for the identification and subsequent complete characterization of the U-49900.

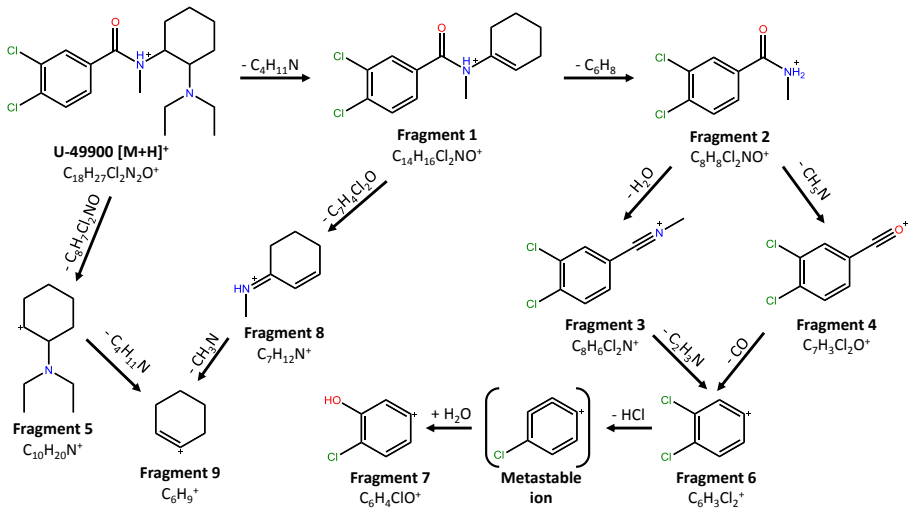


Figure 4. Proposed CID fragmentation pathway for the U-49900.

Nuclear magnetic resonance

Different NMR experiments were performed in order to identify the compound. The first step was to perform ^{1}H NMR (**Figure 5**, top) and COSY (**Figure 5**, bottom) experiments (400 MHz, $CDCl_3$). COSY is a bidimensional NMR experiment (2D-NMR) which shows through-bond correlations between the hydrogens of the molecule. As the compound was tentatively identified by UHPLC-HRMS, most of the NMR signals were easily identified. For ^{1}H NMR, signals between δ = 7.5 and 8.0 ppm corresponded to the aromatic hydrogens, identifying each signal based on its multiplicity and chemical shift. Doublets corresponded to the hydrogens of carbon marked as 15 in **Figure 5** (C15) and C16 hydrogens. The C15 would correspond to the doublet with the highest chemical shift (δ = 7.89 ppm) due to the proximity of the chlorine atom; the C16

would correspond to the other doublet (δ = 7.45 ppm). The third signal, a singlet, corresponded to C12 hydrogen (δ = 7.72 ppm). COSY spectrum showed a correlation between C15 and C16 and no correlation with C12, as expected.

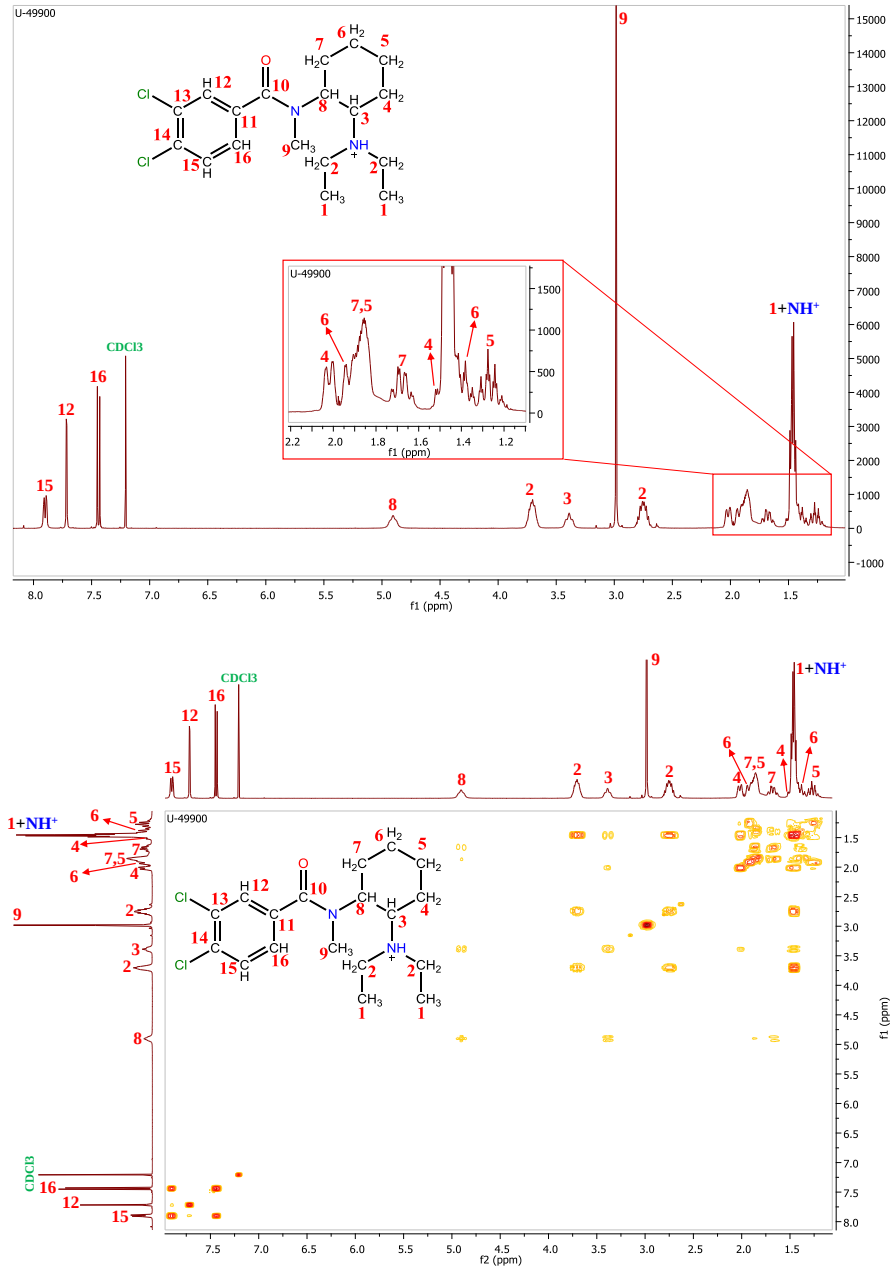


Figure 5. Top ^{1}H NMR spectrum for U-49900 with signal assignation based on compound structure. **Bottom** COSY spectrum of U-49900.

The signal at δ = 4.90 ppm would correspond to the C8 hydrogen based on the multiplicity and chemical shift. Similarly, the signal at δ = 3.39 ppm might correspond to the hydrogen in C3. A correlation between C3 and C8 hydrogens was observed in COSY. The most intense signal in ^{1}H NMR was the singlet at δ = 2.98 ppm, produced by the methyl group named as C9 in **Figure 5**, without correlations with other hydrogens.

The CH_3 of both ethyl groups bonded to the cyclohexylamine were expected to be found as a triplet around δ = 1.5 ppm, with an integration corresponding to 6 hydrogens. Even so, the signal at δ = 1.46 ppm was a quadruplet with an integration of 7 hydrogens. Signals corresponding to protonated amines are expected to be between 0.5 and 5 ppm, and it is possible that the observed quadruplet was produced by a triplet (corresponding to CH_3) and a singlet (corresponding to NH^+). In order to prove that, an additional ^{1}H NMR experiment was performed, using deuterated water as solvent (400 MHz, D_2O). The use of deuterated water as solvent promotes protonatable groups to exchange deuterium from the solvent. These signals from ^{1}H NMR spectrum are therefore removed as deuterium does not produce resonance in ^{1}H NMR. The resulting signal was a triplet whose integration corresponded to 6 hydrogens, therefore confirming our hypothesis. ^{1}H NMR spectra acquired using $CDCl_3$ and D_2O comparison can be found in **Figure S2**. So, the signal at δ = 1.46 ppm was assigned to the hydrogens of C1 and the proton of the amine (**Figure 5**).

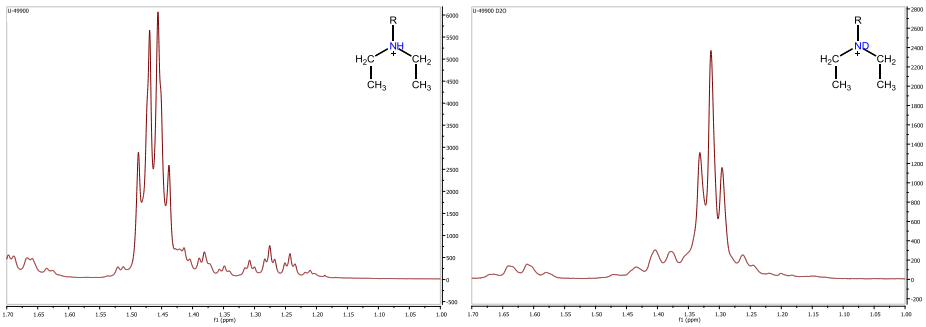


Figure S2. ^{1}H NMR signal for hydrogens of the methyl groups of diethylamine group. **Left** Signal obtained when using $CDCl_3$ for dissolve the sample. **Right** Signal obtained when using D_2O for dissolving the sample.

Signals at δ = 2.75 and 3.70 ppm would correspond to the CH_2 of the ethyl groups (C2), but producing different chemical shifts. This would indicate that the ethyl groups presented a different electronic surrounding. The protonation of the amine could produce an intramolecular hydrogen bond between this proton and the oxygen atom or the nitrogen atom of the amide group. The formation of this bond would hamper the free rotation of the *N*-cyclohexyl bond, producing the diastereotopicity of the C2 hydrogens. Once the signals produced by C2 hydrogens were identified, the correlation with C1 hydrogens was confirmed after performing an accurate study of the COSY spectrum. Finally, low-intense signals between δ = 0 and 2 ppm corresponded to CH_2 cyclohexyl hydrogens (C4-C7), as is explained in the ^{13}C NMR and HSQC study.

The second step was to perform ^{13}C NMR (**Figure 6**, top) and HSQC (**Figure 6**, bottom) experiments (400 MHz, $CDCl_3$). HSQC is a 2D NMR experiment which establishes the through-bond correlation between the carbon atoms and their bonded hydrogens. HSQC spectra work with a colour code: CH_3 and CH groups are represented in one colour, while CH_2 is represented in another one. In this case, in the HSQC spectrum of U-49900 CH_3/CH are represented in red-yellow spots, while CH_2 is represented in blue spots (**Figure 6**, bottom). Most ^{13}C NMR signals were assigned based on the assignation previously performed in ^{1}H NMR and looking for the correlation between carbon and hydrogen signals in HSQC. Quaternary carbon atoms, which have no hydrogens and thus no signal in ^{1}H NMR, were identified based on the chemical shift. Signal at δ = 171.46 ppm corresponded to the carbon atom of the amide group (C10). The three signals in the aromatic region (between δ = 100 and 140 ppm) which did not have correlation with ^{1}H NMR signals corresponded to the functionalized carbon atoms of the benzene ring. Signals at δ = 133.97 and 135.80 ppm were assigned to the carbons bonded to chlorine atoms (C13 and C14), while signal at δ = 132.37 ppm corresponded to C11.

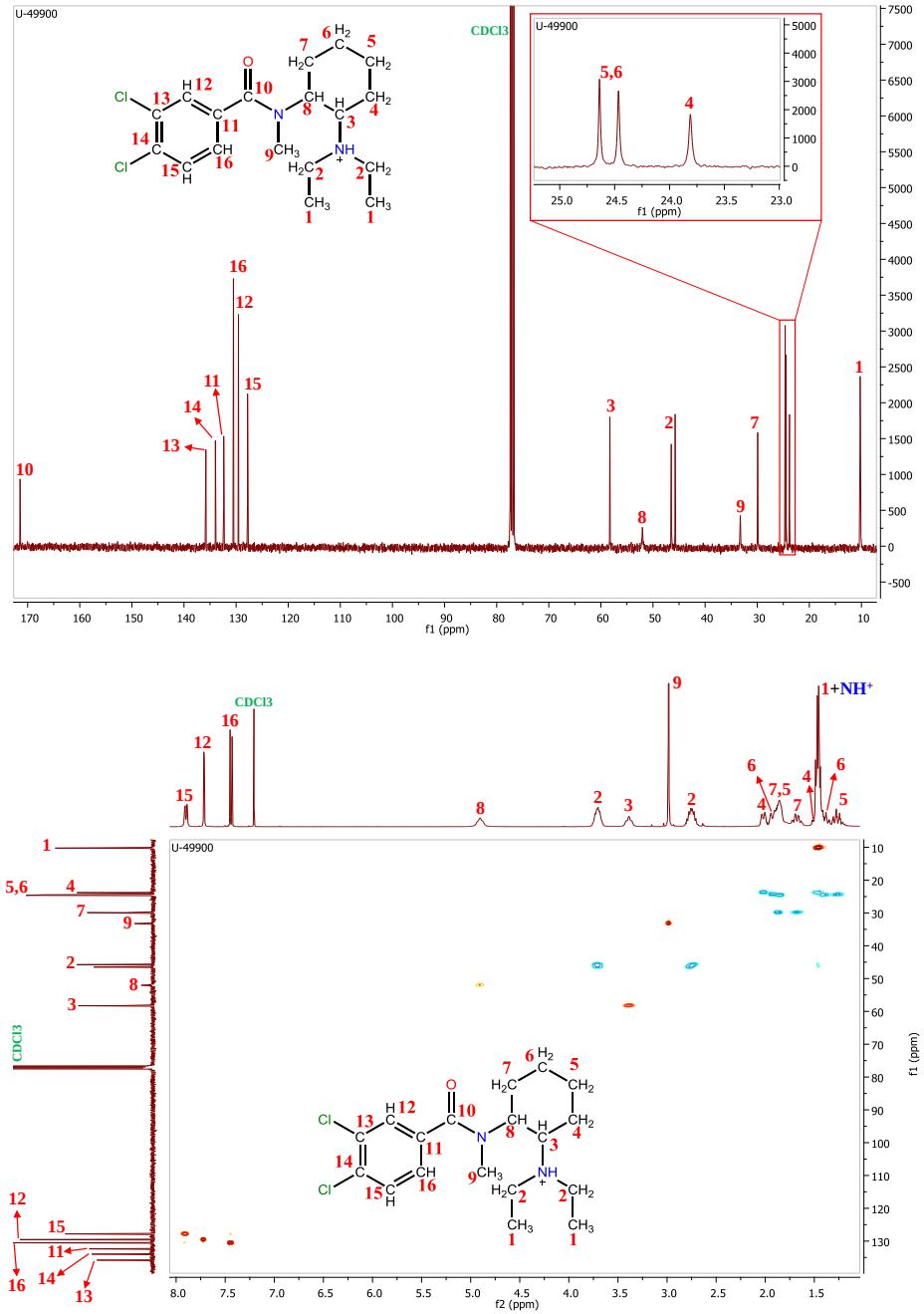


Figure 6. Top 13C-NMR spectrum for U-49900. **Bottom** HSQC spectrum of U-49900.

An accurate analysis of the HSQC spectrum confirmed the previously explained supposition: the hydrogens of C2 are diastereotopic. ^{13}C NMR spectrum showed a slight change in the chemical shift of both C2 carbons, probably due to the proximity of one of them to the nitrogen atom of the amide group. HSQC also showed the correlation between ^{13}C NMR signals corresponding to C2 carbon, and the two observed signals in ^{1}H NMR related previously to C2 hydrogens, reinforcing the supposition of the amine protonation. Finally, signals corresponding to CH_2 of the cyclohexyl (signals between $\delta = 0$ and 2 ppm in 1H-NMR) presented diastereotopicity. This was observed in HSQC spectrum as a double ^{1}H NMR signal corresponding to a single ^{13}C NMR signal. In other words, there were two different hydrogens in each carbon atom of the cyclohexane. This double signal was easy to justify, as the hydrogens of a CH_2 in a cyclohexane can present two different orientations: axial and equatorial. The different orientation of the hydrogens produces diastereotopicity in hydrogens theoretically equivalents.

The combination of COSY and HSQC spectra allowed a complete NMR characterization of the compound, increasing the confidence on the tentative identification of the structure of U-49900. **Table 1** and **Figure 7** show the assignation of ^{1}H and ^{13}C NMR signals once stablished the correlation between atoms and signals.

In order to increase the confidence on the NMR characterization, a HMBC experiment was performed in order to establish correlations over 2 heteronuclear bonds (**Figure S3**).

The combination of UHPLC-HRMS and NMR experiments allow the complete identification of the compound. Nevertheless, an additional analytical technique was applied in order to unequivocally confirm the identity of U-49900.

Table 1. ^{1}H and ^{13}C NMR signal assignment.

1H-NMR signal assignment			13C-NMR signal assignment	
Hydrogen	**δ (ppm)**	**Multiplicity**	**Carbon**	**δ (ppm)**
1+NH^{+}	1.47	Quadruplet	1	10.25 – 10.15
2	2.75 – 3.70	Multiplet	2	45.75 – 46.49
3	3.39	Triplet	3	58.27
4	1.51 – 2.01	-	4	23.81
5	1.28 – 1.86	-	5	24.64
6	1.44 – 1.91	-	6	24.46
7	1.68 – 1.86	-	7	29.91
8	4.90	Triplet	8	52.04
9	2.98	Singlet	9	33.24
10	-	-	10	171.46
11	-	-	11	132.37
12	7.72	Singlet	12	129.54
13	-	-	13	135.80
14	-	-	14	133.97
15	7.89	Doublet	15	127.79
16	7.45	Doublet	16	130.51

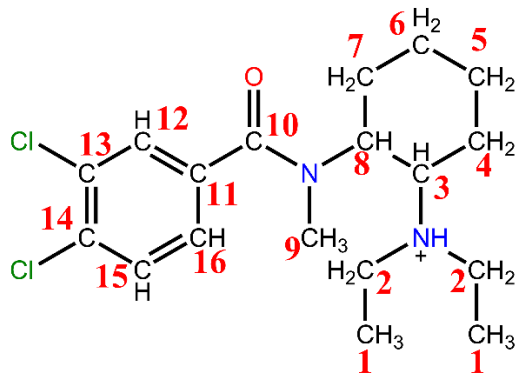


Figure 7. ^{1}H and ^{13}C NMR signal assignment.

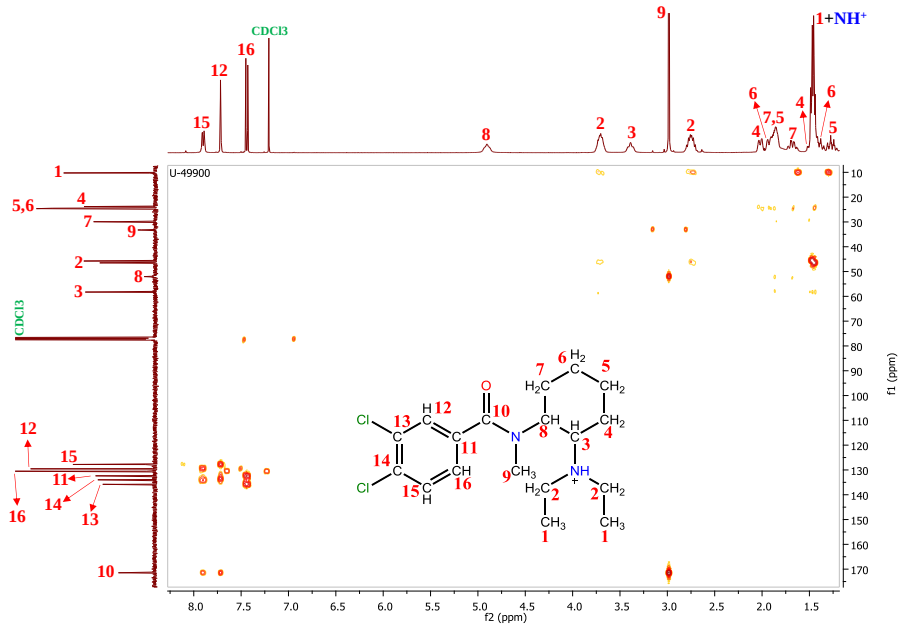


Figure S3. HMBC spectrum of U-49900. Correlations over 2 bonds are showed between ^{1}H and ^{13}C NMR signals.

Single-crystal X-ray diffraction

The unambiguous confirmation of the structure of U-49900 was performed by single-crystal X-ray diffraction analysis. Crystal evaluation and diffraction data were collected by using an Agilent Supernova diffractometer equipped with an Atlas CCD detector. No instrument or crystal instabilities were observed during data collection. Structure of U-49900 was solved by charge flipping methods using Superflip and refined by the full-matrix method on the basis of F^2 with the program SHELXL-2013, using the OLEX software package [21–23]. Absorption correction based on the multiscan method was applied [24]. The graphic was performed with the Diamond visual crystal structure information system software [25]. Crystal data and structure refinement information are summarized in **Table 2**. All non-hydrogen atoms were refined anisotropically and the H atoms were positioned geometrically, assigned isotropic thermal parameters and allowed to ride on their respective parent carbon atoms.

Table 2. Crystallographic data for U-49900.

Parameter	**Data**
Empirical formula	$C_{18}H_{26}Cl_2N_2O \cdot HCl \cdot H_2O$
Formula weight	411.78
Temperature (K)	200.0(10)
Crystal system	Triclinic
Space group	P-1
Unit cell dimensions	
a, Å	7.30052(13)
b, Å	8.14672(13)
c, Å	18.8525(3)
α, º	78.9257(14)
β, º	82.3114(14)
γ, º	66.9588(16)
Volume ($Å^3$)	1010.46(3)
Z	2
ρ_{calc} (mg/mm^3)	1.353
Absorption coefficient μ (mm^{-1})	4.218
F(000)	436.0
Crystal size (mm^3)	0.174 x 0.12 x 0.095
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection (º)	9.58 to 134.152
Index ranges	$-8 \leq h \leq 8$
	$-9 \leq k \leq 9$
	$-22 \leq l \leq 22$
Reflections collected	27700
Independent reflections	3616 [R_{int} = 0.0321, R_{sigma} = 0.0153]
Absorption correction	Multi-scan
Refinement method	Full-matrix least-squares on F^2
Data/restrains/parameters	3616/0/232
Goodness of fit on F^2	1.040

Table 2. Crystallographic data for U-49900 (continuation).

Final R indices [I > 2σ(I)]	R_1 = 0.0288
	wR_2 = 0.0754
R indices (all data)	R_1 = 0.0320
	wR_2 = 0.0784
Largest difference in peak/hole ($e \cdot A^{-3}$)	0.30/-0.23

U-49900 structure was refined in the triclinic space group P-1 with cell dimensions of a = 73.0052(13) Å, b = 8.14672(13) Å, c = 18.8525(3) Å and α = 78.9257(14)º, β = 82.3114(14)º and γ = 66.9588(16)º. **Figure 8** shows the ORTEP representation of the structure with the atom-numbering scheme. Protonation of the diethylamine group observed after performing NMR analyses was confirmed in the solid structure. This protonation produces the stereotopicity of the ethyl groups bonded to the nitrogen atom. However, the centrosymmetric P-1 space group evidences the racemic nature of the compound. Additionally, X-ray analysis demonstrated the high purity degree of this new substance and the high similarity to U-47700, and allowed the unequivocally identification of U-49900.

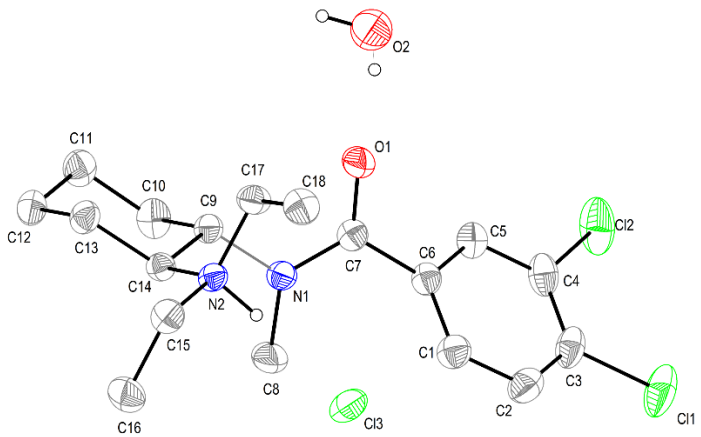


Figure 8. ORTEP representation (ellipsoids at the 50 % probability level) of the U-49900 chloride protonated salt with the atom-numbering scheme. Selected bond lengths [Å]: N1-C7 1.356(9); N1-C8 1.466(9); N1-C9 1.479(9); N2-C14 1.528(9); N2-C15 1.512(9); C3-Cl1 1.732(9); C7-O1 1.234(9); C7-C6 1.502(11). Hydrogen atoms, except that of the protonated amine, have been omitted for clarity.

All data regarding single-crystal X-ray crystallography and structure refinement were included in the Cambridge Crystallographic Data Centre (CCDC). This information can be found free of charge in the CCDC 1546594 file available at www.ccdc.cam.ac.uk/data_request/cif.

Additional characterization

FTIR and UV analyses were performed in order to obtain additional information about the compound. These spectra can be found in **Figure S4** and **S5,** respectively. Moreover, the melting point range was determined and established to be between 169-171 ºC. Finally, CD measurements were carried out to check the optical activity of U-49900. No Cotton effects were observed, confirming the racemic nature of this new drug.

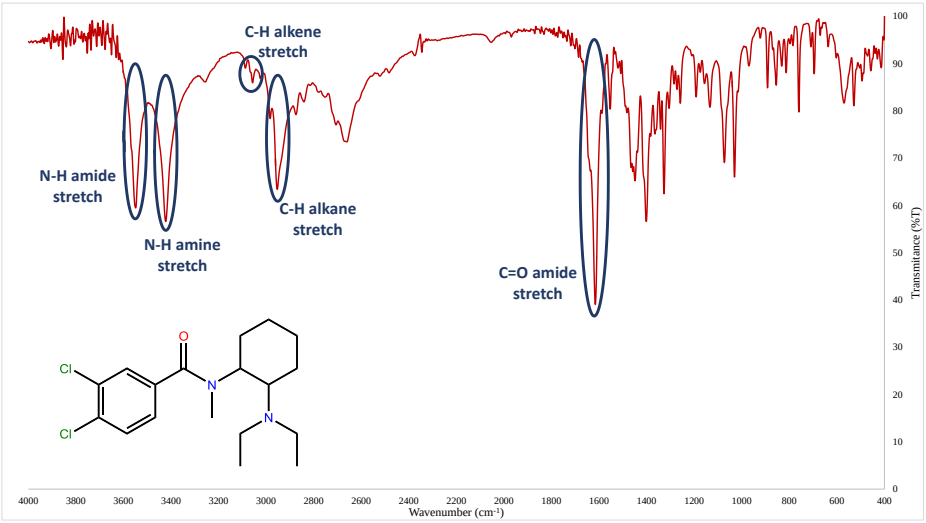


Figure S4. FTIR spectrum of U-49900. C=O band of the amide group is observed at 1600-1650 cm^{-1}. Aliphatic C-H bands are observed down to 3000 cm^{-1}. Aromatic C-H bands are observed up to 3000 cm^{-1}. N-H band of the amine is observed around 3400 cm^{-1}. N-H band of the amide is observed around 3500 cm^{-1}.

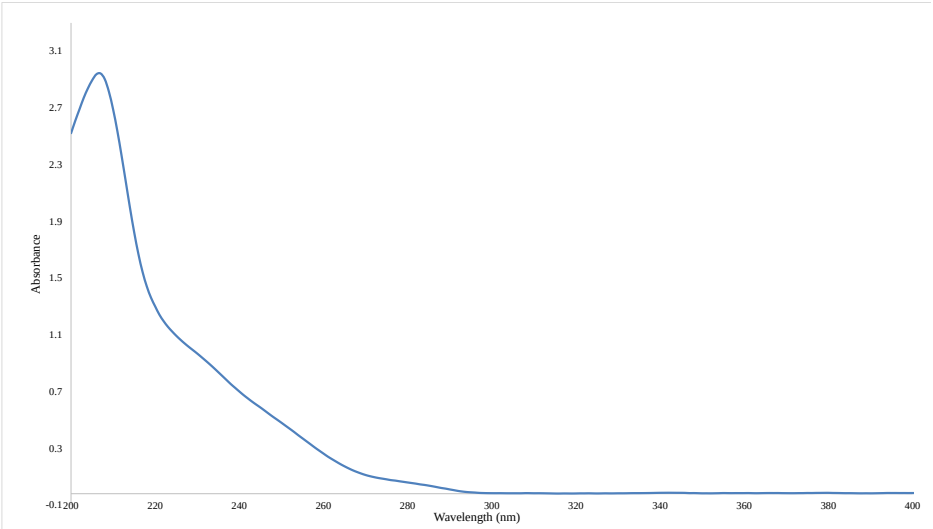


Figure S5. UV spectrum of the U-49900.

Psychoactive effects and availability of the drug as described by consumers

U-49900 is first mentioned online on November 2nd, 2016 on Bluelight, a popular drug forum, by a user announcing its availability and querying other users about possible effects. Users expressed concerns about its possible health hazards, as it is a close analogue to U-47700, which has been linked to damaged veins, damage to nasal and rectal passages, violent withdrawals, and other concerning effects [26,27]. Most forum threads reference the same post made by the anonymous user [28]. In the post, the user reports concerning side effects like pain upon insufflation, loss of taste, loss of smell, neurologic pain on the left side of the body, loss of the sense of touch, and a foam-like discharge from the lungs.

Reports of effects are few and far between, as most users that inquire about effects are linked to the aforementioned post and subsequently refrain from consuming the substance [29]. A user on the Swedish forum Flashback reports classic opioid effects from a dose of 50 mg intravenous [30]. Users on the same Swedish forum have tried U-49900 at doses of 5-10 milligrams and report no activity, whereas U-47700 is active in those amounts.

Discussion

In this work, the combination of different analytical techniques has allowed the unequivocal characterization of the novel opioid derivative U-49900. The use of HRMS, NMR and single-crystal X-ray diffraction has proved to be a powerful approach for the identification of NPS when reference standards are not available. Moreover, information obtained by GC-MS, FTIR and UV are also provided, in order to facilitate the identification of this compound in forensic laboratories with routine equipment. Although the present methodology could be used for the elucidation of most of organic compounds, it is especially useful for monitoring NPS due to the continuous on-going of these substances. This fact is the main handicap for obtaining reference standards, making the combination of powerful techniques necessary for unequivocal identification of NPS.

Although U-49900 is reported to be active, the doses taken are much larger than U-47700. Less than a year has passed since its first appearance on fora, but users' experiences seem to indicate that U-49900 is yet another lacklustre successor to U-47700 [29].

It is interesting to note that users of fora dedicated to NPS and other drug discussion seem to have a self-regulating system for novel compounds. Although these users are treading unknown territory, they are quick to share effects and side effects of NPS, and when such effects are concerning, they promptly share it with others and tend to steer clear of the substances discussed. One notable example is MT-45, a synthetic opioid developed in the 1970s by Dainippon Pharmaceutical Co [31], which surfaced on internet shops late 2012 [5,32], and has been involved in several non-fatal as well as fatal intoxications around the world. Users were quick to realize that among various side effects, MT-45 caused hearing loss and unconsciousness, and popularity dwindled well before its regulation in some European countries (like the UK and Czech Republic [33]) around 2015. It's possible that this is due to the negative side effects being widely reported on and shared across NPS-focused fora.

With U-49900 a similar trend appears to be taking place, as reports of use have been slow to surface, and most users that inquire about it are warned about potential side effects and resolve to stay away from the substance.

Possibly one of the most curious aspects of U-49900 is that, aside from bearing a striking structural similarity to U-47700, the name itself is a clear reference to this compound. It is, in fact, quite peculiar that the first appearance of this compound comes from a Chinese retailer [34], and not from old studies or patents, as tends to be the case for most NPS. U-49900 is likely a deliberate attempt to find a similar compound to U-47700, and demonstrates at least a rudimentary grasp of pharmacology, seeing as the modification is minor, and in theory the compound should retain similar activity to its parent compound. A similar case is methoxetamine, an NPS structurally similar to ketamine and other arylcyclohexylamines, which surfaced on online shops before being mentioned on any scientific literature. There is an enigmatic interview with a British biochemist that went by the pseudonym "M" [35] who claimed to have had a hand in the ideation and synthesis of this compound [36]. This suggests that for some NPS, the research and development stage is conducted in countries that have a market for NPS, and their production is then outsourced to Chinese laboratories. It is plausible that this is also the case for U-49900, and that there is a development process that took place in a European or North American country.

So far, there are no reported deaths related to U-49900, but at least 46 deaths were reported in the US alone with U-47700 being involved. Although this may appear to be a high figure, it pales in comparison to illicit fentanyl and its analogues, perhaps the closest contenders to the U-XXXXX family for use by opioid users, which have been involved in hundreds of deaths in the US alone [37–39]. It is clear that U-47700 is a harmful substance, with a much lower safety margin than other opioids or opiates, but the fact that the dosage is more manageable than fentanyl analogues, and the ratio of euphoric effects to respiratory depression suggests that users may be less prone to overdosing in the quest for the effects they are accustomed to with classical opiates.

This is especially important in US, where there is an important problem with opioid and opiate abuse. Users tend to start with pharmaceutical derivatives, such as codeine or tramadol, which are perceived as less harmful and their use is less stigmatized [40]. Some eventually migrate to more potent and euphoric derivatives, such as oxycodone or oxymorphone. When a dependence develops and the cost of the habit becomes prohibitive, some users may transition to illegal opioids and opiates, such as heroin, fentanyl analogues and/or novel opioid derivatives, such as non-pharmaceutical fentanyl, U-47700, or U-49900. These compounds tend to have lower safety margins than the more studied classical opiates. It is therefore important to evaluate the repercussions of scheduling novel opioids without contemplating future derivatives. It seems that scheduling these compounds as they surface has not been effective at curbing novel opioid abuse. A similar phenomenon was observed for synthetic cannabinoids, where the first generation (JWH-018, etc.) were prone to seizures, causing dependence, and other complications, but the latest generation, the result of several waves of prohibition, are more potent, more prone to causing complications, and have been involved in a striking amount of deaths [41–43].

After the unequivocal identification of U-49900, metabolism and toxicological studies would be needed to get comprehensive information on this drug. The study of metabolic pathways of NPS is crucial for establishing the consumption biomarkers of these substances in urine for their monitoring in intoxication and overdoses cases [44]. However, obtaining urine or serum from intoxication cases is troublesome, as the source of the intoxication is commonly unknown. In the case of U-47700, a possible metabolism pathway has been proposed in literature [14] (**Figure S6**), including 10 tentative metabolites. The major metabolites were the *N*-desmethyl and *N*,*N*-bisdesmetyl derivatives, which were found in a urine sample from a subject after U-47700 overdose.

In view of the difficulties of performing metabolism studies in humans, the in-vivo approach using animals plays an important role. The use of different animal models (mice or rats) has demonstrated its efficiency for obtaining potential

consumption markers of NPS in human urine samples [45,46]. So, future research will be focused on metabolites identification of U-49900 using *in vivo* models (similarly to the analogue U-47700). Information provided on these or similar studies will be useful for monitoring this compound in consumers and in possible intoxication cases.

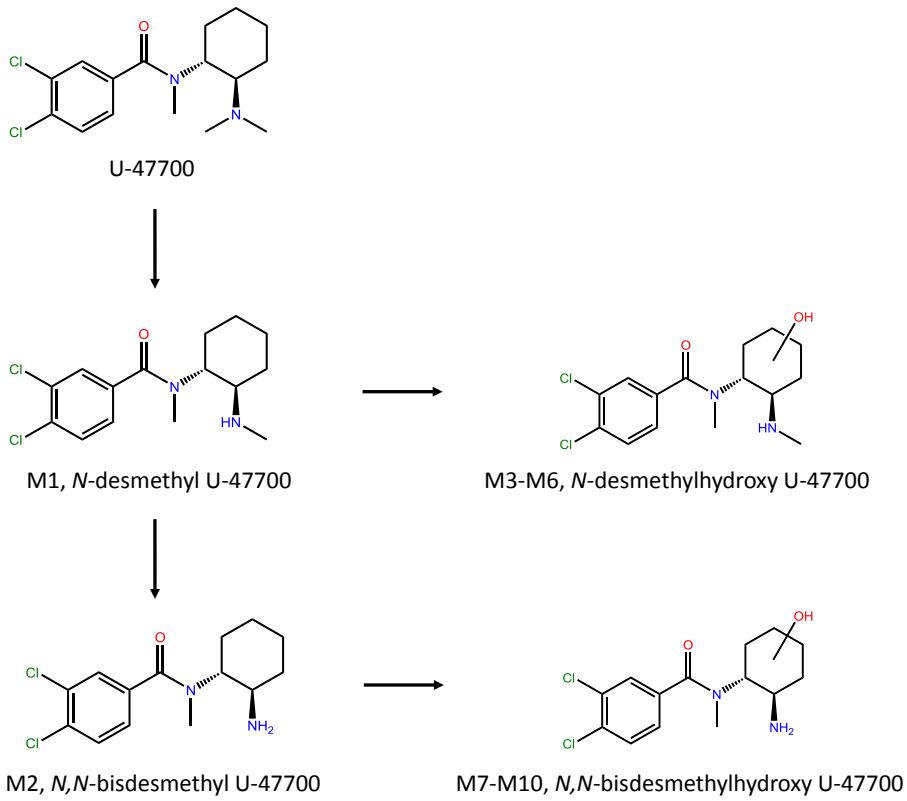


Figure S6. Proposed metabolic pathway of U-47700.

As a final note, a new opioid analogue related to U-47700 and identified as 3,4-dichloro-*N*-(2-(diethylamino)cyclohexyl)-*N*-methylbenzamide (also known as U-49900) has been identified and characterized in a drug sample from Spain. Characterization of the compound consisted of GC-MS analysis, UHPLC-HRMS, NMR, 2D NMR analyses and single-crystal X-ray diffraction, unequivocally confirming its chemical structure. Additionally, FTIR, UV and CD spectra, as well as melting point range were measured in order to obtain a

complete characterization of U-49900. The information reported in this work about this new opioid analogue will be useful to enhance the Early Warning Systems and for future toxicological studies.

It seems that demand for novel opioids does not decrease when a compound is scheduled, and if the supply for a certain substance dwindles, users are bound to seek an alternative. AH-7291 was dangerous, U-47700 has been proven to be more widespread and even more problematic, and the newest generation of derivatives, such as U-49900 and U-51754 do not seem to be of less concern. While it is clear that a solution needs to be found, perhaps quickly and systematically scheduling new compounds as they gain popularity can be complemented with other prevention measures in response to the growing problem of NPS abuse.

Future work on U-49900 will be focused on the metabolic pathway of this opioid in urine samples from *in vivo* experiments, as well as on the proposal of potential consumption markers. These studies will be useful for monitoring U-49900 in consumers and in suspicious intoxication cases.

Methods

Drug sample

The suspect sample (consisting on white powder) was submitted by an anonymous user to Energy Control for its analysis. Additional information about Energy Control can be seen elsewhere [47].

Reagents and chemicals

For GC-MS analysis, HPLC-grade methanol (MeOH) was purchased from Panreac (Panreac, Barcelona, Spain). For UHPLC-HRMS analysis, HPLC-grade water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH),

HPLC-grade acetonitrile (ACN), formic acid (HCOOH), acetone, and sodium hydroxide (NaOH) were acquired from Scharlau (Scharlab, Barcelona, Spain). Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA). For NMR analysis, deuterated chloroform ($CDCl_3$) and deuterated waters (D_2O) were purchased from Sigma-Aldrich. For single-crystal X-ray analysis, GC ultra-trace analysis grade dichloromethane (stabilized with ethanol) and GC ultra-trace analysis grade n-hexane were purchased from Scharlau. For FTIR analysis potassium bromide (KBr) was purchased from Scharlau. For UV and CD analyses, ACN was purchased from Scharlau.

Sample treatment

For GC-MS analysis, 10 mg of bulk sample were sonication-assisted dissolved with 10 mL of MeOH during 15 min. The extract was then centrifuged to remove insoluble material and afterwards directly injected into GC-MS system.

For UHPLC-HRMS analysis, 10 mg of sample were extracted with 1 mL of acetone in an ultrasonic bath for 15 min, following the sample treatment described in literature [48,49]. After centrifugation, the supernatant was ten thousand-fold diluted with HPLC-grade water, and 20 µL of the extract were injected in the UHPLC-HRMS system.

For NMR analysis, approximately 15 mg of sample were dissolved in 0.6 mL of $CDCl_3$. Additionally, 15 mg of sample were dissolved in 0.6 mL of D_2O in order to obtain a ^1H NMR spectrum without the protonated amine signal.

The recrystallization was performed by dissolving 10 mg of sample in 1 mL of dichloromethane in a glass vial, and dropwise addition of n-hexane, taking care to avoid mixing both solvents. The process was performed over-night, by diffusion of n-hexane in dichloromethane and thus, precipitating the compound and crystallizing it. Needle-shaped crystals were obtained and used for X-ray diffraction.

For FTIR analysis, the sample was mixed with KBr in a ratio 5:95 and homogenized in an agate mortar. Finally, the mixture was compressed under a pressure of 5000 kg/cm^2.

For UV and CD analyses, the compound was dissolved in ACN at 10^{-4} M, preparing the appropriate volume of dissolution.

Instrumentation

For GC-MS analysis, an Agilent 7890B gas chromatograph was coupled to a 5977A quadrupole mass spectrometer detector (Agilent Technologies, Santa Clara, CA, USA) using an electron ionization (EI) interface. The gas chromatograph was fitted with a G4513A auto-sampler injector (Agilent). Insert liners packed with silanized glass wool were used, and the injector and the interface were operated at 280 ºC. 1 µL of sample was injected in split mode, with a split ratio 1:10, into a 30 m 0.25 mm i.d., 0.25 µm film thickness 5% phenylmethylsilicone column (HP-5MS, Agilent). Helium was used as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially maintained at 90 ºC for 2 min and programmed to reach 320 ºC at 20 ºC per min. It was finally maintained at 320 ºC for 9.5 min (total run time was 21.5 min). The mass spectrometer was operated in electronic ionization mode at 70 eV. MS system worked in SCAN acquisition mode, acquiring from *m/z* 40 to 400 Da. Analytical data were acquired and processed using MassHunter B.06.00 (Agilent) operation software.

LC-HRMS analysis was performed using an ACQUITY UPLC ultra-high performance liquid chromatography (UHPLC) system (Waters, Mildford, MA, USA) coupled to a XEVO G2 QTOF hybrid quadrupole time-of-flight (QTOF) mass spectrometer (Waters Micromass, Manchester, UK) with an orthogonal Z-spray electrospray ionization (ESI) interface operating in positive ionization mode. The chromatographic separation was performed using a CORTECS C18 (Waters) 2.7 µm particle size analytical column 100 x 2.1 mm at a flow rate of 0.3 mL/min. The column temperature was set to 40 ºC. The mobile phases used

were H_2O with 0.01% HCOOH (A) and MeOH with 0.01% HCOOH (B). The mobile phase gradient was performed as follows: 10% of B at 0 min, 90% of B at 14 min linearly increased, 90% of B at 16 min, and finally 10% B at 18 min in order to return to initial conditions. The injection volume was 10 µL. Nitrogen (Praxair, Valencia, Spain) was used as desolvation and nebulizing gas. The desolvation gas flow was set at 1000 L/h. The TOF resolution was ~20000 at FWHM at *m/z* 556. The range acquired by the MS system was from *m/z* 50 to 1000. A capillary voltage of 0.7 kV and a cone voltage of 20 V were used during all the chromatographic run. Argon 99.995% (Praxair, Valencia, Spain) was used as a collision gas. The interface temperature was set to 650 ºC and the source temperature to 120 ºC. For MS^E experiments, two acquisition functions with different collision energy were created. The low energy function (LE) used a collision energy of 4 eV in order to obtain information about the protonated molecule and adducts (if present), while the high energy function (HE) applied a collision energy ramp from 15 to 40 eV, in order to promote fragmentation of the compounds 48. Calibration of the mass-axis was performed daily from *m/z* 50 to 1000 using a 1:1 mixture of 0.05 M NaOH:5% HCOOH, diluted 1:25 with ACN:H_2O 80:20 mixture. For accurate mass measurement, a 2 µg/mL leucine enkephalin solution in ACN:H_2O 50:50 with 0.1% HCOOH was used as lock-mass, pumped at a flow rate of 20 µL/min. The leucine enkephalin protonated molecule (*m/z* 556.2771) was used for recalibrating the mass axis and ensure an accurate mass during all the chromatographic run. MS data were acquired in centroid mode using MassLynx data station operation software, version 4.1 (Waters).

NMR analyses were performed using a Bruker Ascend 400 MHz spectrometer equipped with a SampleCase autosampler (Bruker, Etlingen, Germany), performing data acquisition at 303 K using $CDCl_3$. The residual solvents signals at δ = 7.24 ppm for ^1H ($CHCl_3$) and at δ = 77.23 ppm for ^{13}C ($CDCl_3$) were used as internal references. Characterization of the compound was performed using 5 gradient-enhanced experiments: ^1H NMR, ^{13}C NMR, correlated spectroscopy

(COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC). NMR experiment data were collected using the Bruker Icon NMR 5.0.5 software (Bruker). MestreNova program was used for raw data processing (Mestrelab Research, Santiago de Compostela, Spain).

For single-crystal X-ray diffraction crystallography, an Agilent SuperNova diffractometer (Agilent Technologies) was used. The diffractometer was equipped with an Atlas CCD detector (Agilent Technologies), and CuKα radiation (λ = 1.54184 Å) was used. Sample was kept at 199.95 K during data collection. Experimental data was acquired using the SHELXS-2013 software (Yale University, New Haven, CT, USA), using the OLEX software package (Olex AS, Trondheim, Norway).

For FTIR analysis, a Jasco FT/IR-6200 FTIR spectrometer (Jasco Inc., Easton, MD, USA) was used. Data acquisition was performed at 23 ºC between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1} and performing 32 acquisitions.

For UV and CD analyses, a Jasco J-810 spectrophotometer (Jasco Inc., Easton, MD, USA) was used. Data acquisition was performed at 23 ºC between 200 and 400 nm, with a resolution of 1 nm, a scanning speed of 100 nm/min and performing 2 acquisitions.

Acknowledgements

D.F-S., JV.S., F.H. and M.I. acknowledge financial support from Generalitat Valenciana (Group of Excellence Prometeo II 2014/023) and from Ministerio de Economía y Competitividad in Spain (Project: CTQ2015-65603-P). D.F-S., JV.S., F.H. and M.I. also acknowledge NPS-Euronet (HOME/2014/JDRUG/AG/DRUG/7086), co-funded by the European Commission. This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein. The authors are very grateful to the

Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I (UJI) for the use of NMR, X-ray diffractometer, FTIR and UV. They also wish to thank Cristian Vicent (SCIC), Gabriel Peris (SCIC), José Pedra (SCIC) and Florenci V. González (UJI) for skillful technical assistance and useful comments. D.F-S. acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (Grant FPU15/02033). X.C., M.V. and I.F. acknowledge the grants from Subdirecció General de Drogodependències, Departament de Salut, Generalitat de Catalunya and Plan Nacional sobre Drogas in Spain.

Author contributions

M.V., I.F., JV.S., F.H. and M.I. conceived the work. D.F-S., X.C., M.V., I.F., JV.S. and M.I. designed research approach. D.F-S., I.F., E.G. and M.I. performed experiments and analyzed raw data. D.F-S., X.C., I.F., E.G. and M.I. analyzed all data together and wrote the manuscript. M.V., JV.S. and F. H. provided useful comments and feedback for the manuscript.

Competing interests: The authors declare no potential conflict of interest.

References

1. Griffiths, P., Evans-Brown, M. & Sedefov, R. Getting up to speed with the public health and regulatory challenges posed by new psychoactive substances in the information age. Addiction 108, 1700–1703 (2013).

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

3. Katselou, M., Papoutsis, I., Nikolaou, P., Spiliopoulou, C. & Athanaselis, S. Old opioids, new concerns: the case of acetylfentanyl. Forensic Toxicol. 34, 201–212 (2016).

4. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2016. EMCDDA–Europol Jt. Publ. 80 (2016). doi:10.2810/88175

5. Helander, A., Bäckberg, M. & Beck, O. MT-45, a new psychoactive substance associated with hearing loss and unconsciousness. Clin. Toxicol. 52, 901–904 (2014).

6. Harper, N. J. & Veitch, G. B. A. 1-(3,4-dichlororbenzamidomethyl)-cyclohexyldimethylamine. Patent No. US3975443A. Allen & Hanburys Limited, London, England. (1976).

7. Kjellgren, A. & Jacobsson, K. The Quest for Well-Being and Pleasure: Experiences of the Novel Synthetic Opioids AH-7921 and MT-45, as Reported by Anonymous Users Online. J. Addict. Res. Ther. 7, (2016).

8. Szmuszkovicz, J. Analgesic N-(2-aminocycloaliphatic)benzamides. Patent No. US4098904A. The Upjohn Company, Michigan, USA. (1978).

9. Novel opioid, U-47700-Mega Thread and FAQ. Date accessed 2017-02-23. bluelight.org (2014). Available at: http://bluelight.org/vb/threads/739960-Novel-opioid-U-47700-Mega-Thread-and-FAQ. (Accessed: 23rd February 2017)

10. Department of Justice. Drug Enforcement Administration (DEA). Schedules of Controlled Substances: Temporary Placement of U-47700 Into Schedule I. Federal Register 81 (173) 61636–61638 (2016). Available at: https://www.deadiversion.usdoj.gov/fed_regs/rules/2016/fr1114.htm.

11. World Health Organization. U-47700 Critical Review Report. 38th Expert Committee on Drug Dependence, convened from 14 to 18 November 2016, at WHO headquarters in Geneva. 38th Expert Comm. Drug Depend. 14–18 (2016).

12. Elliott, S. P., Brandt, S. D. & Smith, C. The first reported fatality associated with the synthetic opioid 3,4-dichloro- N -[2-(dimethylamino)cyclohexyl]- N -methylbenzamide (U-47700) and implications for forensic analysis. Drug Test. Anal. 8, 875–879 (2016).

13. Domanski, K. et al. Two cases of intoxication with new synthetic opioid, U-47700. Clin. Toxicol. 3650, 1–5 (2016).

14. Jones, M. J., Hernandez, B. S., Janis, G. C. & Stellpflug, S. J. A case of U-47700 overdose with laboratory confirmation and metabolite identification. Clin. Toxicol. 3650, 1–5 (2016).

15. Schneir, A., Metushi, I. G., Sloane, C., Benaron, D. J. & Fitzgerald, R. L. Near death from a novel synthetic opioid labeled U-47700: emergence of a new opioid class. Clin. Toxicol. 3650, 1–4 (2016).

16. Commision on Narcotic Drugs. Extract from the Report of the 38th Expert Committee on Drug Dependence, convened from 14 to 18 November 2016, at WHO headquarters in Geneva. Annex 1. 38th Expert Comm. Drug Depend. 10192, (2016).

17. U-47700. Date accessed 2017-02-23. psychonautwiki.org Available at: https://psychonautwiki.org/wiki/U-47700. (Accessed: 23rd February 2017)

18. Novel opioid U-51754. Date accessed 2017-02-23. bluelight.org (2016). Available at: http://www.bluelight.org/vb/threads/793107-Novel-opioid-U-51754. (Accessed: 23rd February 2017)

19. Fabregat-Safont, D. et al. Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test. Anal. (2017). doi:10.1002/dta.2182

20. Alechaga, É., Moyano, E. & Galceran, M. T. Ion-molecule adduct formation in tandem mass spectrometry. Anal. Bioanal. Chem. 408, 1269–1277 (2016).

21. Dolomanov, O. V., Bourhis, L. J., Gildea, R. J., Howard, J. A. K. & Puschmann, H. OLEX2: A complete structure solution, refinement and analysis program. J. Appl. Crystallogr. 42, 339–341 (2009).

22. Palatinus, L. & Chapuis, G. SUPERFLIP - A computer program for the solution of crystal structures by charge flipping in arbitrary dimensions. J. Appl. Crystallogr. 40, 786–790 (2007).

23. Sheldrick, G. M. A short history of SHELX. Acta Crystallogr. Sect. A Found. Crystallogr. 64, 112–122 (2007).

24. Clark, R. C. & Reid, J. S. The analytical calculation of absorption in multifaceted crystals. Acta Crystallogr. Sect. A Found. Crystallogr. 51, 887–897 (1995).

25. Brandenburg, K. & Putz, H. DIAMOND. Cryst. Impact GbR, Bonn, Ger. (1999).

26. U-47700 P.S.A. Date accessed 2017-02-23. reddit.com (2016). Available at: https://www.reddit.com/r/researchchemicals/comments/3yc7hr/u47700_psa/. (Accessed: 23rd February 2017)

27. Anyone else have infections, abscesses from IV'ing U47700?. Date accessed 2017-02-23. reddit.com (2016). Available at: https://www.reddit.com/r/researchchemicals/comments/4kt9jk/anyone_else_have_infections_abscesses_from_iving. (Accessed: 23rd February 2017)

28. U-49900 - Experiment and Warning. Date accessed 2017-02-23. reddit.com (2016). Available at: https://www.reddit.com/r/researchchemicals/comments/5gbo3e/u49900_experiment_and_warning. (Accessed: 23rd February 2017)

29. U-49900 - New Op RC. Date accessed 2017-02-23. reddit.com (2016). Available at: https://www.reddit.com/r/researchchemicals/comments/5e1bzy/u49900_new_op_rc. (Accessed: 23rd February 2017)

30. Ny RC opiat U-49000 [U-49900]. Date accesses 2017-02-23. flashback.org (2016). Available at: https://www.flashback.org/t2785232p2. (Accessed: 23rd February 2017)

31. Nishimura, H. et al. 1-substituted-4-(1,2-diphenylethyl)-piperazine derivatives and their salts and the preparation thereof. Patent No. US3957788A. Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. 77, (1976).

32. MT-45 (1-cyclohexyl-4-(1,2-diphenylethyl)piperazine). Date accessed 2017-02-23. bluelight.org (2012). Available at: http://www.bluelight.org/vb/threads/640564-MT-45-(1-cyclohexyl-4-(1-2-diphenylethyl)piperazine. (Accessed: 23rd February 2017)

33. Government of the United Kingdom. A change to the Misuse of Drugs Act 1971: control of MT-45 and 4,4'-DMAR. Date accessed 2017-02-23. gov.uk (2015). Available at: https://www.gov.uk/government/publications/circular-0032015-a-change-to-the-misuse-of-drugs-act-1971-control-of-mt-45-and-44-dmar/a-change-to-the-misuse-of-drugs-act-1971-control-of-mt-45-and-44-dmar. (Accessed: 23rd February 2017)

34. U-49900 HCL from lsresearchchemlab.com. Date accessed 2017-02-23. lsresearchchemlab.com Available at: http://www.lsresearchchemlab.com/u-49900-hcl.html. (Accessed: 23rd February 2017)

35. Interview with a Ketamine Chemist. Date accessed 2017-02-23. vice.com (2011). Available at: https://www.vice.com/en_us/article/interview-with-ketamine-chemist-704-v18n2. (Accessed: 23rd February 2017)

36. Morris, H. & Wallach, J. From PCP to MXE: a comprehensive review of the non-medical use of dissociative drugs. Drug Test. Anal. 6, 614–632 (2014).

37. Centers for Disease Control and Prevention. Increases in Fentanyl Drug Confiscations and Fentanyl-related Overdose Fatalities. Accessed 2017-02-23. emergency.cdc.gov (2015). Available at: https://emergency.cdc.gov/han/han00384.asp. (Accessed: 23rd February 2017)

38. Centers for Disease Control and Prevention. Increases in Fentanyl-Related Overdose Deaths — Florida and Ohio, 2013–2015. Accessed 2017-02-23. cdc.gov (2016). Available at: Increases in Fentanyl-Related Overdose Deaths — Florida and Ohio, 2013–2015. (Accessed: 23rd February 2017)

39. Lozier, M. J. et al. Acetyl Fentanyl, a Novel Fentanyl Analog, Causes 14 Overdose Deaths in Rhode Island, March–May 2013. J. Med. Toxicol. 11, 208–217 (2015).

40. Lucyk, S. N. & Nelson, L. S. Novel Synthetic Opioids: An Opioid Epidemic Within an Opioid Epidemic. Ann. Emerg. Med. 69, 91–93 (2017).

41. Tournebize, J., Gibaja, V. & Kahn, J.-P. Acute effects of synthetic cannabinoids: Update 2015. Subst. Abus. 7077, 1–23 (2016).

42. Fattore, L. & Fratta, W. Beyond THC: The New Generation of Cannabinoid Designer Drugs. Front. Behav. Neurosci. 5, 1–12 (2011).

43. Adams, A. J. et al. 'Zombie' Outbreak Caused by the Synthetic Cannabinoid AMB-FUBINACA in New York. N. Engl. J. Med. 376, 235–242 (2017).

44. Ibáñez, M. et al. Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry. Anal. Bioanal. Chem. 408, 151–164 (2016).

45. Welter, J., Meyer, M. R., Kavanagh, P. & Maurer, H. H. Studies on the metabolism and the detectability of 4-methyl-amphetamine and its isomers 2-methyl-amphetamine and 3-methyl-amphetamine in rat urine using GC-MS and LC-(high-resolution)-MS n. Anal. Bioanal. Chem. 406, 1957–1974 (2014).

46. Meyer, M. R., Vollmar, C., Schwaninger, A. E., Wolf, E. U. & Maurer, H. H. New cathinone-derived designer drugs 3-bromomethcathinone and 3-fluoromethcathinone: studies on their metabolism in rat urine and human liver microsomes using GC-MS and LC-high-resolution MS and their detectability in urine. J. Mass Spectrom. 47, 253–262 (2012).

47. Quintana, P. et al. The hidden web and the fentanyl problem: Detection of ocfentanil as an adulterant in heroin. Int. J. Drug Policy 40, 78–83 (2017).

48. Ibañez, M. et al. Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends. J. Mass Spectrom. 48, 685–694 (2013).

49. Ibañez, M. et al. Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. TrAC - Trends Anal. Chem. 57, 107–117 (2014).

2.4. Research article III

Forensic Toxicology (2018) 36:447–457
https://doi.org/10.1007/s11419-018-0422-0

ORIGINAL ARTICLE

Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance

David Fabregat-Safont[1] · Xoán Carbón[2] · Cristina Gil[2] · Mireia Ventura[2] · Juan V. Sancho[1] · Félix Hernández[1] · Maria Ibáñez[1]

Received: 7 March 2018 / Accepted: 21 April 2018 / Published online: 9 May 2018

Abstract

Purpose In this work, the identification and characterization of the novel synthetic cathinone 5-PPDI found in a suspect drug sample were performed.

Methods The suspect sample was analyzed by gas chromatography–mass spectrometry (GC–MS), Fourier-transformed infrared (FTIR) spectroscopy, ultra-high performance liquid chromatography–high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy.

Results The fragmentation observed in GC–MS and the identification of functional groups by FTIR was not enough for compound identification. After an exhaustive analysis of the accurate-mass fragmentation observed in HRMS, the compound was tentatively identified as the novel cathinone 5-PPDI. Finally, five different NMR experiments were used for the unequivocal identification and complete characterization of the compound. In addition, the origin of this cathinone was investigated in depth.

Conclusions The analytical data provided in this work will be useful for the identification of 5-PPDI by forensic laboratories. In addition, the origin of this cathinone has been investigated, which could be of interest for the identification of future synthetic cathinones prepared following the similar synthesis route.

Keywords 5-PPDI · Synthetic cathinones · 1-(2,3-Dihydro-1*H*-inden-5-yl)-2-(pyrrolidin-1-yl)butan-1-one · High-resolution mass spectrometry · NMR spectroscopy · FTIR spectroscopy

Introduction

According to the last report from the European Monitoring Centre for Drug and Drug Addiction (EMCDDA), 14 novel cathinones were reported in the European Union in 2016. Synthetic cathinones represent the second largest novel psychoactive substance (NPS) class, with 118 compounds currently being monitored by EMCDDA. These compounds were the most commonly seized NPS in 2015, representing the third part of the total number of seizures [1]. Of these, many are pyrovalerone analogs; they are cathinones that contain the pyrrolidine moiety (42 substances) [1]. The compound reported in this paper, 5-PPDI, newly appeared as a new pyrovalerone analog that produces effects in humans and is not controlled. It is the indane analog of α-PBP, a drug that is currently controlled in the USA, China, and other countries [2, 3]. It is likely that it shares the same synthetic route, albeit with different precursors, as other pyrovalerone derivatives (α-bromination of the pentan-1-one precursor to form the 2-bromopentan-1-one intermediate, and reaction with pyrrolidine to yield the substance), and; therefore, the synthesis is easy to carry out by a facility that has the means to manufacture α-PVP [4].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11419-018-0422-0) contains supplementary material, which is available to authorized users.

✉ Maria Ibáñez
ibanezm@uji.es

[1] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071 Castellón, Spain

[2] Energy Control (Asociación Bienestar y Desarrollo), c/Independencia 384, 08041 Barcelona, Spain

Forensic Toxicology, 2018; 36:447

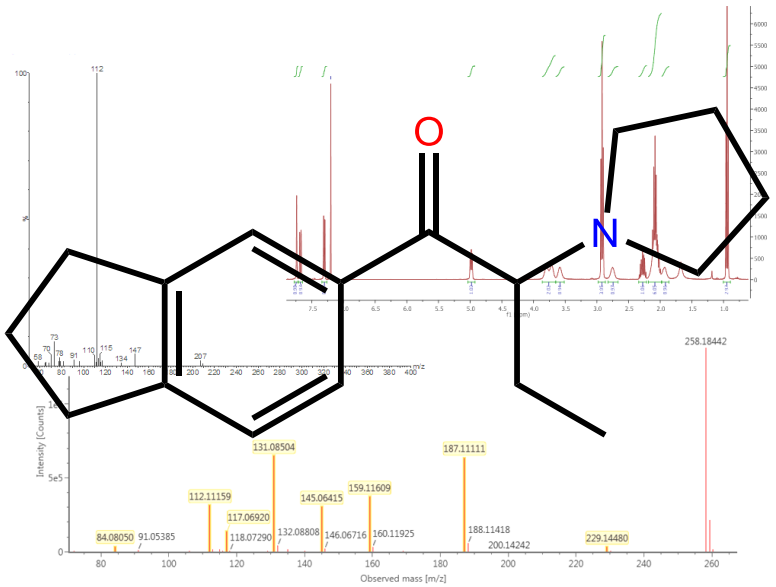
Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance

David Fabregat-Safont [1], Xoán Carbón [2], Cristina Gil [2], Mireia Ventura [2], Juan V. Sancho [1], Félix Hernández [1], María Ibáñez [1*]

[1] Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12071 Castellón, Spain.

[2] Energy Control (Asociación Bienestar y Desarrollo), c/ Independencia 384, 08041 Barcelona, Spain.

Graphical abstract



Abstract

Purpose In this work, the identification and characterization of the novel synthetic cathinone 5-PPDI found in a suspect drug sample was performed.

Methods The suspect sample was analysed by gas chromatography–mass spectrometry (GC–MS), Fourier-transformed infrared spectroscopy (FTIR), ultra-high performance liquid chromatography–high-resolution mass spectrometry (UHPLC–HRMS) and nuclear magnetic resonance (NMR).

Results The fragmentation observed in GC–MS and the identification of functional groups by FTIR were not enough for compound identification. After an exhaustive analysis of the accurate-mass fragmentation observed in HRMS, the compound was tentatively identified as the novel cathinone 5-PPDI. Finally, five different NMR experiments were used for the unequivocal identification and complete characterization of the compound. In addition, the origin of this cathinone was investigated in depth.

Conclusions The analytical data provided in this work will be useful for the identification of 5-PPDI by forensic laboratories. In addition, the origin of this cathinone has been investigated, which could be of interest for the identification of future synthetic cathinones prepared following the same synthesis route than that employed for obtaining 5-PPDI.

Keywords 5-PPDI; Synthetic cathinones; 1-(2,3-dihydro-1*H*-inden-5-yl)-2-(pyrrolidin-1-yl)butan-1-one; High-resolution mass spectrometry; NMR spectroscopy; FTIR spectroscopy.

Introduction

According to the last report from the European Monitoring Centre for Drug and Drug Addiction (EMCDDA), 14 novel cathinones were reported in the European Union in 2016. Synthetic cathinones represent the second largest novel psychoactive substances (NPS) family, with 118 compounds currently being monitored by EMCDDA. These compounds were the most commonly seized NPS in 2015, representing the third part of the total number of seizures [1]. Of these, many are pyrovalerone analogues; they are cathinones that contain the pyrrolidine moiety (42 substances) [1]. The compound reported in this paper, 5-PPDI, newly appeared as a new pyrovalerone analog that produces effects in humans and is not controlled. It is the indane analog of α-PBP, a drug that is currently controlled in the USA, China, and other countries [2, 3]. It is likely that it shares the same synthetic route, albeit with different precursors, as other pyrovalerone derivatives (α-bromination of the pentan-1-one precursor to form the 2-bromopentan-1-one intermediate, and reaction with pyrrolidine to yield the substance), and therefore the synthesis is easy to carry out by a facility that has the means to manufacture α-PBP [4].

Monitoring and identification of NPS is still handicapped due to this wide range of structures along with their high turn-out rate. For this reason, it is essential to keep developing analytical approaches for their characterization [5–7].

The most commonly used analytical techniques in toxicological routine laboratories are Fourier-transformed infrared (FTIR) spectroscopy and gas chromatography–mass spectrometry (GC–MS), with the predominating ionization source being electron ionization (EI) [8]. FTIR is especially useful for NPS analysis when attenuated total reflectance (ATR) is used, allowing a direct analysis with a small amount of recoverable sample. The use of ATR-FTIR has recently demonstrated its potential for direct classification of NPS in seizures through the use of multivariate discriminant analysis, allowing compound identification with a cost-effective and rapid analysis (2 min per sample) [9, 10].

Nevertheless, this methodology can only be applied if the compound spectrum has been previously acquired, which limits its suitability for monitoring emerging NPS. GC-MS is probably the most frequently used instrumental technique in the field of toxicology, where its applicability for cathinone analysis has been widely reported [11–14]. Although GC–MS provides a way to quickly identify a compound by the use of EI spectrum libraries, the frequent emergence of novel cathinone derivatives proves a serious drawback. First of all, most of the novel cathinones that have been detected recently are not listed in spectral libraries. Additionally, these cathinone derivatives tend to produce very similar (or identical) fragmentation patterns, and the identification of the molecular ion is commonly difficult due to the high fragmentation produced by an EI source [13].

Recent studies dealing with the analysis of synthetic cathinones have been carried out by ultra-high performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS), using electrospray ionization (ESI) interface as the ionization source. These studies have demonstrated the potential of this technique for cathinone identification in legal high samples, usually employing a hybrid quadrupole time-of-flight (QTOF) mass analyser [15, 16]. The QTOF instrument allows for a tentative compound identification even without the use of reference standards. Moreover, the applicability of the "non-target" approach for unknown compounds present in these samples has also been demonstrated [17].

When no reference standard is available, the use of UHPLC–HRMS is not enough for compound identification, and thus, additional spectroscopic techniques must be used. Nuclear magnetic resonance (NMR) is one of the most useful techniques for structural elucidation (including synthetic cathinones), allowing the differentiation of the substitutional isomerism without the use of reference standards [18–20]. Thus, the combination of UHPLC–HRMS and NMR allows the identification and complete characterization of unknown (or unreported) NPS [17, 21–24].

In this work, an unknown white powder (suspected to contain a synthetic cathinone) was received in our laboratory. After analysis by GC–MS and ATR-FTIR, the compound could not be identified. Analysis by UHPLC-HRMS allowed a tentative compound identification of the unreported synthetic cathinone 1-(2,3-dihydro-1*H*-inden-5-yl)-2-(pyrrolidin-1-yl)butan-1-one, sold in several webpages as 5-PPDI. The analysis of this cathinone by NMR in combination with HRMS data provided enough information for the unequivocal compound identification.

Materials and methods

Drug sample

The suspect sample was submitted by an anonymous user to Energy Control's drop-in service for its analysis. Additional information about Energy Control can be seen elsewhere [25].

Reagents and chemicals

For GC–MS analysis, GC-grade n-hexane and GC-grade acetone were purchased from Scharlau (Scharlab, Barcelona, Spain). For UHPLC-HRMS analysis, HPLC-grade water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC-grade methanol, HPLC-grade acetonitrile, formic acid, acetone, and sodium hydroxide (NaOH) were acquired from Scharlau. Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA). For NMR analysis, deuterated chloroform ($CDCl_3$) was purchased from Sigma-Aldrich. For FTIR analysis potassium bromide (KBr) was purchased from Scharlau.

Sample treatment

For FTIR analysis, the sample was directly analysed by ATR-FTIR spectroscopy.

For GC-MS analysis, 10 mg of sample were extracted with 1 mL of acetone in an ultrasonic bath for 15 min. After centrifugation, the supernatant was five thousand-fold diluted with GC-grade n-hexane, and 1 µL of the extract were injected in the GC-MS system.

For UHPLC-HRMS analysis, 10 mg of sample were extracted with 1 mL of acetone in an ultrasonic bath for 15 min. After centrifugation, the supernatant was ten thousand-fold diluted with HPLC-grade water, and 20 µL of the extract were injected in the UHPLC-HRMS system.

For NMR analysis, approximately 15 mg of sample were dissolved in 0.6 mL of $CDCl_3$.

Instrumentation

For FTIR analysis, a Jasco FT/IR-6200 FTIR spectrometer (Jasco Inc., Easton, MD, USA) equipped with a Specac Silver Gate ATR accessory (Specac, Orpington, UK) was used. Data acquisition was performed at 23 ºC between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1} and performing 32 acquisitions.

For GC–MS analysis, an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 7683 autosampler (Agilent Technologies) was coupled to a Quattro Micro GC triple quadrupole mass spectrometer (Micromass, Boston, MA, USA) using an electron ionization (EI) interface. The injector and the interface were operated at 250 ºC. A 1-µL aliquot of sample was injected in splitless mode using deactivated liners into a 30 m x 0.25 mm i.d., 0.25 µm film thickness DB-5MS column (Agilent Technologies). Helium (99.999%; Praxair, Valencia, Spain) was used as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially maintained at 90 ºC for 1 min and programmed to reach 300 ºC at 20 ºC/min. It was finally maintained at 300 ºC for 1.5 min (total run time was 12 min). The mass

spectrometer was operated in electronic ionization mode at 70 eV. MS system worked in scan acquisition mode, acquiring from m/z 50 to 400 Da. Analytical data were acquired and processed using MassLynx data station operation software (version 4.0; Waters, Mildford, MA, USA).

UHPLC–HRMS analysis was performed using an ACQUITY UHPLC system (Waters) coupled to a XEVO G2 QTOF hybrid QTOF mass spectrometer (Waters Micromass, Manchester, UK) with an orthogonal Z-spray ESI interface operating in positive ionization mode. The chromatographic separation was performed using a CORTECS C18 (Waters) analytical column (100 x 2.1 mm i.d., 2.7 µm particle size; Waters) at a flow rate of 0.3 mL/min. The column temperature was set to 40 ºC. The mobile phases used were H_2O with 0.01% formic acid (A) and methanol with 0.01% formic acid (B). The mobile phase gradient was performed as follows: 10% of B at 0 min, 90% B at 14 min linearly increased, 90% B at 16 min, and finally 10% B at 18 min in order to return to initial conditions. The injection volume was 20 µL. Nitrogen (Praxair) was used as desolvation and nebulizing gas. The desolvation gas flow was set at 1000 L/h. The TOF resolution was ~20000 at full width at half maximum at *m/z* 556. The range acquired by the MS system was *m/z* 50 to 1000. A capillary voltage of 0.7 kV and a cone voltage of 20 V were used during all the chromatographic run. Argon 99.995% (Praxair) was used as a collision gas. The interface temperature was set to 650 ºC and the source temperature to 120 ºC. For MS^E experiments, two acquisition functions with different collision energy were created. The low energy function used a collision energy of 4 eV in order to obtain information about the protonated molecule and adducts (if present), while the high energy function applied a collision energy ramp from 15 to 40 eV, in order to promote fragmentation of the compounds. Calibration of the mass-axis was performed daily from m/z 50 to 1000 using 0.05 M NaOH/5% formic acid (1:1, v/v), diluted 25-fold with acetonitrile/H_2O mixture (80:20, v/v). For accurate mass measurement, a 2 µg/mL leucine enkephalin solution in acetonitrile/H_2O with 0.1% formic acid (50:50, v/v) was used as lock-mass, and pumped at a flow rate of 20 µL/min. The

leucine enkephalin protonated molecule (*m/z* 556.2771) was used for recalibrating the mass axis and ensure an accurate mass during all the chromatographic run. UHPLC-HRMS data were acquired in continuum mode using MassLynx data station operation software (version 4.1; Waters) and processed with UNIFI scientific information system (version 1.8; Waters).

NMR analyses were performed using a Bruker Ascend 400 MHz spectrometer equipped with a SampleCase autosampler (Bruker, Etlingen, Germany), performing data acquisition at 303 K using $CDCl_3$. The residual solvent signals at $\delta = 7.24$ ppm for ^{1}H ($CHCl_3$) and at $\delta = 77.23$ ppm for ^{13}C ($CDCl_3$) were used as internal references. Characterization of the compound was performed using 5 gradient-enhanced experiments: ^{1}H NMR, ^{13}C NMR, correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC). NMR experiment data were collected using the Bruker Icon NMR 5.0.5 software (Bruker). MestreNova program was used for raw data processing (Mestrelab Research, Santiago de Compostela, Spain).

Results and discussion

Infrared spectroscopy and gas chromatography–mass spectrometry

Preliminary analyses were performed by ATR-FTIR and GC–MS. In the case of FTIR analysis, no spectral databases were available at our laboratory, and therefore only functional groups could be identified. No significant information could be obtained, and only aliphatic (<3000 cm^{-1}) and aromatic (>3000 cm^{-1}) C-H stretching signals, and carbonyl stretching signal (1675 cm^{-1}) were present in the FTIR spectrum. The FTIR spectrum and the identification of the observed bands can be found in **Figure S1**.

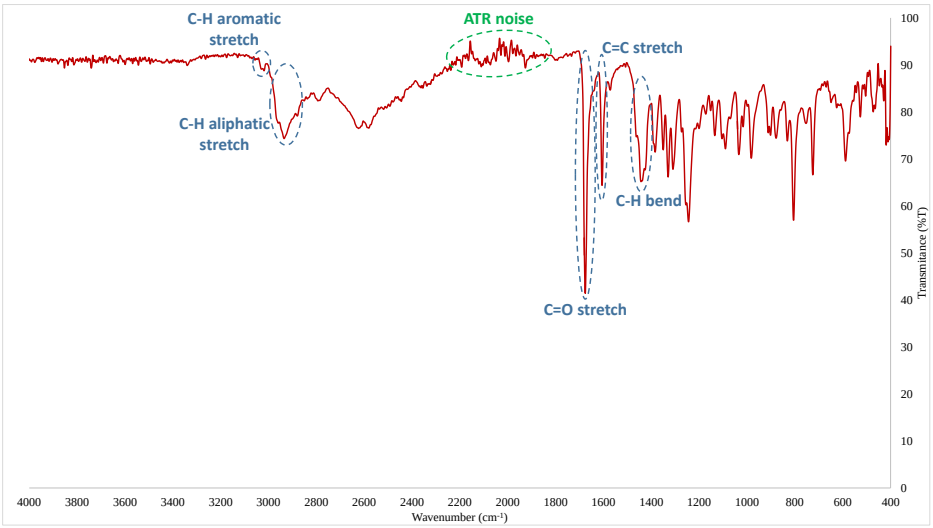


Figure S1. FTIR spectrum of the unknown compound. Characteristic bands are highlighted in blue. ATR noise is highlighted in green.

Analysis by GC–MS revealed the presence of only one organic compound, detectable by this equipment, which presented a chromatographic peak at 9.45 min. When the mass spectrum of this chromatographic peak was extracted (**Figure 1**), no matches were obtained after applying the spectral libraries available at the laboratory (NIST, Cayman Chemical, and a home-made library). The fragmentation spectrum showed only an intense fragment ion at *m/z* 112. No information about the molecular ion could be obtained from the EI spectrum.

The combination of the information provided by FTIR and GC–MS was not enough for compound identification, requiring analysis by HRMS (and NMR) for compound identification.

High-resolution mass spectrometry

The analysis by UHPLC–HRMS confirmed the high purity of the sample, and only a chromatographic peak was observed in the total ion current chromatogram. The low energy function spectrum of this chromatographic peak showed an ion at *m/z* 258.1845, corresponding to the protonated molecule of the compound ($C_{17}H_{24}NO^+$, -2.9 ppm) (**Figure 2a**). The fragmentation observed in the high

energy function spectrum suggested the compound to be a synthetic cathinone (**Figure 2b**). The product ion 2 observed at *m/z* 187.1111 ($C_{13}H_{15}O^+$, -3.4 ppm) suggested the presence of a pyrrolidine moiety (neutral loss of C_4H_9N, 71.0735 Da). This neutral loss has been described for several synthetic cathinones with a pyrrolidine moiety [15, 20, 22, 23]. The product ion 4 (at *m/z* 145.0642, $C_{10}H_9O^+$, -4.5 ppm) indicated that the alkyl chain in the α-carbon of the cathinone should be an ethyl moiety. This fact was in accordance to product ion 1 at *m/z* 229.1448 ($C_{15}H_{19}NO^+$, -5.8 ppm), corresponding to a radical loss of 29.0391 Da (C_2H_5). Finally, product ion 6 at *m/z* 117.0692 ($C_9H_9^+$, -5.8 ppm) was obtained after a CO loss (27.9949 Da) from product ion 4. The double bond equivalence for product ion 6 indicated the presence of 5 insaturations, 4 of them corresponding to the aromatic ring. The remaining one, and the presence of 3 carbon atoms, could be related with the presence of a 2,3-dihydroindene moiety.

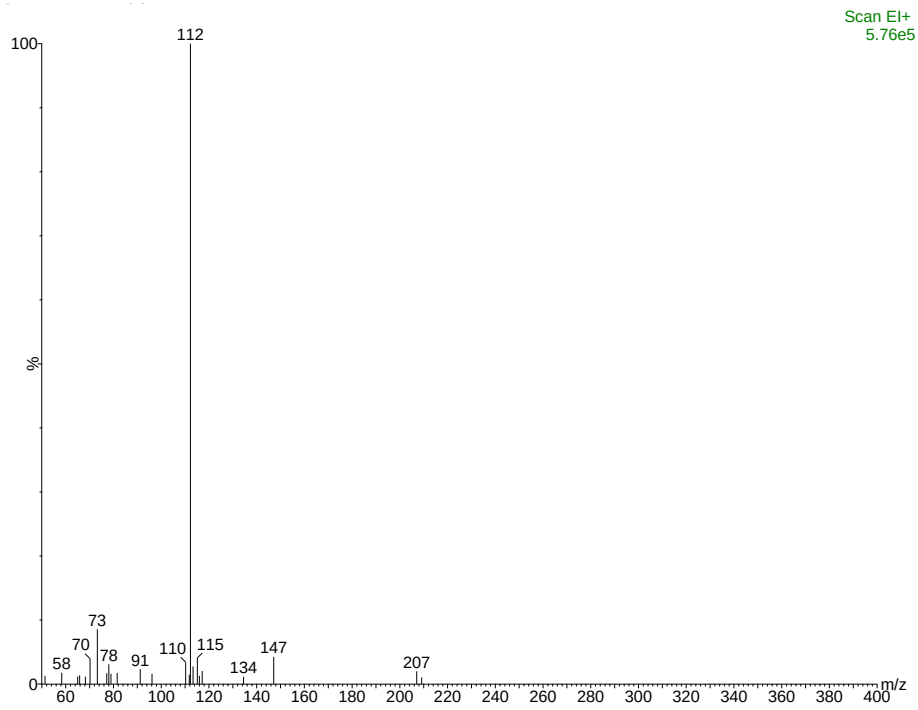


Figure 1. Electron ionization mass spectrum of a chromatographic peak at 9.54 min, corresponding to the unknown compound, obtained by gas chromatography–mass spectrometry.

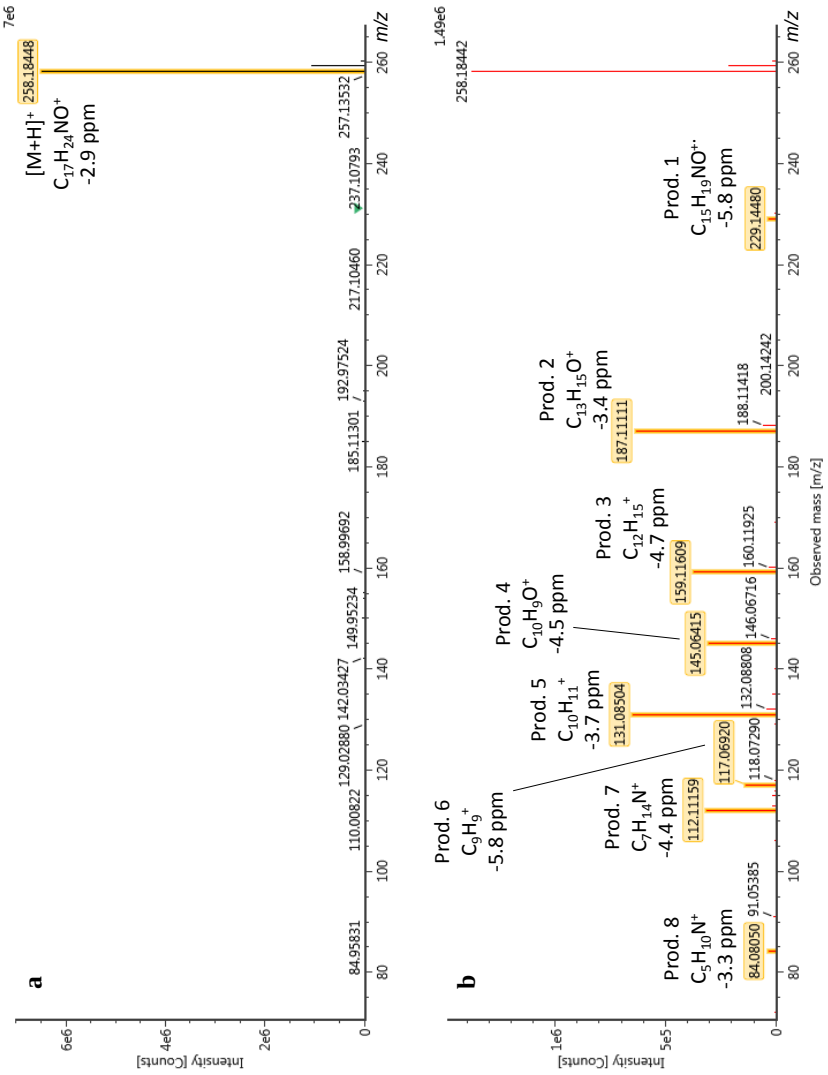


Figure 2. MS^E spectra of the unknown compound. Low energy function (**a**) and high energy function (**b**) spectra of the tentatively identified 5-PPDI.

Thus, a pyrrolidine, an ethyl and a 2,3-dihydroindene moieties would be the three parts of the cathinone structure, being proposed as 1-(2,3-dihydro-1*H*-inden-5-yl)-2-(pyrrolidin-1-yl)butan-1-one. Searching for this systematic name on different websites which sell research chemicals, our putative cathinone was found under the name of 5-PPDI. To the best of our knowledge, this synthetic cathinone has not been reported yet.

Once the compound was tentatively identified as 5-PPDI, the fragmentation pathways for this synthetic cathinone were proposed. As it is shown in **Figure 3**, all the observed product ions could be justified based on the structure of this cathinone. The base peak at *m/z* 112 observed in the EI mass spectrum (**Figure 1**) corresponds to the product ion 7.

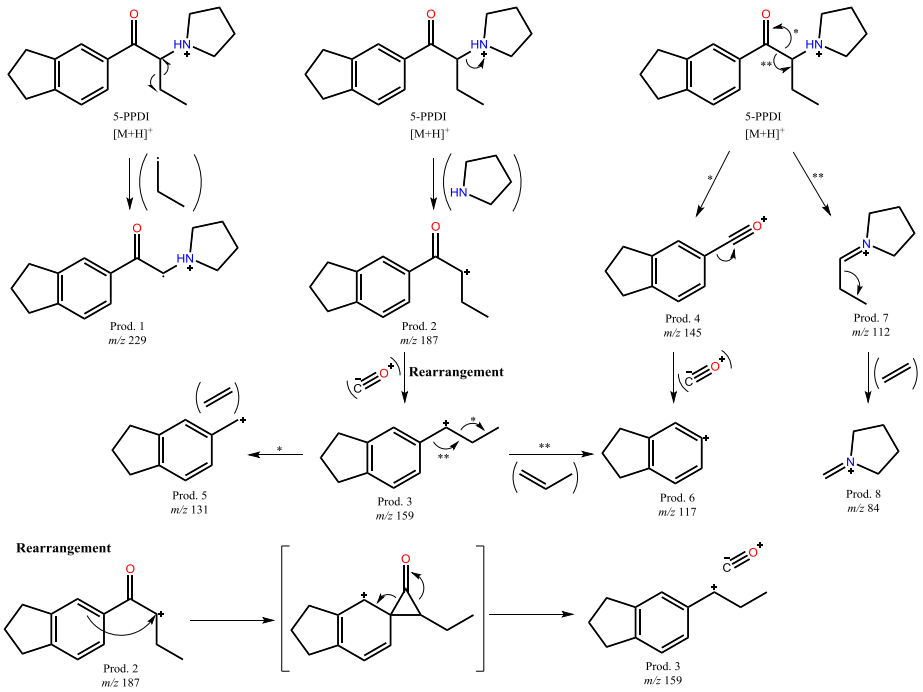


Figure 3. Proposed collision induced dissociation (CID) fragmentation pathways for the 5-PPDI.

Nevertheless, the information obtained by HRMS allowed only a tentative identification. The complete characterization and unequivocal identification of the compound was performed by the combination of different NMR experiments.

Nuclear magnetic resonance

Figure 4 shows the ^{1}H NMR spectrum and the ^{13}C NMR spectrum for the tentatively identified 5-PPDI, and **Table 1** presents signal assignment for ^{1}H and ^{13}C NMR signals.

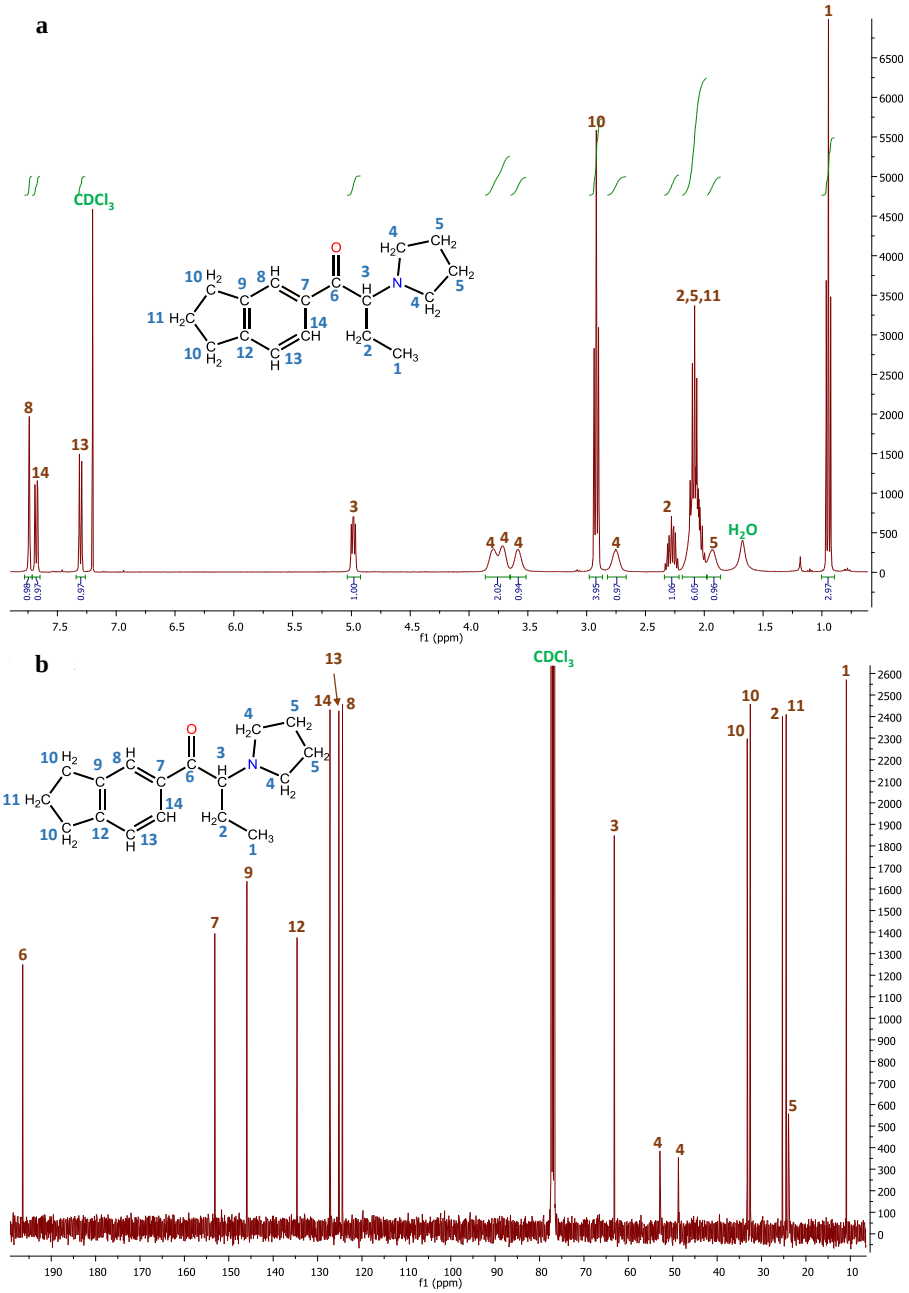
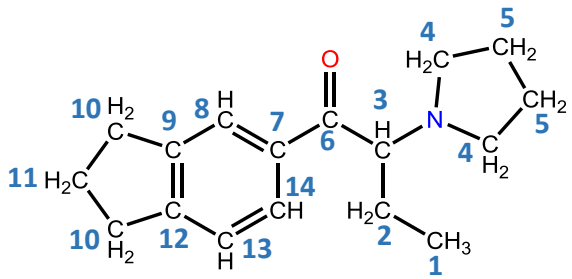


Figure 4. Nuclear magnetic resonance spectra of the unknown substance. **a** ^{1}H spectrum, with proton-signal assignation based on the structure of 5-PPDI. **b** ^{13}C spectrum, with carbon-signal assignation based on its structure.

Table 1. ^{1}H and ^{13}C nuclear magnetic resonance signal assignment.

^{1}H NMR signal assignment			**^{13}C NMR signal assignment**	
Hydrogen	δ (ppm)	Multiplicity	Carbon	δ (ppm)
1	0.94	triplet	1	10.88
2	2.08, 2.29	multiplet	2	25.27
3	4.98	triplet	3	63.16
4	2.75, 3.59, 3.71, 3.79	[a]	4	48.72, 52.88
5	1.93, 2.08	[a]	5	23.92
6	-	-	6	196.43
7	-	-	7	153.16
8	7.74	singlet	8	124.39
9	-	-	9	145.93
10	2.92	triplet	10	32.52, 33.21
11	2.08	[a]	11	24.44
12	-	-	12	134.62
13	7.31	doublet	13	125.21
14	7.67	doublet	14	127.20



δ chemical shift.
[a] multiplicity of these signals could not be stablished

For ^{1}H NMR spectrum, all the observed signals could be justified based on the structure of 5-PPDI without major problems. Nevertheless, some signals presented certain curiosities that should be discussed in more detail. Resonances of hydrogen atoms 4 and 5 presented broad signals, as usual in aliphatic rings

with heteroatoms (for example, the pyrrolidine moiety) [20, 22, 23]. Methylene hydrogens signals which present resonance between δ 1.75 and 2.25 presented overlapping, making the assignation of these signals difficult. These signals were finally assigned after an accurate evaluation of the COSY and HSQC spectra, which can be found in supplementary material (**Figure S2**). The study of HSQC spectra also allowed a direct assignation of ^{13}C NMR signals.

The combination of the NMR experiments and the fragmentation observed in HRMS, allowed the complete characterization of the compound and thus, its identification. Nevertheless, in order to enhance the confidence of compound structure, an additional bidimensional NMR experiment was performed. **Figure 5** shows the HMBC spectrum of 5-PPDI. The multiple bond correlations observed in this experiment confirmed the structure initially proposed. Thus, the compound was unequivocally identified as the synthetic cathinone 5-PPDI.

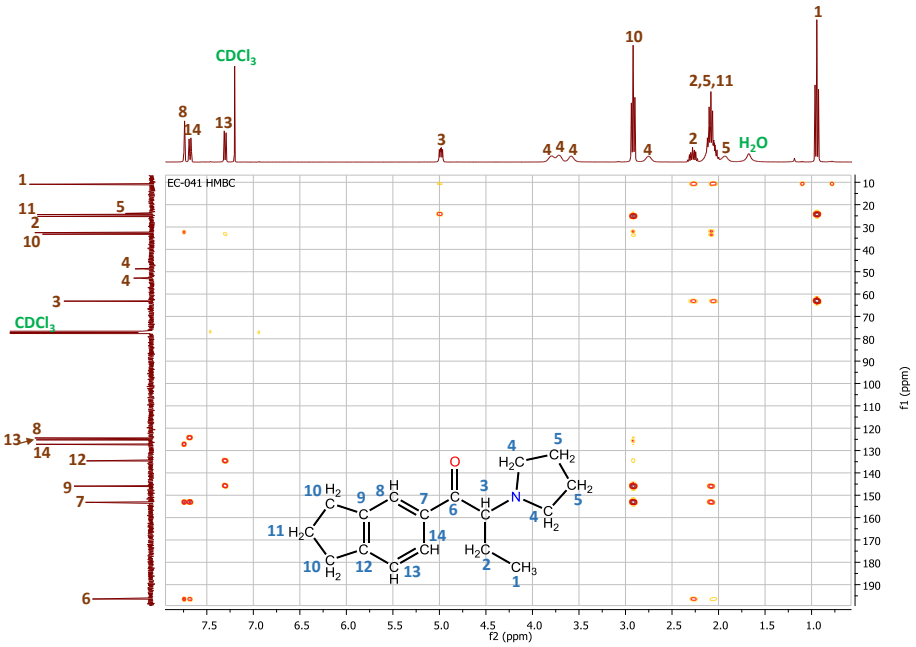


Figure 5. Heteronuclear multiple bond correlation (HMBC) spectra of the compound identified as 5-PPDI.

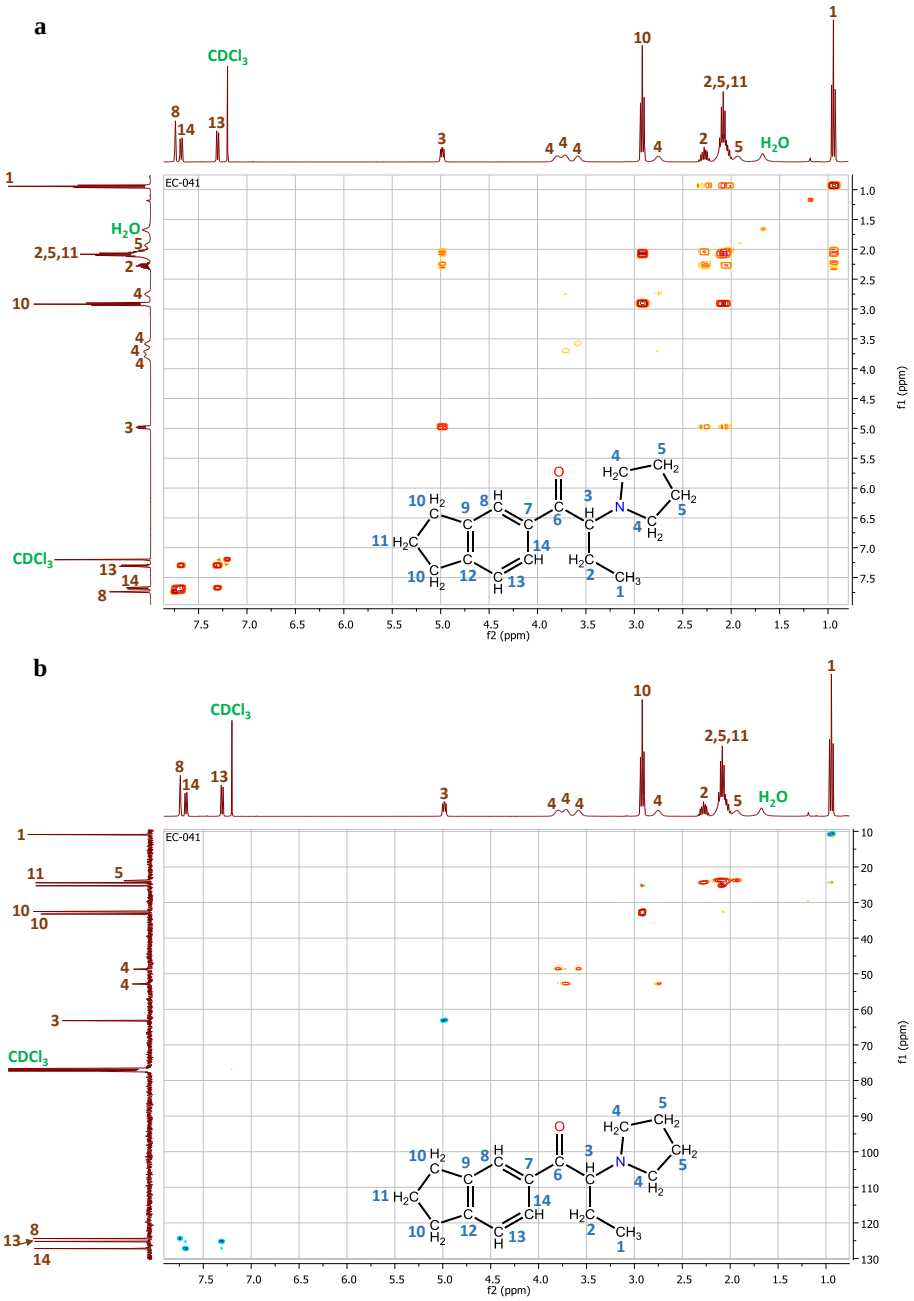


Figure S2. a COSY spectrum of 5-PPDI, showing the correlation between hydrogens. **b** HSQC spectrum of 5-PPDI, linking ^{1}H and ^{13}C NMR signals. CH_3 and CH groups are represented by blue spots, and CH_2 groups are represented by red/yellow spots.

Reasons behind synthesizing 5-PPDI

Structure-activity relationship (SAR) is very difficult to deduce from theoretical data. There is some available information on the SAR of amphetamines, but less for the SAR of synthetic cathinones.

Although SARs of amphetamines and synthetic are not the same, some inferences can be made from modifications in one family to the other. It has been shown that the phenyl ring in pyrovalerone derivatives can be substituted with a benzodioxole and the compound will retain similar activity (α-PVP to MDPV, Fig. 6). Similarly, the benzodioxole moiety in MDA can be substituted with an indane and also will retain similar properties (MDA to 5-APDI). It is therefore a reasonable assumption that the benzodioxole and phenyl moieties are interchangeable in pyrovalerone derivatives and amphetamine analogues. Then, the benzodioxole could be replaced by an indane, substituting the phenyl moiety of α-PBP with an indane moiety which will yield to the active compound 5-PPDI. Replacing the phenyl moiety of α-PBP with a benzodioxole leads to MDPBP, a compound that is, at least, active; it is a logical next step to see if something similar happens with 5-PPDI (**Figure 6**).

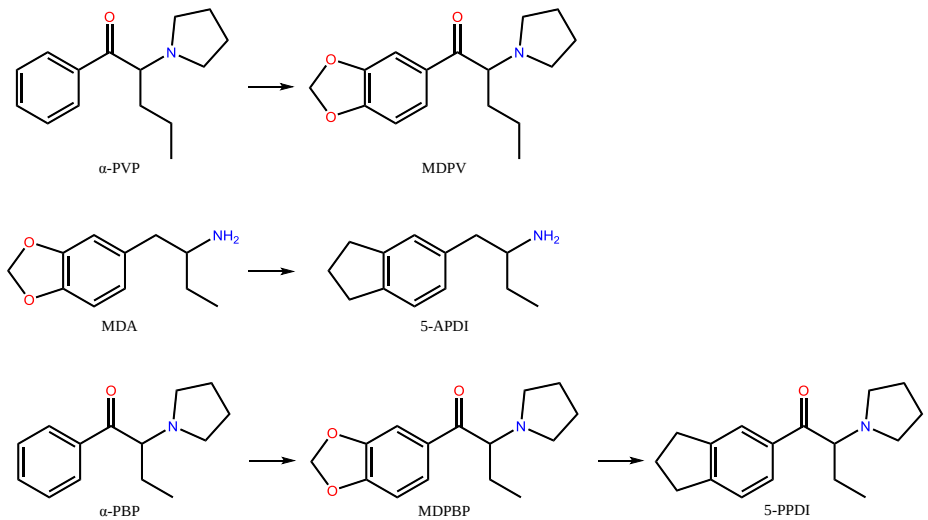


Figure 6. Structures of synthetic cathinones with moiety changes to 5-PPDI.

Because 5-PPDI has not previously appeared in the literature and little is known about it, vendors tend to send it for free with other orders, or even to send a sample at no cost to the consumer in an attempt to get users to describe its effects and generate interest in the substance [26]. It appears that the compound is inactive at dosages similar to other pyrovalerone derivatives, and users tend to not push the dose above which they perceive as safe. Some users report light activity, especially with administration through vaporization of the compound, which is reported to be more potent than oral or nasal use. Reports are mixed however, likely due to factors such as purity, personal tolerance, route of administration, etc., leading to conflicting reports such as one user reporting 20 mg vaporized to be an active dose, and another reporting that 32 mg vaporized to yield no effects. It is also possible that some vendors claim to ship 5-PPDI, but in reality, they ship other compounds, leading to the disparity in effects reported [27, 28].

Conclusions

This work presents the detection and characterization of the novel cathinone 1-(2,3-dihydro-1*H*-inden-5-yl)-2-(pyrrolidin-1-yl)butan-1-one, better known as 5-PPDI. The results obtained in this study remark the limitations of the routine analysis techniques used in forensic laboratories for NPS detection and identification. Thus, FTIR spectroscopy and GC–MS allow a rapid identification of the sample only if the corresponding spectrum has been previously recorded.

In this work, GC–MS revealed that the compound was highly pure, without any other organic compound being detected. Nevertheless, no matches were obtained for the acquired spectrum using commercial libraries, illustrating that this technique is not efficient for structure elucidation of unknown substances or unanalysed compounds; therefore, advanced techniques are required for that aim.

The analysis by UHPLC–HRMS allowed a tentative identification of the compound, based on the accurate-mass fragmentation observed. Nevertheless, due to the lack of an analytical reference standard at the moment of developing this work, the compound could not be unequivocally identified based only on HRMS data. The use of different NMR experiments (^{1}H, ^{13}C, COSY, HSQC and HMBC) confirmed its structure, and after combining this information with that obtained by HRMS, the substance was unequivocally characterized as 5-PPDI.

The analytical data provided in this work will facilitate the detection and identification of this novel synthetic cathinone by forensic and toxicological laboratories, even if they use routine techniques.

Although this compound does not appear to be very potent, and it will be unlikely to see its widespread use, it is interesting to consider that it was synthesized with a clear objective to produce a viable alternative to compounds like α-PBP. Its structure demonstrates some knowledge on pharmacology and SAR of synthetic cathinones, and contributes to clarifying the theory, by which manufacturers of NPS are proficient at finding alternatives to banned compounds. Such a theory casts a doubt on the efficacy of systematically scheduling NPS, because manufacturers have been able to provide alternatives that not only evade legislation, but also are usually active compounds.

Acknowledgements

D. Fabregat-Safont, J.V. Sancho, F. Hernández and M. Ibáñez acknowledge financial support from Generalitat Valenciana (Group of Excellence Prometeo II 2014/023 and from the Ministerio de Economía y Competitividad in Spain (Project: CTQ2015-65603-P). The authors also acknowledge NPS-Euronet (HOME/2014/JDRUG/AG/DRUG/7086), co-funded by the European Union. This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein. The authors are very grateful to the Serveis

Centrals d'Instrumentació Científica (SCIC) of University Jaume I (UJI) for the use of NMR and ATR-FTIR instruments. D. Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (Grant FPU15/02033). X. Carbón, C. Gil and M. Ventura acknowledge the grants from Subdirecció General de Drogodependències, Departament de Salut, Generalitat de Catalunya and Plan Nacional sobre Drogas.

Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. European Monitoring Centre for Drugs and Drug Addiction (2017) European Drug Report 2017. EMCDDA–Europol Jt Publ 88. htpps://doi.org/10.2810/88175

2. Department of Justice. Drug Enforcement Administration (DEA) (2014) Schedules of controlled substances: temporary placement of 10 Synthetic Cathinones Into Schedule I. https://www.federalregister.gov/documents/2014/03/07/2014-04997/schedules-of-controlled-substances-temporary-placement-of-10-synthetic-cathinones-into-schedule-i. Accessed 7 Dec 2017

3. China Food and Drug Administration (2015) 关于印发《非药用类麻醉药品和精神药品列管办法》的通知 公通字〔2015〕27号. http://www.sfda.gov.cn/WS01/CL0056/130753.html. Accessed 7 Dec 2017

4. Casale JF, Hays PA (2012) The characterization of α-pyrrolidinopentiophenone. Microgram J 9:33–38

5. King LA, Kicman AT (2011) A brief history of "new psychoactive substances." Drug Test Anal 3:401–403. htpps://doi.org/10.1002/dta.319

6. Griffiths P, Evans-Brown M, Sedefov R (2013) Getting up to speed with the public health and regulatory challenges posed by new psychoactive substances in the information age. Addiction 108:1700–1703. htpps://doi.org/10.1111/add.12287

7. Brandt SD, King LA, Evans-Brown M (2014) The new drug phenomenon. Drug Test Anal 6:587–597. htpps://doi.org/10.1002/dta.1686

8. Majchrzak M, Celiński R, Kuś P, Kowalska T, Sajewicz M (2018) The newest cathinone derivatives as designer drugs: an analytical and toxicological review. Forensic Toxicol 36:33-50. htpps://doi.org/10.1007/s11419-017-0385-6

9. Coelho Neto J (2015) Rapid detection of NBOME's and other NPS on blotter papers by direct ATR-FTIR spectrometry. Forensic Sci Int 252:87–92. htpps://doi.org/10.1016/j.forsciint.2015.04.025

10. Pereira LSA, Lisboa FLC, Neto JC, Valladão FN, Sena MM (2017) Direct classification of new psychoactive substances in seized blotter papers by ATR-FTIR and multivariate discriminant analysis. Microchem J 133:96–103. htpps://doi.org/10.1016/j.microc.2017.03.032

11. Kudo K, Usumoto Y, Usui K, Hayashida M, Kurisaki E, Saka K, Tsuji A, Ikeda N (2014) Rapid and simultaneous extraction of acidic and basic drugs from human whole blood for reliable semi-quantitative NAGINATA drug screening by GC–MS. Forensic Toxicol 32:97–104. doi: 10.1007/s11419-013-0215-4

12. Namera A, Kawamura M, Nakamoto A, Saito T, Nagao M (2015) Comprehensive review of the detection methods for synthetic cannabinoids and cathinones. Forensic Toxicol 33:175–194. htpps://doi.org/10.1007/s11419-015-0270-0

13. Zuba D (2012) Identification of cathinones and other active components of “legal highs” by mass spectrometric methods. Trends Anal Chem 32:15–30. htpps://doi.org/10.1016/j.trac.2011.09.009

14. Kohyama E, Chikumoto T, Tada H, Kitaichi K, Horiuchi K, Ito T (2016) Differentiation of the isomers of N-alkylated cathinones by GC-EI-MS-MS and LC-PDA. Anal Sci 32:831–837. htpps://doi.org/10.2116/analsci.32.831

15. Ibañez M, Sancho J V., Bijlsma L, van Nuijs ALN, Covaci A, Hernández F (2014) Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. Trends Anal Chem 57:107–117. htpps://doi.org/10.1016/j.trac.2014.02.009

16. Fornal E (2013) Identification of substituted cathinones: 3,4-methylenedioxy derivatives by high performance liquid chromatography–quadrupole time of flight mass spectrometry. J Pharm Biomed Anal 81–82:13–19. htpps://doi.org/10.1016/j.jpba.2013.03.016

17. Fabregat-Safont D, Fornís I, Ventura M, Gil C, Calzada N, Sancho JV, Hernández F (2017) Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test Anal 9:1073–1080. htpps://doi.org/10.1002/dta.2182

18. Westphal F, Junge T, Rösner P, Fritschi G, Klein B, Girreser U (2007) Mass spectral and NMR spectral data of two new designer drugs with an α-aminophenone structure: 4′-methyl-α-pyrrolidinohexanophenone and 4′-methyl-α-pyrrolidinobutyrophenone. Forensic Sci Int 169:32–42. htpps://doi.org/10.1016/j.forsciint.2006.07.024

19. Kuś P, Kusz J, Książek M, Pieprzyca E, (2017) Spectroscopic characterization and crystal structures of two cathinone derivatives: N-ethyl-2-amino-1-phenylpropan-1-one (ethcathinone) hydrochloride and N-ethyl-2-amino-1-(4-chlorophenyl)propan-1-one (4-CEC) hydrochloride. Forensic Toxicol 35:114–124. htpps://doi.org/10.1007/s11419-016-0345-6

20. Majchrzak M, Rojkiewicz M, Celiński R, Kuś P, Sajewicz M (2016) Identification and characterization of new designer drug 4-fluoro-PV9 and α-PHP in the seized materials. Forensic Toxicol 34:115–124. htpps://doi.org/10.1007/s11419-015-0295-4

21. Qian Z, Jia W, Li T, Hua Z, Liu C (2017) Identification and analytical characterization of four synthetic cannabinoids ADB-BICA, NNL-1, NNL-2, and PPA(N)-2201. Drug Test Anal 9:51–60. htpps://doi.org/10.1002/dta.1990

22. Liu C, Jia W, Li T, Hua Z, Qian Z (2017) Identification and analytical characterization of nine synthetic cathinone derivatives N-ethylhexedrone, 4-Cl-pentedrone, 4-Cl- α -EAPP, propylone, N-ethylnorpentylone, 6-MeO-bk-MDMA, α -PiHP, 4-Cl- α -PHP, and 4-F- α -PHP. Drug Test Anal 9:1162–1171. htpps://doi.org/10.1002/dta.2136

23. Apirakkan O, Frinculescu A, Shine T, Parkin MC, Cilibrizzi A, Frascione N, Abbate V (2018) Analytical characterization of three cathinone derivatives, 4-MPD, 4F-PHP and bk-EPDP, purchased as bulk powder from online vendors. Drug Test Anal 10:372-378. htpps://doi.org/10.1002/dta.2218

24. Fabregat-Safont D, Carbón X, Ventura M, Fornís I, Guillamón E, Sancho JV, Hernández F, Ibáñez M (2017) Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci Rep 7:6338. htpps://doi.org/10.1038/s41598-017-06778-9

25. González D, Ventura M, Caudevilla F, Torrens M, Farré M (2013) Consumption of new psychoactive substances in a Spanish sample of research chemical users. Hum Psychopharmacol Clin Exp 28:332–340. htpps://doi.org/10.1002/hup.2323

26. Flashback (2015) Ny RC "5-PPDI" Någon info om detta?. https://www.flashback.org/t2540560. Accessed 7 Dec 2017

27. Reddit (2015) 5-PPDI. https://www.reddit.com/r/researchchemicals/comments/2qhdkl/5ppdi/. Accessed 7 Dec 2017

28. Reddit (2015) 5-PPDI experiment #2. https://www.reddit.com/r/researchchemicals/comments/2u9hzw/5ppdi_experiment_2/. Accessed 7 Dec 2017

2.5. Research article IV

www.nature.com/scientificreports

SCIENTIFIC REPORTS

OPEN

Characterization of a recently detected halogenated aminorex derivative: *para*-fluoro-4-methylaminorex (4′F-4-MAR)

Received: 5 July 2018
Accepted: 20 May 2019
Published online: 05 June 2019

D. Fabregat-Safont[1], X. Carbón[2], M. Ventura[2], I. Fornís[2], F. Hernández[1] & M. Ibáñez[1]

Despite the fact that 33% of the new psychoactive substances seized in 2015 were synthetic cathinones, the number of these derivatives has been decreasing in the last years, probably as a consequence of the unfavourable effects reported by users. Thus, the list of possible cathinone analogues is expected to get shorter, and it is likely that the same moiety changes applied for the preparation of synthetic cathinones will be applied in the near future to other stimulants in the search for favourable alternatives to controlled substances. This is evidenced by the increase in newly reported substances belonging to stimulant classes other than cathinones. One of the possible candidates for a new backbone from which to base new stimulants is aminorex, which is classified as a Schedule I substance by the Drug Enforcement Administration. Three derivatives have been reported until now: 4-methylaminorex or 4-MAR (also categorized as a Schedule I substance), para-methyl-4-methylaminorex (4,4′-DMAR) and 3′,4′-methylenedioxy-4-methylaminorex (MDMAR). Recently, the new halogenated 4-MAR derivative, para-fluoro-4-methylaminorex, characterised in this work (and abbreviated as pF-4-methylaminorex or 4′F-4-MAR) was detected by the Slovenian police. In the present work, 4′F-4-MAR has been characterized by high resolution mass spectrometry and nuclear magnetic resonance in a sample obtained from an anonymous consumer. This research shows that the same modifications applied for the preparation of synthetic cathinones are being used to prepare new stimulants based on the aminorex backbone.

The use of synthetic stimulants has increased in Europe in the last years, based on the seizures reported by the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) in its 2017 Report[1]. According to this report, 33% of the substances seized in 2015 were synthetic cathinones, and 6% phenethylamines[1]. Synthetic cathinones are, nowadays, the second largest group of new psychoactive substances (NPS) monitored by the EMCDDA, and the most frequently seized NPS in 2015[1]. In the last years, the characterization of novel synthetic cathinones has been an on-going topic for forensic laboratories, showing that modifications of the cathinone moieties are the most frequent option for the production of novel stimulants[2–9]. Nonetheless, the number of new synthetic cathinones reported every year is decreasing[1], and the effects profile of the newest cathinone derivatives as reported by users have been unfavourable (especially when compared to older cathinone analogues)[10]. While the cathinone backbone appears to be capable of tolerating highly bulky as well as polar substitutions and additions while retaining psychoactivity, it seems that the list of possible analogues is getting shorter. Based on this trend, it is expected that in the next years, these modifications will be keep being applied to other stimulant classes in the search for favourable (to users) alternatives to controlled substances. This is evidenced by the increase in newly reported substances belonging to stimulant classes like the phenethylamines and piperazines[1].

Over the past few years, the cathinone family has been the most common starting point for the production of analogs that belong in the stimulants class[11]. Several other substance families have been used, in much lesser instances, as a backbone to produce analogs, such as amphetamine (2-FA, 3-FA, 4-FA)[12], phenmetrazine (3-FPM)[13], methylphenidate[14], etc. There are not many cases analogs of aminorex[15], so it remains a possible choice for producers of NPS to explore. Aminorex (Fig. 1) was patented by McNeil Laboratories in 1964 (US Patent

[1]Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castelló de la Plana, Spain. [2]Energy Control (Asociación Bienestar y Desarrollo), c/Independencia 384, 08041, Barcelona, Spain. Correspondence and requests for materials should be addressed to M.I. (email: ibanezm@uji.es)

Scientific Reports, 2019; 9:8314

Characterization of a recently detected halogenated aminorex derivative: *para*-fluoro-4-methylaminorex (4'F-4-MAR)

D. Fabregat-Safont [1], X. Carbón [2], M. Ventura [2], I. Fornís [2], J.V. Sancho [1], F. Hernández [1] & M. Ibáñez [1*]

[1] Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12071 Castelló de la Plana, Spain.

[2] Energy Control (Asociación Bienestar y Desarrollo), c/ Independencia 384, 08041 Barcelona, Spain.

Abstract

Despite the fact that 33% of the new psychoactive substances seized in 2015 were synthetic cathinones, the number of these derivatives has been decreasing in the last years, probably as a consequence of the unfavourable effects reported by users. Thus, the list of possible cathinone analogues is expected to get shorter, and it is likely that the same moiety changes applied for the preparation of synthetic cathinones will be applied in the near future to other stimulants in the search for favourable alternatives to controlled substances. This is evidenced by the increase in newly reported substances belonging to stimulant classes other than cathinones. One of the possible candidates for a new backbone from which to base new stimulants is aminorex, which is classified as a Schedule I substance by the Drug Enforcement Administration. Three derivatives have been reported until now: 4-methylaminorex or 4-MAR (also categorized as a Schedule I substance), *para*-methyl-4-methylaminorex (4,4'-DMAR) and 3',4'-methylenedioxy-4-methylaminorex (MDMAR). Recently, the new halogenated 4-MAR derivative, *para*-fluoro-4-methylaminorex, characterised in this work (and abbreviated as pF-4-methylaminorex or 4'F-4-MAR) was detected by the Slovenian police. In the present work, 4'F-4-MAR has been characterized by high

resolution mass spectrometry and nuclear magnetic resonance in a sample obtained from an anonymous consumer. This research shows that the same modifications applied for the preparation of synthetic cathinones are being used to prepare new stimulants based on the aminorex backbone.

Keywords Aminorex; *para*-fluoro-4-methylaminorex; 4'F-4-MAR; Synthetic stimulants; High-resolution mass spectrometry; Nuclear magnetic resonance.

The use of synthetic stimulants has increased in Europe in the last years, based on the seizures reported by the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) in its 2017 Report [1]. According to this report, 33% of the substances seized in 2015 were synthetic cathinones, and 6% phenethylamines [1]. Synthetic cathinones are, nowadays, the second largest group of new psychoactive substances (NPS) monitored by the EMCDDA, and the most frequently seized NPS in 2015 [1]. In the last years, the characterization of novel synthetic cathinones has been an on-going topic for forensic laboratories, showing that modifications of the cathinone moieties are the most frequent option for the production of novel stimulants [2–9]. Nonetheless, the number of new synthetic cathinones reported every year is decreasing [1], and the effects profile of the newest cathinone derivatives as reported by users have been unfavourable (especially when compared to older cathinone analogues) [10]. While the cathinone backbone appears to be capable of tolerating highly bulky as well as polar substitutions and additions while retaining psychoactivity, it seems that the list of possible analogues is getting shorter. Based on this trend, it is expected that in the next years, these modifications will be keep being applied to other stimulant classes in the search for favourable (to users) alternatives to controlled substances. This is evidenced by the increase in newly reported substances belonging to stimulant classes like the phenethylamines and piperazines [1].

Over the past few years, the cathinone family has been the most common starting point for the production of analogues that belong in the stimulants class [11]. Several other substance families have been used, in much lesser instances, as a backbone to produce analogues, such as amphetamine (2-FA, 3-FA, 4-FA) [12], phenmetrazine (3-FPM) [13], methylphenidate [14], etc. There are not many cases analogues of aminorex [15], so it remains a possible choice for producers of NPS to explore. Aminorex (**Figure 1**) was patented by McNeil Laboratories in 1964 (US Patent 3,161,650) as a new central nervous system stimulant [16]. Aminorex was used in different countries as an appetite suppressor [17], but was rapidly withdrawn due to the pulmonary hypertension it produced, which was linked with several

deaths [18,19]. In the United States, aminorex is classified as a Schedule I substance by the Drug Enforcement Administration (DEA) [20]. The most well-known aminorex derivative is 4-methylaminorex or 4-MAR (**Figure 1**), also patented by McNeil Laboratories in 1966 [21]. Several years ago, it was discovered that 4-MAR produces stimulant effects similar to those of cocaine [22–24], as well as several neurochemical effects complications derived from its use [25], leading to a fatality related to this compound [26]. For this reason, 4-MAR is also categorized as a Schedule I substance in the U.S. [20].

In 2014, *para*-methyl-4-methylaminorex (4,4'-DMAR) (**Figure 1**), a novel 4-MAR derivative, was reported and characterized [15,27]. Together with Europol, EMCDDA published an early warning notification in 2014, notifying of the involvement of 4,4'-DMAR in several deaths in Europe, 8 of them in Hungary (June 2013) and 18 in the United Kingdom (between June and December 2013) [28]. Additionally, EMCDDA-Europol published a joint report providing all the information available for 4,4'-DMAR [29]. One year later, the European Union decided to apply control measures to this aminorex derivative [30]. Nevertheless, in 2014 another 4-MAR derivative was reported and characterized, the 3',4'-methylenedioxy-4-methylaminorex or MDMAR (**Figure 1**) [31]. At the time of writing this paper, no additional information has been found for this compound.

The development of aminorex derivatives by modification of the aromatic ring has probably been carried out based on the "successful" synthesis of novel synthetic cathinones that use the same methodology. Two of the reported derivatives presented a *para*-methyl (4,4'-DMAR) and a 3,4-dioxane (MDMAR) moieties. These substitutions can be easily found in well-known synthetic cathinones: using the structure of methcathinone [32], mephedrone (4-methyl-methcathinone or 4-MMC) [33] was synthetized by adding a *para*-methyl moiety, and methylone (3,4-methylenedioxy-N-methylcathinone or bk-MDMA) [34] by adding a 3,4-dioxane moiety (**Figure 2**). Moreover, 4-FMC (4-fluoromethcathinone or flephedrone) [35] and the recently reported 4-CMC (4-chloromethcathinone or clephedrone) [36], were also synthetized based on different

aromatic substitutions of methcathinone (**Figure 2**). In this case, halogen atoms were used as para-substituents of the aromatic ring, producing novel active stimulants. In the light of this information, it would not be surprising that, in the next years, para-methoxy or para-chloro 4-MAR derivatives will appear in the streets.

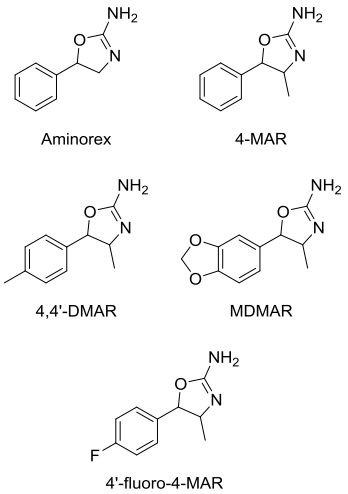


Figure 1. Structure of aminorex and its reported derivatives, including the new derivative reported in this work.

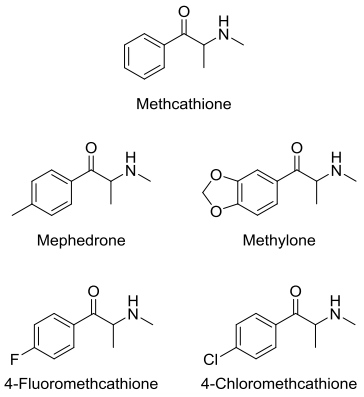


Figure 2. Structure of methcathinone and its reported derivatives.

Recently, a new halogenated 4-MAR derivative was detected by the Slovenian Police [37], and reported in literature [38]. In the present study, *para*-fluoro-4-methylaminorex was fully characterized in a sample obtained from an anonymous Polish consumer. The compound was named as 4'F-4-MAR (or pF-4-methylaminorex), following a similar nomenclature to that used for other aminorex derivatives. Its characterization was performed by ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) and two-dimension nuclear magnetic resonance (NMR) experiments. Analyses with gas chromatography coupled to mass spectrometry with electron ionization (GC-MS), Fourier-transformed infrared spectroscopy (FTIR), as well as ^{1}H and ^{13}C NMR experiments were also performed in order to provide as much information as possible. Additionally, this research illustrates

how the same moiety changes applied for the preparation of synthetic cathinones are being used to prepare new stimulants based on the 4-MAR structure. All analytical information provided in this work will be useful for the identification of 4'F-4-MAR or for future 4-MAR derivatives in forensic laboratories.

Results

Identifying the 4'F-4-MAR

When the compound was injected into the UHPLC-HRMS, a single chromatographic peak at 2.63 min was observed in the total ion chromatogram. **Figure 3** shows the low energy and high energy spectra obtained by HRMS for this chromatographic peak. The low energy spectrum corresponding to this peak showed an ion at *m/z* 195.0933, which would correspond to the protonated molecule of the compound ($[M+H]^+$) (**Figure 3A**). The elemental composition for $[M+H]^+$ was determined to be $C_{10}H_{12}FN_2O^+$ (-0.1 mDa) based on the accurate-mass observed. According to the aminorex elemental composition ($C_9H_{10}N_2O$) [31], this putative derivative should contain an extra methyl group and a fluorine atom on the aminorex structure. The high energy spectrum of the chromatographic peak showed six important fragment ions (**Figure 3B**). Fragment 1 ($C_9H_{11}FN^+$, -0.2 mDa) corresponds to a CHNO loss. This specific loss has been reported in literature for two aminorex derivatives, the *para*-methyl-4-methylaminorex (4,4'-DMAR) and the 3′,4′-methylenedioxy-4-methylaminorex (MDMAR) [15,31]. The loss of CHNO from $[M+H]^+$ produces the formation of an *N*-epoxide structure on the molecule, in the same way than 4,4'-DMAR and MDMAR [15,31]. Fragment 3 ($C_9H_8F^+$, -0.1 mDa) would derive from Fragment 1 after an ammonia molecule loss, according to its elemental composition. Fragment 5 ($C_9H_7^+$, -0.1 mDa) produced after a HF molecule loss from Fragment 3, did not provide any information about the position of the fluorine atom or of the methyl group. Nevertheless, Fragment 6 ($C_7H_6F^+$, 0.0 mDa) would correspond to a fluorotropylium ion. This fragment ion is in

concordance with the tropylium ion observed for the 4,4'-DMAR [15]. Fragment 6 would indicate that the fluorine atom could be on the aromatic ring. Nevertheless, the accurate-mass fragmentation observed was not enough for confirming the identity of the compound.

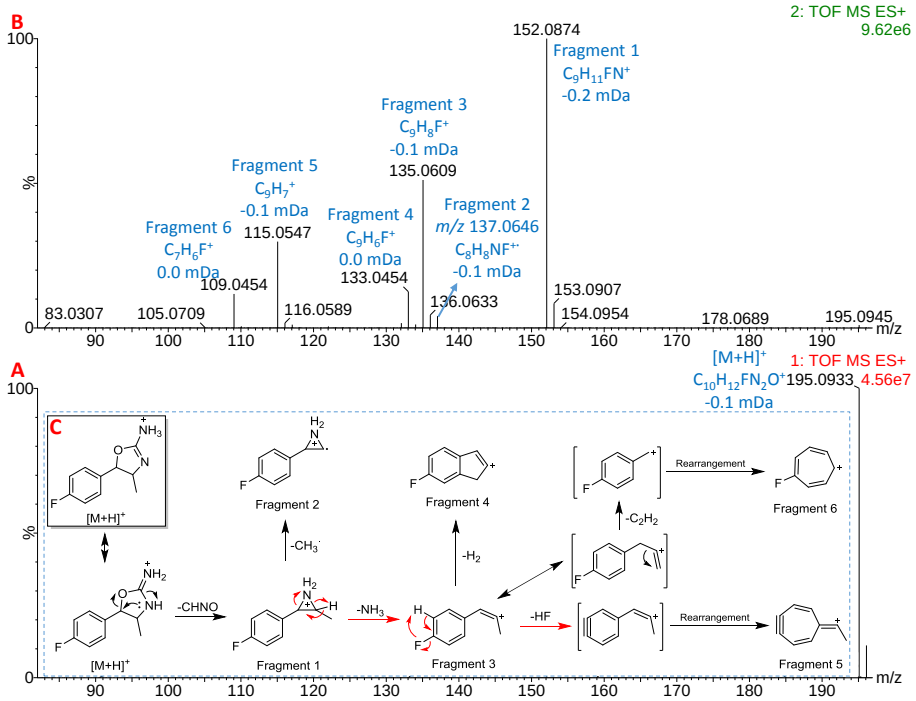


Figure 3. UHPLC-HRMS information of the putative aminorex derivative. **A** Low energy spectrum. **B** High energy spectrum. **C** Proposed fragmentation pathway once identified the new aminorex derivative.

In order to find the location of the methyl group and establish the position of the fluorine atom in the aromatic ring, two two-dimension NMR experiments (COSY and HSQC) were performed. **Figure 4** shows the COSY spectrum (**A**) and the HSQC spectrum (**B**) of the putative aminorex derivative. The doublet at $\delta = 1.32$ ppm, which integration was 3 in the COSY spectrum (**Figure 4A**, and thus in a ^{1}H spectrum) indicated the presence of a methyl group in the structure, as supposed based on the elemental composition ($C_{10}H_{11}FN_2O$) determined by HRMS. The HSQC spectrum established that the carbon atom of this methyl was at $\delta = 21.50$ ppm in the ^{13}C spectrum (**Figure 4B**), as expected for this moiety.

According to the couplings observed in the COSY, this methyl group is near to a tertiary carbon (multiplet at δ = 3.90 ppm in ^{1}H spectrum -**Figure 4A**- which integrates for 1, and δ = 68.31 ppm in ^{13}C -**Figure 4B**-). Moreover, this tertiary atom is coupled to another tertiary atom (doublet at δ = 4.90 ppm in ^{1}H spectrum –**Figure 4A**– which integrates for 1, and δ = 88.28 ppm in ^{13}C –**Figure 4B**–), which multiplicity indicates that it has only one hydrogen to be coupled (the first tertiary carbon mentioned). The high δ observed for this second tertiary atom indicates that it should be near to electronegative moieties such as oxygen atoms, nitrogen atoms or aromatic rings. These observations established the methyl group to be on the position 4 of the 4,5-dihydrooxazol ring, similarly to 4-MAR [22], 4,4'-DMAR [15,31], and MDMAR [15,31]. Moreover, this position was in concordance with the CHNO loss observed in HRMS (Fragment 1 in **Figure 3**), which can only occur if the methyl group is in this position. Once the position of the methyl group was established, the position of the fluorine atom on the aromatic ring was determined. The aromatic signals observed in the COSY spectrum (signals at δ = 7.07 and 7.31 in the ^{1}H spectrum) presented the typical pattern of a *para*-substituted aromatic ring. Based on this fact and considering the fluorotropylium ion observed in HRMS (Fragment 6 in **Figure 3**), the fluorine atom should be in position 4 of the aromatic ring. With this information, the novel aminorex derivative should be the *para*-fluoro-4-methylaminorex, which could be known as 4'F-4-MAR based on the nomenclature used for the other aminorex derivatives reported previously.

Once the structure of this compound was established, all the NMR signals observed in the COSY and HSQC (and thus the ^{1}H and ^{13}C signals) were successfully assigned to the proposed structure. **Figure 4A** and **B** show the signal assignation based on the structure of 4'F-4-MAR. The fragmentation pathway of 4'F-4-MAR was also proposed based on the accurate-mass fragmentation (**Figure 3C**).

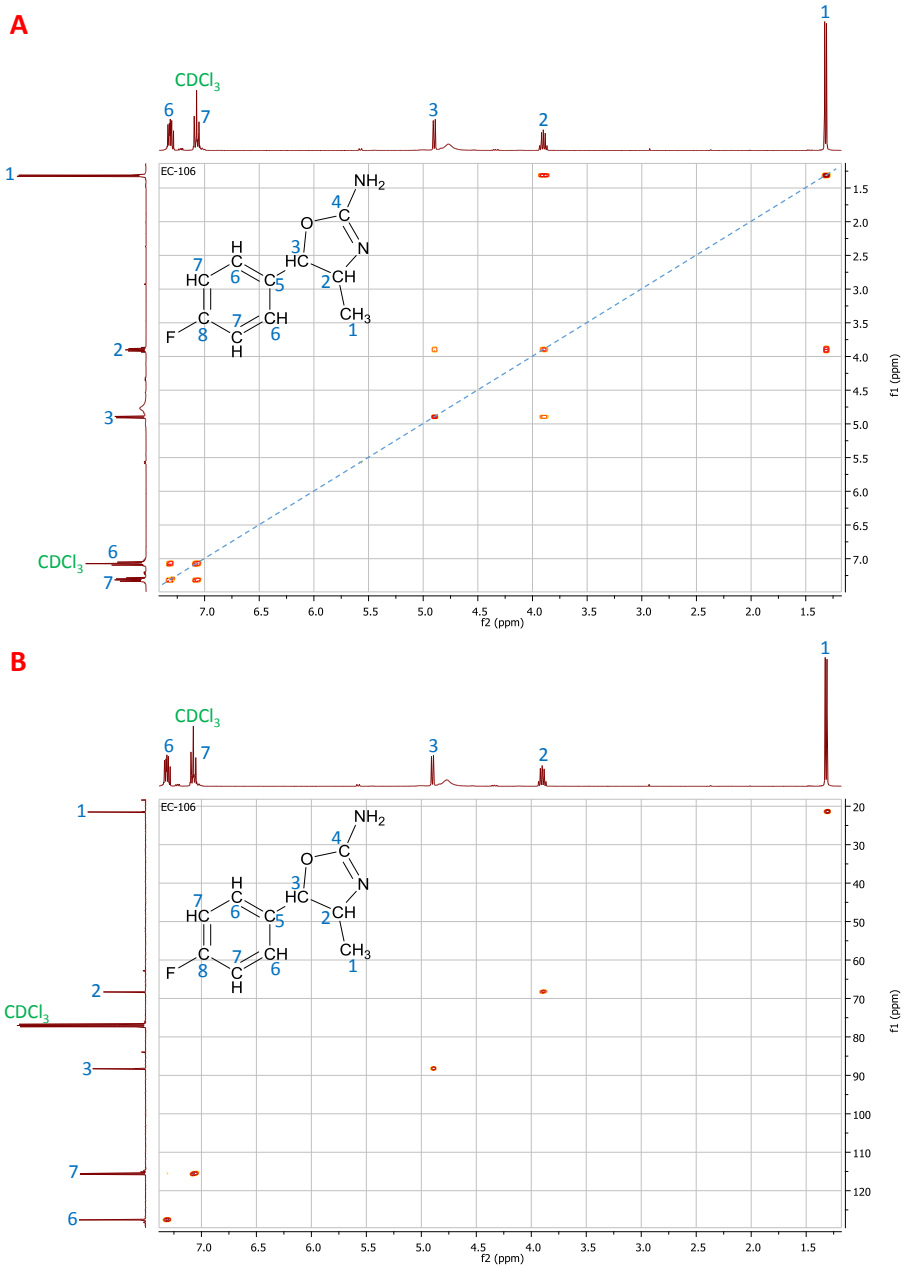


Figure 4. Two-dimension NMR experiments, with signal assignation, performed for the identification of the novel aminorex derivative. **A** COSY spectrum. **B** HSCQ spectrum.

In order to provide a complete characterization of the compound and facilitate the identification of 4'F-4-MAR by forensic laboratories, GC-EI-MS and FTIR analyses were also carried out. The EI spectrum and FTIR spectrum can be found in **Figure S1** and **Figure S2**, respectively. The ^{1}H and ^{13}C NMR spectra are also provided (**Figure S3** and **Figure S4**, respectively).

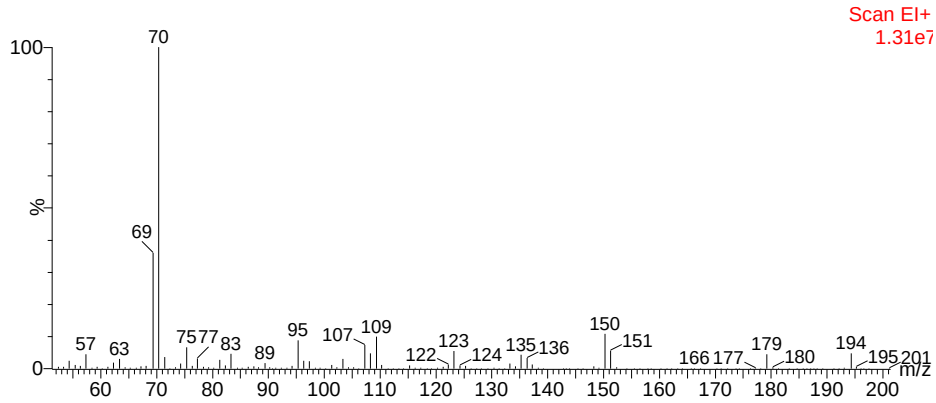


Figure S1. GC-(EI)MS spectrum of the 4'-fluoro-4-MAR.

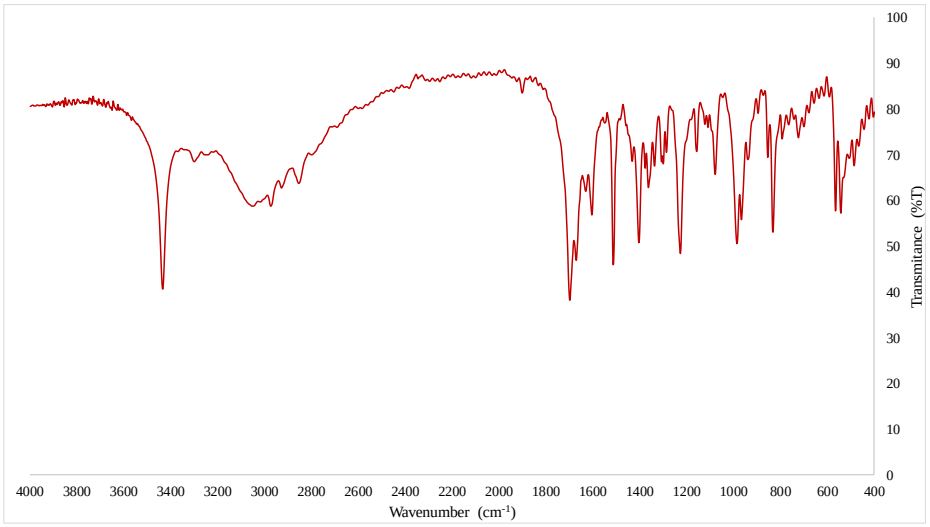


Figure S2. FTIR spectrum of the 4'-fluoro-4-MAR.

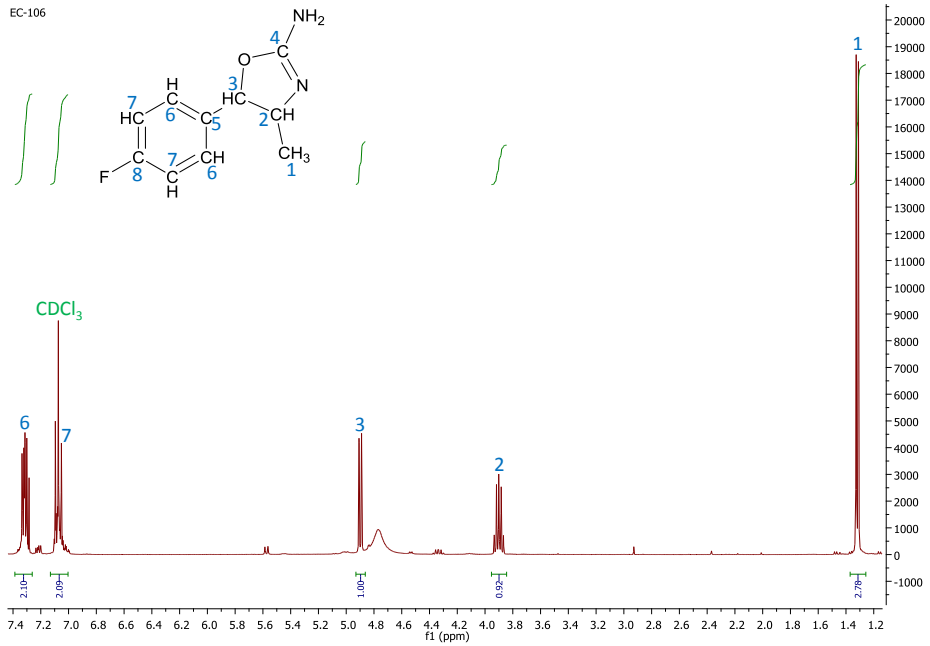


Figure S3. ^{1}H NMR spectrum of the 4'-fluoro-4-MAR.

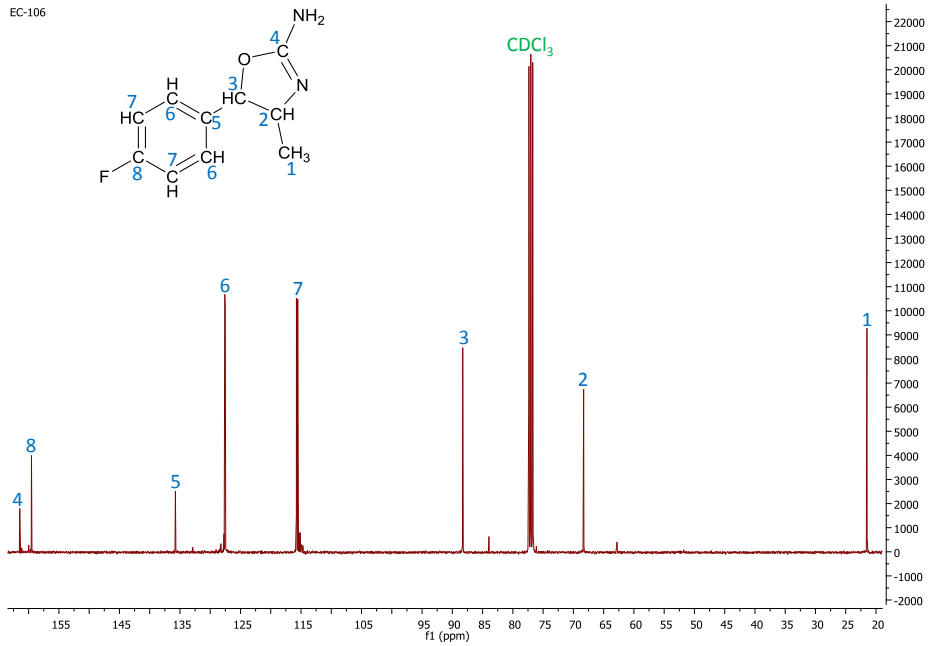


Figure S4. ^{13}C NMR spectrum of the 4'-fluoro-4-MAR.

Discussion

A decrease in the number of new cathinones detected every year has been observed in the last few years, together with an increase in other groups of stimulants such as phenethylamines [1]. It appears that the list of possible cathinone analogues that have not been synthesized is dwindling, and producers of NPS are struggling to find substitutions that will yield an active compound [39]. Although theoretically the possibilities are nearly endless, there is a limit to the substitutions that the cathinone backbone can tolerate and still yield an active compound. It appears that the limit has nearly been reached, and producers appear to be exploring other backbones from which to create new psychoactive substances.

One example is amphetamine. In the past year, we have seen the first detection of 2-FEA, 3-FEA, and 4-FEA, three new compounds based on the amphetamine backbone [40]. These compounds follow the same modification pattern of the aromatic ring to create a new analogue [41]. Nevertheless, they have reached limited popularity due to their lower effects and large required doses respect to amphetamine, as each carbon added to the amine chain of amphetamine appears to make the compound less potent [42].

The aminoindane family has also been explored with the same pattern of limited success, with compounds such as MDAI, 5-IAI, or 2-AI being reported [43]. The same can be observed with the phenmetrazine (e.g. 3-FPM) [13]. These seem to yield compounds that are either not potent or popular enough to make their production profitable. The logical next step by producers is to continue exploring possible new families from which to create new analogues.

There are three distinct advantages that likely motivated the decision of synthesizing another aminorex analog, specifically 4'F-4-MAR: ease of synthesis, available data on effects, and users appeal.

There are discussions on synthesis forums, dating to around 2003, where a functioning synthesis is discussed and perfected [44]. For that synthesis, the described precursor is the same one used in 4-FA synthesis [45], which means that the precursor for an NPS that is banned in several countries can be repurposed to produce a new substance not currently under strict restrictions.

Additionally, as can be seen in the archived hive thread referenced above about the synthesis of 4'F-4-MAR, bioassays by the forum users are positive, describing pleasant effects at fairly low doses and no immediate negative side effects [38]. This is very useful for producers because it removes some of the uncertainty regarding the recreational potential of this substance. Often there is no available data about effects of a new NPS on humans, and favourable *in vitro* and/or animal studies do not translate well to human effects.

The Aminorex family appears to be well-liked by forum users, as it is not explicitly mentioned in existing analog laws like the German NpSG [46] and because of an expectation of recreational potential stemming from the effects and popularity of 4-methylaminorex before it was banned [47]. Consequently, users will likely be willing to sample 4'F-4-MAR, and not much marketing will surely be needed by producers or vendors.

As final note, an unknown sample suspected to contain an aminorex derivative has been analysed by HRMS, allowing the tentative identification of the recently reported para-fluoro-4-methylaminorex or 4'F-4-MAR. The unequivocal identification and structure confirmation was performed by two-dimension NMR experiments (COSY and HSQC). The compound was also analysed by GC-EI-MS, FTIR, ^{1}H and ^{13}C NMR in order to provide additional information to forensic laboratories.

Based in the glorification of the effects of 4,4'-DMAR and 4-MAR by users on online forums after they were banned, it is reasonable to think that the aminorex family is an attractive candidate for the new stimulant backbone that producers are looking for [47–50]. 4'F-4-MAR appears to be the next analog that has been

released. If the users evaluate this compound positively in forum discussions and demand is high, it is likely that producers will manufacture further analogues. As such, it is possible that the number of stimulants based on the cathinone backbone reported every year will keep decreasing while the number of stimulants based on aminorex backbone will continue to rise.

Data available on aminorex analogues from forums, where advanced pharmacology and synthesis are discussed, allows for a rare glimpse of the possible factors that influence the process of NPS production and distribution. Our findings support the theory that this process is relatively complex and involves a more-than-rudimentary grasp on chemistry, pharmacology, and business concepts by producers of these novel psychoactive substances.

The information provided in this paper will be of help to analytical/forensic laboratories for the identification of 4'F-4-MAR or for potential 4-MAR derivatives that may appear in the near future.

Methods

Research chemical sample

The research chemical sample was deferred by an anonymous user to Energy Control's [51] for its analysis. This sample was suspected to be a novel aminorex derivative based on the information provided by the anonymous user, so this was the premise in order to identify it.

Reagents and chemicals

For GC-MS analysis, GC-grade n-hexane and GC-grade acetone were purchased from Scharlau (Scharlab, Barcelona, Spain). For FTIR analysis, potassium bromide (KBr) was purchased from Scharlau. HPLC-grade water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol (MeOH) and acetonitrile (ACN), acetone, formic acid (HCOOH) and sodium

hydroxide (NaOH) were acquired from Scharlau (Scharlab, Barcelona, Spain). Leucine enkephalin was bought from Sigma-Aldrich (St. Louis, MO, USA). Deuterated chloroform ($CDCl_3$) was purchased from Sigma-Aldrich.

Sample treatment

For FTIR analysis, sample was prepared with 5% of the unknown compound and 95% of potassium bromide, homogenized in an agate mortar and compressed under a pressure of 5000 kg/cm^2.

For GC-MS analysis, 10 mg of sample were extracted with 1 mL of acetone in an ultrasonic bath for 15 min. After centrifugation, the supernatant was five thousand-fold diluted with GC-grade n-hexane, and 1 µL of the extract were injected in the GC-MS system.

For UHPLC-HRMS analysis, 10 mg of sample were extracted with 1 mL of acetone in an ultrasonic bath for 15 min. After centrifugation, the supernatant was ten thousand-fold diluted with HPLC-grade water, and 20 µL of the extract were injected in the UHPLC-HRMS system.

For NMR analysis, approximately 15 mg of sample were dissolved in 0.6 mL of $CDCl_3$.

Instrumentation

For FTIR analysis, a Jasco FT/IR-6200 FTIR spectrometer (Jasco Inc., Easton, MD, USA) was used. Data acquisition was performed at 23 ºC between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1} and performing 32 acquisitions.

For GC-MS analysis, an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 7683 autosampler (Agilent Technologies) was coupled to a Quattro Micro GC triple quadrupole mass spectrometer (Micromass, Boston, USA) using an electron ionization (EI) interface. The injector and the interface were operated at 250 ºC. 1 µL of sample was injected in splitless mode using deactivated liners into a

30 m 0.25 mm i.d., 0.25 μm film thickness DB-5MS column (Agilent). Helium (99.999%, Praxair, Valencia, Spain) was used as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially maintained at 90 ºC for 1 min and programmed to reach 300 ºC at 20 ºC per min. It was finally maintained at 300 ºC for 1.5 min (total run time was 12 min). The mass spectrometer was operated in electronic ionization mode at 70 eV. MS system worked in SCAN acquisition mode, acquiring from *m/z* 50 to 400 Da. Analytical data were acquired and processed using MassLynx data station operation software (version 4.0, Waters).

For UHPLC-HRMS analysis, an ACQUITY UPLC system (Waters, Mildford, MA, USA) coupled to a XEVO G2 QTOF mass spectrometer (Waters Micromass, Manchester, UK) was used. A CORTECS C18 (Waters) analytical column (100 x 2.1 mm, 2.7 μm particle size) at a flow rate of 0.3 mL/min was used. The column temperature was set to 40 ºC. The mobile phases used were H_2O and MeOH, both with 0.01% HCOOH. The initial percentage of organic mobile phase was changed as follows: 10% at 0 min, 90% at 14 min linearly increased, 90% at 16 min, and return to initial conditions at 18 min. The injection volume was 20 μL. Nitrogen (Praxair) was used as desolvation (1000 L/h) and nebulizing gas. TOF resolution was ~20000 at FWHM (at *m/z* 556). A mass range from *m/z* 50 to 1000 was acquired. The capillary and cone voltage were set to 0.7 kV and 20 V, respectively. Argon 99.995% (Praxair) was used as a collision gas. The interface temperature was set to 650 ºC and the source temperature to 120 ºC.

In MS^E experiments, the low energy function (LE) used a collision energy of 4 eV while the high energy function (HE) applied a collision energy ramp from 15 to 40 eV. On this way, information about the protonated molecule and adducts (if present) and fragment ions [48] were obtained in a single analysis.

Calibration of the mass-axis was daily performed using a mixture of 0.05 M NaOH:5% HCOOH (50:50), 25-fold diluted with ACN:H_2O (80:20). A 2 μg/mL leucine enkephalin solution in ACN:H_2O (50:50) with 0.1% HCOOH was used

as lock-mass. UHPLC-HRMS data were acquired in continuum mode (MassLynx, version 4.1, Waters) and processed with UNIFI (version 1.8, Waters).

A Bruker Ascend 400 MHz spectrometer equipped with a SampleCase autosampler (Bruker, Etlingen, Germany) was used for NMR analysis. Data acquisition was performed at 303 K using $CDCl_3$. The residual solvents signals at $\delta = 7.24$ ppm for ^{1}H ($CHCl_3$) and at $\delta = 77.23$ ppm for ^{13}C ($CDCl_3$) were used as internal references. Characterization of the compound was performed using ^{1}H NMR, ^{13}C NMR, correlated spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC). NMR experiment data were collected using the Bruker Icon NMR 5.0.5 software (Bruker). MestreNova program was used for raw data processing (Mestrelab Research, Santiago de Compostela, Spain).

Acknowledgements

D.F-S., F.H. and M.I. acknowledge financial support from Generalitat Valenciana (Group of Excellence Prometeo II 2014/023, from Ministerio de Economía y Competitividad (Project: CTQ2015-65603-P, Spain) and from University Jaume I (UJI-B2018-19). Authors also acknowledge NPS-Euronet (HOME/2014/JDRUG/AG/DRUG/7086, European Union). D.F-S. acknowledges Ministerio de Educación, Cultura y Deporte for his predoctoral grant (Grant FPU15/02033, Spain). X.C., M.V. and I.F. acknowledge the grants from Subdirecció General de Drogodependències, Departament de Salut, Generalitat de Catalunya and Plan Nacional sobre Drogas. This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein.

Author contributions

M.V., I.F, F.H. and M.I. conceived the work. D.F-S., X.C., M.V., I.F., and M.I. designed research approach. D.F-S., X.C. and I.F. performed experiments and analyzed raw data. D.F-S., X.C., I.F., and M.I. analyzed all data together and wrote the manuscript. M.V. and F. H. provided useful comments and feedback for the manuscript.

Competing interests: The authors declare no potential conflict of interest.

References

1. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2017. EMCDDA–Europol Jt. Publ. 88 (2017). doi:10.2810/610791

2. Fornal, E. Identification of substituted cathinones: 3,4-Methylenedioxy derivatives by high performance liquid chromatography–quadrupole time of flight mass spectrometry. J. Pharm. Biomed. Anal. 81–82, 13–19 (2013).

3. Qian, Z., Jia, W., Li, T., Liu, C. & Hua, Z. Identification and analytical characterization of four synthetic cathinone derivatives iso-4-BMC, β -TH-naphyrone, mexedrone, and 4-MDMC. Drug Test. Anal. 9, 274–281 (2017).

4. Liu, C., Jia, W., Li, T., Hua, Z. & Qian, Z. Identification and analytical characterization of nine synthetic cathinone derivatives N -ethylhexedrone, 4-Cl-pentedrone, 4-Cl- α -EAPP, propylone, N -ethylnorpentylone, 6-MeO-bk-MDMA, α -PiHP, 4-Cl- α -PHP, and 4-F- α -PHP. Drug Test. Anal. 9, 1162–1171 (2017).

5. Brandt, S. D., Daley, P. F. & Cozzi, N. V. Analytical characterization of three trifluoromethyl-substituted methcathinone isomers. Drug Test. Anal. 4, 525–529 (2012).

6. Doi, T. et al. Identification and characterization of a-PVT, a-PBT, and their bromothienyl analogs found in illicit drug products. Forensic Toxicol. 34, 76–93 (2016).

7. Apirakkan, O. et al. Analytical characterization of three cathinone derivatives, 4-MPD, 4F-PHP and bk-EPDP, purchased as bulk powder from online vendors. Drug Test. Anal. 1–7 (2017). doi:10.1002/dta.2218

8. Gaspar, H. et al. 4F-PBP (4'-fluoro-α-pyrrolidinobutyrophenone), a new substance of abuse: Structural characterization and purity NMR profiling. Forensic Sci. Int. 252, 168–176 (2015).

9. Fabregat-Safont, D. et al. Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. 36, 447–457 (2018).

10. Assi, S., Gulyamova, N., Kneller, P. & Osselton, D. The effects and toxicity of cathinones from the users' perspectives: A qualitative study. Hum. Psychopharmacol. Clin. Exp. 32, e2610 (2017).

11. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2018. EMCDDA Publ. (2018). doi:10.2810/88175

12. Johansen, S. S. & Hansen, T. M. Isomers of fluoroamphetamines detected in forensic cases in Denmark. Int. J. Legal Med. 126, 541–547 (2012).

13. McLaughlin, G. et al. Test purchase, synthesis and characterization of 3-fluorophenmetrazine (3-FPM) and differentiation from its ortho - and para - substituted isomers. Drug Test. Anal. 9, 369–377 (2017).

14. Davidson, C., Raby, C. A. R., Barrese, V. & Ramsey, J. In Vitro Neurochemical Assessment of Methylphenidate and Its "Legal High" Analogs 3,4-CTMP and Ethylphenidate in Rat Nucleus Accumbens and Bed Nucleus of the Stria Terminalis. Front. Psychiatry 9, 1–9 (2018).

15. Brandt, S. D. et al. Characterization of a novel and potentially lethal designer drug (±)-cis-para-methyl-4-methylaminorex (4,4'-DMAR, or 'Serotoni'). Drug Test. Anal. 6, 684–695 (2014).

16. McNeil Laboratories. 2-AMINO-5-ARYLOXAZOLINE PRODUCTS (US Patent Office 3,161,650). US Pat. Off. (1964).

17. Weigle, D. S. Pharmacological Therapy of Obesity: Past, Present, and Future. J. Clin. Endocrinol. Metab. 88, 2462–2469 (2003).

18. Gaine, S. P., Rubin, L. J., Kmetzo, J. J., Palevsky, H. I. & Traill, T. A. Recreational Use of Aminorex and Pulmonary Hypertension. Chest 118, 1496–1497 (2000).

19. Fishman, A. P. Aminorex to Fen/Phen: An Epidemic Foretold. Circulation 99, 156–161 (1999).

20. Drug Enforcement Administration. Lists of: Scheduling Actions, Controlled Substances, Regulated Chemicals. U.S. Dep. Justice (2018).

21. McNeil Laboratories. 2-AMINO-5-ARYLOXAZOLINE COMPOSITIONS AND METHODS OF USING SAME (US Patent Office 3,278,382). US Pat. Off. (1966).

22. Young, R. & Glennon, R. A. Cocaine-stimulus generalization to two new designer drugs: Methcathinone and 4-methylaminorex. Pharmacol. Biochem. Behav. 45, 229–231 (1993).

23. Russell, B. R., Beresford, R. A., Schmierer, D. M., McNaughton, N. & Clark, C. R. Stimulus properties of some analogues of 4-methylaminorex. Pharmacol. Biochem. Behav. 51, 375–378 (1995).

24. Glennon, R. A. & Misenheimer, B. Stimulus properties of a new designer drug: 4-methylaminorex ("U4Euh"). Pharmacol. Biochem. Behav. 35, 517–521 (1990).

25. Bunker, C. F., Johnson, M., Gibb, J. W., Bush, L. G. & Hanson, G. R. Neurochemical effects of an acute treatment with 4-methylaminorex: a new stimulant of abuse. Eur. J. Pharmacol. 180, 103–111 (1990).

26. Davis, F. T. & Brewster, M. E. A fatality involving U4Euh, a cyclic derivative of phenylpropanolamine. J. Forensic Sci. 33, 549–553 (1988).

27. Maier, J. et al. The psychostimulant (±)-cis-4,4′-dimethylaminorex (4,4′-DMAR) interacts with human plasmalemmal and vesicular monoamine transporters. Neuropharmacology 138, 282–291 (2018).

28. EMCDDA-Europol. Link suspected between deaths and new psychoactive substance: 4-methylaminorex, para-methyl derivative. EARLY Warn. Notif. (2014).

29. EMCDDA-Europol. EMCDDA–Europol Joint Report on a new psychoactive substance: 4,4′-DMAR (4-methyl-5-(4-methylphenyl)-4,5-dihydrooxazol-2-amine). EMCDDA–Europol Jt. Publ. (2014).

30. European Union. COUNCIL IMPLEMENTING DECISION (EU) 2015/1873. Off. J. Eur. Union 32–34 (2015).

31. McLaughlin, G. et al. Synthesis, characterization, and monoamine transporter activity of the new psychoactive substance 3′,4′-methylenedioxy-4-methylaminorex (MDMAR). Drug Test. Anal. 7, 555–564 (2015).

32. Calkins, R. F., Aktan, G. B. & Hussain, K. L. Methcathinone: The Next Illicit Stimulant Epidemic? J. Psychoactive Drugs 27, 277–285 (1995).

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

34. Bossong, M. G., Van Dijk, J. P. & Niesink, R. J. M. Methylone and mCPP, two new drugs of abuse? Addict. Biol. 10, 321–323 (2005).

35. Archer, R. P. Fluoromethcathinone, a new substance of abuse. Forensic Sci. Int. 185, 10–20 (2009).

36. Taschwer, M., Weiß, J. A., Kunert, O. & Schmid, M. G. Analysis and characterization of the novel psychoactive drug 4-chloromethcathinone (clephedrone). Forensic Sci. Int. 244, e56–e59 (2014).

37. National Forensic Laboratory of Slovenia. ANALYTICAL REPORT. pF-4-methylaminorex (C10H11FN2O). European Project RESPONSE 2

38. Maier, J., Mayer, F. P., Brandt, S. D. & Sitte, H. H. DARK Classics in Chemical Neuroscience: Aminorex Analogues. ACS Chem. Neurosci. 9, 2484–2502 (2018).

39. Fabregat-Safont, D. et al. Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017).

40. Yanini, Á., Armenta, S., Esteve-Turrillas, F. A., Galipienso, N. & de la Guardia, M. Identification and characterization of the new psychoactive substance 3-fluoroethamphetamine in seized material. Forensic Toxicol. 36, 404–414 (2018).

41. Grifell, M. et al. Patterns of use and toxicity of new para-halogenated substituted cathinones: 4-CMC (clephedrone), 4-CEC (4-chloroethcatinone) and 4-BMC (brephedrone). Hum. Psychopharmacol. Clin. Exp. 32, e2621 (2017).

42. 3-fea. bluelight.org (2017). Available at: http://bluelight.org/vb/threads/813802-3-fea/page2. (Accessed: 24th June 2018)

43. Pinterova, N., Horsley, R. R. & Palenicek, T. Synthetic Aminoindanes: A Summary of Existing Knowledge. Front. Psychiatry 8, (2017).

44. Synthesis of para-fluoro-(4-methylaminorex). chemistry.mdma.ch Available at: https://chemistry.mdma.ch/hiveboard/methods/000464621.html. (Accessed: 24th June 2018)

45. 4-Fluoroamphetamine Synthesis. erowid.org Available at: https://erowid.org/archive/rhodium/chemistry/pfa.spicybrown.html. (Accessed: 24th June 2018)

46. Bundesgesetzblatt Jahrgang. Gesetz zur Bekämpfung der Verbreitung neuer psychoaktiver Stoffe. 2016, 2615–2622 (2016).

47. 2'-Fluoro-4-Methylaminorex. He's done it. reddit.com (2018). Available at: https://www.reddit.com/r/researchchemicals/comments/7xuj76/2fluoro4methylaminorex_hes_done_it/. (Accessed: 24th June 2018)

48. Euphoria of the Dick. 4-Methylaminorex. erowid.org (2018). Available at: https://erowid.org/experiences/exp.php?ID=106177. (Accessed: 2nd May 2019)

49. 4-MAR (U4EA). sixthseal.com (2003). Available at: http://sixthseal.com/2003/04/4-mar-u4ea/. (Accessed: 2nd May 2019)

50. The synthesis of 2'-Fluoro-4-Methylaminorex. reddit.com (2018). Available at: https://www.reddit.com/r/TheeHive/comments/7xvd5d/the_synthesis_of_2fluoro4methylaminorex/. (Accessed: 2nd May 2019)

51. González, D., Ventura, M., Caudevilla, F., Torrens, M. & Farre, M. Consumption of new psychoactive substances in a Spanish sample of research chemical users. Hum. Psychopharmacol Clin Exp 28, 332–340 (2013).

2.6. Discussion of the results obtained

For an adequate monitoring of the NPS that are being consumed, the analysis of the consumption products provided by users to the European drug checking services (such as Energy Control in Spain) as well as the analysis of seizures is crucial. Nevertheless, these drug checking services and routine forensic laboratories are usually equipped with GC-MS and FTIR instruments. As commented in the introduction of the chapter, these instruments allow the tentative identification of the active compound in the sample using different spectral libraries. Nevertheless, for the most recent compounds, especially those unreported, powerful techniques such as HRMS and NMR are needed.

In all the studies included in this chapter, the combination of different analytical techniques was required for the unequivocal identification of the studied compounds, as no analytical reference standards were available by the time of writing these research articles. Due to the lack of information about these novel compounds, preliminary analyses by GC-MS and/or FTIR did not retrieve any result, needing powerful analytical techniques such as HRMS and NMR. Although the analytical characterisation of novel NPS is very useful for forensic and toxicological laboratories, it is also important to explore the origin and reasons of synthesising new compounds. For that, in the studies included in this chapter, the background of the origin of non-previously reported compounds was investigated in depth.

In the **research article I**, where a putative NPS is elucidated and characterised, the GC-MS did not allow the identification of the compound due to the high fragmentation obtained by EI. Although the analysis by HRMS provided very useful information about the moieties present in the unknown molecule, the final identification was performed by NMR, considering also the information provided by HRMS. Nevertheless, as the elucidated structure was not found in any database, drug forum or research chemical supplier, single-crystal X-ray diffraction was also performed for an unequivocal structure identification.

So, in this study is shown that in some cases, the use of MS techniques could not be enough for the elucidation of an unknown compound.

In **research articles II** and **IV**, information about the supposed active compounds present in the samples (U-49900 and 4'F-4-MAR, respectively) was provided by the anonymous users that provided the samples to the drug testing service. Moreover, information on both compounds was found in different web forums that discuss about NPS dosage and psychoactive effects. Thus, as the chemical structure of these NPS was known, the HRMS was enough for a tentative identification of the compounds. Nevertheless, as the analytical reference standards were not available by the time of writing those articles, different NMR experiments were also performed.

Finally, in the **research articles III**, the only information obtained (after GC-MS analysis) was the presence of a synthetic cathinone in the powder sample based on the observed fragmentation. In this case, HRMS was enough for the elucidation of its structure, and NMR was used for the unequivocal identification, as no analytical reference standard was available at this time. Once identified the compound structure, it was searched in different research chemical suppliers' webpages, as well as different web forums, finding the 5-PPDi name.

Although the identification of the compounds described in this chapter was performed by HRMS and NMR, information about GC-MS, FTIR, and in some cases ultra-violet spectroscopy, was also provided for a complete characterisation.

These four unreported and/or uncharacterised novel NPS were found during the collaboration with Energy Control for the identification of the active compounds present in research chemical samples provided by anonymous users to its drug checking service. **Table 2.1** shows a summary of the compounds identified, including the (in some cases erroneous) identification provided by GC-MS and number of detections.

Table 2.1. Summary of the identified NPS during the collaboration with Energy Control.

Identification by GC-MS	Identification by HRMS and NMR	Family	Detections
3-FEA	3-FEA	Amphetamine	1
4-fluoromethamphetamine	4-fluoroamphetamine	Amphetamine	2
4-fluoromethamphetamine	4-fluoromethamphetamine	Amphetamine	3
6-APB	6-APB	Amphetamine	2
6-EAPB	6-EAPB	Amphetamine	4
6-MBPB	6-MBPB	Amphetamine	1
Methamnetamine	Methamnetamine	Amphetamine	1
Unknown	Novel NPS reported in research article I	Amphetamine	1
NSI-189	NSI-189	Antidepressant	1
3-MeO-PCP	3-MeO-PCP	Arylcyclohexylamine	1
4-OH-EPT	4-OH-EPT	Arylcyclohexylamine	1
4-MeO-PCP	4-OH-PCP	Arylcyclohexylamine	1
Unknown	3-Hidroxiphenazepam	Benzodiazepine	1
N-desmethyl-flunitrazepam	*N*-desmethyl-flunitrazepam	Benzodiazepine	1
4-Methoxybutyrfentanyl	4-Methoxybutyrfentanyl	Opioid	1
U-47700	U-47700	Opioid	1
Unknown	U-49900	Opioid	1
2-fluoro-ketamine	2-fluoro-ketamine	Pharmaceutical	1
Olmesartan	Olmesartan	Pharmaceutical	1
25I-NBF	25I-NBF	Phenethylamine	1
2C-B	2C-B	Phenethylamine	2
HDMP-28	HEDP-28	Piperidine	1
Isoprophylphenidate	Isoprophylphenidate	Piperidine	1
MDP2P glycidate	MDP2P glycidate	Precursor	1
Unknown	Synthetic cathinone precursor	Precursor	1
3-FMP	3-FMP	Stimulant	1
Aminorex derivative	4'F-4-MAR	Stimulant	1
Unknown cannabinoid	5F-ABICA	Synthetic cannabinoid	1
Unknown	5F-AB-PINACA	Synthetic cannabinoid	1
Unknown	5F-APP-PICA	Synthetic cannabinoid	1
Unknown cannabinoid	5F-MDMB-PINACA	Synthetic cannabinoid	1
Unknown	AB-CHIMINACA Metabolite 2	Synthetic cannabinoid	5
AMB-CHMINACA	AMB-CHMINACA	Synthetic cannabinoid	1
Unknown cannabinoid	AMB-FUBINACA	Synthetic cannabinoid	2

Table 2.1. Summary of the identified NPS during the collaboration with Energy Control (continuation).

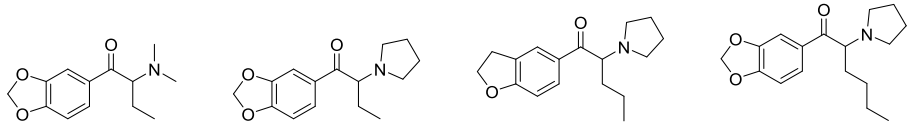
Identification by GC-MS	Identification by HRMS and NMR	Family	Detections
EG-018	EG-018	Synthetic cannabinoid	1
MAB-CHMINACA	MAB-CHMINACA	Synthetic cannabinoid	1
MDMB-CHMICA	MDMB-CHMICA	Synthetic cannabinoid	1
JWH-018 benzimidazole analogue	THJ-018	Synthetic cannabinoid	1
Unknown cannabinoid	THJ-2201	Synthetic cannabinoid	1
4-ANPP	3,4-dimethylethcathinone	Synthetic cathinone	1
Unknown	3-FEC	Synthetic cathinone	1
3-MEC	3-MEC	Synthetic cathinone	1
4-MEC	3-MEC	Synthetic cathinone	3
4-Cl-PVP	4-CEC	Synthetic cathinone	2
4-Cl-PVP	4-Cl-PVP	Synthetic cathinone	2
4-Cl-α-PPP	4-Cl-α-PPP	Synthetic cathinone	10
4-fluoromethcathinone	4-fluoromethcathinone	Synthetic cathinone	1
4-F-*N*-ethyl-pentedrone	4-fluoromethcathinone	Synthetic cathinone	1
4-fluoropentedrone	4-fluoropentedrone	Synthetic cathinone	1
4F-PHP	4F-PHP	Synthetic cathinone	1
4-MeO-PV9	4-MeO-PV9	Synthetic cathinone	1
Mexedrone	4-methoxy-*N*,*N*-dimethylcathinone	Synthetic cathinone	3
4-MEC	4-methyl-*N*,*N*-dimethylcathinone	Synthetic cathinone	2
4-methylpentedrone	4-methyl-*N*-ethylpentedrone	Synthetic cathinone	1
4-methyl-*N*-ethyl-pentedrone	4-methyl-*N*-ethyl-pentedrone	Synthetic cathinone	1
4-metilpentedrone	4-metilpentedrone	Synthetic cathinone	1
Unknown	5-MDPHP	Synthetic cathinone	1
Unknown	5-PPDi	Synthetic cathinone	1
Buphedrone	Buphedrone	Synthetic cathinone	1
Dibutylone	Dibutylone	Synthetic cathinone	1
Dimetylone	Dimetylone	Synthetic cathinone	1
MDPBP	MDPBP	Synthetic cathinone	1
TH-PVP	MPHP	Synthetic cathinone	2
N-ethyl-hexedrone	*N*-ethyl-hexedrone	Synthetic cathinone	1
4-Cl-*N*-ethyl-pentedrone	*N*-ethyl-hexedrone	Synthetic cathinone	1
N-ethyl-Pentylone	*N*-ethyl-Pentylone	Synthetic cathinone	1
N-etyl-pentedrone	*N*-etyl-pentedrone	Synthetic cathinone	1
Pentedrone	Pentedrone	Synthetic cathinone	1

Table 2.1. Summary of the identified NPS during the collaboration with Energy Control (continuation).

Identification by GC-MS	Identification by HRMS and NMR	Family	Detections
Pentylone	Pentylone	Synthetic cathinone	1
Thiothinone	Thiothinone	Synthetic cathinone	1
TH-PVP	TH-PVP	Synthetic cathinone	1
α-PVP	α-PVP	Synthetic cathinone	1
MDPH	Dibutylone	Synthetic cathinone	1[a]
	MDPBP	Synthetic cathinone	
	5-DBFPV	Synthetic cathinone	
	MDPHP	Synthetic cathinone	
α-PVT	α-PVT	Synthetic cathinone	1
4-AcO-DMT	4-AcO-DMT	Tryptamine	2

[a] These compounds were found in a sample as a mixture

It is important to remark that almost all the samples analysed presented a high purity, determined by the absence of additional signals in UHPLC-HRMS and NMR analyses. Nevertheless, it is also important to comment the research chemical sample with four different synthetic cathinones (**Table 2.1[a]**). The structures of these cathinones (**Figure 2.2**) illustrate that they are related compounds, and it is possible that this research chemical sample was prepared after a non-controlled synthesis reaction.



Dibutylone MDPBP 5-DBFPV MDPHP

Figure 2.2. Structures of the related synthetic cathinones found in a research chemical sample.

During this thesis, the analysis of the legal highs available in a smartshop of the city of Valencia (Valencian region, Spain) was continued. The analysis of the products sold in this smartshop through its webpage started in our laboratory in

2012, with the analysis of the herbal blends [17]. After that, the developed strategy was applied to the remaining products available by this time [9].

Figure 2.3 shows the packaging of the herbal blends, pills and powder samples analysed by UHPLC-HRMS. In this case, analysis by NMR was not possible due to the presence of impurities, and the compounds were only tentatively identified based on the accurate-mass fragmentation, and the HRMS data available in the literature for the detected compounds.

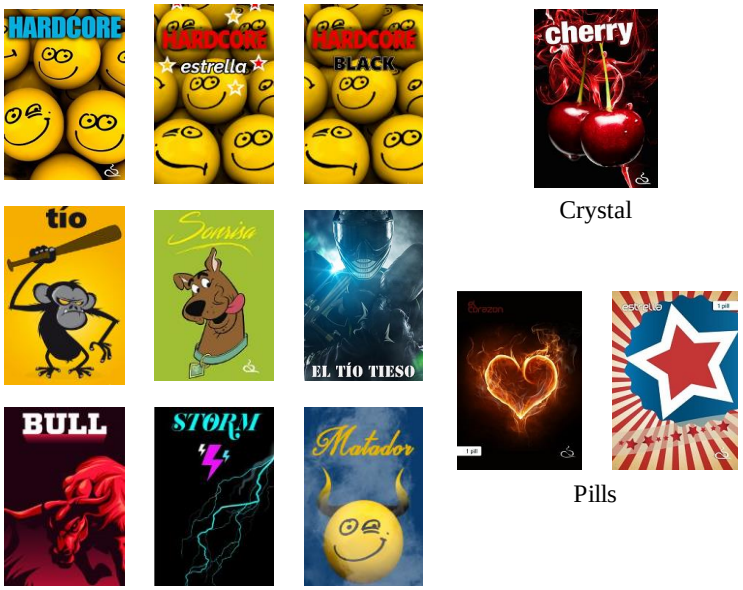


Figure 2.3. Packaging of the different legal highs purchased and analysed by UHPLC-HRMS.

The analysis of these products, specially the herbal blends, revealed that products with different packaging presented the same composition. This fact may be encouraged because some consumers like to try new products and, for them, a new packaging means a new product. **Table 2.2** lists the tentatively identified compounds in these samples.

Table 2.2. Active compounds tentatively identified in the legal high samples purchased from the smart shop.

Legal high	Compound	Family
Hardcore *Hardcore black* *Hardcore estrella*	XLR-11[a]	Synthetic cannabinoid
	UR-144[a]	Synthetic cannabinoid
	UR-144 *N*-(5-chloropentyl) analogue	Synthetic cannabinoid
Tío *Sonrisa* *El Tío Tieso*	XLR-11[a]	Synthetic cannabinoid
	UR-144[a]	Synthetic cannabinoid
	F5-APINACA (or 5F-AKB48)	Synthetic cannabinoid
Bull *Storm*	DMBA-CHMINACA	Synthetic cannabinoid
Matador	5F-ADB	Synthetic cannabinoid
Cherry	4-Methylbuphedrone	Synthetic cathinone
Corazón	5-MeO-MiPT[b]	Tryptamine
Estrella (1)	5-MeO-MiPT[b]	Tryptamine
Estrella (2)	4-MeO-PCP[a]	Arylcyclohexylamine
	4-AcO-DMT	Tryptamine
	5-MeO-DiPT	Tryptamine
	5-MeO-MiPT	Tryptamine

[a] Major compound present in the sample.
[b] Confirmed with the analytical reference standard.

It is important to point out that herbal blends sold with different packaging presented the same composition (for example, *Harcore*, *Hardcore black* and *Hardcore estrella*). In the case of the pill *Estrella (2)*, which was the second pill purchased with the same name, different compounds were found as a mixture, being the major compound the arylcyclohexylamine 4-MeO-PCP. For the first pill purchased with this name, *Estrella (1)*, only the tryptamine 5-MeO-MiPT was found. Some of the compounds identified in these research chemical samples and legal highs were afterwards selected for performing the studies included in **Chapters 4** and **5**, which deal with the study of the *in vitro* and *in vivo* metabolism and pharmacokinetics using pHH and mice/rats, respectively. Additionally, as the herbal blends were purchased in a smartshop located in València, the same products were related to the studies included in the **Chapter 6**, for the study of SCRA consumption among teenagers in the Valencian region.

2.7. Literature

1. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2019. (2019).

2. NEW Net. T.E.D.I - Trans-European Drug Information project. Available at: http://www.safernightlife.org/tedi. (Accessed: 17th March 2020)

3. Brunt, T. M. et al. Drug testing in Europe: monitoring results of the Trans European Drug Information (TEDI) project. Drug Test. Anal. 9, 188–198 (2017).

4. Trans-European Drug Information project. Guidelines for Drug Checking Methodology. 1–29 (2012).

5. European Project RESPONSE. Forensic Drugs Analyses - European project RESPONSE. Available at: https://www.policija.si/eng/about-the-police/organization/general-police-directorate/national-forensic-laboratory/project-response. (Accessed: 17th March 2020)

6. European Project RESPONSE. NPS AND RELATED COMPOUNDS - ANALYTICAL REPORTS. Available at: https://www.policija.si/apps/nfl_response_web/seznam.php. (Accessed: 17th March 2020)

7. Mardal, M. et al. HighResNPS.com: An Online Crowd-Sourced HR-MS Database for Suspect and Non-targeted Screening of New Psychoactive Substances. J. Anal. Toxicol. 43, 520–527 (2019).

8. Cayman Chemical. Cayman Spectral Library. Available at: https://www.caymanchem.com/forensics/publications/csl. (Accessed: 17th March 2020)

9. Ibañez, M. et al. Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. TrAC - Trends Anal. Chem. 57, 107–117 (2014).

10. Strano Rossi, S. et al. An analytical approach to the forensic identification of different classes of new psychoactive substances (NPSs) in seized materials. Rapid Commun. Mass Spectrom. 28, 1904–1916 (2014).

11. Majchrzak, M., Celiński, R., Kuś, P., Kowalska, T. & Sajewicz, M. The newest cathinone derivatives as designer drugs: an analytical and toxicological review. Forensic Toxicol. 36, 33–50 (2018).

12. Qian, Z., Jia, W., Li, T., Hua, Z. & Liu, C. Identification and analytical characterization of four synthetic cannabinoids ADB-BICA, NNL-1, NNL-2, and PPA(N)-2201. Drug Test. Anal. 9, 51–60 (2017).

13. Qian, Z., Jia, W., Li, T., Liu, C. & Hua, Z. Identification and analytical characterization of four synthetic cathinone derivatives iso-4-BMC, β -TH-naphyrone, mexedrone, and 4-MDMC. Drug Test. Anal. 9, 274–281 (2017).

14. Dolomanov, O. V., Bourhis, L. J., Gildea, R. J., Howard, J. A. K. & Puschmann, H. OLEX2: A complete structure solution, refinement and analysis program. J. Appl. Crystallogr. 42, 339–341 (2009).

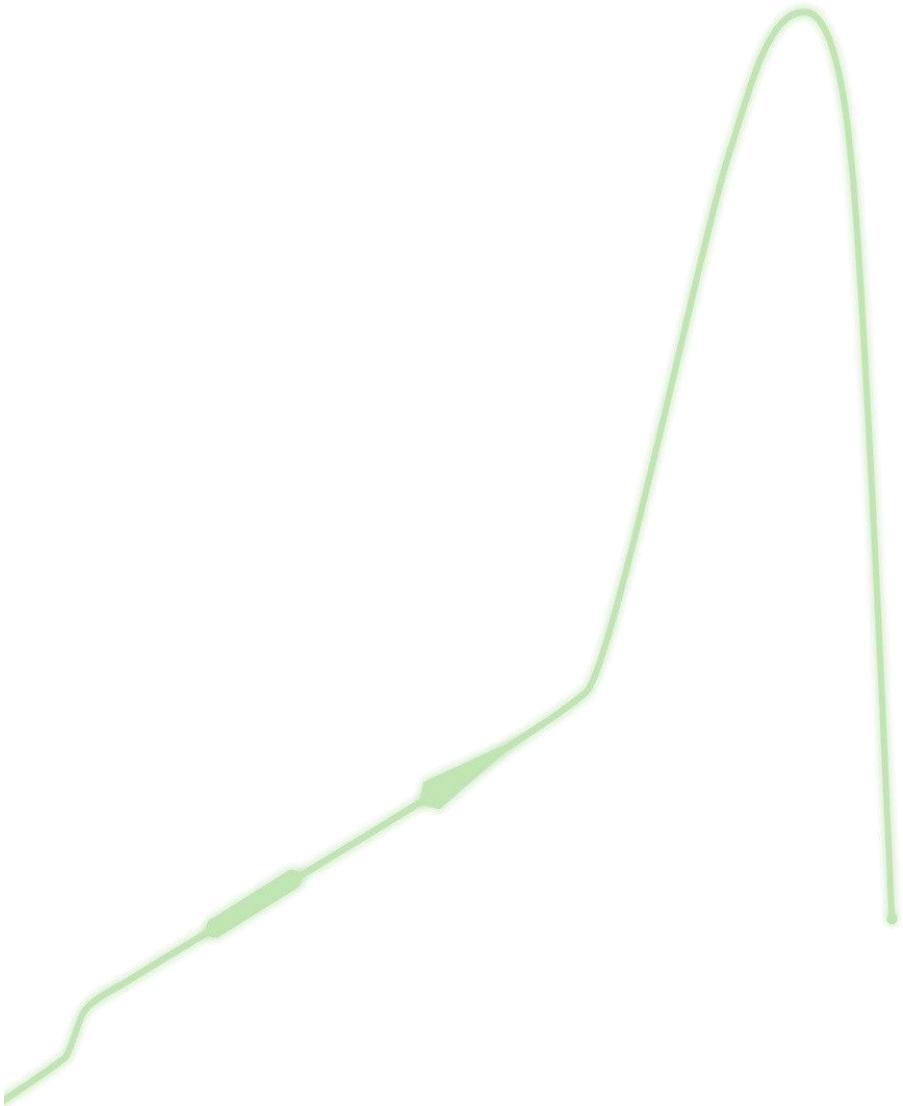
15. Nycz, J. E., Pazdziorek, T., Malecki, G. & Szala, M. Identification and derivatization of selected cathinones by spectroscopic studies. Forensic Sci. Int. 266, 416–426 (2016).

16. Kuś, P., Kusz, J., Książek, M., Pieprzyca, E. & Rojkiewicz, M. Spectroscopic characterization and crystal structures of two cathinone derivatives: N-ethyl-2-amino-1-phenylpropan-1-one (ethcathinone) hydrochloride and N-ethyl-2-amino-1-(4-chlorophenyl)propan-1-one (4-CEC) hydrochloride. Forensic Toxicol. 35, 114–124 (2017).

17. Ibáñez, M. et al. Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends. J. Mass Spectrom. 48, 685–694 (2013).

CHAPTER 3

NEW ANALYTICAL STRATEGIES BASED ON TRIPLE QUADRUPOLE FOR NPS DETERMINATION



CHAPTER 3

NEW ANALYTICAL STRATEGIES BASED ON TRIPLE QUADRUPOLE FOR NPS DETERMINATION

3.1. Introduction

3.2. Research article V

"Rapid tentative identification of synthetic cathinones in seized products taking profit of the full capabilities of triple quadrupole analyser".
Forensic Toxicology, 2019; 37:34.

3.3. Research article VI

"Direct and fast screening of new psychoactive substances using medical swabs and atmospheric solids analysis probe-triple quadrupole with data-dependent acquisition".
Journal of the American Society for Mass Spectrometry, 2020; 31:1610.

3.4. Discussion of the results obtained

3.5. Literature

3.1. Introduction

As commented in **Chapter 2**, monitoring the NPS that are being consumed through the analysis of seizures, research chemicals and legal highs is crucial for understanding changes in the psychoactive substances market. As previously discussed, advanced analytical techniques such as HRMS and NMR are mandatory for the elucidation and unequivocal identification of unknown compounds and novel NPS that have not been reported yet. Nevertheless, these analytical techniques are expensive, complex, and require highly specialised personnel, being difficult their implementation in routine laboratories, such as the drug checking services. In those laboratories, the use of GC-MS and FTIR are the most commonly used analytical techniques [1–3]. Nevertheless, toxicological and forensic laboratories are usually also equipped with LC-MS/MS systems, especially triple quadrupole instruments, due to their high sensitivity and specificity for compound identification and quantification at trace levels when using SRM acquisition mode. Thus, the use of LC-MS/MS has been widely described for the pre-target analysis and quantification of NPS in different matrices such as urine [4], brain tissue [5], hair [6], and even wastewater [7]. However, LC-MS/MS is not usually applied for the analysis of research chemicals/legal highs as it is more effective the use of analytical techniques that allow performing wide-scope screening analysis using, for example, EI spectral libraries (GC-MS), IR spectral libraries (FTIR) and/or accurate-mass compound databases (HRMS).

Hence, the development of suspect screening methodologies using MS/MS instruments would be highly useful for toxicological/forensic laboratories when performing the analysis of consumption products. These methodologies can be developed using the additional working modes of the QqQ instrument described in **Chapter 1**. Most of the application of the QqQ are based on the SRM acquisition mode, as explained previously. Nevertheless, other less-used working modes, such as precursor ion scan and neutral loss scan, have been described in the literature for performing suspect screening. This is the case of the detection and identification of corticosteroid metabolites in urine [8] or bis-sulphate

metabolites in urine [9]. An additional QqQ working mode is the product ion scan, used for the selection of the most adequate SRM transitions to be monitored when optimizing a pre-target method and for the elucidation of a certain ion based on the observed fragmentation [10]. The fragmentation spectra obtained using this acquisition mode can be used for compound identification using spectral libraries, similarly to the processing applied during GC-MS analysis. The product ion scan can be obtained by specifying the precursor ion to be fragmented or using a DDA acquisition mode. The DDA when using QqQ instruments has the same basis of the DDA in Q-HRMS instruments: the precursor ions to be selected for MS/MS analysis are selected based on the information obtained after a survey scan acquisition, as explained in **Chapter 1**. The scan and automatic MS/MS are performed in a single analysis, so it is not necessary a second sample analysis for obtaining the product ion spectra, as long as the ion of interest had been automatically selected for MS/MS acquisition.

Together with the use of QqQ instruments for the suspect screening of NPS in consumption products, forensic and toxicological laboratories need rapid methodologies that allow the analysis of a high number of samples in a short time. In this field, ambient ion sources, such as the ASAP source described in **Chapter 1**, present an attractive opportunity as they allow the direct MS analysis of the sample. The use of ambient sources has been described for the determination of NPS in different matrices, such as the desorption electrospray (DESI) for the analysis of legal highs [11], the direct analysis in real time (DART) for seizure analysis [12], as well as the ASAP for determining amphetamines in authentic urine samples [13].

In this chapter, two different methodologies based on the additional working modes of the QqQ instrument for the suspect screening of NPS are presented and discussed. On the one hand, **research article V** presents the use of precursor ion scan and neutral loss scan acquisition modes for the suspect screening and elucidation of synthetic cathinones in research chemical samples without stablishing target compounds. Synthetic cathinones present similar fragmentation

pathways [14] that allow the proposal of common product ions and neutral losses to be monitored for compound identification. On the other hand, **research article VI** presents a suspect screening strategy for the tentative identification of different NPS based on the observed fragmentation spectra. For that, DDA acquisition mode was used, together with an in-house built spectral library. Moreover, this study presents the use of a modified ASAP source, by means of a medical swab instead of the typical glass capillary used in this source. The use of the swab also allows the determination of NPS in surfaces, such as the finger of a potential consumer.

3.2. Research article V

Forensic Toxicology (2019) 37:34–44
https://doi.org/10.1007/s11419-018-0432-y

ORIGINAL ARTICLE

Rapid tentative identification of synthetic cathinones in seized products taking advantage of the full capabilities of triple quadrupole analyzer

David Fabregat-Safont[1] · Juan V. Sancho[1] · Félix Hernández[1] · María Ibáñez[1]

Received: 8 May 2018 / Accepted: 27 June 2018 / Published online: 4 July 2018

Abstract

Purpose In this work, a new approach for synthetic cathinone identification in seized products, consisting of a rapid pseudo-target screening using liquid chromatography coupled to low-resolution tandem mass spectrometry (MS/MS), is proposed based on typical common product ions and neutral losses observed for this drug class.

Methods The term "pseudo-target" screening indicates that although a pre-defined target compound list is not used, the search is limited to synthetic cathinones with expected common moieties. A total of 22 neutral losses and 36 common product ions were monitored and used for cathinone identification.

Results In order to test the approach, 14 blind samples were analyzed and the results compared with high-resolution mass spectrometry data. From the data obtained, the different moieties of the cathinones (and therefore their structures) could be derived, allowing their tentative identification.

Conclusions This methodology will be useful for the first and rapid synthetic cathinone detection in laboratories that have low-resolution MS/MS instrumentation.

Keywords Synthetic cathinones · "Pseudo-target" screening · Low-resolution tandem mass spectrometry · Tentative identification · Precursor ion scan · Neutral loss scan

Introduction

It is undeniable that there is epochal rising of the so-called "new psychoactive substances" (NPS) in the last decade. The European Monitoring Centre for Drug and Drug Addiction (EMCDDA) recently highlighted the increasing presence of synthetic cathinones in seized products. These compounds have effects similar to those of stimulant drugs such as amphetamines, cocaine and MDMA [1, 2]. In 2015, around 80,000 seizures of NPS were reported in Europe, 33% of them corresponding to synthetic cathinones [3]. They form the second largest group of NPS controlled by the EMCDDA, with a total of 118 cathinones being currently monitored (14 of them detected for the first time in 2016) [3].

The continuous proliferation of NPS makes difficult the establishment of updated regulatory laws [4]. Thus, when a certain compound is banned in several countries, new derivatives rapidly emerge to cover its demand. This fact has been recently described for synthetic cannabinoids, showing the changes in the composition of legal highs in UK after the 2013 legislative ban [5]. The continuous emergence of novel derivatives is an analytical challenge for NPS identification in consumption samples.

The United Nations Office on Drugs and Crime (UNODC) ST/NAR/49 manual [6] established some recommendations for the analysis of synthetic cathinones by different analytical techniques, such as gas chromatography (GC) coupled to mass spectrometry (MS) using electron ionization (EI), and liquid chromatography (LC) coupled to low-resolution tandem mass spectrometry (MS/MS) using an electrospray interface (ESI). Both techniques, together with advanced

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11419-018-0432-y) contains supplementary material, which is available to authorized users.

✉ María Ibáñez
ibanezm@uji.es

[1] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071 Castellón de la Plana, Castellón, Spain

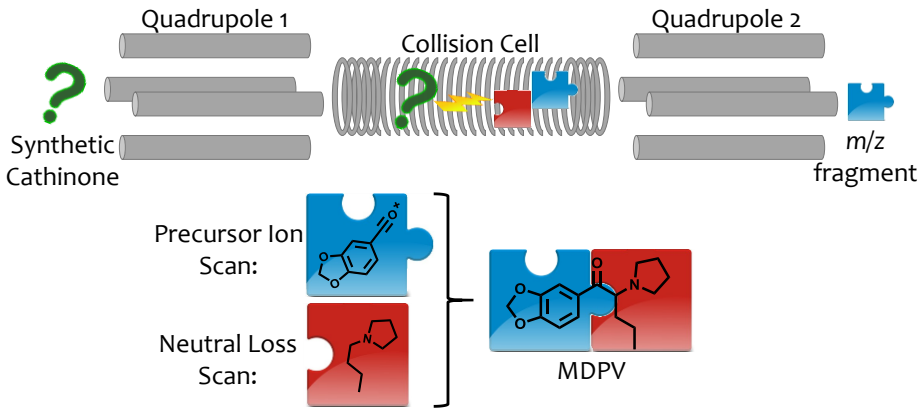
Forensic Toxicology, 2019; 37:34

Rapid tentative identification of synthetic cathinones in seized products taking profit of the full capabilities of triple quadrupole analyser

David Fabregat-Safont [1], Juan V. Sancho [1], Félix Hernández [1], María Ibáñez [1*]

[1] Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12071 Castellón, Spain.

Graphical abstract



Abstract

Purpose In this work, a new approach for synthetic cathinone identification in seized products, consisting of a rapid pseudo-target screening using liquid chromatography coupled to low-resolution tandem mass spectrometry is proposed, based on typical common product ions and neutral losses observed for this drug family.

Methods The term "pseudo-target" screening indicates that although a pre-defined target compound list is not used, the search is limited to synthetic cathinones with expected common moieties. A total of 25 neutral losses and 35 common product ions were monitored and used for cathinone identification.

Results In order to test the approach, 14 blind samples were analysed and the results compared with high-resolution mass spectrometry data. From the data obtained, the different moieties of the cathinones (and therefore their structures) could be derived, allowing their tentative identification.

Conclusions This methodology will be useful for the first and rapid synthetic cathinone detection in laboratories that have low-resolution MS/MS instrumentation.

Keywords Synthetic cathinones; "Pseudo-target" screening; Low-resolution tandem mass spectrometry; Tentative identification; Precursor ion scan; Neutral loss scan.

Introduction

It is undeniable that there is epochal rising of the so-called "new psychoactive substances" (NPS) in the last decade. The European Monitoring Centre for Drug and Drug Addiction (EMCDDA) recently highlighted the increasing presence of synthetic cathinones in seized products. These compounds have similar effects to stimulant drugs such as amphetamines, cocaine and MDMA [1, 2]. In 2015, around 80,000 seizures of NPS were reported in Europe, the 33% of them corresponding to synthetic cathinones [3]. They form the second largest group of NPS controlled by the EMCDDA, with a total of 118 cathinones being currently monitored (14 of them detected for the first time in 2016) [3].

The continuous proliferation of NPS makes difficult the establishment of updated regulatory laws [4]. Thus, when a certain compound is banned in several countries, new derivatives rapidly emerge to cover its demand. This fact has been recently described for synthetic cannabinoids, showing the changes in the composition of legal highs in UK after the 2013 legislative ban [5]. The continuous emergence of novel derivatives is an analytical challenge for NPS identification in consumption samples.

The United Nations Office on Drugs and Crime (UNODC) ST/NAR/49 manual [6] establishes some recommendations for the analysis of synthetic cathinones by different analytical techniques, such as gas chromatography (GC) coupled to mass spectrometry (MS) using electron ionization (EI), and liquid chromatography (LC) coupled to low-resolution tandem mass spectrometry (MS/MS) using electrospray interface (ESI). Both techniques, together with advanced analytical techniques such as high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR), are the most commonly used for identify NPS in seized materials [7].

Several GC–EI-MS methodologies have been developed for synthetic cathinone identification in consumption products [8–11], taking profit of the use of standardized spectral libraries. Nevertheless, the success in unknown compound

elucidation is problematic due to the high fragmentation in EI, which usually leads to the absence of the molecular ion in the spectrum, and the lack of standardized mass spectra for new unknown compounds.

The use of soft ionisation in ESI LC-based methods, allows the obtention of the protonated molecule of the compound (and/or its adducts), facilitating compound identification [12]. This is notably favoured when using LC–HRMS due to the accurate-mass full-acquisition, making HRMS the preferred technique for tentative identification of active ingredients in seized materials and legal high samples [13]. Different strategies have been described using quadrupole time-of-flight (QTOF) MS, illustrating the tentative identification of cathinones without the use of reference standards [13] and the elucidation of novel derivatives in legal high samples [7, 14–17].

The high cost and expensive maintenance of LC–HRMS instruments together with the complexity of use, make this technique less extended than LC–low-resolution MS/MS in forensic and toxicological laboratories. The most common working mode, especially when using triple quadrupole (QqQ), is selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM), as it provides excellent sensitivity, selectivity and method robustness. For this reason, LC–MS/MS has become the most used analytical technique for the determination of cathinones in a vast variety of matrices, such as human hair [18, 19], blood [20], and even influent wastewater samples [21]. Nevertheless, SRM can only be used for the determination of targeted cathinones, for which the reference standards need to be available at the laboratory.

LC–MS/MS with QqQ also allows additional working modes, which could be especially useful for detection and elucidation of analyte related-compound, such as precursor ion scan (PIS) and neutral loss scan (NLS). Although these possibilities are well known, they are much less explored than SRM in most forensic laboratories, as the wide majority of methods published deal with quantitative analysis making use of SRM LC–MS/MS methods. Using PIS,

specific product ions are selected in the second quadrupole (MS2), while the first quadrupole (MS1) scans a determined mass range, fragmenting all the ions in the collision cell. This is especially useful for families of compounds which produce a common fragment ion, regardless their structures [22]. On the NLS working mode, the MS1 and MS2 are continuously scanning a selected mass range and thus, fragmenting all ions in the collision cell. Nevertheless, there is a fixed mass offset between MS1 and MS2, corresponding to a specific neutral loss related to a certain family of compounds (for example, a mass offset of 36 Da would correspond to a HCl molecule loss) [22]. The applicability of PIS and NLS working modes has been reported for the detection of unknown steroids [22, 23], through the combination of different PIS and NLS, or for the detection of bis-sulfate metabolites [24], by monitoring a specific neutral loss (NL). However, up to our knowledge, these modes have never been applied to the analysis of NPS.

The similar core-structure of cathinones promotes a common fragmentation pathway, independently of the functional groups existing in the cathinone derivative [25]. This common fragmentation, in combination with the PIS and NLS working modes, gives an excellent opportunity for the detection and tentative identification of non-specific cathinones by QqQ instruments.

In this work, a rapid pseudo-target screening strategy based on monitoring cathinone-typical common product ions and NLs has been developed by flow injection analysis (FIA) coupled to QqQ. The "pseudo-target" term refers to a methodology developed not for specific compounds (target analysis), but for the detection and tentative identification of a certain family, in this case, synthetic cathinones. This strategy has allowed the tentative identification of different cathinones in research chemicals after only 2 min analysis without the need of analytical reference standards or the use of HRMS systems. The presented methodology will be useful for forensic laboratories without HRMS instruments, allowing these laboratories performing a screening in seized products for the tentative identification of synthetic cathinones using low-resolution MS/MS.

Materials and methods

Reagents and chemicals

HPLC-grade water (H_2O) was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC-grade methanol, formic acid (HCOOH) and acetone were acquired from Scharlau (Scharlab, Barcelona, Spain). Research chemicals including synthetic cathinones were provided by Energy Control (Asociación Bienestar y Desarrollo, Barcelona, Spain) [26].

For LC-HRMS analysis, HPLC-grade water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC-grade methanol, HPLC-grade acetonitrile, formic acid, acetone, and sodium hydroxide were acquired from Scharlau (Scharlab, Barcelona, Spain). Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample treatment

Approximately, 10 mg of powder or seized material containing synthetic cathinones was extracted with 1 mL of acetone in an ultrasonic bath for 15 min. After centrifugation, the supernatant was ten thousand-fold diluted with HPLC-grade water, until a concentration of approximately 1 mg/L. Samples were submitted by anonymous users to Energy Control's drop-in service for their analysis. Additional information about Energy Control can be seen elsewhere [26].

Flow injection analysis–MS/MS instrumentation

A Waters Acquity UPLC H-Class ultra-high performance liquid chromatography system (Waters, Mildford, MA, USA) was coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Manchester, UK) using an orthogonal Z-Spray electrospray ionization (ESI) interface operating in positive ionization mode.

The chromatographic system was used without chromatographic column as a FIA setup. The flow carrier stream was a mixture of H_2O/methanol (1:1 v/v with 0.01% HCOOH at 0.3 mL/min). The injection volume was 30 µL. The total run time per sample was 2 min. A capillary voltage of 3.2 kV and a cone voltage of 30 V were used. Nitrogen (Praxair, Valencia, Spain) was used as desolvation and nebulizing gas. Cone gas flow was set to 250 /h, and desolvation gas flow to 1200 L/h. The interface temperature was set to 150 ºC, and the desolvation temperature to 650 ºC. For operating in MS/MS mode, argon (99.995%; Praxair, Valencia, Spain) was used as collision gas. The *m/z* scanned in MS1 ranged from *m/z* 150 to 324.

MS data were acquired and processed using MassLynx data station operation software, version 4.1 (Waters).

UHPLC-HRMS instrumentation

A Waters Acquity UPLC ultra-high performance liquid chromatography system (Waters, Mildford, MA, USA) was coupled to a Xevo G2 QTOF hybrid quadrupole-time of flight mass spectrometer (Waters, Manchester, UK) using an orthogonal Z-Spray electrospray ionization (ESI) interface operating in positive ionization mode.

The chromatographic separation was performed using a CORTECS C18 (Waters) 2.7 µm particle size analytical column 100 x 2.1 mm at a flow rate of 0.3 mL/min. The column temperature was set to 40 ºC. The mobile phases used were H_2O with 0.01% HCOOH (A) and methanol with 0.01% HCOOH (B). The mobile phase gradient was performed as follows: 10% of B at 0 min, 90% of B at 14 min linearly increased, 90% of B at 16 min, and finally 10% B at 18 min in order to return to initial conditions. The injection volume was 10 µL. Nitrogen (Praxair, Valencia, Spain) was used as desolvation and nebulizing gas. The desolvation gas flow was set at 1000 L/h. The TOF resolution was ~20000 at FWHM at *m/z* 556. The range acquired by the MS system was from *m/z* 50 to 1000. A capillary voltage of 0.7 kV and a cone voltage of 20 V were used during all the

chromatographic run. Argon 99.995% (Praxair, Valencia, Spain) was used as a collision gas. The interface temperature was set to 600 ºC and the source temperature to 120 ºC.

For MS^E experiments, two acquisition functions with different collision energy were created. The low energy function (LE) used a collision energy of 4 eV in order to obtain information about the protonated molecule and adducts (if present), while the high energy function (HE) applied a collision energy ramp from 15 to 40 eV, in order to promote fragmentation of the compounds. Calibration of the mass-axis was performed daily from *m/z* 50 to 1000 using a 1:1 mixture of 0.05 M NaOH:5% HCOOH, diluted 1:25 with acetonitrile/H_2O 80:20 v/v mixture). For accurate mass measurement, a 2 µg/mL leucine enkephalin solution in acetonitrile/H_2O (50:50 v/v with 0.1% HCOOH) was used as lock-mass, pumped at a flow rate of 20 µL/min. The leucine enkephalin protonated molecule (*m/z* 556.2771) was used for recalibrating the mass axis and ensure an accurate mass during all the chromatographic run.

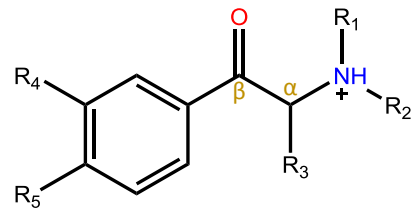
MS data were acquired in continuum mode using MassLynx data station operation software, version 4.1 (Waters). MS data were processed using UNIFI scientific information system software, version 1.8 (Waters).

Results and discussion

Synthetic cathinones structure and typical fragmentation

The EMCDDA webpage provides information about the most common substitutions and moieties found on synthetic cathinones [27]. **Figure 1** shows the general structures of this family of NPS. They feature a phenethylamine core with an alkyl group attached to the alpha carbon, and a ketone group attached to the beta carbon. Five different amine functionalizations (R_1, R_2) have been described for cathinones: methyl, dimethyl, ethyl, isopropyl and the pyrrolidine moieties. For the alkyl chain (R_3), lengths from 1 to 4 carbon atoms can be found.

The aromatic ring presents up to 7 different substitutes (R_4, R_5), including halogen atoms and alkyl substitutions. Additional moieties, such as the replacement of the aromatic ring by a thiophene moiety (described for some recently described cathinones such as thiothinone [28] or α-PVT [29]) can also be found.



R_1: H, methyl (if R_2 is methyl)

R_2: H, methyl, ethyl, isopropyl

R_1+R_2: pyrrolidine

R_3: C_nH_{2n+2} (n=1-4)

R_4: H, halogen atom (if R_5 is H), methyl (if R_5 is H or methyl), ethyl (if R_5 is H), methoxy (if R_5 is H or methoxy)

R_5: H, halogen atom (if R_4 is H), methyl (if R_4 is H or methyl), ethyl (if R_4 is H), methoxy (if R_4 is H or methoxy)

R_4+R_5: 3,4-methylenedioxy, cyclopentane, cyclohexane

Figure 1. Typical structures of synthetic cathinones.

Due to their structures (**Figure 1**), cathinones have two protonatable centres feasible for ESI: the keto function, which can be protonated mainly when conjugated with double bonds, and the amine group. Hence, cathinones can be detected by positive ionization mode.

According to the information available in the literature [25, 30, 31] and the analyses performed in our own laboratory by HRMS [13], synthetic cathinones present very similar fragmentation pathways using collision-induced dissociation (CID). One of the most observed fragmentation is promoted when the protonation is produced in the carbonyl group, losing a water molecule (18 Da) (NLS18) [25, 30] and generating an insaturation on the structure (**Figure 2a**). Other commonly observed fragmentation involves the amine moiety, which is typically lost as a neutral molecule (NLS_1, **Figure 2c**) [25, 30]. Additionally, the loss of one (or more) functionalizations of the amine as alkylic chain (R_1 or R_2) [30] (**Figure 2b**), or the loss of the amine moiety together with the alpha alkyl chain (NLS_2, **Figure 2f**), which moreover is usually found as product ion [25] (PIS_1, **Figure 2e**) is possible. The alpha alkyl chain can be lost as radical alkylic chain (R_3), but

this fragmentation presents low intensity (**Figure 2d**). Finally, the aromatic ring can be detected with its substitutions and the carbonyl group [25] (PIS_2, **Figure 2f**), or without one of the substituents in case of some alkyl moieties and halogen atoms [25]. In this way, the occurrence of either some product ions or NLs can be directly associated with the presence of a characteristic nucleus in the detected cathinone.

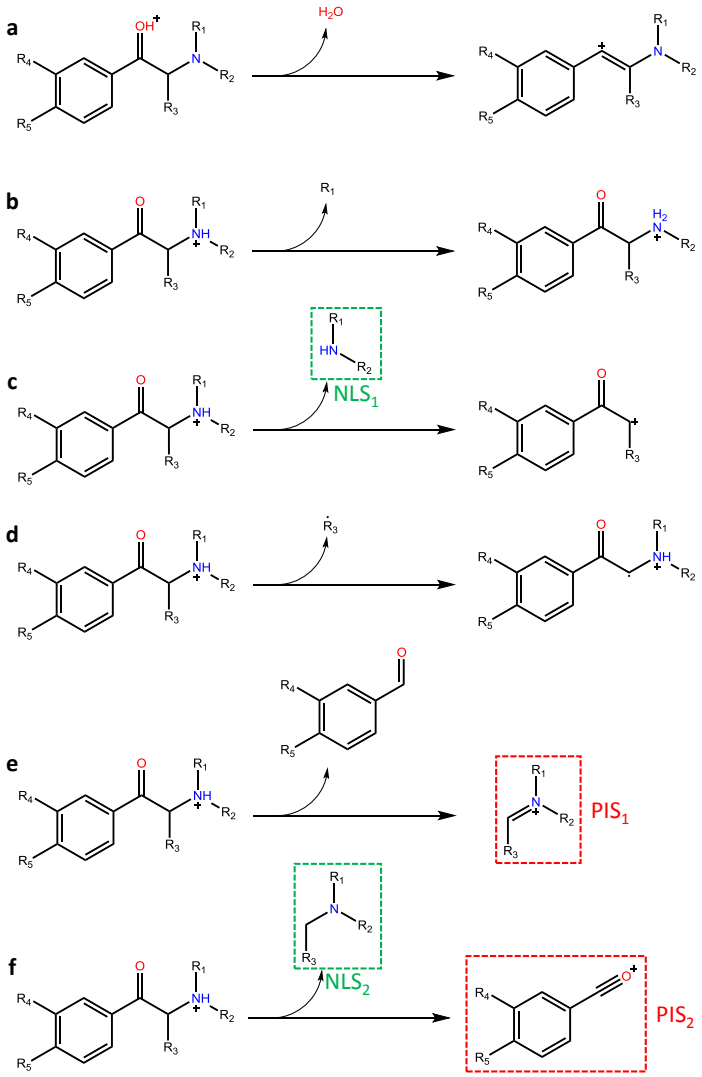


Figure 2. Typical common fragmentation pathway observed for synthetic cathinones. Neutral losses used for neutral loss scan (NLS) and common product ions used for precursor ion scan (PIS) used in this strategy are highlighted.

Neutral loss scan (NLS) and precursor ion scan (PIS) experiments

As commented in the "Introduction" section, in NLS experiments, the mass spectrometer scans a certain mass range in MS1, fragments the ions in the collision cell, and then scans in MS2 a predetermined range corresponding to a CID loss of a specific mass. Therefore, MS2 scans were performed with a certain mass-offset between MS1 and MS2 corresponding to the neutral loss occurred [22]. This working mode allowed the identification of all precursor ions that produced a NL, usually related to a specific moiety (for example, an 18 Da NL would correspond to a water molecule loss).

In PIS experiments, the mass spectrometer scanned a certain mass range in MS1, fragmented the ions in the collision cell, and then selected a specific product ion in MS2 [22]. This working mode allowed the identification of all precursor ions that had a certain product ion, usually related to a specific compound family or structure (for example, a fragment ion at *m/z* 91 (PIS91) would correspond to the tropylium ion, and thus the presence of a methylbenzene moiety).

In this work, several common product ions and NLs were evaluated from the study of product ion spectra of model compounds: butylone, 3',4'-methylenedioxypyrovalerone (MDPV), methedrone, methedrone, methylone, 4'-methyl-α-pyrrolidinopropiophenone (4'-MePPP), ethcathinone, 4'-chloro-α-pyrrolidinopropiophenone (4-CPrC or 4-chloro-PPP), 4-chloroethcathinone (4-CEC), 4-chloro-α-pyrrolidinovalerophenone (4-Cl-PVP), 4-methyl-*N*,*N*-dimethylcathinone (4-methyl-*N*,*N*-DMC), 4-methoxy-*N*,*N*-dimethylcathinone (4-methoxy-*N*,*N*-DMC), 4'-methyl-α-pyrrolidinohexiophenone (MPHP), *N*,*N*-dimethylpentylone (bk-DMBDP), 3',4'-methylenedioxy-α-pyrrolidinobutyrophenone (MDPBP), 5-dihydrobenzofuranpyrovalerone (5-DBFPV), 3',4'-methylenedioxy-α-pyrrolidinohexiophenone (MDPHP), 4-fluoromethcathinone (4-FMC), dimethylone, 3-methylethcathinone (3-MEC), 4-fluoro-N-isopropyl pentedrone (4-fluoro-IPV), 4-fluoro-pentedrone, 4'-fluoro-α-pyrrolidinohexanophenone (4-fluoro-PHP), α-pyrrolidinopentiothiophenone (α-

PVT), thiothinone, and the recently reported 3',4'-trimethylene-α-pyrrolidinobutiophenone (5-PPDi) [32]. Additionally, the fragmentation of more than 40 cathinones, available on the literature, was assessed [25, 30].

As an example, experiments NLS71 and PIS70 were specific for the detection of cathinones with a pyrrolidine moiety. Compounds with this moiety usually presented a product ion at *m/z* 70 corresponding to the pyrrolidine, and a product ion at *m/z* $[M+H-71]^+$ corresponding to the NL of this moiety. In a similar way, compounds which showed analytical signal for PIS139 would have a chlorine atom in the aromatic ring. In this case, the characteristic isotope pattern would also be observed on the acquired spectrum.

According to the results obtained from the fragmentation of selected cathinones, 35 methods based on PIS (**Figure 3**) and 25 methods based on NLS (**Figure 4**) were initially selected for the detection of unknown synthetic cathinones.

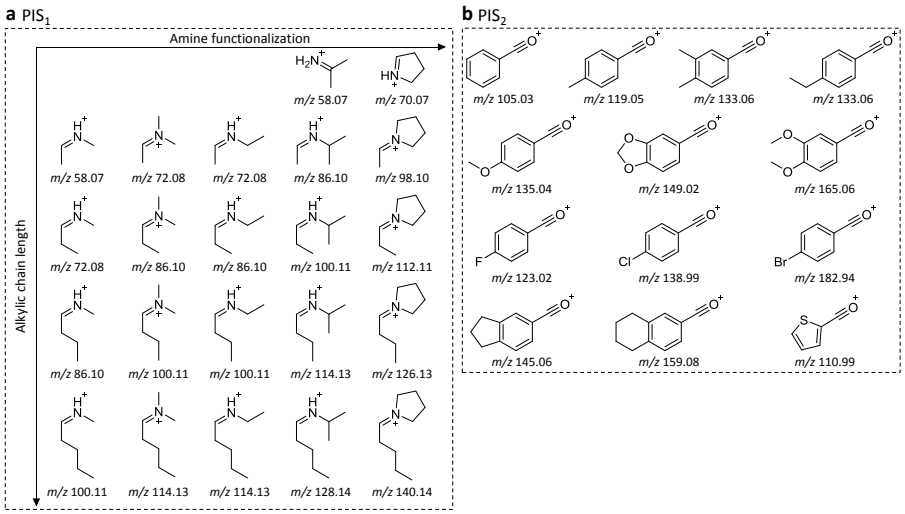


Figure 3. Common fragments monitored in the PIS strategy. **a** Common fragments derived from the amine moiety and alkylic chain. **b** Common fragments involving the aromatic ring.

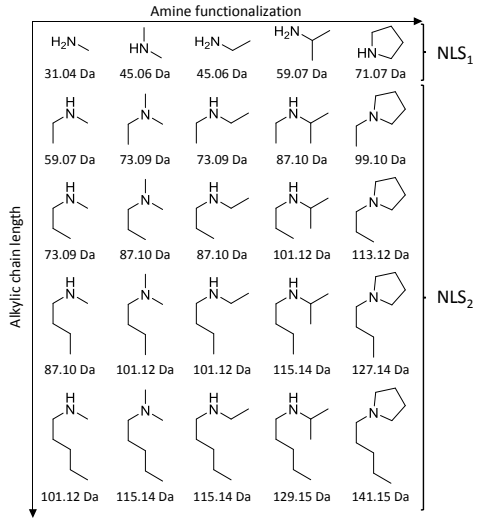


Figure 4. Neutral losses monitored in the NLS strategy.

Table 1 shows the MS/MS methods finally chosen to monitor the different NLs and common product ions considered. As can be seen, 13 NLS and 23 PIS experiments were selected, as some NLs and product ions related with the amine moiety share the same nominal mass (e.g., PIS72, PIS86, NL73, NL101, NL115…) (see **Figure 3** and **4**).

This pseudo-target screening would allow the tentative identification of 260 synthetic cathinones. This value represents the maximum number of cathinone structures which could be obtained after combining all the monitored moieties. Of these, only 118 are currently being monitored by EMCDDA [3].

FIA-MS/MS optimization

The typical high purity of seizures and research chemicals shows that the identification of their major active compounds does not require a chromatographic separation, allowing sample analysis by FIA-MS/MS. In this work, the flow carrier stream was H_2O/methanol (1:1 v/v with 0.01% HCOOH) at 0.3 mL/min. The mixture of H_2O and MeOH ensures the complete sweep along

of the substance, and the small amount of HCOOH enhances the ionization efficiency, as cathinones present a basic amine moiety.

Table 1. Precursor ion scan (PIS) and neutral loss scan (NLS) selected for the pseudo-target screening strategy.

Working mode	**Scan number**	**MS2**
PIS	1	Fixed at *m/z* 58
	2	Fixed at *m/z* 70
	3	Fixed at *m/z* 72
	4	Fixed at *m/z* 86
	5	Fixed at *m/z* 98
	6	Fixed at *m/z* 100
	7	Fixed at *m/z* 105
	8	Fixed at *m/z* 111
	9	Fixed at *m/z* 112
	10	Fixed at *m/z* 114
	11	Fixed at *m/z* 119
	12	Fixed at *m/z* 123
	13	Fixed at *m/z* 126
	14	Fixed at *m/z* 128
	15	Fixed at *m/z* 133
	16	Fixed at *m/z* 135
	17	Fixed at *m/z* 139
	18	Fixed at *m/z* 140
	19	Fixed at *m/z* 145
	20	Fixed at *m/z* 149
	21	Fixed at *m/z* 159
	22	Fixed at *m/z* 165
	23	Fixed at *m/z* 183

Cone voltage: 30 eV

MS1: scanning from *m/z* 15 to 324

Collision energy: 25 eV

Table 1. Precursor ion scan (PIS) and neutral loss scan (NLS) selected for the pseudo-target screening strategy (continuation).

Working mode	Scan number	MS2
NLS	24	Mass offset of 141 Da
	25	Mass offset of 129 Da
	26	Mass offset of 127 Da
	27	Mass offset of 115 Da
	28	Mass offset of 113 Da
	29	Mass offset of 101 Da
	30	Mass offset of 99 Da
	31	Mass offset of 87 Da
	32	Mass offset of 73 Da
	33	Mass offset of 71 Da
	34	Mass offset of 59 Da
	35	Mass offset of 45 Da
	36	Mass offset of 31 Da

Cone voltage: 30 eV

MS1: scanning from *m/z* 15 to 324

Collision energy: 25 eV

For the mass spectrometer optimization, analytical standards of MDPV, methylone, methedrone and mephedrone were tested. Capillary voltage was tested between 0.5 and 3.2 kV, at intervals of 0.5 kV, having the most intense at 3.2 kV. On a similar way, cone voltage was tested between 10 and 30 V, at intervals of 5 V. On this case, no significant differences were observed, but a slightly higher signal was obtained at 30 V. Collision energy was tested between 15 and 40 eV, at intervals of 5 eV, and using a 15-40 eV collision energy ramp. An intensity compromise between the different PISs and NLSs of the four cathinone standards was observed at 25 eV.

Application to research chemical samples

In order to check the applicability of the developed strategy, 14 samples were randomly selected from a total of 26 (previously analysed by HRMS but all blind for the operator) and analysed by FIA–MS/MS for synthetic cathinones identification.

After analysis by FIA–MS/MS, data obtained was carefully evaluated in order to elucidate a compound structure. It has to be taken into account that no chromatography is employed; thus, the functions that present an injection band and characteristic of the FIA system, confirm the presence of the related product ions or NLs. Below some illustrative examples are shown.

Example 1

Only PIS111 and PIS58 (corresponding to product ions at *m/z* 111 and 58, respectively) retrieved analytical signals (**Figure 5a**). The PIS111 was related to the presence of a thiophene ring bonded to the carbonyl moiety. Nevertheless, the PIS58 and the NLS59 (**Figure. 5b**) could correspond, in principle, to two possible structures. Hopefully, the NLS31 (corresponding to methylamine) directly assigned the PIS58 and the NLS59, as isopropylamine could not produce these fragments. No additional NLS were positive for this cathinone, discarding again the possibility of the isopropylamine moiety. The combination of the two parts of the molecule (PIS111 and NLS59) allowed to identify the synthetic cathinone as thiothinone [28]. Finally, the spectrum was extracted (**Figure 5c**), showing the protonated molecule of thiothinone at *m/z* 170 and the characteristic isotope pattern of a sulphur atom.

Example 2

Unluckily, in some occasions, compound identification was not as direct as shown in *Example 1*, and a detailed evaluation of the results was required. **Figure 6** shows the identification of a synthetic cathinone by FIA–MS/MS. In **Figure 6a**, all the positive PIS functions are shown (PIS140, PIS139, PIS128, PIS126,

PIS112, PIS72 and PIS70), illustrating the main drawbacks of the pseudo-target screening strategy. PIS140 and PIS112 presented much lower intensities than PIS126. These ±14 Da differences are produced by fragmentations involving methylene moieties, but they can be discarded (a priori) based on their intensities.

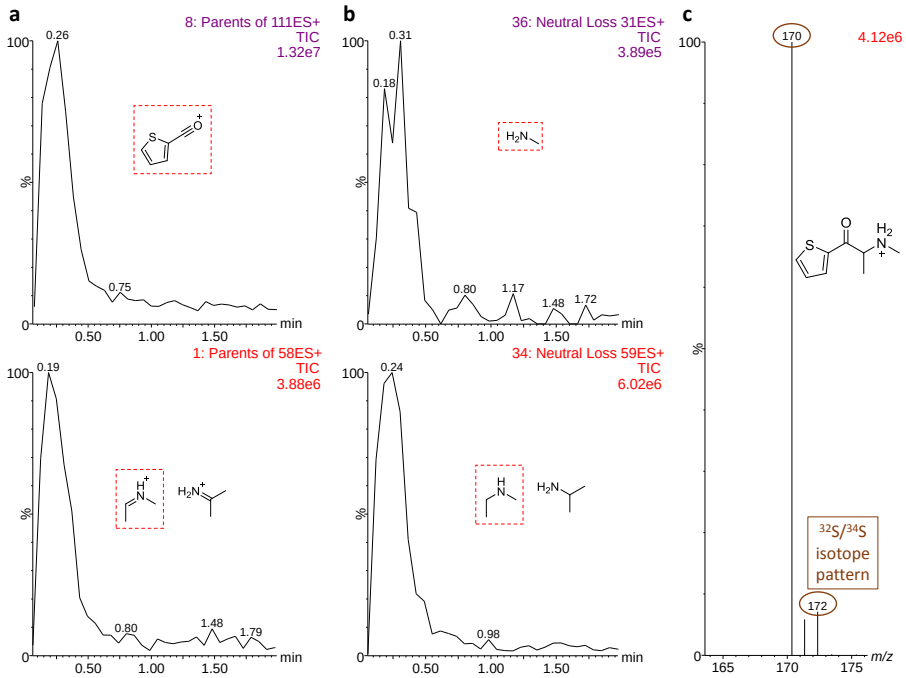


Figure 5. Identification of thiothinone by flow injection analysis-tandem mass spectrometry FIA–MS/MS using the pseudo-target screening strategy. **a** Total Ion Chromatogram (TIC) of PIS and **b** TIC of NLS, which retrieved analytical signals. **c** Spectrum obtained from NLS 59 Da.

On the same way, PIS128 can correspond to the ring opening or to the hydrogenation of the insaturation present in product ion at *m/z* 126 (PIS126), which probably occurs in the collision cell. In a similar way, this could also occur to PIS72 (which could in principle correspond to three different amine moieties) and PIS70. For a more confident identification, PIS139 presented a unique structure, and the presence of a chlorine atom on the aromatic ring could be assured. Regarding NLs (**Figure 6b**), NLS71 confirmed the presence of the pyrrolidine moiety, and the direct assignation of the PIS70 and rejection of the

PIS72, were made as previously discussed. Additionally, NLS127 was in concordance with PIS126. NLS129 and NLS113 were discarded in front of NLS127, based on the lower intensity observed. Finally, **Figure 6c** shows the spectrum obtained from the NLS70, where the characteristic isotope pattern of a chlorine atom was observed. The combination of all information allowed to confirm the identity of the synthetic cathinone to be the 4-chloro-α-pyrrolidinovalerophenone or 4-chloro-PVP, a novel cathinone recently identified [33]. Additionally, the **Figure S1** shows the HRMS spectra for the 4-chloro-PVP.

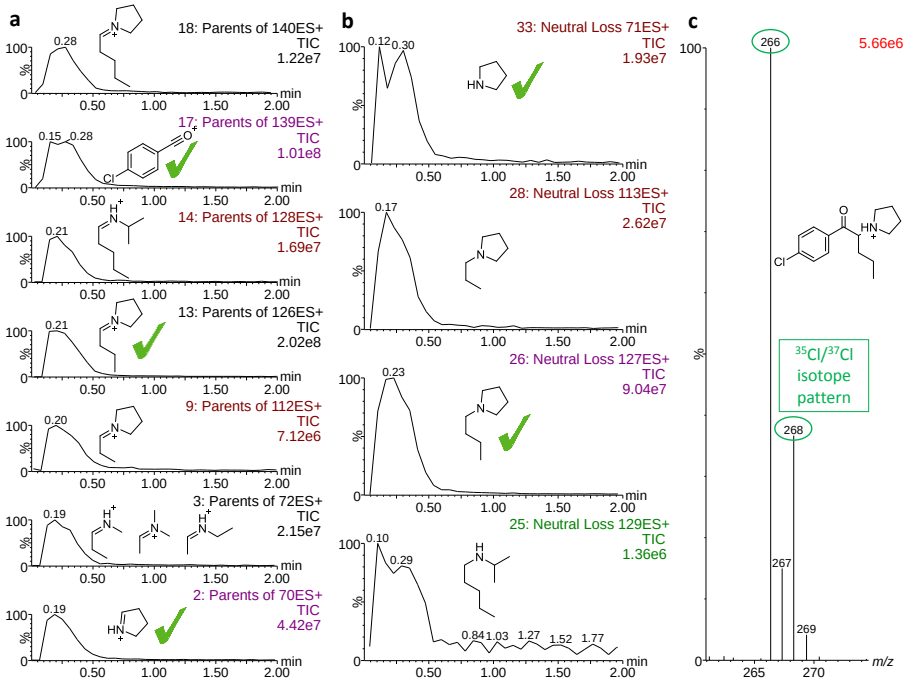


Figure 6. Identification of 4-Cl-PVP by FIA–MS/MS using the pseudo-target screening strategy. **a** TIC of PIS and **b** TIC of NLS, which retrieved analytical signals. **c** Spectrum obtained from NLS70.

In a similar way, the remaining samples were analysed and processed. A total of 14 research chemical samples were analysed by FIA–MS/MS using the pseudo-target screening strategy.

The cathinones identified in these samples can be found in **Table 2**. Additionally, positive and "false positives" PIS and NLS observed for these cathinones can be found in **Table S1**.

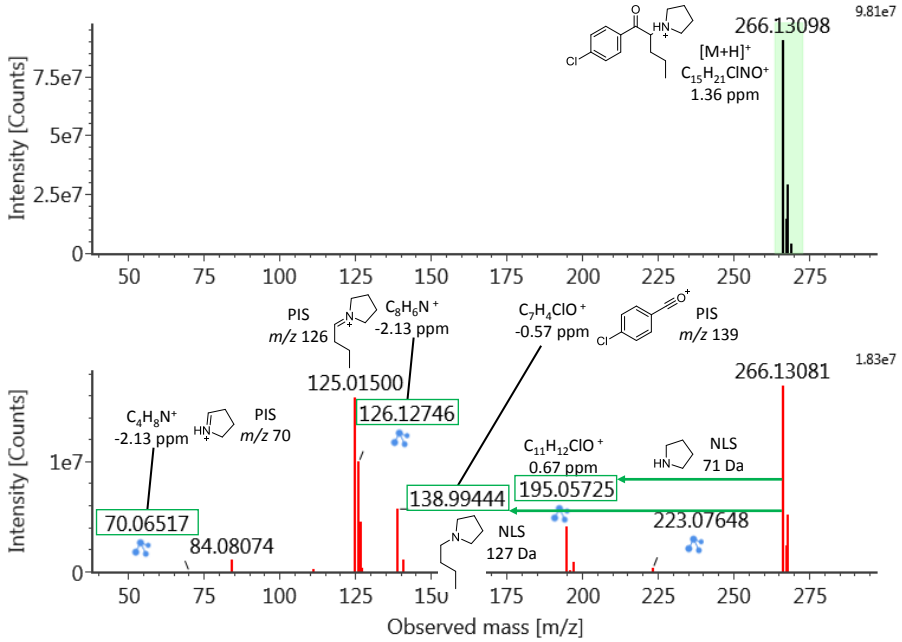


Figure S1. HRMS spectra of 4-Cl-PVP. **Top** Low energy spectrum, showing the protonated molecule of the compound. **Bottom** High energy spectrum showing the accurate-mass fragments (used for PIS) and the neutral loss observed (used for NLS).

General remarks and special considerations

For the correct identification of the alkyl chain and amine functionalization, it is necessary to study both NLS corresponding to the amine (R_1 and R_2 in **Figure 1**) (NLS31, NLS45, NLS59, NLS71), and NLS/PIS from the alkyl chain (R_3) bonded to the amine group. For example, the synthetic cathinone 4-FMC was positive for PIS58, which could correspond to two possible product ions (as it can be seen in **Figure 3a**). However, the specific NLS31 (**Figure 4**) revealed that the correct amine functionalization was a methyl group instead of an isopropyl.

Some compounds with short alkyl chain, such as thiothinone and 4-FMC, were directly identified by FIA–MS/MS. In some cases, and similarly to HRMS instruments, pseudo-target screening could not differentiate between dimethylamine and ethylamine, as the fragmentation of both moieties was identical, and NMR experiments would be required. This was the case of 4-CEC (or 4-chloro-*N*,*N*-DMC), 4-methyl-*N*,*N*-DMC (or 4-methyl-*N*-ethylcathinone) and 4-methoxy-*N*,*N*-DMC (or 4-methoxy-*N*-ethylcathinone).

Table 2. Cathinones found in the blind samples used for testing the pseudo-target screening strategy.

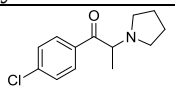
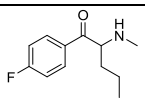
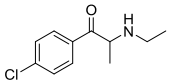
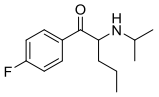
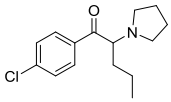
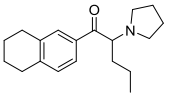
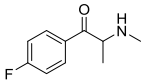
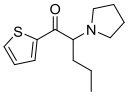
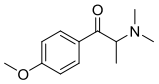
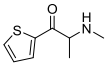
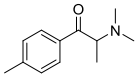
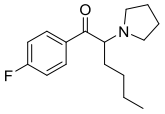
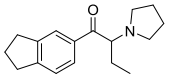
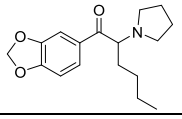
4-CPrC		4-fluoro-pentedrone	
4-CEC		4-fluoro-IPV	
4-Cl-PVP *(example 2)*		TH-PVP	
4-FMC		α-PVT	
4-methoxy-*N*,*N*-DMC		Thiothinone *(example 1)*	
4-methyl-*N*,*N*-DMC		4-fluoro-PHP	
5-PPDi		5-MDPHP	

Table S1. Positives and false positive PIS and NLS seen for the 14 cathinones.

Compound	PIS																						NLS												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
4-CPrC		✓	×		✓												✓													✓			✓			
4-CEC			✓														✓		×	×	×	×								×		✓			✓	
4-Cl-PVP		✓	×						×				✓	×			✓	×							×	✓		×					✓			
4-FMC	✓											✓								×														✓		✓
4-Methoxy-*N*,*N*-DMC	×		✓													✓													×			✓			✓	
4-Methyl-*N*,*N*-DMC	×		✓								✓																		×			✓			✓	
5-PPDi		✓	×						✓										✓		×					×		✓		×			✓			
4-Fluoro-pentedrone				✓								✓				×													×		✓					✓
4-Fluoro-IPV						×				✓		✓		×		×									×		✓		×					✓		
TH-PVP		✓	×										✓						×		✓			×		✓		×					✓			
α-PVT		✓	×		×			✓					✓											×		✓							✓			
Thiothinone	✓							✓																										✓		✓
4-Fluoro-PHP		✓	×									✓	×					✓						✓		×							✓			
5-MDPHP		✓	×													×		✓		✓				✓	×								✓			

✓: Positive PIS/NLS
×: False positive PIS/NLS

Compounds with large alkyl chains, such as 4-fluoro-pentedrone, 4-fluoro-IPV, 4-fluoro-PHP, 5-MDPHP or 4-Cl-PVP, presented some problems in the determination of the amine moiety and alkyl chain, as shown in *Example 2*. These problems are consequence of a CH_2 loss or gain respect of the correct PIS or NLS. This CH_2 loss could be explained by the loss of a methylene during the fragmentation, generating analytical signals in PIS and NLS, which have 14 Da less than the correct one. Similarly, the CH_2 gain could be produced when the product ion or NL comes from the $[M+H-H_2O]^+$ ion, as the fragmentation involves the carbon atom of the ketone moiety.

In a similar way, compounds with a 2,3-methylenedioxyphenyl moiety (such as 5-MDPHP), which produce signal at PIS149, unexpectedly also produce signal at PIS119. The ion at *m/z* 119 is also obtained after fragmentation of the dioxole ring, releasing formaldehyde and leaving an aromatic ring doubly bonded to the oxygen atom [34]. Despite the elemental composition of PIS119 ($C_8H_7O^+$) (see **Figure 3b**) and the above commented ($C_7H_3O_2^+$) are different, the nominal mass of both product ions is the same, and thus the PIS119 leads to a false positive.

Compounds with an alkyl ring fused to the aromatic ring suffer similar problems. As an example, TH-PVP (a novel cathinone derivative recently characterized [35]), which should retrieve signal at PIS159, leads also signal at PIS145, with much lower intensity. The product ion at *m/z* 145 is produced by the fragmentation of the $[M+H-H_2O]^+$ ion, releasing the methyl-tetrahydronaphthalene fragment ($C_{11}H_{13}^+$), which has the same nominal mass as PIS145 ($C_{10}H_9O^+$) (See **Figure 3b**).

Compounds with halogen atoms on the aromatic ring, or small substitutions such as methyl or methoxy groups did not usually present problems on the interpretation of PIS corresponding to the aromatic moiety. Only in one of the studied cases (4-CEC), the chlorine atom was lost as radical, and PIS159 from the aromatic moiety led to a false positive. This is produced, because the $[M+H-H_2O-Cl]^+$ ion ($C_{11}H_{13}N^+$) has the same nominal mass as PIS159 ($C_{11}H_{11}O^+$).

It is interesting to highlight that compounds with chlorine, bromine or sulphur atoms present their characteristic isotope patterns when each spectrum is extracted from an NLS. This will directly allow to discard the false positive commented above. Isotope pattern cannot be evaluated from PIS, as only the most abundant isotope is acquired (for example, PIS139 only acquires ^{35}Cl).

Finally, the compounds with a pyrrolidine moiety provided analytical signals for PIS70 (the correct one) and PIS72, as a consequence of the hydrogenation of *m/z* 70. In this case, the NLS71 is crucial to establish the correct functionalization.

Samples containing synthetic cathinones mixtures

The pseudo-target screening strategy for low resolution MS/MS presented in this work has been developed for its application in legal highs and research chemicals with high purity. All the samples analysed in our laboratory by UHPLC–HRMS and NMR (more than 100) presented only single NPS on its composition, without additives or secondary products. Nevertheless, we are aware that some seizures or legal highs could contain two or more synthetic cathinones in their composition, and therefore the approach presented should be modified including a chromatographic separation and another option.

In order to detect the presence of cathinone mixtures by FIA–MS/MS, a scan function was added to the screening strategy (scan time of 0.017 s, from *m/z* 150 to 324), not affecting the sensitivity of the PIS and NLS monitored. This approach was tested with two artificial mixtures prepared in the laboratory, containing both of them two synthetic cathinones. As an example, **Figure 7** shows the scan function corresponding to a mixture of 4-CPrC and 4-Cl-PVP at a concentration of approximately 0.5 mg/L. It can be easily observed the presence of two chlorinated compounds. In this case, a general chromatographic separation could be easily used, in order to obtain a tentatively identification of both cathinones applying the pseudo-target screening strategy.

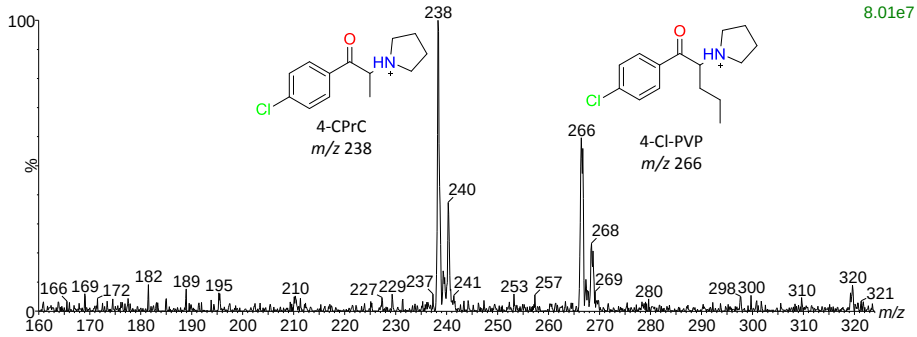


Figure 7. Scan function of one prepared mixture containing 4-CPrC and 4-Cl-PVP at a concentration of approximately 0.5 mg/L.

Conclusions

In this work, a pseudo-target screening strategy has been developed for rapid synthetic cathinone identification by FIA–MS/MS. The methodology applied is based on the common fragmentation pathways observed for synthetic cathinones, and combines 25 NLs and 35 common product ions, which include a wide majority of possible substitutions present in this family of NPS.

Once analysed the substance by FIA–MS/MS under PIS and NLS working modes, the tentative identification of the cathinones was feasible. With the acquisition of the reference standards, such tentative identification can be finally confirmed. Most of the compounds tested could be directly and rapidly identified using this strategy; however, some of them, such as cathinones with long alkyl chain, would need a detailed data processing to discard false positives in some PIS and NLS experiments.

The strategy presented in this work will be useful for forensic laboratories equipped with low-resolution MS/MS equipment only. Although this pseudo-target screening approach has demonstrated its applicability for identification of described synthetic cathinones, it should be periodically updated to cover novel cathinones that might appear in the near future.

Acknowledgements

Authors acknowledge financial support from Generalitat Valenciana (Group of Excellence Prometeo II 2014/023) and from the Ministerio de Economía y Competitividad in Spain (Project: CTQ2015-65603-P). Authors also acknowledge NPS-Euronet (HOME/2014/JDRUG/AG/DRUG/7086), co-funded by the European Union. This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein. D. Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (Grant FPU15/02033). Authors also acknowledge Energy Control for providing the research chemical samples used for testing the pseudo-target screening strategy.

Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Prosser JM, Nelson LS (2012) The toxicology of bath salts: A review of synthetic cathinones. J Med Toxicol 8:33–42. https://doi.org/10.1007/s13181-011-0193-z

2. Karila L, Megarbane B, Cottencin O, Lejoyeux M (2015) Synthetic cathinones: a new public health problem. Curr Neuropharmacol 13:12–20. https://doi.org/10.2174/1570159X13666141210224137

3. European Monitoring Centre for Drugs and Drug Addiction (2017) European drug report 2017. EMCDDA–Europol joint publication 88. https://doi.org/10.2810/88175

4. Brandt SD, King LA, Evans-Brown M (2014) The new drug phenomenon. Drug Test Anal 6:587–597. https://doi.org/10.1002/dta.1686

5. Bijlsma L, Ibáñez M, Miserez B, Ma STF, Shine T, Ramsey J, Hernández F (2017) Mass spectrometric identification and structural analysis of the third-generation synthetic cannabinoids on the UK market since the 2013 legislative ban. Forensic Toxicol 35:376–388. https://doi.org/10.1007/s11419-017-0368-7

6. United Nations Office on Drugs and Crime (UNODC) (2015) Recommended methods for the identification and analysis of synthetic cathinones in seized materials (ST/NAR/49). https://www.unodc.org/documents/scientific/STNAR49_Synthetic_Cathinones_E.pdf. Accessed 29 Nov 2017.

7. Majchrzak M, Celiński R, Kuś P, Kowalska T, Sajewicz M (2017) The newest cathinone derivatives as designer drugs: an analytical and toxicological review. Forensic Toxicol 36:33-52. https://doi.org/10.1007/s11419-017-0385-6

8. Brandt SD, Daley PF, Cozzi NV (2012) Analytical characterization of three trifluoromethyl-substituted methcathinone isomers. Drug Test Anal 4:525–529. https://doi.org/10.1002/dta.382

9. Tsujikawa K, Mikuma T, Kuwayama K, Miyaguchi H, Kanemori T, Iweta YT, Inoue H (2013) Identification and differentiation of methcathinone analogs by gas chromatography-mass spectrometry. Drug Test Anal 5:670–677. https://doi.org/10.1002/dta.1437

10. Nic Daeid N, Savage KA, Ramsay D, et al (2014) Development of gas chromatography–mass spectrometry (GC–MS) and other rapid screening methods for the analysis of 16 ‘legal high’ cathinone derivatives. Sci Justice 54:22–31. https://doi.org/10.1016/j.scijus.2013.08.004

11. Carnes S, O'Brien S, Szewczak A, Tremesu-Cayel L, Rowe WF, McCord B, Lurie IS (2017) Comparison of ultra-high performance supercritical fluid chromatography, ultra-high performance liquid chromatography, and gas chromatography for the separation of synthetic cathinones. J Sep Sci 40:3545–3556. https://doi.org/10.1002/jssc.201700349

12. De Vijlder T, Valkenborg D, Lemière F, Romijin EP, Laukens K, Cuyckens F (2017) A tutorial in small molecule identification via electrospray ionization-mass spectrometry: The practical art of structural elucidation. Mass Spectrom Rev 1–23. https://doi.org/10.1002/mas.21551

13. Ibañez M, Sancho J V., Bijlsma L, van Nuijs A, Covaci A, Hernández F (2014) Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. Trends Anal Chem 57:107–117. https://doi.org/10.1016/j.trac.2014.02.009

14. Strano Rossi S, Odoardi S, Gregori A, Pelao G, Ripeni L, Ortar G, Serpelloni G, Romelo FS (2014) An analytical approach to the forensic identification of different classes of new psychoactive substances (NPSs) in seized materials. Rapid Commun Mass Spectrom 28:1904–1916. https://doi.org/10.1002/rcm.6969

15. Lobo Vicente J, Chassaigne H, Holland MV., Reniero F, Kolár K, Tirendi S, Vandecasteale I, Vinakier I, Guilln C (2016) Systematic analytical characterization of new psychoactive substances: A case study. Forensic Sci Int 265:107–115. https://doi.org/10.1016/j.forsciint.2016.01.024

16. Liu C, Jia W, Li T, Hua Z, Qian Z (2017) Identification and analytical characterization of nine synthetic cathinone derivatives N-ethylhexedrone, 4-Cl-pentedrone, 4-Cl-α-EAPP, propylone, N-ethylnorpentylone, 6-MeO-bk-MDMA, α-PiHP, 4-Cl-α-PHP, and 4-F-α-PHP. Drug Test Anal 9:1162–1171. https://doi.org/10.1002/dta.2136

17. Qian Z, Jia W, Li T, Liu C, Hua z (2017) Identification and analytical characterization of four synthetic cathinone derivatives iso-4-BMC, β -TH-naphyrone, mexedrone, and 4-MDMC. Drug Test Anal 9:274–281. https://doi.org/10.1002/dta.1983

18. Boumba VA, Di Rago M, Peka M, Drummer OH, Gerostamoulos M (2017) The analysis of 132 novel psychoactive substances in human hair using a single step extraction by tandem LC/MS. Forensic Sci Int 279:192–202. https://doi.org/10.1016/j.forsciint.2017.08.031

19. Lendoiro E, Jiménez-Morigosa C, Cruz A, Páramo M, López-Rivadella M, de Castro A (2017) 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 9:96–105. https://doi.org/10.1002/dta.1948

20. Adamowicz P, Tokarczyk B (2016) Simple and rapid screening procedure for 143 new psychoactive substances by liquid chromatography-tandem mass spectrometry. Drug Test Anal 8:652–667. https://doi.org/10.1002/dta.1815

21. Bade R, Bijlsma L, Sancho JV, Baz-Lomba JA , Castiglioni S, Castrignanò E, Causanilles A, Gracia-Lor E, Kasprzyk-Hordern B, Kinyua J, McCall AK, van Nuijs ALN, Ort C, Plósz BG, Ramin P, Rousis NI, Ryu Y, Thomas KV, de Voogt P, Zuccato E, Hernández F (2017) Liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in influent wastewater of eight European cities. Chemosphere 168:1032–1041. https://doi.org/10.1016/j.chemosphere.2016.10.107

22. Marcos J, Pozo OJ (2016) Current LC–MS methods and procedures applied to the identification of new steroid metabolites. J Steroid Biochem Mol Biol 162:41–56. https://doi.org/10.1016/j.jsbmb.2015.12.012

23. Pozo OJ, Ventura R, Monfort N, Segura J, Delbeke FT (2009) Evaluation of different scan methods for the urinary detection of corticosteroid metabolites by liquid chromatography tandem mass spectrometry. J Mass Spectrom 44:929–944. https://doi.org/10.1002/jms.1568

24. McLeod MD, Waller CC, Esquivel A, Balcells G, Ventura R, Segura J, Pozo OJ (2017) Constant ion loss method for the untargeted detection of bis-sulfate metabolites. Anal Chem 89:1602–1609. https://doi.org/10.1021/acs.analchem.6b03671

25. Fornal E (2014) Study of collision-induced dissociation of electrospray-generated protonated cathinones. Drug Test Anal 6:705–715. https://doi.org/10.1002/dta.1573

26. González D, Ventura M, Caudevilla F, Torrens M, Farré M (2013) Consumption of new psychoactive substances in a Spanish sample of research chemical users. Hum Psychopharmacol Clin Exp 28:332–340. https://doi.org/10.1002/hup.2323

27. European Monitoring Centre for Drugs and Drug Addiction 2015 Synthetic cathinones drug profile. http://www.emcdda.europa.eu/publications/drug-profiles/synthetic-cathinones. Accessed 28 Nov 2017

28. Gambaro V, Casagni E, Dell'Acqua L, Rosa G, Terborini L, Visconti GL, Demartin F (2016) Identification and characterization of a new designer drug thiothinone in seized products. Forensic Toxicol 34:174–178. https://doi.org/10.1007/s11419-015-0289-2

29. Doi T, Asada A, Takeda A, Tagami T, Kategi M, Matsuta S, Kamato H, Kawagucho M, Satauki Y, Sawaka Y, Obane H (2016) Identification and characterization of a-PVT, a-PBT, and their bromothienyl analogs found in illicit drug products. Forensic Toxicol 34:76–93. https://doi.org/10.1007/s11419-015-0288-3

30. Zuba D (2012) Identification of cathinones and other active components of ‘legal highs’ by mass spectrometric methods. Trends Anal Chem 32:15–30. https://doi.org/10.1016/j.trac.2011.09.009

31. Fornal E (2013) Identification of substituted cathinones: 3,4-methylenedioxy derivatives by high performance liquid chromatography–quadrupole time of flight mass spectrometry. J Pharm Biomed Anal 81–82:13–19. https://doi.org/10.1016/j.jpba.2013.03.016

32. Fabregat-Safont D, Carbón X, Gil C, Ventura M, Sancho JV, Hernández F, Ibañez M (2018) Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. https://doi.org/10.1007/s11419-018-0422-0

33. Qian Z, Jia W, Li T, Hua Z, Liu C (2017) Identification of five pyrrolidinyl substituted cathinones and the collision-induced dissociation of electrospray-generated pyrrolidinyl substituted cathinones. Drug Test Anal 9:778–787. https://doi.org/10.1002/dta.2035

34. Ibáñez M, Pozo ÓJ, Sancho JV, Orengo T, Haro G, Hernández F (2016) Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers’ urine by high-resolution mass spectrometry. Anal Bioanal Chem 408:151–164. https://doi.org/10.1007/s00216-015-9088-1

35. Nycz JE, Pazdziorek T, Malecki G, Szala M (2016) Identification and derivatization of selected cathinones by spectroscopic studies. Forensic Sci Int 266:416–426. https://doi.org/10.1016/j.forsciint.2016.06.034

3.3. Research article VI

ACS Partner Journal

Journal of the American Society for Mass Spectrometry

pubs.acs.org/jasms

Application Note

Direct and Fast Screening of New Psychoactive Substances Using Medical Swabs and Atmospheric Solids Analysis Probe Triple Quadrupole with Data-Dependent Acquisition

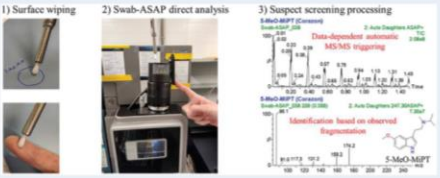
David Fabregat-Safont, Daniela Felis-Brittes, Maria Mata-Pesquera, Juan V. Sancho, Félix Hernández, and María Ibáñez*

Cite This: *J. Am. Soc. Mass Spectrom.* 2020, 31, 1610−1614

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: New psychoactive substances (NPS) have become a serious public health problem, as they are continuously changing their structures and modifying their potency and effects on humans, and therefore, novel compounds are unceasingly appearing. One of the major challenges in forensic analysis, particularly related to the problem of NPS, is the development of fast screening methodologies that allow the detection of a wide variety of compounds in a single analysis. In this study, a novel application of the atmospheric solids analysis probe (ASAP) using medical swabs has been developed. The swab−ASAP was coupled to a triple quadrupole mass analyzer working under a data-dependent acquisition mode in order to perform a suspect screening of NPS in different types of samples as well as on surfaces. The compounds were automatically identified based on the observed fragmentation spectra using an in-house built MS/MS spectra library. The developed methodology was applied for the identification of psychoactive substances in research chemicals and herbal blends. The sensitivity of the method, as well as its applicability for surface analysis, was also assessed by identifying down to 1 μg of compound impregnated onto a laboratory table. Another remarkable application was the identification of cathinones and synthetic cannabinoids on the fingers of potential consumers. Interestingly, our data showed that NPS could be identified on the fingers after being in contact with the product and even after cleaning their hands by shaking off with a cloth. The methodology proposed in this paper can be applied for routine analyses of NPS in different matrix samples without the need to establish a list of target compounds prior to analysis.



KEYWORDS: *new psychoactive substances, medical swab, atmospheric solids analysis probe, data-dependent acquisition, ambient ionization mass spectrometry*

INTRODUCTION

During 2018, 52 new psychoactive substances (NPS) were reported for the first time, with approximately one compound each week.[1,2] This trend has been observed during the past decade, and nowadays, more than 700 different NPS are currently being monitored.[1,2] The continuous increase of novel compounds increases the need for analytical methodologies that allow their fast analysis and identification.

Chromatography coupled to mass spectrometry is the most powerful analytical technique for the analysis of NPS[3] in a wide variety of matrices such as seizures, legal substances, and biological tissues and fluids.[4] In recent years, the development of ambient mass ionization sources that allow the fast and direct analysis of samples, without any sample treatment, has posed a new promising scenario in forensic analysis.[3] Among the most commonly used for identification of psychoactive substances, direct analysis in real time (DART),[5,6] desorption electrospray,[7] the recently developed swab touch spray,[8] and the atmospheric solids analysis probe (ASAP) can be highlighted, based on an atmospheric chemical ionization (APCI)-modified source, which has already proven its potential in toxicological analysis.[9−11] The ASAP source has demonstrated its applicability when coupled to high-resolution mass spectrometry (HRMS) and also to tandem mass spectrometry (MS/MS) with a triple quadrupole.[9,12]

In this work, a rapid and efficient analytical methodology based on a modified ASAP-MS/MS system has been developed for the suspect screening of a wide variety of NPS and has been applied to different cases related to the consumption of these substances. The glass capillary was replaced by a medical swab in order to allow the determination

Received: March 30, 2020
Revised: May 26, 2020
Accepted: May 28, 2020
Published: May 28, 2020

https://dx.doi.org/10.1021/jasms.0c00112
J. Am. Soc. Mass Spectrom. 2020, 31, 1610−1614

Journal of the American Society for Mass Spectrometry, 2020; 31:1610

Direct and fast screening of new psychoactive substances using medical swabs and atmospheric solids analysis probe-triple quadrupole with data-dependent acquisition

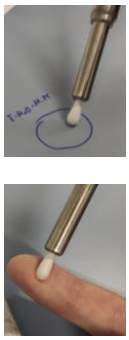
David Fabregat-Safont, Daniela Felis-Brittes, Maria Mata-Pesquera, Juan V. Sancho, Félix Hernández, María Ibáñez *

Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, Avda Sos Baynat s/n, 12071 Castellón, Spain.

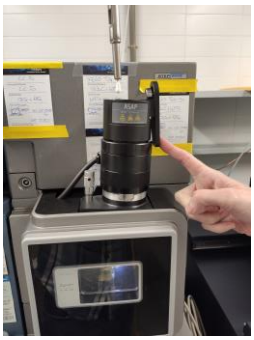
Graphical abstract

Direct and fast screening of new psychoactive substances using medical swabs and atmospheric solids analysis probe-triple quadrupole with data-dependent acquisition.
David Fabregat-Safont, Daniela Felis-Brittes, Maria Mata-Pesquera, Juan V. Sancho, Félix Hernández, María Ibáñez

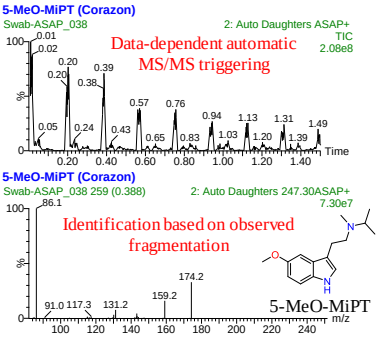
1) Surface wiping



2) Swab-ASAP direct analysis



3) Suspect screening processing



Abstract

New psychoactive substances (NPS) have become a serious public health problem, as they are continuously changing their structures, modifying their potency and effects on humans, and therefore novel compounds are unceasingly appearing. One of the major challenges in forensic analysis, particularly related to the problematic of NPS, is the development of fast screening methodologies that allow the detection of a wide variety of compounds in a single analysis. In this study, a novel application of the atmospheric solids analysis probe (ASAP) using medical swabs has been developed. The swab-ASAP was coupled to a triple quadrupole mass analyser working under data-dependent acquisition mode in order to perform a suspect screening of NPS in different types of samples as well as in surfaces. The compounds were automatically identified based on the observed fragmentation spectra using an in-house built MS/MS spectra library. The developed methodology was applied to the identification of psychoactive substances in research chemicals and herbal blends. The sensitivity of the method, as well as its applicability for surface analysis, was also assessed by identifying down to 1 µg of compound impregnated into a laboratory table. Another remarkable application was the identification of cathinones and synthetic cannabinoids in the fingers of potential consumers. Interestingly, our data showed that NPS could be identified in the fingers after being in contact with the product and even after cleaning their hands by shaking off with a cloth. The methodology proposed in this paper can be applied for routine analyses of NPS in different matrix samples without the need to establish a list of target compounds prior to analysis.

Keywords New psychoactive substances; Medical swab; Atmospheric solids analysis probe; Data-dependent acquisition; Ambient ionization mass spectrometry.

Introduction

During 2018, 52 new psychoactive substances (NPS) were reported for the first time, around one compound each week [1, 2]. This trend has been observed during the last decade, and nowadays more than 700 different NPS are currently being monitored [1, 2]. The continuous rising of novel compounds increases the need of analytical methodologies that allow their fast analysis and identification.

Chromatography coupled to mass spectrometry is the most powerful analytical technique for the analysis of NPS [3] in a wide variety of matrices such as seizures, legal highs, and biological tissues and fluids [4]. In the last years, the development of ambient mass ionisation sources that allow the fast and direct analysis of samples, without any sample treatment, has posed a new promising scenario in forensic analysis [3]. Among the most commonly used for identification of psychoactive substances it can be highlighted the direct analysis in real time (DART) [5, 6], desorption electrospray [7], the recently developed swab touch spray [8], and the atmospheric solids analysis probe (ASAP), based on an atmospheric chemical ionisation (APCI) modified source, which have already proved its potential in toxicological analysis [9–11]. The ASAP source has demonstrated its applicability when coupled to high-resolution mass spectrometry (HRMS) but also to tandem mass spectrometry (MS/MS) with triple quadrupole [9, 12].

In this work, a rapid and efficient analytical methodology based on a modified ASAP-MS/MS system, has been developed for the suspect screening of a wide variety of NPS, and has been applied to different cases related to the consumption of these substances. The glass capillary was replaced by a medical swab in order to allow the determination of NPS in surfaces, including the fingers of a potential consumer. For suspect screening, a data-dependent acquisition (DDA) mode was used in the triple quadrupole instrument to obtain the fragmentation spectra of the compounds. The acquired product ion spectra were then automatically searched in an in-house MS/MS spectra database for compound identification.

The applicability of this methodology for tentative identification of selected NPS was tested in different matrices with emphasis on the sensitivity and reliability of the identification.

Materials and methods

Reagents and chemicals

Herbal blends, powders and pills were purchased in a local smart-shop and were previously analysed by UHPLC-HRMS for compound identification [13]. Research chemicals had been provided by Energy Control and analysed by UHPLC-HRMS and nuclear magnetic resonance for compound identification [14]. HPLC-grade methanol was purchased from Scharlau (Scharlab, Barcelona, Spain). Medical swabs were purchased from neoLab (neoLab Migge GmbH, Heidelberg, Germany).

Sample treatment

For direct analysis, a medical swab was placed on the ASAP probe and gently wiped in the sample or surface. 100 µL of methanol was added to the swab, and introduced into the ASAP holder for sample analysis.

Instrumentation

Samples were analysed using a Xevo TQ-S mass spectrometer (Waters Corp, Manchester, UK) with a triple quadrupole mass analyser, equipped with an ASAP source (Waters Corp, Manchester, UK). The corona pin current was 2.0 µA in positive ionization mode, and the cone voltage 30 V. Source temperature was stablished at 150 ºC, and desolvation temperature 450 ºC. Nitrogen (Praxair, Valencia, Spain) was used as cone and desolvation gas at 150 and 800 L/h, respectively. MS/MS was operated in DDA acquisition mode. Survey MS scan data were acquired from *m/z* 170 to 450 with a scan time of 50 ms. For automatic MS/MS, the switch threshold was $5 \cdot 10^5$ counts/s, acquiring data from

m/z 60 to 450, with a scan time of 50 ms, using a 25 eV collision induced-dissociation (CID) energy (argon 99.995%; Praxair), an isolation window of 1 Da and an exclusion time of 10 s for the previously detected precursor ion. Only one *m/z* value was selected for MS/MS in each survey MS scan. The total run time was 1.5 min.

Data were acquired using MassLynx data station operation software (v4.1; Waters), and processed using MassLynx and MS Search (v2.0; NIST, USA) for automatic compound identification based on DDA MS/MS data. For compound identification, experimental MS/MS spectrum was directly processed with MassLynx, searching in the fragmentation spectra database generated in our laboratory. For automatic search, MS Search software must be installed together with MassLynx.

Results and discussion

Acquisition parameters optimization

The ASAP probe allows the use of a desolvation temperature ramp. Desolvation temperature can be stablished at 70 ºC, and rapidly increased until 600 ºC in 20-30 s. This process allows the analytes with higher volatility to be analysed by ASAP-MS, as the probe is at low temperature at the beginning of the acquisition. Application of desolvation ramp temperature in ASAP sources can be found in literature, for example, applied to petroleomics for the analysis of different fractions [12]. When a swab is used, the evaporation of the sample does not occur as faster as using the glass capillary, and a fixed desolvation temperature can be used for obtaining closer analyses. **Figure S1** shows the total ion chromatogram (TIC) of a MS scan from *m/z* 50 to 450 of a blank swab with 100 µL of methanol, acquired during 3 min using a 70-450 ºC ramp desolvation temperature (**A**), and a 450 ºC fixed desolvation temperature (**B**). It can be observed that using the ramp temperature, it takes around 60 s to start the evaporation and ionization of the

compounds from swab, while using a fixed temperature the evaporation starts at around 30 s. For this reason, a fixed desolvation temperature at 450 ºC, and an acquisition time of 1.5 min were selected for the analysis using swab-ASAP.

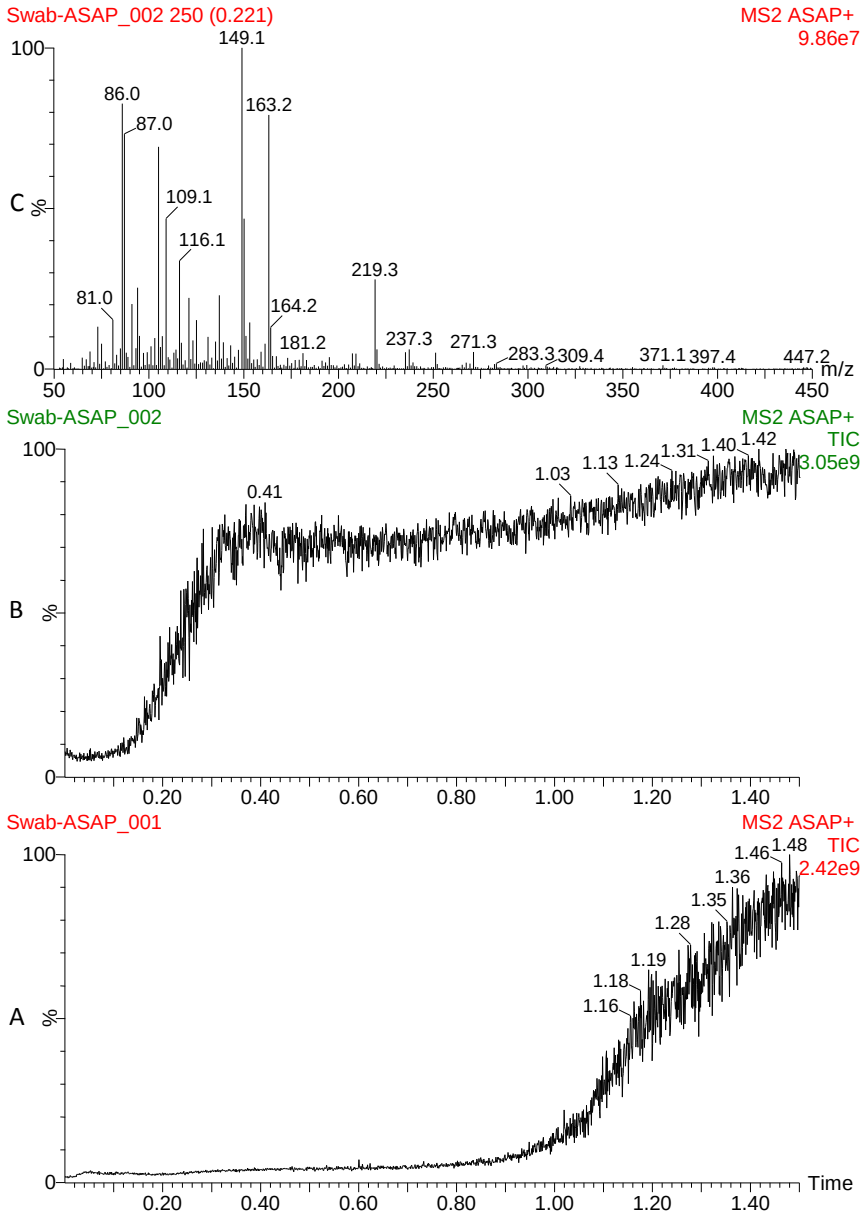


Figure S1. Swab-ASAP source conditions optimization. **A** Scan of a blank swab using a desolvation ramp temperature from 70 to 450 ºC. **B** Scan of a blank swab using a fixed desolvation temperature maintained at 450 ºC. **C** Spectrum of a blank swab.

When using DDA acquisition mode, it is important to stablish an m/z exclusion list to avoid unnecessary MS/MS switches produced by ions coming from the system. Nevertheless, for the analysis of NPS (and using our swabs) it was not considered necessary. **Figure S1C** shows the MS scan spectrum from a blank swab with 100 µL of methanol and 450 ºC desolvation temperature. It can be observed that major ions coming from the system were observed below *m/z* 170. Anyway, if the swab type is changed, the presence of background ions should be assessed. Limiting the survey MS scan from *m/z* 170 to 450 does not affect the detection of known NPS, as their protonated molecules present m/z values within this range.

Once stablished the survey MS scan parameters, the MS/MS acquisition parameters were explored. The *m/z* range was stablished from 60 to 450, in order to detect all the product ions produced. A collision energy of 25 eV was selected as a compromise value, in order to obtain enough product ions with a wide *m/z* range for all NPS. The acquisition of MS/MS data also allows the use of on-line spectral MS/MS databases for the tentative identification of the compound, such as METLIN (https://metlin.scripps.edu/) or MassBank (https://massbank.eu/), if the spectrum of the compound of interest is available in these databases.

As the signal produced by the evaporation/ionization of the compounds from the swab was constant during all the analysis time, only one *m/z* value was selected for automatic MS/MS in each survey scan. Additionally, an exclusion time of 10 s for the previously detected precursor ion was stablished, in order to allow the detection of NPS mixtures, even at different concentration. These parameters should not be used for DDA acquisition with chromatographic separation, as the peaks produced are only of few seconds. **Figure S2A** shows the TIC of the automatic CID MS/MS acquisition of an herbal blend sample containing two synthetic cannabinoids. The observed "peaks" are produced when the MS/MS of the protonated cannabinoids are automatically acquired, and it can be also observed the 10 s exclusion time from the last measured *m/z* value. The extracted ion chromatograms (EIC) of specific product ions of these compounds illustrate

that the exclusion time used allows the automatic MS/MS acquisition for both compounds, even in mixtures. **Figure S2B** shows the automatic MS/MS spectra acquired for the protonated molecules of JWH-081 and JWH-203, illustrating that the method is suitable for the analysis of mixtures.

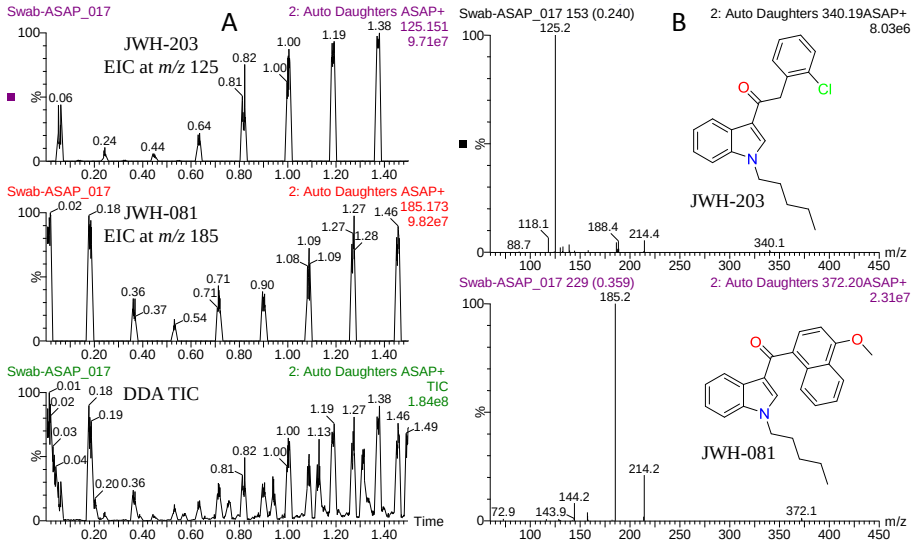


Figure S2. Detection of two synthetic cannabinoids in an herbal blend sample by swab-ASAP-MS/MS DDA. **A** TIC of the DDA function, EIC of product ion *m/z* 185 from JWH-081, and EIC of product ion *m/z* 125 from JWH-203. **B** Automatic MS/MS spectra of JWH-203 and JWH-081 present in the sample.

Building the spectra library

Once optimized the swab-ASAP-MS/MS DDA parameters, the MS/MS spectra of all NPS available in our laboratory were acquired, in order to create an MS/MS library for a direct compound identification.

Solid samples were directly analyzed by swab-ASAP-MS/MS, following the procedure described in the sample treatment section. Once acquired the DDA data, MS/MS spectra acquired were manually checked, selecting one spectrum for automatic library search. The library was created using the MassLynx Library utilities. For each compound, MS/MS spectrum, name, elemental composition and molecular weight were included in the library.

Up to 90 compounds, including synthetic cannabinoids, synthetic cathinones, amphetamines, tryptamines and opioids were included in the spectral library.

Once the library was completed, it was indexed and then converted to the NIST format, required by the MS Search software.

Application to blind samples and detection/identification of NPS in different surfaces

In order to demonstrate the applicability of the developed methodology for identifying the active compounds, different experiments were performed to assess selectivity and sensitivity of the swab-ASAP-MS/MS DDA.

Firstly, the identification of NPS present in blind research chemicals and legal highs samples was tested. The selected products included herbal blends, pills, crystal and powder samples, containing NPS of different families. **Figure 1** shows the identification of the synthetic cathinone butylone in a legal high sample named *Euforia*, purchased in a local smartshop through its webpage [13]. The automatic MS/MS function shows the presence of a certain ion with a high response (**Figure 1A**). When the MS/MS spectrum was searched in the database using the MS Search, only one compound presented a match higher than 800 (minimum value for considering a compound tentatively identified), as shown in **Figure 1B**.

The applicability of this approach was supported by the analysis of several research chemicals containing synthetic cathinones, synthetic cannabinoids, opioids or tryptamines. For example, the synthetic opioid U-47700 was tentatively identified in a powder sample by swab-ASAP-MS/MS DDA. In this case, the swab was wiped into the plastic bag that contained the product. The opioid was tentatively identified with a match of 859.

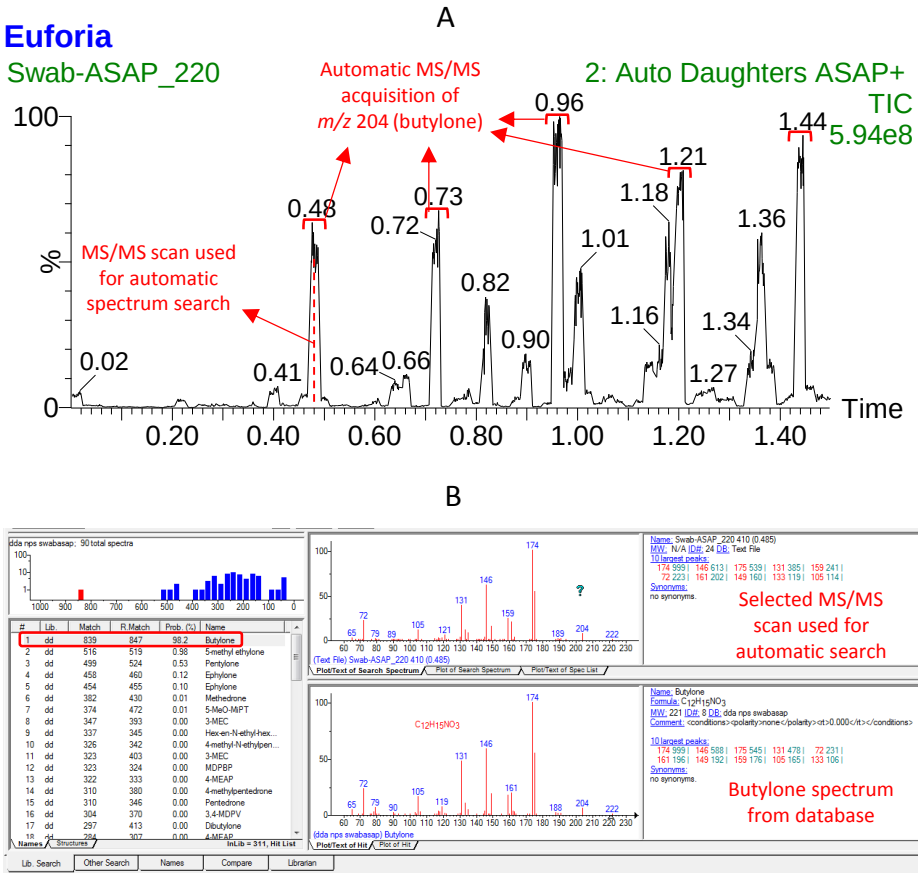


Figure 1. Identification of the synthetic cathinone butylone in a legal high sample. **A** Automatic MS/MS function generated during DDA analysis. **B** Compound identification using MS Search v2.0 and the in-house built database.

The high sensitivity observed when analysing these products, encouraged us to perform sensitivity tests using the swab-ASAP. For this purpose, a small amount of compound was placed onto the laboratory table using a certain volume from a stock solution at high concentration (e.g., 10 µL from a 1 mg/mL stock solution). In order to simulate a real situation, the solvent was allowed to evaporate before applying the swab. In this experiment, 12 µg of the synthetic cannabinoid AMB-FUBINACA and 12 µg of the tryptamine 5-MeO-MiPT were satisfactorily identified, as it can be observed in **Figure 2A** and **2B**.

Moreover, the methodology allowed the identification of 1 µg of the synthetic cathinone 3,4-MDPV placed onto the laboratory table as shown in **Figure 2C**.

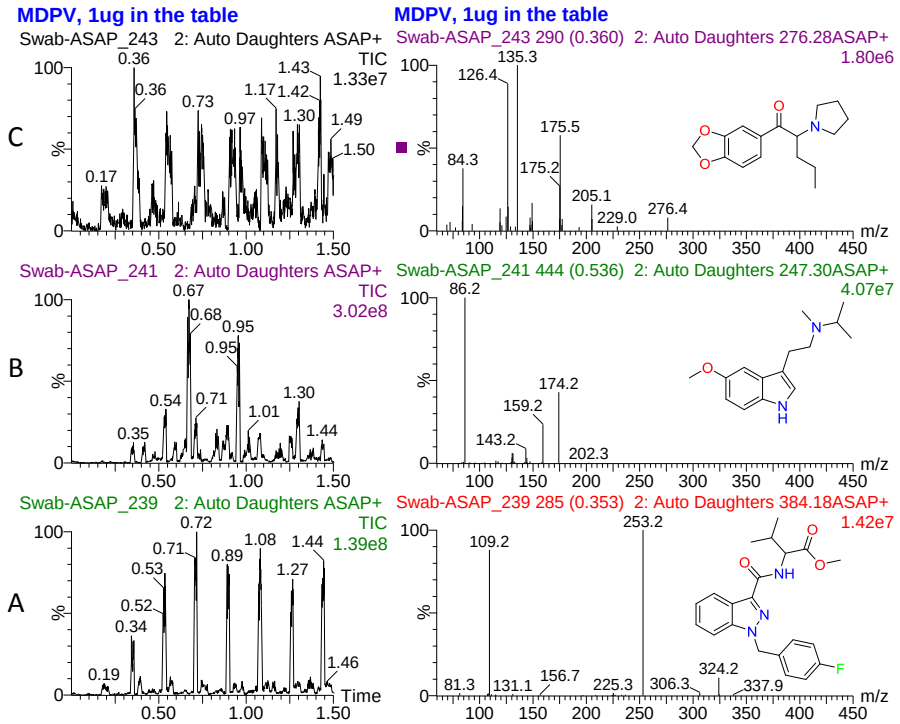


Figure 2. Analysis of NPS placed in the laboratory table. **A** 12 µg of the synthetic cannabinoid AMB-FUBINACA. **B** 12 µg of the tryptamine 5-MeO-MiPT. **C** 1 µg of the cathinone MDPV.

To complete the whole set of experiments, the swab-ASAP-MS/MS DDA analysis was applied to the detection and identification of NPS in fingers of potential consumers who had touched legal highs with their hands. Experiments consisted on the simulation of somebody snorting a powder sample or preparing a cigarette with an herbal blend, cleaning subsequently his hands by shaking off with a cloth. After cleaning their hands no traces of powder or herb were observed, being apparently clean. However, the analysis of the finger surface by swab-ASAP-MS/MS DDA revealed the presence of intense peaks.

Based on the observed fragmentation, it was possible to identify α-PVP after "snorting" (**Figure 3A**), as well as the synthetic cannabinoids XLR-11 and UR-144 after "preparing the cigarette" (**Figure 3B**).

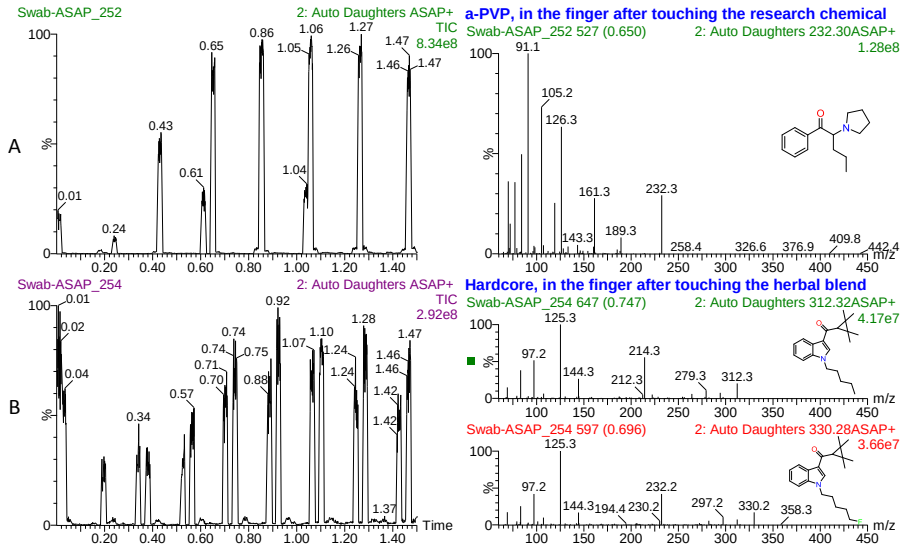


Figure 3. Analysis of NPS in the finger of a volunteer who had touched different products and had cleaned his hands by shaking off with a cloth. **A** Identification of the cathinone α-PVP after being in contact with a powder sample. **B** Identification of the cannabinoids UR-144 and XLR-11 (halogenated compound) after being in contact with an herbal blend sample.

This approach has proved its potential for the rapid suspect screening of the compounds present in legal highs and research chemicals, as well as its applicability for detecting NPS in surfaces, such as the fingers of a potential consumer. Nevertheless, it must be continuously updated, including more and more fragmentation spectra for NPS, especially for novel compounds. The major handicap is, nowadays, the lack of on-line spectral libraries for NPS when using QqQ instruments, similarly to those available for HRMS [15].

Identification of isomeric compounds

The different NPS families are formed by compounds with a similar core, and some modifications in their structure. For example, synthetic cathinones are based on the structure of cathinone, and hundreds of compounds have been

developed by modifying the three main moieties of the cathinone: *N*-functionalization, α-alkyl chain, and aromatic ring substitution. So, an important number of isomeric compounds can be found by modifying and combining these moieties, some of which can be differentiated based on their MS/MS spectra.

As illustrative example, **Figure S3** shows the identification of the two structural isomeric synthetic cathinones *N*-ethyl-4-methylpentedrone (or 4-MEAP) (**A**) and *N*-ethyl-hexedrone (**B**). Both compounds present the same elemental composition, but differ on the position of one methyl: N-ethyl-4-methylpentedrone has a 3-carbon atoms α-alkyl chain length and one methyl group in the aromatic ring, while *N*-ethyl-hexedrone has a 4-carbon atoms α-alkyl chain length without aromatic ring substitution. As it can be observed in **Figure S3**, both compounds can be successfully differentiated based on the observed MS/MS spectra, obtaining only one compound with a match higher than 800.

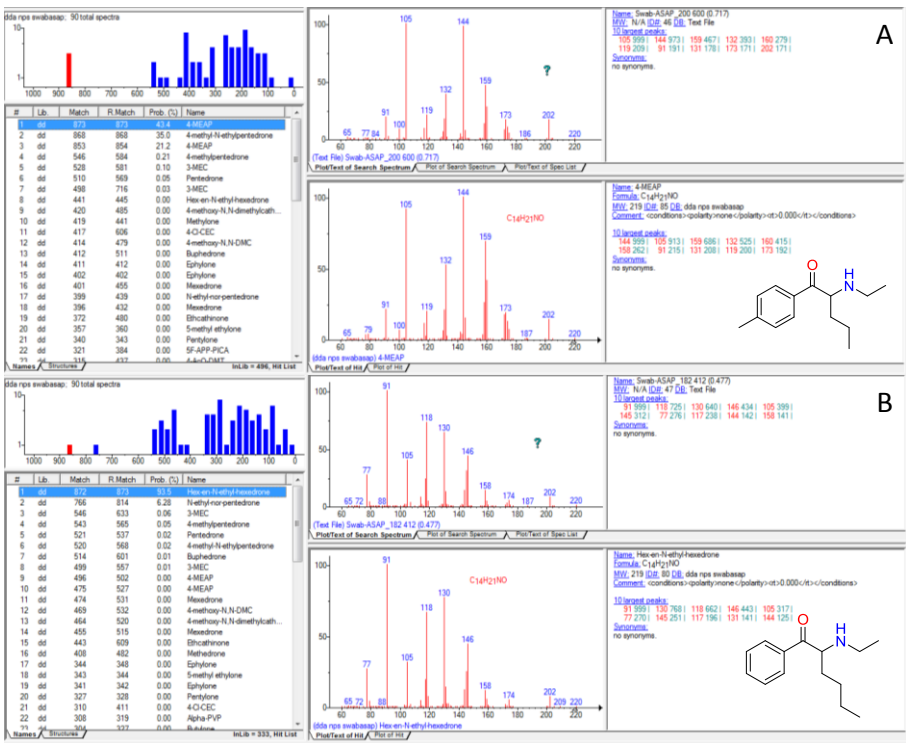


Figure S3. Differentiation of isomeric synthetic cathinones by swab-ASAP-MS/MS. **A** Identification of 4-MEAP. **B** Identification of *N*-ethyl-hexedrone.

Nevertheless, other isomeric synthetic cathinones could not be differentiated with this technique as they present the same CID fragmentation. This is the case of the cathinones *N*,*N*-dimethyl-pentylone and *N*-ethyl-pentylone, which are only differentiated by the *N*-functionalization. As the most significative fragmentation of cathinones is the amine disconnection from the α-carbon, it is not possible to differentiate between both compounds. In this case, additional analytical techniques such as nuclear magnetic resonance are needed.

Conclusions

The developed methodology, based on swab-ASAP-MS/MS DDA, for the suspect screening of NPS in seizures and different surfaces in contact with a variety of products has demonstrated its applicability with high sensitivity and selectivity. It has allowed the identification of different families of NPS in several legal highs and research chemicals tested. The identification was fast, without the need of any sample treatment, and it was automatically performed by searching the acquired fragmentation spectra in an in-house built spectra database. The use of medical swabs was also tested for the analysis of different surfaces that were in contact with NPS, supporting the suitability of this approach for detecting low amounts of these compounds in a laboratory table, as well as in the fingers of a person who used legal highs. The methodology proposed in this work should be continuously updated by including the fragmentation spectra of novel NPS. This would allow to build a wide spectra database that would notably facilitate the routine monitoring of the ever-changing NPS market. Additionally, future work will include the possibility of the use of swab-ASAP-MS/MS for quantification purposes, using calibration curves or isotope pattern deconvolution for those compounds which isotopically-labelled standard is available.

Acknowledgements

Authors acknowledge financial support from the University Jaume I (UJI-B2018-19) and the Generalitat Valenciana (PROMETEO/2019/040). D. Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (FPU15/02033).

Competing Interests

The authors declare that they have no conflict of interest.

Author contribution

D.F-S. and M.I. conceived the work. D.F-S., D.F-B. and M.M-P. performed sample treatment, instrumental analysis and data process. D.F-S., D.F-B and M.I. interpreted and discussed the results. F.H. and J.V.S. contributed with reagents and analytical tools. D.F-S and M.I. wrote the first draft of the manuscript. J.V.S, and F.H. provided useful comments and feedback for the manuscript.

References

1. European Monitoring Centre for Drugs and Drug Addiction: European Drug Report 2019. (2019)

2. United Nations Office on Drugs and Crime: World Drug Report 2019. (2019)

3. Harper, L., Powell, J., Pijl, E.M.: An overview of forensic drug testing methods and their suitability for harm reduction point-of-care services. Harm Reduct. J. 14, 52 (2017). https://doi.org/10.1186/s12954-017-0179-5

4. Pasin, D., Cawley, A., Bidny, S., Fu, S.: Current applications of high-resolution mass spectrometry for the analysis of new psychoactive substances: a critical review. Anal. Bioanal. Chem. 409, 5821–5836 (2017). https://doi.org/10.1007/s00216-017-0441-4

5. Gwak, S., Almirall, J.R.: Rapid screening of 35 new psychoactive substances by ion mobility spectrometry (IMS) and direct analysis in real time (DART) coupled to quadrupole time-of-flight mass spectrometry (QTOF-MS). Drug Test. Anal. 7, 884–893 (2015). https://doi.org/10.1002/dta.1783

6. Nie, H., Li, X., Hua, Z., Pan, W., Bai, Y., Fu, X.: Rapid screening and determination of 11 new psychoactive substances by direct analysis in real time mass spectrometry and liquid chromatography/quadrupole time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 30, 141–146 (2016). https://doi.org/10.1002/rcm.7629

7. Vircks, K.E., Mulligan, C.C.: Rapid screening of synthetic cathinones as trace residues and in authentic seizures using a portable mass spectrometer equipped with desorption electrospray ionization. Rapid Commun. Mass Spectrom. 26, 2665–2672 (2012). https://doi.org/10.1002/rcm.6390

8. Pirro, V., Jarmusch, A.K., Vincenti, M., Cooks, R.G.: Direct drug analysis from oral fluid using medical swab touch spray mass spectrometry. Anal. Chim. Acta. 861, 47–54 (2015). https://doi.org/10.1016/j.aca.2015.01.008

9. Crevelin, E.J., Salami, F.H., Alves, M.N.R., De Martinis, B.S., Crotti, A.E.M., Moraes, L.A.B.: Direct Analysis of Amphetamine Stimulants in a Whole Urine Sample by Atmospheric Solids Analysis Probe Tandem Mass Spectrometry. J. Am. Soc. Mass Spectrom. 27, 944–947 (2016). https://doi.org/10.1007/s13361-016-1349-5

10. Jagerdeo, E., Wriston, A.: Rapid analysis of forensic-related samples using two ambient ionization techniques coupled to high-resolution mass spectrometers. Rapid Commun. Mass Spectrom. 31, 782–790 (2017). https://doi.org/10.1002/rcm.7844

11. Jagerdeo, E., Clark, J.A., Leibowitz, J.N., Reda, L.J.: Rapid analysis of forensic samples using an atmospheric solid analysis probe interfaced to a linear ion trap mass spectrometer. Rapid Commun. Mass Spectrom. 29, 205–212 (2015). https://doi.org/10.1002/rcm.7097

12. Tose, L. V, Murgu, M., Vaz, B.G., Romão, W.: Application of Atmospheric Solids Analysis Probe Mass Spectrometry (ASAP-MS) in Petroleomics: Analysis of Condensed Aromatics Standards, Crude Oil, and Paraffinic Fraction. J. Am. Soc. Mass Spectrom. 28, 2401–2407 (2017). https://doi.org/10.1007/s13361-017-1764-2

13. Ibañez, M., Sancho, J. V., Bijlsma, L., Van Nuijs, A.L.N., Covaci, A., Hernandez, F.: Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. TrAC - Trends Anal. Chem. 57, 107–117 (2014). https://doi.org/10.1016/j.trac.2014.02.009

14. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Guillamón, E., Sancho, J. V., Hernández, F., Ibáñez, M.: Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017). https://doi.org/10.1038/s41598-017-06778-9

15. Mardal, M., Andreasen, M.F., Mollerup, C.B., Stockham, P., Telving, R., Thomaidis, N.S., Diamanti, K.S., Linnet, K., Dalsgaard, P.W.: HighResNPS.com: An Online Crowd-Sourced HR-MS Database for Suspect and Non-targeted Screening of New Psychoactive Substances. J. Anal. Toxicol. 43, 520–527 (2019). https://doi.org/10.1093/jat/bkz030

3.4. Discussion of the results obtained

The main objective of the studies included in this chapter was to provide additional resources for NPS analysis to those laboratories equipped with QqQ instruments (with no access to HRMS equipment) as well as delving into less-used working modes of this analyser. Therefore, these studies are focused on the development of fast analytical strategies for the suspect screening, and even non-target screening, of NPS in seizures, research chemicals, legal highs, and even surfaces.

On the one hand, **research article V** presents the use of precursor ion scan and neutral loss scan for the suspect screening and tentative identification of synthetic cathinones in research chemicals, based on the common fragmentation pathways of these compounds. In this case, flow-injection analysis (FIA) was used as sample introduction technique in order to obtain fast analyses, but other direct analysis interfaces could also be used, such as ASAP. On the other hand, **research article VI** uses a DDA working mode for an automatic MS/MS spectrum (product ion scan) acquisition of the active compounds present in legal highs, research chemicals and surfaces. These spectra are used for compound identification based on an automatic search into a spectral database. A modified ASAP source, which replaces the crystal capillary of the ASAP probe by a medical swab, was used, also allowing the analysis of surfaces such as tables, or the fingers of a potential NPS user.

The methodology developed in **research article V** is based on the fact that synthetic cathinones present similar fragmentation pathways, as studied and reported by Emilia Fornal [14]. Nevertheless, this behaviour is also observed for other NPS families, for example different fentanyl analogues, as illustrated by Carolina Noble and colleagues [15], and for several third-generation SCRA, reported by María Ibáñez & Lubertus Bijlsma and co-authors [16]. In the three studies previously indicated, the CID fragmentation of the selected compounds was evaluated after UHPLC-HRMS analysis, using in all the cases QTOF

instruments (6538 Q-TOF from Agilent [14], Xevo G2-S QTOF from Waters [15], and Xevo G2 QTOF from Waters [16]). In the case of synthetic cathinones, Fornal recognised different typical fragmentations for synthetic cathinones, as summarised in **research article V** (**Figure 2**), but being the most important the bond disconnections indicated in **Figure 3.1A**: carbonyl-α carbon, and amine-α carbon. Similar behaviour can be observed for fentanyl analogues, as described by Noble [15] and presented in **Figure 3.1B**. In that study, 34 fentanyl analogues were identified based on the predicted fragment ions from fentanyl-specific CID cleavages, being one of the most important the fragmentation on the C- N bond between the phenylamide moiety and the piperidine ring [15]. Finally, the SCRA mass spectrometric behaviour was carefully investigated by Ibáñez & Bijlsma, based on the fragmentation spectra of 27 compounds [16]. As indicated in that study, and also described in **research article VII** (**Chapter 4**), SCRA present similar fragmentation pathways, being the most common bond disconnections those illustrated in **Figure 3.1C**.

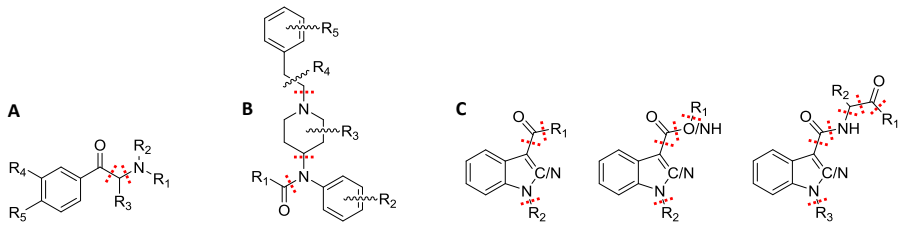


Figure 3.1. Common CID cleavages observed for different NPS families. **A** Synthetic cathinones. **B** Fentanyl derivatives. **C** SCRA.

The common fragmentation pathway observed for these compounds allows the search for common fragments and neutral losses during HRMS analysis, in order to detect related compounds in seizures, as well as to perform suspect screening of their derivatives in forensic samples and during metabolism experiments. Nevertheless, the use of HRMS instruments presents some drawbacks, as the interpretation of the obtained results requires high-skilled scientists, and the high-cost maintenance of this equipment. For these reasons, **research article V** uses the additional working modes available in QqQ mass analysers, commonly found

in forensic and toxicological laboratories, for synthetic cathinone identification in research chemical samples. Based on the information available for fentanyl derivatives and SCRA, a similar methodology based on precursor ion scan and neutral loss scan modes can also be developed for this family. Moreover, the FIA used as sample introduction technique can be replaced by ambient sources such as ASAP, DESI [11], DART [12], swab-touch spray [17], among others, for a fast compound identification in seizures.

The application of the QqQ mass analyser with non-typical working modes for the fast identification of NPS is also the focus of **research article VI**. The DDA acquisition mode is widely-used in HRMS instruments, especially in the hybrid Q-Orbitrap mass analysers, such as the Q Exactive from Thermo Scientific [18,19]. One of the benefits of DDA when compared to DIA, is the acquisition of pure product ion spectra after precursor ion isolation, instead of the fragmentation spectra obtained with DIA that contain the fragment ions coming from all the compounds present in that moment in the collision cell. Once obtained the product ion spectrum of an unknown compound, this information can be used for its tentative identification using spectral libraries (such as NIST) or online compound databases with fragmentation information (MassBank). The product ion spectra obtained with the QqQ can be directly compared with fragmentation spectra obtained by HRMS (in this case, Xevo G2 QTOF instrument working in MSE acquisition mode). As showed in **Figure 3.2**, the fragmentation obtained for the SCRA XLR-11 and for the synthetic cathinone TH-PVP are totally comparable. In spite of the slight variation on the ion abundance (the QqQ acquired the MS/MS spectra at a fixed collision energy of 25 eV, while QTOF instrument in MSE used a collision energy ramp from 15 to 40 eV), these results showed that the spectra obtained by HRMS instruments can be used for compound identification when using the analytical strategy described in **research article VI**.

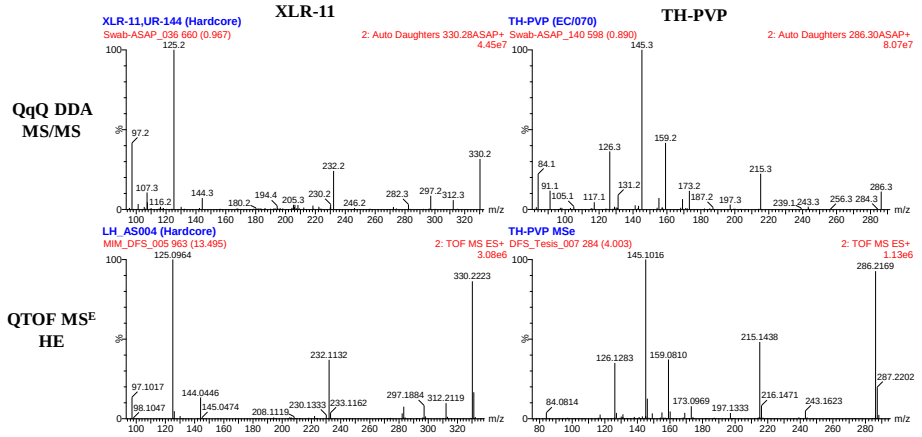


Figure 3.2. Comparative of the fragmentation spectra obtained by QqQ working in DDA acquisition mode (top), and QTOF in MS^E (bottom) for the SCRA XLR-11 (left) and the synthetic cathinone TH-PVP (right).

Another important fact to be commented about **research article VI** is the use of the ASAP source, in combination with medical swabs, for the analysis of a wide variety of samples containing NPS, such as powder, crystal, pills and herbal blends. The ASAP probe is usually equipped with a sealed glass capillary, which is heated using a nitrogen stream at high temperature (the stream used as desolvation gas in ESI and APCI probes). In this work, the glass capillary was replaced by a medical swab. The use of medical swabs as MS sample introduction technique has been described in literature, but using the called "swab-touch spray" [20]. In this ionisation technique, a high voltage is applied to the swab containing the sample and moistened with a certain solvent, usually methanol, isopropanol, or an alcohol:acetonitrile mixture [20]. The tip of the swab generates a Taylor cone, similarly to an ESI source and thus, ionising the compounds.

In our work, no voltage was applied to the swab, but the swab was heated until producing the evaporation of the compounds that were ionised in the corona pin. The use of medical swabs instead of a glass capillary facilitates the analysis of some samples, such as herbal blends. In order to not introduce small leaves and herb pieces into the API source, the swab was applied to the inner surface of the plastic bag containing the herbal blend. Another benefit of using medical swabs

was the analysis of surfaces containing NPS, such as the laboratory table, or the fingers of somebody that had touched the herbs or another product, showing enough sensitivity for detect up to 1 µg of MDPV onto the laboratory table. However, the potential of **research article VI** is the application of DDA acquisition mode for a rapid analysis of seized samples, and additional ambient sources can be used for this purpose, such as those previously indicated as DESI, DART, or the rapid evaporative ionisation mass spectrometry (REIMS) [21].

The studies and information included in **Chapter 3** illustrates the potential of the less-used working modes of the QqQ instrument for performing rapid suspect screening and compound identification of legal highs and research chemicals, being possible its application to routine analysis when fast results are needed, for example in border controls.

3.5. Literature

1. Brunt, T. M. et al. Drug testing in Europe: monitoring results of the Trans European Drug Information (TEDI) project. Drug Test. Anal. 9, 188–198 (2017).

2. Pereira, L. S. A., Lisboa, F. L. C., Neto, J. C., Valladão, F. N. & Sena, M. M. Direct classification of new psychoactive substances in seized blotter papers by ATR-FTIR and multivariate discriminant analysis. Microchem. J. 133, 96–103 (2017).

3. Coelho Neto, J. Rapid detection of NBOME's and other NPS on blotter papers by direct ATR-FTIR spectrometry. Forensic Sci. Int. 252, 87–92 (2015).

4. Pettersson Bergstrand, M., Helander, A. & Beck, O. Development and application of a multi-component LC–MS/MS method for determination of designer benzodiazepines in urine. J. Chromatogr. B 1035, 104–110 (2016).

5. Peters, J. R., Keasling, R., Brown, S. D. & Pond, B. B. Quantification of Synthetic Cathinones in Rat Brain Using HILIC–ESI-MS/MS. J. Anal. Toxicol. 40, 718–725 (2016).

6. Boumba, V. A., Di Rago, M., Peka, M., Drummer, O. H. & Gerostamoulos, D. The analysis of 132 novel psychoactive substances in human hair using a single step extraction by tandem LC/MS. Forensic Sci. Int. 279, 192–202 (2017).

7. Celma, A. et al. Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology. J. Chromatogr. A 1602, 300–309 (2019).

8. Pozo, O. J., Ventura, R., Monfort, N., Segura, J. & Delbeke, F. T. Evaluation of different scan methods for the urinary detection of corticosteroid metabolites by liquid chromatography tandem mass spectrometry. J. Mass Spectrom. 44, 929–944 (2009).

9. McLeod, M. D. et al. Constant Ion Loss Method for the Untargeted Detection of Bis-sulfate Metabolites. Anal. Chem. 89, 1602–1609 (2017).

10. Marcos, J. & Pozo, O. J. Current LC–MS methods and procedures applied to the identification of new steroid metabolites. J. Steroid Biochem. Mol. Biol. 162, 41–56 (2016).

11. Vircks, K. E. & Mulligan, C. C. Rapid screening of synthetic cathinones as trace residues and in authentic seizures using a portable mass spectrometer equipped with desorption electrospray ionization. Rapid Commun. Mass Spectrom. 26, 2665–2672 (2012).

12. Gwak, S. & Almirall, J. R. Rapid screening of 35 new psychoactive substances by ion mobility spectrometry (IMS) and direct analysis in real time (DART) coupled to quadrupole time-of-flight mass spectrometry (QTOF-MS). Drug Test. Anal. 7, 884–893 (2015).

13. Crevelin, E. J. et al. Direct Analysis of Amphetamine Stimulants in a Whole Urine Sample by Atmospheric Solids Analysis Probe Tandem Mass Spectrometry. J. Am. Soc. Mass Spectrom. 27, 944–947 (2016).

14. Fornal, E. Study of collision-induced dissociation of electrospray-generated protonated cathinones. Drug Test. Anal. 6, 705–715 (2014).

15. Noble, C., Weihe Dalsgaard, P., Stybe Johansen, S. & Linnet, K. Application of a screening method for fentanyl and its analogues using UHPLC-QTOF-MS with data-independent acquisition (DIA) in MS E mode and retrospective analysis of authentic forensic blood samples. Drug Test. Anal. 10, 651–662 (2018).

16. Bijlsma, L. et al. Mass spectrometric identification and structural analysis of the third-generation synthetic cannabinoids on the UK market since the 2013 legislative ban. Forensic Toxicol. 35, 376–388 (2017).

17. Pirro, V., Jarmusch, A. K., Vincenti, M. & Cooks, R. G. Direct drug analysis from oral fluid using medical swab touch spray mass spectrometry. Anal. Chim. Acta 861, 47–54 (2015).

18. Cooman, T. & Bell, S. In vitro metabolism of the synthetic cannabinoids PX- 1, PX- 2, and PX- 3 by high- resolution mass spectrometry and their clearance rates in human liver microsomes. Rapid Commun. Mass Spectrom. 33, 1816–1825 (2019).

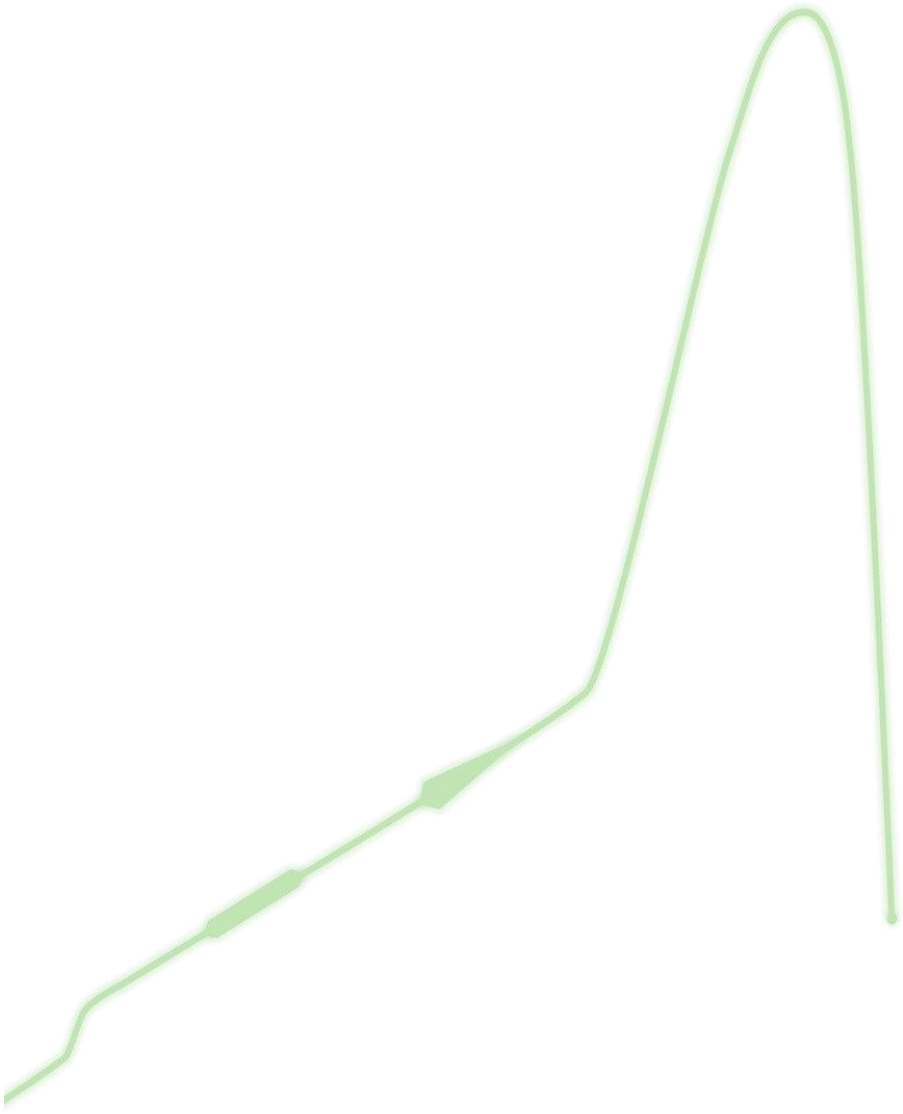
19. Xu, D. et al. Analysis of AMB-FUBINACA Biotransformation Pathways in Human Liver Microsome and Zebrafish Systems by Liquid Chromatography-High Resolution Mass Spectrometry. Front. Chem. 7, 1–9 (2019).

20. Morato, N. M., Pirro, V., Fedick, P. W. & Cooks, R. G. Quantitative Swab Touch Spray Mass Spectrometry for Oral Fluid Drug Testing. Anal. Chem. 91, 7450–7457 (2019).

21. Kosek, V. et al. Ambient mass spectrometry based on REIMS for the rapid detection of adulteration of minced meats by the use of a range of additives. Food Control 104, 50–56 (2019).

CHAPTER 4

THE *IN VITRO* APPROACH: METABOLISM AND POTENCY



CHAPTER 4

THE *IN VITRO* APPROACH: METABOLISM AND POTENCY

4.1. Introduction

4.2. Research article VII

> "Comprehensive investigation on synthetic cannabinoids: Metabolic behaviour and potency testing, using 5F- APP- PICA and AMB-FUBINACA as model compounds".
> *Drug Testing and Analysis, 2019; 11:1358.*

4.3. Research article VIII

> "Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation".
> *Journal of Pharmaceutical Analysis, 2020; 10:147.*

4.4. Discussion of the results obtained

4.5. Literature

4.1. Introduction

As explained in the **Chapter 1** (General introduction), the most used approaches for studying the pharmacological behaviour of NPS, when samples from real consumers or volunteers are not available, are the *in vitro* and the *in vivo* approaches using animals. Both methodologies have advantages and drawbacks, as presented in **Table 1.1**. One of the benefits of working with *in vitro* models is its interesting versatility for an accurate research of the pharmacology of NPS, as almost all the steps can be performed individually. Different pharmacological aspects can be evaluated by *in vitro* approaches, such as the hepatic metabolism [1], protein binding [2], toxicity [3], blood-brain barrier permeability [4], hepatic efflux [5] and receptor affinity [6]. In this thesis, two different applications of the *in vitro* model have been used: the hepatic metabolism and the receptor affinity.

The metabolism experiments using *in vitro* approaches are designed to know what are the metabolites of a certain compound, trying to mimic the metabolic fate that would occur in a real consumer [7]. The aim of identifying these metabolites is to propose potential consumption biomarkers that should be searched for during forensic analysis [8], as well as the assessment of their toxicity and/or psychoactive properties [8]. The metabolism of xenobiotic compounds occurs primarily in the liver, where the compound is detoxified by different biotransformations [9], as explained in **Chapter 1**.

In this sense, different models can be used for metabolism experiments, being the most important ones the single enzymes, pHLM, pS9, pHH and specific cell lines such as HepaRG [10–12]. One of the simplest options is the use of c-DNA-expressed recombinant single human cytochrome P450 (CYP) enzymes for specific biotransformations [13]. However, the gold standard in *in vitro* metabolism studies are the pooled pHH [10,12], as this system presents natural enzyme clusters, co-substrates, drug transporters [14,15], and it is expected to provide the most authentic spectrum of human metabolites [16]. The main disadvantage of using pHH is the high cost, limited availability and variability in the expression of metabolic

enzymes, which limits their applicability for high throughput metabolism [17,18], as well as the need of specific equipment in the laboratory. For all these reasons, the *in vitro* system most used for NPS metabolite profiling is the pHLM together with pS9 [10,12,19–21]. Both systems involve cost-efficiently, fast and easy setups [10–12], but the use of co-substrates is needed for main phases I and II reactions. The use of specific cell lines such as HepaRG has also been described for NPS metabolism profiling, presenting almost the same advantages as pHH but being readily available and having a more stable phenotype [14,22,23].

The *in vitro* models also allow the estimation of the potency of NPS, either by the determination of the receptor affinity for certain NPS families [24], or by the release/uptake inhibition of neurotransmitters [4]. The potency of synthetic cathinones can be assessed by monitoring the inhibition of the monoamine transporters and the monoamine release, as well as by the receptor binding affinities for different compounds [4]. These experiments allow to determine the structure-activity relationships for this type of compounds and thus, to estimate their potency [25]. Nevertheless, one of the most studied and applied *in vitro* experiments for NPS potency determination are the models for determining the SCRA affinity for the CB1 and CB2 receptors [6,8,26]. One of these models, developed by A. Cannaert, C. Stove and colleagues, is based on a G-protein coupled receptor (GPCR) activation assay for evaluating the affinity of selected SCRAs for CB1 and CB2 receptors [6]. The potency is determined by comparison of the luminescence obtained after SCRA-CB activation, with the luminescence obtained for a reference compound, in this case, JWH-018 [6]. This methodology has been successfully applied for determining the potency of different SCRAs and metabolites [6,24], as well as for the determination of SCRA metabolites in urine samples from real consumers [27]. The results obtained with this *in vitro* model allow the establishment of the E_{max} (a measure of efficacy, relative to reference compound) and EC_{50} (a measure of potency) for the studied SCRAs [24].

The same authors have recently developed a similar setup for determining the potency of synthetic opioids based on the activation of the MOR [28], and demonstrating its applicability for up to [22] synthetic opioids such as AH-7921, U-47700, U-49900 and different fentanyl derivatives [29].

In this chapter, the *in vitro* approach for the study of pharmacological aspects of the NPS is explored from two different points of view: its application for metabolite identification and the determination of the potency of NPS. In the **research article VII**, a comprehensive investigation of the SCRAs 5F-APP-PICA (also known as PX-1) and AMB-FUBINACA is performed by the elucidation of the metabolites obtained after pHH incubation and UHPLC-HRMS analysis, and the determination of the potency of both compounds using the assay developed by A. Cannaert and C. Stove [6]. In this study, the prevalence of the metabolites over incubation time has also been studied, in order to propose the most adequate consumption biomarkers that should be searched for in biological samples from consumers. **Research article VIII** presents the metabolic fate of three different synthetic cathinones, including the indanyl-cathinone 5-PPDi (characterised in **research article III**) and one amphetamine using pHH and UHPLC-HRMS. Both studies were carried out during the research stay performed at the Forensic Medicine department of the University of Copenhagen, under the supervision of Dr. Marie Mardal and Prof. Dr. Med. Kristian Linnet.

4.2. Research article VII

Received: 25 February 2019 | Revised: 4 June 2019 | Accepted: 4 June 2019
DOI: 10.1002/dta.2659

RESEARCH ARTICLE

WILEY

Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds

David Fabregat-Safont[1] | Marie Mardal[2] | Carolina Noble[2] | Annelies Cannaert[3] | Christophe P. Stove[3] | Juan V. Sancho[1] | Kristian Linnet[2] | Félix Hernández[1] | María Ibáñez[1]

[1] Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain

[2] Department of Forensic Medicine, Section of Forensic Chemistry, Faculty of Health and Medicinal Sciences, University of Copenhagen, Denmark

[3] Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Correspondence
María Ibáñez, Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain.
Email: ibanezm@uji.es

Funding information
Ministerio de Economía y Competitividad, Grant/Award Number: CTQ2015-65603-P; University Jaume I, Grant/Award Number: UJI-B2018-19; Ministerio de Educación, Cultura y Deporte, Grant/Award Number: FPU15/02033; University of Copenhagen, Grant/Award Number: EST17/00024; National Research and Innovation Agency of Uruguay (ANII); Belgian Science Policy Office (NPSSAY); Ghent University Research Council, Grant/Award Numbers: 01 N00814 and 01 J15517

Abstract

Synthetic cannabinoids (SCs) represented 45% of new psychoactive substances seizures in Europe (data from 2016). The consumption of SCs is an issue of concern due to their still unknown toxicity and effects on human health, the great variety of compounds synthetized, and the continuous modifications being made to their chemical structure to avoid regulatory issues. These compounds are extensively metabolized in the organism and often cannot be detected as the intact molecule in human urine. The monitoring of SCs in forensic samples must be performed by the analysis of their metabolites. In this work, a workflow for the comprehensive study of SC consumption is proposed and applied to 5F-APP-PICA (also known as PX 1 or SRF-30) and AMB-FUBINACA (also known as FUB-AMB or MMB-FUBINACA), based not only on the elucidation of their metabolites but also including functional data using the NanoLuc approach, previously published. Both cannabinoids were completely metabolized by human hepatocytes (12 and 8 metabolites were elucidated by high resolution mass spectrometry for 5F-APP-PICA and AMB-FUBINACA, respectively) and therefore suitable consumption markers are proposed. The bioassays revealed that 5F-APP-PICA presented lower activity than AMB-FUBINACA at CB1 and CB2 receptors, based on the half maximal effective concentration (EC_{50}) and the maximum response (E_{max}). These results are in agreement with the different intoxication cases found in the literature for AMB-FUBINACA.

KEYWORDS

5F-APP-PICA, AMB-FUBINACA, high resolution mass spectrometry, metabolite identification, Synthetic cannabinoids

1 | INTRODUCTION

Synthetic cannabinoids (SCs) are among the most frequently seized new psychoactive substances (NPS), according to the 2018 *European Monitoring Centre for Drug and Drug Addiction* (EMCDDA) report.[1] In 2016, SCs represented 45% of the NPS seizures in Europe, being the largest NPS group monitored by the EMCDDA (179 compounds).[1] Although these compounds can be found as faux hash and e-liquids for vaping,[2] they are typically sold on webpages or in smartshops[3-5] as "herbal blends" or "spice," and used as cannabis substitutions. In

Drug Testing and Analysis, 2019; 11:1358

Comprehensive investigation on synthetic cannabinoids: Metabolic behaviour and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds

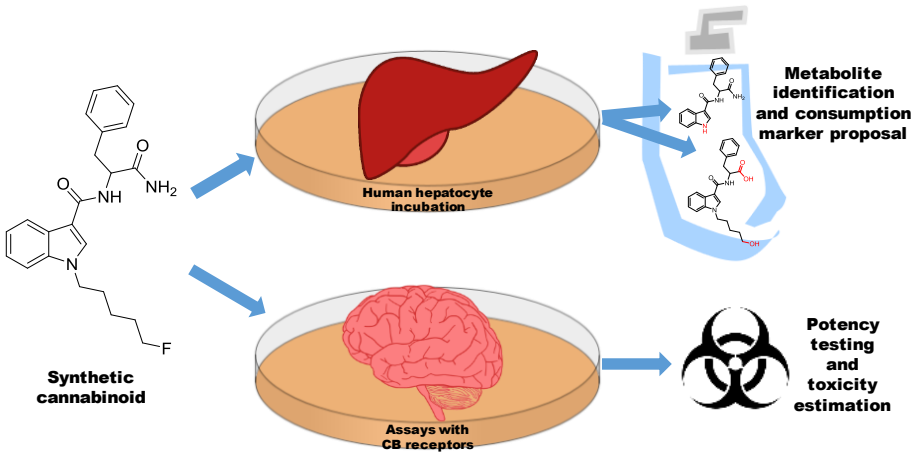
David Fabregat-Safont [1], Marie Mardal [2], Carolina Noble [2], Annelies Cannaert [3], Christophe P. Stove [3], Juan V. Sancho [1], Kristian Linnet [2], Félix Hernández [1] and María Ibáñez [1*]

[1] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain.

[2] Department of Forensic Medicine, Section of Forensic Chemistry, Faculty of Health and Medicinal Sciences, University of Copenhagen, Frederik V's vej 11, 2100 København Ø, Denmark.

[3] Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium.

Graphical abstract



Abstract

Synthetic cannabinoids (SCs) represented 45% of the new psychoactive substances seizures in Europe (data from 2016). The consumption of SCs is an issue of concern due to the still unknown toxicity and effects on human health, the great variety of compounds synthetized and continuous modifications in their chemical structure to avoid regulatory issues. These compounds are extensively metabolised in the organism and therefore often cannot be detected as the intact molecule in human urine. For this reason, the monitoring of SCs in forensic samples must be performed by the analysis of its metabolites. In this work, a workflow for the comprehensive study of SC consumption is proposed and applied to 5F-APP-PICA (also known as PX 1 or SRF-30) and AMB-FUBINACA (also known as FUB-AMB or MMB-FUBINACA), based not only on the elucidation of their metabolites but also including functional data by the use of the NanoLuc approach, previously published. Both cannabinoids were completely metabolised by human hepatocytes (12 and 8 metabolites were elucidated by high resolution mass spectrometry for 5F-APP-PICA and AMB-FUBINACA, respectively) and therefore suitable consumption markers have been proposed. The bioassays revealed that 5F-APP-PICA presented lower activity than AMB-FUBINACA at CB1 and CB2 receptors, based on the half maximal effective concentration (EC_{50}) and the maximum response (E_{max}). These results are in agreement with the different intoxication cases found in literature for AMB-FUBINACA.

Keywords Synthetic cannabinoids; 5F-APP-PICA; AMB-FUBINACA; High resolution mass spectrometry; Metabolite identification.

1. Introduction

Synthetic cannabinoids (SCs) are among the most frequently seized new psychoactive substances (NPS), according to the 2018 European Monitoring Centre for Drug and Drug Addiction (EMCDDA) report [1]. In 2016, SCs represented 45% of the NPS seizures in Europe, being the largest NPS group monitored by the EMCDDA (179 compounds) [1]. Although these compounds can be found as faux hash and e-liquids for vaping [2], they are typically sold on webpages or in smartshops [3–5] as "herbal blends" or "spice", and used as cannabis substitution. In the last years, SCs are also being found as powder [1]. In 2017, 10 new SCs were detected for the first time in Europe [1], illustrating the continuous emergence of this type of substances [1,6]. The constant modifications in the chemical structure of these compounds constitute an analytical challenge for forensic laboratories, which must apply state-of-the-art techniques for compound identification, such as high-resolution mass spectrometry (HRMS) or nuclear magnetic resonance (NMR) [7–9], in most cases without having reference standards available for their rapid confirmation.

It is known that several SCs present more intense psychoactive and side effects than THC, given their full agonism at the cannabinoid receptors CB1 and CB2, while THC only partially activates these [10,11]. The fact that SCs possess higher potency at the CB1 and CB2 could also increase the health risk for drug consumers, as reported in several overdose cases [12–18]. Nevertheless, it has not been confirmed yet that toxicity of SCs is strictly a function of potency and/or efficacy.

As most of the drug tests performed for the treatment of cannabis dependence are based on the detection of THC-COOH (the main urinary metabolite of THC), SCs can be used to by-pass urine controls [19]. Monitoring SC consumption is very important, for example in intoxication cases in hospitals or psychiatric centres. Such control cannot be performed by targeted methodologies directed towards the parent compounds, as these are rapidly metabolised in the liver, resulting in

little or no unaltered compound in urine [20]. This forces analytical laboratories to develop methodologies focused on the major urinary metabolites [21,22], which constitutes an additional challenge for detecting the use of SCs. Ideally, in order to stablish the best consumption biomarkers, urine samples from SC consumers [23–26] should be collected to study their metabolism. However, the availability of this type of samples is mostly limited to medical emergency cases. To circumvent this problem, different strategies can be used. In the *in vivo* approach, healthy animals (typically mice [27] or rats [28–30]) are exposed to the compound of interest, and blood and urine samples are usually collected in order to elucidate the metabolites. Nevertheless, it is possible that some metabolites obtained with an animal model are not found in humans, or the major metabolites identified in humans are not the major ones in other species. As an alternative, *in vitro* approaches using human liver microsomes [31], S9 fractions or hepatocytes [31], have produced reliable human excretory metabolites when studying pharmaceuticals. Human liver microsomes [26,32] or hepatocytes [24,33,34] have already been used for the metabolism study of new synthetic drugs such as SCs. Confirmation of these *in vitro* metabolites via the analysis of authentic samples (especially urine) has proven the suitability of the different *in vitro* strategies to predict human metabolic products [24,32].

As important as the determination of the most suitable consumption biomarkers of SCs is the evaluation of their potency and/or affinity at CB1 and CB2 receptors [35–38], which reveals another important aspect of these drugs and brings new insights on their pharmacology. To this aim, different bioassays have been used for both SCs as well as their metabolites and degradation products.

In order to obtain a complete overview on the problem of SC consumption, a three-step strategy has been used in this work: (a) metabolism study for establishing the metabolic pathway and the most suitable consumption biomarkers; (b) detectability and/or stability of the parent compound and its metabolites during incubation, which could be extrapolated to the excretion through urine in intoxication cases; (c) estimation of their potency using activity-

based CB1 and CB2 receptor bioassays. The proposed strategy has been applied to the 1-pentyl-1*H*-indazole-3-carboxamide derivative 5F-APP-PICA (also known as PX 1 or SRF-30), and the (4-fluorophenyl)methyl-1*H*-indazole-3-carboxamide derivative AMB-FUBINACA (also known as FUB-AMB or MMB-FUBINACA), which are amongst the most common SCs seized in powder and smoking mixtures in Spain and Europe [39], respectively. Several phase I and phase II metabolites have been identified thanks to the use of accurate-mass data provided by HRMS, which has allowed the proposal of useful biomarkers of consumption.

2. Experimental

2.1. Reagents and chemicals

5F-APP-PICA and AMB-FUBINACA were kindly provided by Energy Control (Asociación Bienestar y Desarrollo, Barcelona, Spain). Compound purity was tested by nuclear magnetic resonance. 5F-Py-PICA (internal standard, IS) was kindly provided by the Slovenian National Forensic Laboratory.

For hepatocyte incubation, diclofenac was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue solution (0.4%), fetal bovine serum (FBS), Leibovitz's L-15 medium, Gibco cryopreserved hepatocyte recovery medium, methanol, acetonitrile, ultrapure water, Pierce® LTQ Velos ESI positive Ion calibration solution, Pierce™ ESI negative ion calibration solution, and formic acid (LC–MS grade) were obtained from Fisher Scientific (Leicestershire, UK). The pooled human hepatocytes (pHH) from a pool of 10 were purchased from Lonza (Basel, Switzerland), and stored in liquid nitrogen until use.

For cannabinoid receptor bioassays, JWH- 018 was purchased from LGC (Wesel, Germany). Poly- D- lysine and fetal bovine serum (FBS) were from Sigma- Aldrich (Steinheim, Germany). Opti- MEM I Reduced Serum, Dulbecco's modified eagle medium (DMEM), trypsin, penicillin,

amphotericin B, streptomycin, and glutamine were supplied by Thermo Fisher Scientific (Pittsburg, PA, USA). Nano- Glo® Live Cell substrate furimazine and Nano- Glo® dilution buffer were purchased from Promega (Madison, WI, USA). Methanol and DMSO were purchased from Fisher Scientific.

2.2. Pooled human hepatocyte incubation

Incubation with pooled human hepatocytes was performed at 10 μM of each SC during 180 min, with collection of aliquots at 0, 60 and 180 min. Briefly, cryopreserved pooled human hepatocytes were rapidly thawed in a water bath at 37 °C, mixed with 50 mL preheated recovery medium, and centrifuged at 168 g for 20 min to remove dead hepatocytes. After aspiration of the supernatant, the hepatocytes were washed by resuspending the pellet in 20 mL growth medium (L-15 with 10% FBS), and centrifuging for 5 min at 50 g. Then, the supernatant was aspirated and the resulting pellet was re-suspended in growth medium for cell counting by the trypan blue exclusion method. The measured viability was 71%. The hepatocyte suspension was diluted to 106 viable hepatocytes/mL incubation medium. 5F-APP-PICA, AMB-FUBINACA, or diclofenac (positive control) were added at a final concentration of 10 μM; the maximum organic content of the incubation was 0.3%. The incubations were performed in duplicate for each condition in 96-well plates, at 350 rpm and 37 °C, on a Thermomixer comfort (Eppendorf, Hamburg, Germany). Controls without the addition of hepatocytes were run simultaneously to identify hydrolysis products and artefacts. Aliquots of 20 μL were collected after 0 (with a latency between 2-3 min), 60, and 180 min of incubation and mixed with 80 μL of an ice-cold acetonitrile solution containing 100 ng/mL of IS. The extracts were frozen until analysis.

2.3. Instrumentation

Extracts were analysed using a Dionex Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) system from Thermo Scientific (Germering, Germany) coupled to a Q Exactive high-resolution mass spectrometer from

Thermo Scientific (Bremen, Germany) equipped with a hybrid quadrupole-Orbitrap mass analyser.

Chromatographic separation was performed using an Acquity HSS C18 1.8 μm, 2.1×150 mm column from Waters (Wexford, Ireland), which was maintained at 40 °C. The mobile phase consisted of 5 mM ammonium formiate buffer with 0.1% v/v formic acid (solvent A) and 0.1% v/v formic acid in acetonitrile (solvent B), which was delivered at a flow rate of 0.25 mL/min. A 14 min gradient was used for the identification of metabolites, starting at 5% B (0–0.5 min), increasing to 99% B (0.5–10 min), where it remained isocratic for 2 min and followed by re-equilibration for 2 min. The autosampler temperature was 5 °C. The injection volume was 3 μL.

UHPLC was coupled to the HRMS using a heated electrospray ionization source (HESI-II) (Thermo Scientific, Bremen, Germany) working in positive (ESI^+) and negative (ESI^-) ionization modes. The capillary temperature was 350 °C, and the spray voltage was 4.0 kV in ESI^+ and -4.0 kV in ESI^-. Data were acquired using data-dependent acquisition (dd-MS^2, DDA) and parallel reaction monitoring (PRM, MS/MS). Full-scan data (FTMS) were collected in a scan-range of *m/z* 200–900 using a resolution of 70,000 FWHM, an automatic gain control (AGC) target of 10^6 ions, a maximum injection time of 50 ms and an isolation window of *m/z* 1. DDA MS/MS was acquired at a resolution of 35,000 FWHM, while PRM were acquired at 17,500 FWHM, using an AGC target of $2e^5$ ions and a maximum IT of 25 ms. Nitrogen was used as the collision gas at normalized collision energy (NCE) of 10, 30 or 50 eV.

The instrument was externally calibrated to a mass accuracy of ±5 ppm using the recommended calibration solutions for this instrument (Pierce® LTQ Velos ESI^+ ion calibration solution, Pierce™ ESI^- ion calibration solution, Thermo Scientific), purchased from Thermo Scientific. The instrument was controlled by XCalibur 4.0 software (Thermo Scientific, MA, Waltham, USA).

Data processing was performed using Compound Discoverer 2.0 software (Thermo Scientific) for a preliminary compound identification, and FreeStyle 1.3 (Thermo Scientific) for working with raw data.

2.4. Cannabinoid receptor bioassays

The potency and efficacy (the latter relative to JWH-018, used here as a reference) were estimated via the calculation of the half maximal effective concentration (EC_{50}) and the maximum response (E_{max}), respectively. Both parameters were determined using live cell-based reporter assays based on the application of the NanoLuc Binary Technology, that evaluates the interaction between the cytosolic protein β-arrestin 2 (βarr2) to CB1 and CB2 expressed in human embryonic kidney (HEK) 293T cells. Details regarding the development of the stable CB1 and CB2 cell lines used here and the experimental conditions have been reported elsewhere [40,41]. In brief, cells were seeded on a poly-D-lysine-coated 96-well plate at 5×10^4 cells/well and incubated overnight at 37 °C and 5% CO_2. Following 24 h, the cells were washed twice with 150 µL Opti-MEM I Reduced Serum and finally 100 µL of Opti-MEM I was added to each well. The Nano-Glo® Live Cell Reagent (Promega), a nonlytic detection reagent containing the live cell permeable furimazine substrate, was prepared by diluting the substrate 20× in Nano-Glo® LCS dilution buffer, and 25 µL was added to each well. The plate was placed in a luminometer and after stabilization of the signal (~20 min), 10 µL of 13.5× stock solutions (concentration range: 0.01–10 µM) of the 5F-APP-PICA or AMB-FUBINACA in 50% methanol in Opti-MEM I was added and the luminescence was continuously measured for 2 h (n=5-7). Replicates of solvent (50% methanol in Opti-MEM I) were run in all experiments as negative controls and were used to correct the signal. The final concentration of methanol (3.7%) did not pose a problem given the advantage of the short readout time of the assay.

Curve fitting of concentration-effect curves via nonlinear regression (four parameter logistic fit) was employed to determine EC_{50} (measure of potency) and

E_{max} values (measure of efficacy). The E_{max} values are normalized to the E_{max} value of JWH-018 (100%), used as a reference in our study, and hence are a 'relative measure' of efficacy.

3. Results and discussion

The proposed fragmentation pathway for the SCRA and their metabolites are included in the text, while the MS/MS spectra for these compounds can be found at the end of this research article.

3.1. Fragmentation of the synthetic cannabinoids

It is expected that parent compounds and metabolites present very similar fragmentation pathways. So, an accurate study of the MS fragmentation of the parent compound is extremely useful for metabolite elucidation. The presence of common fragments between parent and metabolite indicates that the biotransformation has not occurred in this part of the molecule. The remaining fragments observed for metabolites usually present a mass shift when compared to the corresponding fragments of the parent compound, commonly corresponding to the biotransformation. So, the identification of these mass fragments is crucial to correctly locate the position in which the biotransformation has occurred. This methodology has been successfully used for NPS metabolite identification [42,43]. Nevertheless, it has to be taken into account that some biotransformations can affect the whole fragmentation pathway of the metabolite, which may become very different from the parent.

Figure S1 shows the MS/MS spectra of 5F-APP-PICA ($C_{23}H_{27}FN_3O_2^+$, *m/z* 396.2083, 0.18 ppm) at 10 eV (top) and 50 eV (bottom) collision energy. **Table S1** lists its fragments, including accurate mass, elemental composition and mass error. The terminal amide moiety easily breaks similarly to ADB-PINACA, 5F-AB-PICA and AB-FUBINACA, producing the loss of an ammonia molecule (Fragment 1, $C_{23}H_{24}FN_2O_2^+$, *m/z* 379.1815, -0.38 ppm). The Fragment 2 ($C_{14}H_{15}FNO^+$, *m/z* 232.1132, -0.39 ppm) would correspond to the disconnection

of the peptide bond of the amide moiety linked to the indole ring. This fragmentation is also observed for ADB-PINACA, 5F-AB-PICA and AB-FUBINACA (which have an indazole group instead of indole) and, APICA and SDB-006 (indole ring). Fragment 2 could also come from the protonated molecule ($[M+H]^+$) when the protonation is produced in the amide moiety linked to the indole ring. Finally, this fragment ion at *m/z* 232, consisting of the indole ring with the *N*-alkyl moiety and the carbonyl moiety, indicates an N-dealkylation and consequent release of the indazole with the carbonyl moiety (Fragment 3, $C_9H_6NO^+$, *m/z* 144.0444, -0.02 ppm). This *N*-dealkylation has also been reported for AB-FUBINACA and ADB-PINACA. Nevertheless, the cyclopentylium ion (Fragment 4, $C_5H_9^+$, *m/z* 69.0706, 11.17 ppm) has not been observed for related compounds when a QTOF instrument is used [6]. This could be consequence of the different geometry of the collision cell used in both instruments. **Figure S3** shows the proposed fragmentation pathway for 5F-APP-PICA, once evaluated the observed fragmentation.

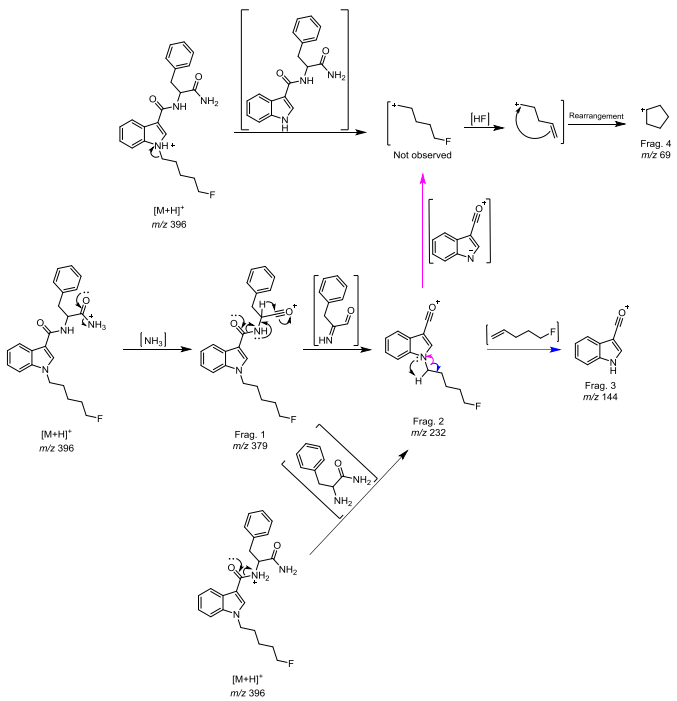


Figure S3. Proposed fragmentation pathway for 5F-APP-PICA based on the observed MS/MS fragmentation.

Figure S2 and **Table S2** show the mass spectrometric behaviour of AMB-FUBINACA ($C_{21}H_{23}FN_3O_3^+$, *m/z* 384.1717, -0.35 ppm) at 10 eV (top) and 30 eV (bottom). The first fragment corresponds to the ester bond disconnection and subsequent loss of methanol (Fragment 1, $C_{20}H_{19}FN_3O_2^+$, *m/z* 352.1455, -0.12 ppm), equivalent to the ammonia loss observed for 5F-APP-PICA. Subsequently, a CO loss is produced (Fragment 2, $C_{19}H_{19}FN_3O^+$, *m/z* 324.1507, 0.09 ppm). After that, the peptide bond is disconnected, releasing the indazole ring bonded to the carbonyl moiety (Fragment 4, $C_{15}H_{10}FN_2O^+$, *m/z* 253.0771, -0.14 ppm). Peptide bond can also be disconnected from the $[M+H]^+$ protonated in the amide moiety, as explained in **Figure S4**. This behaviour has also been reported for ADB-FUBINACA and AB-FUBINACA [26,44]. Finally, an *N*-dealkylation is produced, resulting in a fluorotropylium ion (Fragment 5, $C_7H_6F^+$, *m/z* 109.0451, 3.45 ppm), also reported for cannabinoids with a fluorobenzyl moiety [26,44]. Similarly to Fragment 4, the fluorotropylium fragment ion could be produced directly from the protonated molecule after an *N*-alkyl disconnection (**Figure S4**). An additional fragment is observed at *m/z* 271.0877 (Fragment 3, $C_{15}H_{12}FN_2O_2^+$, -0.09 ppm), obtained after the addition of a water molecule to Fragment 4. The generation of adducts between unstable ions and neutral molecules, especially water molecules, has been previously reported for Q-Orbitrap instruments [45]. As stated by those authors, these adducts are less promoted in QTOF instruments, due to the different geometry of the collision cell.

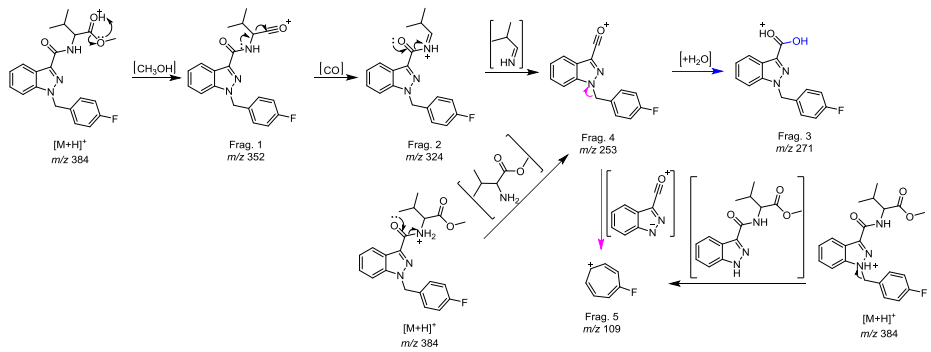


Figure S4. Proposed fragmentation pathway for AMB-FUBINACA based on the observed MS/MS fragmentation.

Although there are differences in the fragmentation of both compounds, a generic fragmentation pathway can be proposed based on most of the observed ions (see **Figure 1**). The information about the fragmentation pathway of both synthetic cannabinoids can be used by forensic laboratories for the identification of these SCs, but also for elucidation of similar cannabinoids, as most present similar fragmentation [6,44–46].

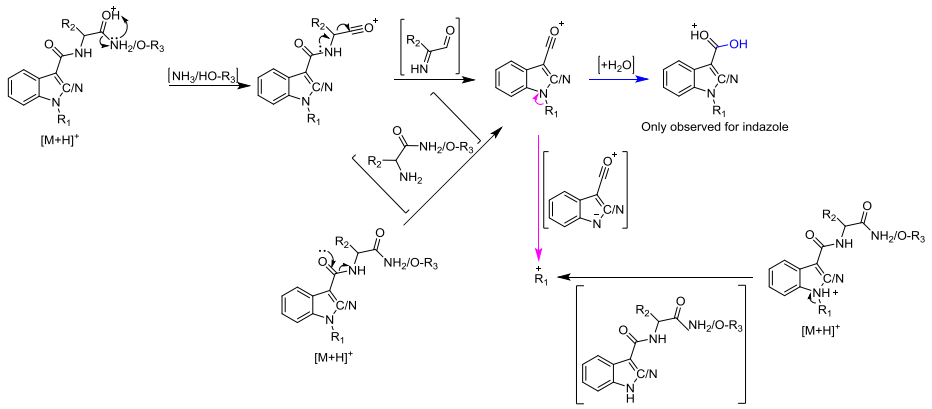


Figure 1. Common fragmentation pathway for 5F-APP-PICA and AMB-FUBINACA. Moieties that are different between both cannabinoids are indicated with a "/".

Table S1. MS/MS fragmentation of 5F-APP-PICA and its metabolites, including retention time, ionization mode, and elemental composition, accurate mass and mass error (in ppm) for (de)protonated molecule and fragment ions.

Metabolite	Retention time (min)	ESI	$[M+H]^{+/-}$ (*m/z*)	Elemental composition	Mass error (ppm)	Fragment ion (*m/z*)	Elemental composition	Mass error (ppm)	Collision energy (eV)
5F-APP-PICA	7.94	+	396.2083	$C_{23}H_{27}FN_3O_2^+$	0.18	379.1815	$C_{23}H_{24}FN_2O_2^+$	-0.38	10
						232.1132	$C_{14}H_{15}FNO^+$	-0.39	30
						144.0444	$C_9H_6NO^+$	-0.02	50
						69.0706	$C_5H_9^+$	11.17	50
M1	6.70	+	394.2116	$C_{23}H_{28}N_3O_3^+$	-2.31	377.1851	$C_{23}H_{25}N_2O_3^+$	-2.33	10
						230.1170	$C_{14}H_{16}NO_2^+$	-1.93	30
						144.0441	$C_9H_6NO^+$	-2.24	50
						116.0493	$C_8H_6N^+$	-1.37	50
						87.0809	$C_5H_{11}O^+$	5.11	50
						69.0705	$C_5H_9^+$	8.96	50

Table S1. MS/MS fragmentation of 5F-APP-PICA and its metabolites, including retention time, ionization mode, and elemental composition, accurate mass and mass error (in ppm) for (de)protonated molecule and fragment ions (continuation).

Metabolite	Retention time (min)	ESI	$[M+H]^{+/-}$ (*m/z*)	Elemental composition	Mass error (ppm)	Fragment ion (*m/z*)	Elemental composition	Mass error (ppm)	Collision energy (eV)
M2	6.63	+	408.1908	$C_{23}H_{26}N_3O_4^+$	-2.42	391.1642	$C_{23}H_{23}N_2O_4^+$	-2.68	10
						244.0963	$C_{14}H_{14}NO_3^+$	-1.95	30
						144.0441	$C_9H_6NO^+$	-1.82	50
						116.0495	$C_8H_6N^+$	0.27	50
						101.6000	$C_5H_9O_2^+$	2.66	50
						83.0496	$C_5H_7O^+$	5.78	50
						55.0550	$C_4H_7^+$	14.33	50
M3	8.32	+	397.1916	$C_{23}H_{26}FN_2O_3^+$	-1.61	232.1128	$C_{14}H_{15}FNO^+$	-2.03	30
						144.0441	$C_9H_6NO^+$	-2.24	50
						116.0494	$C_8H_6N^+$	-0.78	50
						69.0705	$C_5H_9^+$	8.96	50
M4	7.07	+	395.1958	$C_{23}H_{27}N_2O_4^+$	-1.85	230.1171	$C_{14}H_{16}NO_2^+$	-2.2	30
						144.0441	$C_9H_6NO^+$	-2.24	50
						87.0808	$C_5H_{11}O^+$	4.59	50
						69.0705	$C_5H_9^+$	9.18	50
M5	6.98	+	409.1751	$C_{23}H_{25}N_2O_5^+$	-1.61	244.0964	$C_{14}H_{14}NO_3^+$	-1.76	30
						144.0442	$C_9H_6NO^+$	-1.61	50
						116.0494	$C_8H_6N^+$	0.2	50
						101.0600	$C_5H_9O_2^+$	2.66	50
						83.0497	$C_5H_7O^+$	2.5	50
						55.0552	$C_4H_7^+$	14.47	50
M6	6.32	–	306.1250	$C_{18}H_{16}N_3O_2^-$	4.14	189.0663	$C_{10}H_9N_2O_2^-$	2.36	10
						116.0494	$C_8H_6N^-$	-0.91	50
M7	6.74	–	307.1089	$C_{18}H_{15}N_2O_3^-$	3.9	190.0504	$C_{10}H_8NO_3^-$	2.63	10
						164.0708	$C_9H_{10}NO_2^-$	1.19	10
						147.0441	$C_9H_7O_2^-$	0.6	50
						116.0493	$C_8H_6N^-$	-1.11	50
						72.0077	$C_2H_2NO_2^-$	-4.79	50
M1-Gluc	5.89	–	568.2294	$C_{29}H_{34}N_3O_9^-$	0.73				
M2-Gluc	5.80	–	582.2098	$C_{29}H_{32}N_3O_{10}^-$	2.78	406.1774	$C_{23}H_{24}N_3O_4^-$	3.19	10
						116.0494	$C_8H_6N^-$	-0.32	50
M3-Gluc	7.24	+	357.2238	$C_{29}H_{34}FN_2O_9^+$	-0.93	555.2118	$C_{29}H_{32}FN_2O_9^+$	-3.47	10
						397.1918	$C_{23}H_{26}FN_2O_3^+$	-1.07	10
						232.1128	$C_{14}H_{15}FNO^+$	-1.77	30
						144.0441	$C_9H_6NO^+$	-1.71	50
M4-Gluc	6.19	+	571.2290	$C_{29}H_{35}N_2O_{10}^+$	0.67	230.1172	$C_{14}H_{16}NO_2^+$	-1.53	30
						144.0440	$C_9H_6NO^+$	-2.24	50
M5-Gluc	6.14	+	585.2079	$C_{29}H_{33}N_2O_{11}^+$	0	224.0963	$C_{14}H_{14}NO_3^+$	-1.95	30
						144.0440	$C_9H_6NO^+$	-2.77	50
						101.0601	$C_5H_9O_2^+$	3.64	50

Table S2. MS/MS fragmentation of AMB-FUBINACA and its metabolites, including retention time, ionization mode, and elemental composition, accurate mass and mass error (in ppm) for (de)protonated molecule and fragment ions.

Metabolite	Retention time (min)	ESI	$[M+H]^{+/-}$ (*m/z*)	Elemental composition	Mass error (ppm)	Fragment ion (*m/z*)	Elemental composition	Mass error (ppm)	Collision energy (eV)
AMB-FUBINACA	9.73	+	384.1717	$C_{21}H_{23}FN_3O_3^+$	-0.35	352.1455	$C_{20}H_{19}FN_3O_2^+$	-0.12	10
						324.1507	$C_{19}H_{19}FN_3O^+$	0.09	30
						271.0877	$C_{15}H_{12}FN_2O_2^+$	-0.09	30
						253.0771	$C_{15}H_{10}FN_2O^+$	-0.14	30
						109.0451	$C_7H_6F^+$	3.45	50
M1	8.55	+	370.1560	$C_{20}H_{21}FN_3O_3^+$	-0.3	352.1455	$C_{20}H_{19}FN_3O_2^+$	-0.3	10
						324.1506	$C_{19}H_{19}FN_3O^+$	-0.29	30
						271.0876	$C_{15}H_{12}FN_2O_2^+$	-0.43	30
						253.0770	$C_{15}H_{10}FN_2O^+$	-0.5	30
						109.0452	$C_7H_6F^+$	2.82	50
						72.0815	$C_4H_{10}N^+$	10.43	30
M2	7.33	+	386.1508	$C_{20}H_{21}FN_3O_4^+$	-0.63	368.1400	$C_{20}H_{19}FN_3O_3^+$	-1.31	10
						322.1344	$C_{19}H_{17}FN_3O^+$	-1.82	30
						271.0874	$C_{15}H_{12}FN_2O_2^+$	-1.33	30
						253.0769	$C_{15}H_{10}FN_2O^+$	-1.1	30
						109.0451	$C_7H_6F^+$	3.17	50
M3	7.50	+	386.1513	$C_{20}H_{21}FN_3O_4^+$	0.71	368.1409	$C_{20}H_{19}FN_3O_3^+$	1.01	10
						350.1290	$C_{20}H_{17}FN_3O_2^+$	-2.67	30
						310.1349	$C_{18}H_{17}FN_3O^+$	-0.52	30
						271.0876	$C_{15}H_{12}FN_2O_2^+$	-0.54	30
						253.0769	$C_{15}H_{10}FN_2O^+$	-1.1	30
						109.0453	$C_7H_6F^+$	2.82	50
M4	8.75	+	386.1507	$C_{20}H_{21}FN_3O_4^+$	-0.95	340.1459	$C_{19}H_{19}FN_3O_2^+$	1.01	10
						269.0719	$C_{15}H_{10}FN_2O_2^+$	-0.57	30
						109.0451	$C_7H_6F^+$	2.89	50
M5	8.45	+	400.1674	$C_{21}H_{23}FN_3O_4^+$	1.69	382.1555	$C_{21}H_{21}FN_3O_3^+$	-1.57	10
						350.1297	$C_{20}H_{17}FN_3O_2^+$	-0.2	30
						271.0875	$C_{15}H_{12}FN_2O_2^+$	-0.99	30
						253.0771	$C_{15}H_{10}FN_2O^+$	-0.2	30
						109.0450	$C_7H_6F^+$	2.89	50
M6	6.31	+	262.1185	$C_{13}H_{16}N_3O_3^+$	-0.57	216.1131	$C_{12}H_{14}N_3O^+$	0.02	10
						163.0501	$C_8H_7N_2O_2^+$	-0.48	50
						145.0396	$C_8H_5N_2O^+$	-0.52	50
						72.0815	$C_4H_{10}N^+$	10.22	50
M6-Methyl	7.06	+	276.1342	$C_{14}H_{18}N_3O_3^+$	-0.19	230.1288	$C_{13}H_{16}N_3O^+$	-0.15	10
						177.0658	$C_9H_9N_2O_2^+$	-0.5	50
						159.0552	$C_9H_7N_2O^+$	-0.52	50
						72.0815	$C_4H_{10}N^+$	10.22	50

Table S2. MS/MS fragmentation of AMB-FUBINACA and its metabolites, including retention time, ionization mode, and elemental composition, accurate mass and mass error (in ppm) for (de)protonated molecule and fragment ions (continuation).

Metabolite	Retention time (min)	ESI	$[M+H]^{+/-}$ (*m/z*)	Elemental composition	Mass error (ppm)	Fragment ion (*m/z*)	Elemental composition	Mass error (ppm)	Collision energy (eV)
M1-Gluc	7.17	+	546.1881	$C_{26}H_{29}FN_3O_9^+$	-0.23	528.1772	$C_{26}H_{27}FN_3O_8^+$	-0.8	10
						370.1558	$C_{20}H_{21}FN_3O_3^+$	-0.87	10
						324.1503	$C_{19}H_{19}FN_3O^+$	-0.1	30
						271.0876	$C_{15}H_{12}FN_2O_2^+$	-0.43	50
						253.0771	$C_{15}H_{10}FN_2O^+$	-0.26	50
						109.0452	$C_7H_6F^+$	3.31	50
						72.0815	$C_4H_{10}N^+$	10.75	30

Tables SI1 and **SI2** show the fragmentation observed for all the 5F-APP-PICA and AMB-FUBINACA metabolites, respectively, in ESI^+ and ESI^-, including the chromatographic retention time, and the elemental composition and mass error of the (de)protonated molecule and its fragments.

3.2. Analytical strategy for metabolite identification

Once samples were injected into the UHPLC-HRMS system using data dependent acquisition mode (DDA), Compound Discoverer 2.0 software was used for data processing. A list of expected metabolites was obtained based on presumed biotransformations. The software automatically performed extracted ion chromatograms (EIC) (with a mass window of ±5 ppm) for the (de)protonated molecule of the expected metabolites. Compounds that were present in incubation samples but not in blanks nor stability samples were considered as potential metabolites.

MS/MS spectra of the potential metabolites, acquired during DDA, were studied using FreeStyle 1.3 software. Those potential metabolites that presented a plausible fragmentation (i.e. the MS/MS spectra fit with the expected biotransformation) were considered for a re-analysis by UHPLC-HRMS using parallel reaction monitoring acquisition (PRM) at three different collision energies (10, 30 and 50 eV). If not, they were directly discarded.

Based on the observed PRM MS/MS fragmentation, the position of the biotransformation on the structure was determined. Finally, fragmentation pathways were proposed for all metabolites.

3.3. 5F-APP-PICA metabolites

The above strategy allowed the identification of 7 phase I metabolites of 5F-APP-PICA. Regarding phase II metabolites, 5 compounds were found, all of them corresponding to glucuronides (Gluc) of the main phase I metabolites. An exhaustive description on the identification of all metabolites based on the observed fragmentation, including fragmentation pathways, can be found then.

Phase I metabolites

As it has been commented, 7 different phase I metabolites were identified for 5F-APP-PICA. The 5 major metabolites (M1-M5) were initially identified in ESI^+, and also detected in ESI^- but showing lower sensitivity. The acquisition in ESI^- allowed the identification of 2 additional minor metabolites (M6-M7), not observed in ESI^+.

M1-M5 presented the same fragment at *m/z* 144 (also present in 5F-APP-PICA, see **Table S1**), corresponding to the indole ring bonded to the carbonyl moiety ($C_9H_6NO^+$). Moreover, these metabolites (except M4) presented a fragment at *m/z* 116 corresponding to the indole ring ($C_8H_6N^+$). These two fragments evidenced that the indole moiety was not changed during hepatocyte incubation, unlike other synthetic cannabinoids for which the hydroxylation of the indole moiety has been reported [31,44]. Regarding M6 and M7 the fragment ion at *m/z* 116 ($C_8H_6N^-$) was also observed, indicating that this moiety remained unaltered. Thus, the biotransformations should be placed in the alkylic chain, both amide moieties, and/or the aromatic ring bonded to the α carbon atom of amide moieties.

M1 showed a $[M+H]^+$ at *m/z* 394.2116 ($C_{23}H_{28}N_3O_3^+$, -2.31 ppm), which would correspond to an oxidative defluorination. This hypothesis was confirmed after fragmentation evaluation. Fragment 2 (*m/z* 230.1170, $C_{14}H_{16}NO_2^+$, -1.93 ppm)

corresponded to the indole ring, with the carbonyl moiety and the *N*-alkylic chain. The corresponding fragment was also present in 5F-APP-PICA (*m/z* 232). Moreover, Fragment 5 (*m/z* 87.0809, $C_5H_{11}O^+$, 5.11 ppm) corresponded to the *N*-alkyl disconnection and release of the 5-hydroxypentanylium ion, confirming the position of the hydroxyl group in the alkylic chain. After the dehydration of Fragment 5, cyclopentylium ion (*m/z* 69.0705, $C_5H_9^+$, 8.96 ppm) was observed for both M1 and 5F-APP-PICA. Other observed fragments (see **Table S1**) could be justified based on the proposed structure of M1. MS/MS spectra at 10 and 50 eV for M1, and the proposed fragmentation pathway can be found in **Figure S5** and **Figure S6**, respectively.

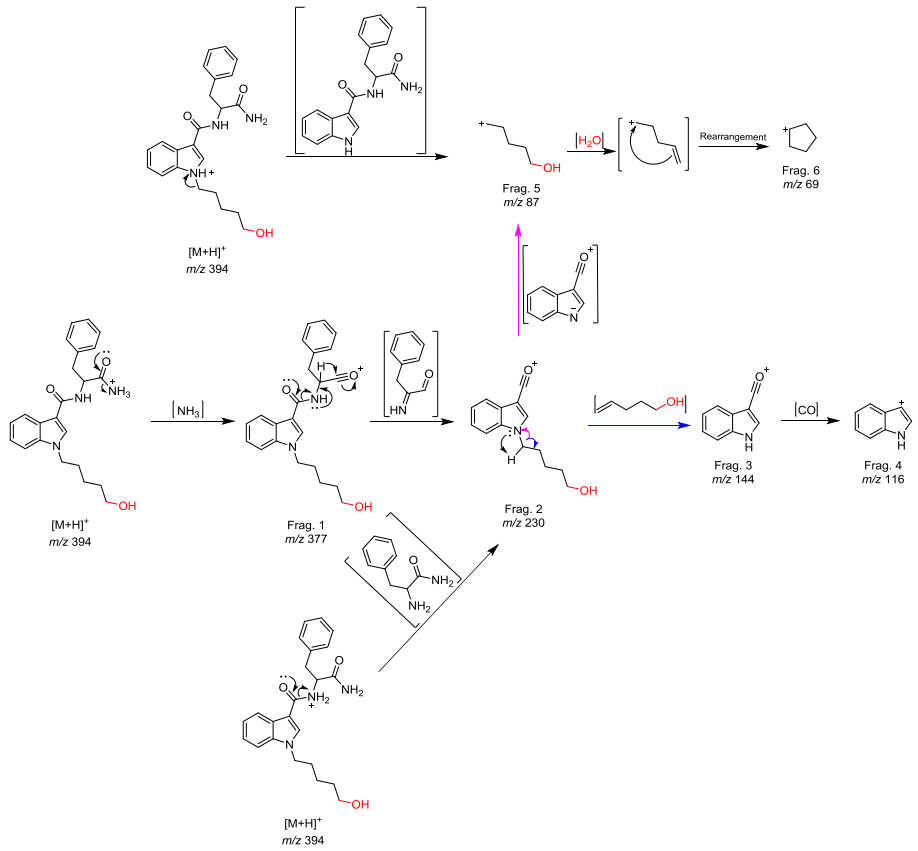


Figure S6. Proposed fragmentation pathway for 5F-APP-PICA M1 based on the observed MS/MS fragmentation.

M2 $[M+H]^+$ was found at *m/z* 408.1908 ($C_{23}H_{26}N_3O_4^+$, -2.42 ppm), and corresponded to the oxidation of the hydroxyl group present in the *N*-alkyl chain of M1. Similarly to M1, fragments at *m/z* 244.0963 ($C_{14}H_{14}NO_3^+$, -1.95 ppm) *m/z* 101.6000 ($C_5H_9O_2^+$, 2.66 ppm) established the position of the biotransformation to be in the alkylic chain. Fragment 6 (*m/z* 83.0496, $C_5H_7O^+$, 5.78 ppm) was produced after Fragment 5 dehydration (similarly to M1), while Fragment 7 (*m/z* 55.0550, $C_4H_7^+$, 14.33 ppm) was produced after a CO loss from Fragment 6. These fragmentations can only be justified if there is a carboxylic acid moiety in the *N*-alkyl chain. Additional fragments (see **Table S1**) were justified based on the proposed structure. In **Figure I7**, MS/MS spectra at 10 and 50 eV can be observed, and **Figure S8** shows the proposed fragmentation pathway for M2.

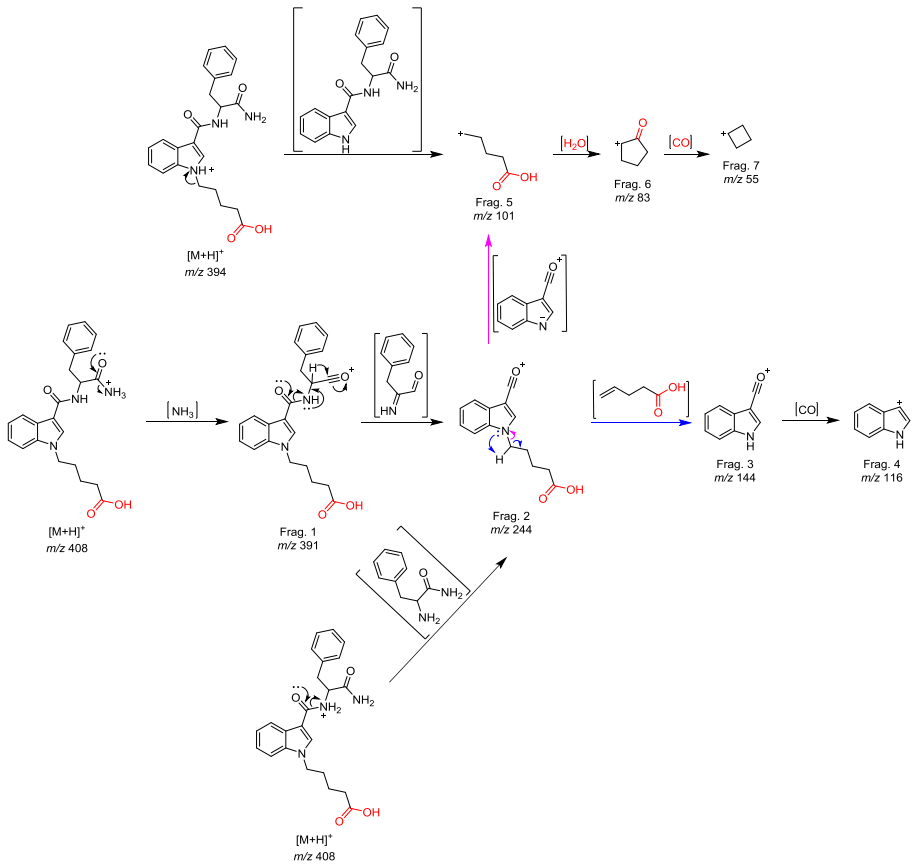


Figure S8. Proposed fragmentation pathway for 5F-APP-PICA M2 based on the observed MS/MS fragmentation.

M3 $[M+H]^+$ was observed at *m/z* 397.1916 ($C_{23}H_{26}FN_2O_3^+$, -2.42 ppm), corresponding to an oxidative deamination from 5F-APP-PICA. The four observed fragments were the same observed for 5F-APP-PICA (see **Table S1**): *m/z* 232, 144, 116, and 69. Nevertheless, 5F-APP-PICA, and also M1 and M2, presented the loss of an ammonia molecule which was not observed for M3, indicating that the oxidative deamination occurred in the terminal amide moiety. Finally, the fragmentation pathway was proposed (**Figure S10**) based on the MS/MS fragmentation at 10 and 50 eV (**Figure S9**).

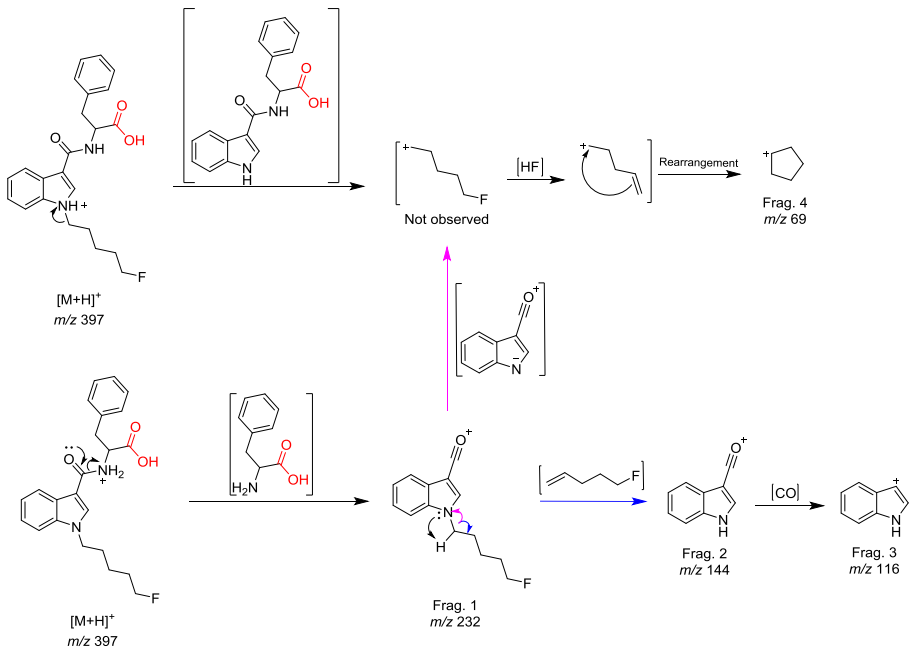


Figure S10. Proposed fragmentation pathway for 5F-APP-PICA M3 based on the observed MS/MS fragmentation.

M4 presented a $[M+H]^+$ at *m/z* 395.1958 ($C_{23}H_{27}N_2O_4^+$, -1.85 ppm), corresponding to an oxidative defluorination plus an oxidative deamination. This metabolite shared some fragments with M1 (see **Table S1**), concretely at *m/z* 230, 144, 87, and 69. Only the fragment ion corresponding to the indole ring (*m/z* 116) and the corresponding to the ammonia loss were not present in M4. This behaviour indicates that M4 correspond to the oxidative defluorination of the *N*-

alkylic chain, followed by oxidative deamination of the terminal amide moiety (or vice versa). In other words, M4 comes from M1 after an oxidative deamination, or from M3 after an oxidative defluorination. Once all the fragments were justified based on the structure of the metabolite, a plausible fragmentation pathway was proposed (**Figure S12**). Additionally, MS/MS spectra at 10 and 50 eV can be checked in **Figure S11**.

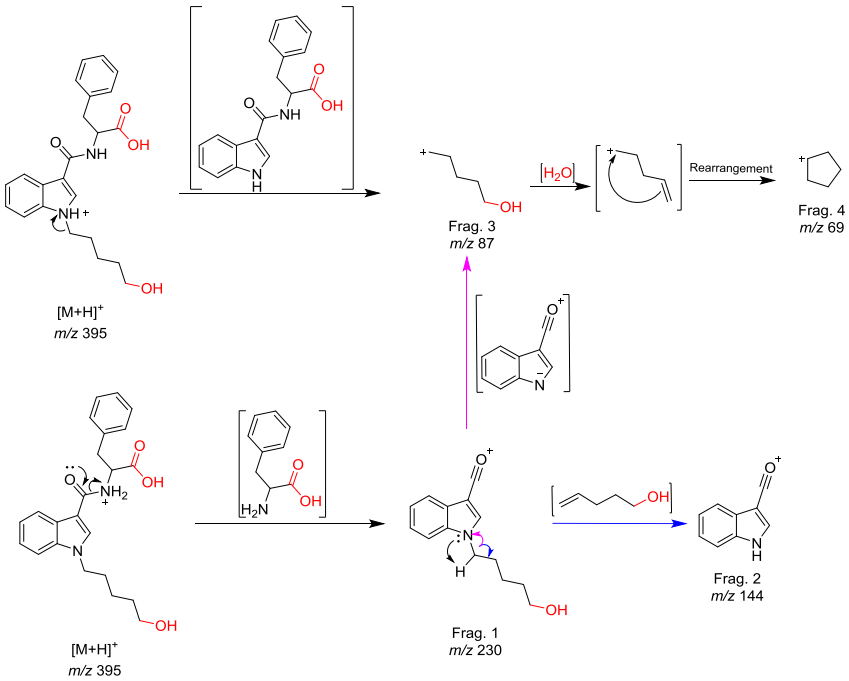


Figure S12. Proposed fragmentation pathway for 5F-APP-PICA M4 based on the observed MS/MS fragmentation.

M5 $[M+H]^+$ was observed at *m/z* 409.1751 ($C_{23}H_{25}N_2O_5$+, -1.61 ppm), sharing 6 fragment ions with M2 (see **Table S1** and **Figure S13**): *m/z* 244, 144, 116, 101, 83, and 55. Ammonia loss was not observed for this metabolite. Based on this information, this metabolite could be produced after the oxidation of the hydroxyl group present in the *N*-alkylic chain of M4, or after the oxidative deamination of M2. Thus, M5 presented two carboxylic acid moieties in both sides of the molecule. Finally, the fragmentation pathway of M5 was proposed, based on compound structure and observed fragmentation (**Figure S14**).

M6 and M7 were only observed in ESI^-, presenting low sensitivity. M6 $[M–H]^-$ was detected at m/z 306.1250 ($C_{18}H_{16}N_3O_2^-$, 4.14 ppm). As explained previously, the detection of the fragment at m/z 116 (indole ring) revealed that this moiety was not affected during hepatocyte incubation. Based on the elemental composition, the biotransformation should correspond to the *N*-dealkylation of 5F-APP-PICA. Only two fragment ions were observed (see **Table S1**), being Fragment 1 *(m/z* 189.0663, $C_{10}H_9N_2O_2$–, 2.36 ppm) the loss of the indole ring as a neutral loss, and Fragment 2 the indole ring itself (*m/z* 116.0494, $C_8H_6N^-$, -0.91 ppm). **Figure S15** shows MS/MS spectra at 10 and 50 eV. The fragmentation pathway proposed can be seen in **Figure S16**.

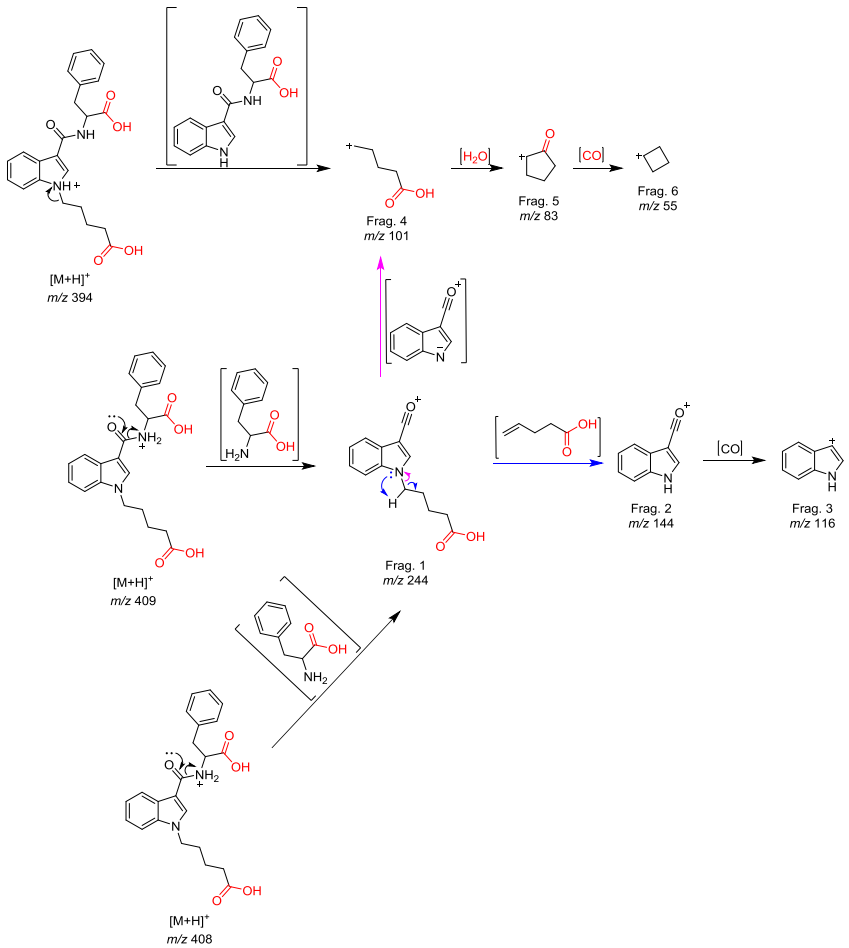


Figure S14. Proposed fragmentation pathway for 5F-APP-PICA M5 based on the observed MS/MS fragmentation.

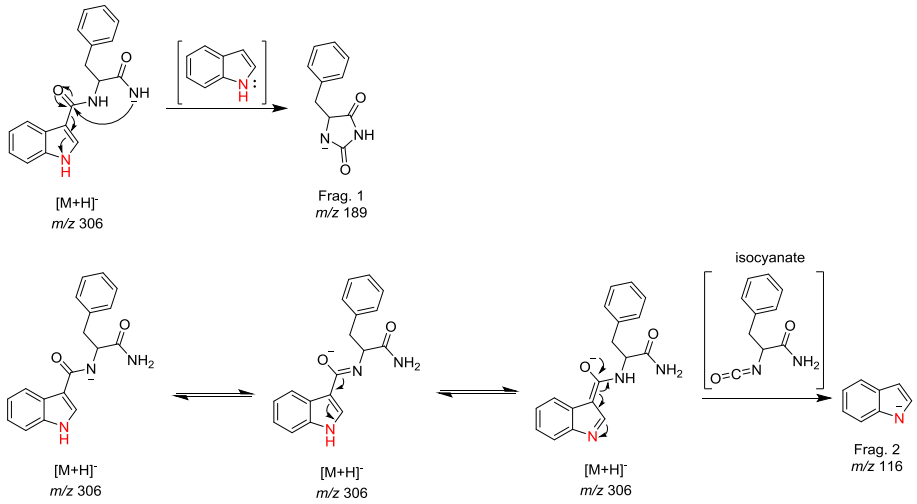


Figure S16. Proposed fragmentation pathway for 5F-APP-PICA M6 based on the observed MS/MS fragmentation.

M7 was the last phase I metabolite identified, which $[M–H]^-$ was found at *m/z* 307.1089 ($C_{18}H_{16}N_2O_3^-$, 3.90 ppm). The fragment at *m/z* 190.0504 ($C_{10}H_8NO_3^-$, 3.90 ppm) (see **Table S1**) would be equivalent to *m/z* 189 in M6, whereas the fragment at *m/z* 116 was common to M6. Following the same metabolic pathway than M3, M4 and M5, and based on the elemental composition calculated, M7 was obtained after an oxidative deamination from M6 (the terminal amide moiety changed to a carboxylic acid). The other three observed fragments (Fragments 2, 3 and 5, **Table S1**) confirmed the hypothesis of the oxidative deamination from M6. Finally, a fragmentation pathway was proposed (**Figure S18**), justifying all the fragments observed in MS/MS spectra at 10 and 50 eV (**Figure S17**).

Phase II metabolites

Once identified the phase I metabolites, the phase II metabolites were searched for by considering different conjugations to phase I metabolites. Glucuronide, sulphate, phosphate and glutathione conjugates were investigated in both ionisation modes. Up to 5 glucuronides conjugates were detected in the DDA acquisition and thus, acquired in MS/MS in order to confirm the structure of the phase II metabolites (**Table S1**).

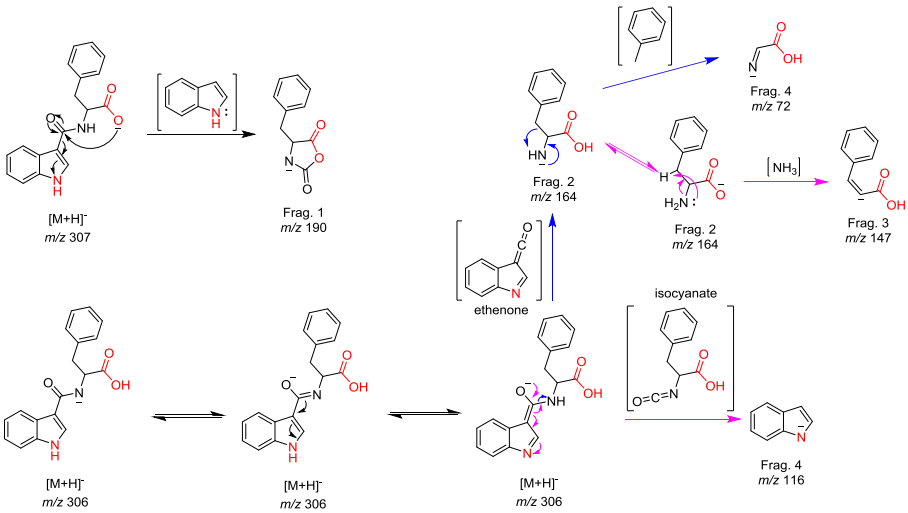


Figure S18. Proposed fragmentation pathway for 5F-APP-PICA M7 based on the observed MS/MS fragmentation.

M1-Gluc and M2-Gluc were only detected in ESI$^-$. For M1-Gluc, only the [M–H]$^-$ was observed, without any fragment ion, probably due to its low sensitivity or abundance. M2-Gluc showed 2 fragment ions: Fragment 1 at *m/z* 406, corresponding to the loss of the glucuronide (and thus, releasing the [M–H]$^-$ of M2), and Fragment 2 at *m/z* 116, corresponding to the indole ring. MS/MS spectra at 10 eV for M1-Gluc, and at 10 and 50 eV for M2-Gluc for both phase II metabolites are shown in **Figure S19** and **Figure S20**, respectively.

M3-Gluc, M4-Gluc and M5-Gluc were detected in both ionisation modes, but presenting a higher sensitivity in ESI$^+$. All the fragments ions were shared with their corresponding phase I metabolites (see **Table S1**), being highly specific and locating the unequivocal position of the glucuronidation for metabolites M4 and M5 which possess two possible conjugation sites. So, M3, M4 and M5 presented conjugation on the carboxylic acid moiety linked to the amide moiety. MS/MS spectra at 10 and 50 eV corresponding to these three metabolites can be found in **Figure S21** (M3-Gluc), **Figure S22** (M4-Gluc) and **Figure S23** (M5-Gluc).

3.3.1. Metabolic behaviour

After elucidating the structures for all metabolites, the metabolic pathway for 5F-APP-PICA using human hepatocyte incubation was proposed (**Figure 2**). As an aliquot of the incubation was taken at 0 (a 'lag time' of 2-3 minutes should be taken into account), 60 and 180 min, the prevalence of 5F-APP-PICA and its metabolites in the incubation was also studied. **Figure 3** shows the relative presence of all the identified metabolites, including the parent compound, normalizing all the responses to the one obtained for 5F-APP-PICA at 0 min. For metabolites detected in ESI^+, 5F-APP-PICA $[M+H]^+$ response was used, while metabolites observed in ESI^- were plotted relative to 5F-APP-PICA $[M–H]^-$ response. Obviously, an important limitation here is that this approach assumes similar ionisation and fragmentation efficiencies for the parent compounds and the metabolites, which is not really the case, as can also be deduced from the fact that the sum of the obtained % (strongly) deviates from 100%. Still, this approach allows to get an idea about the appearance and further conversion of metabolites.

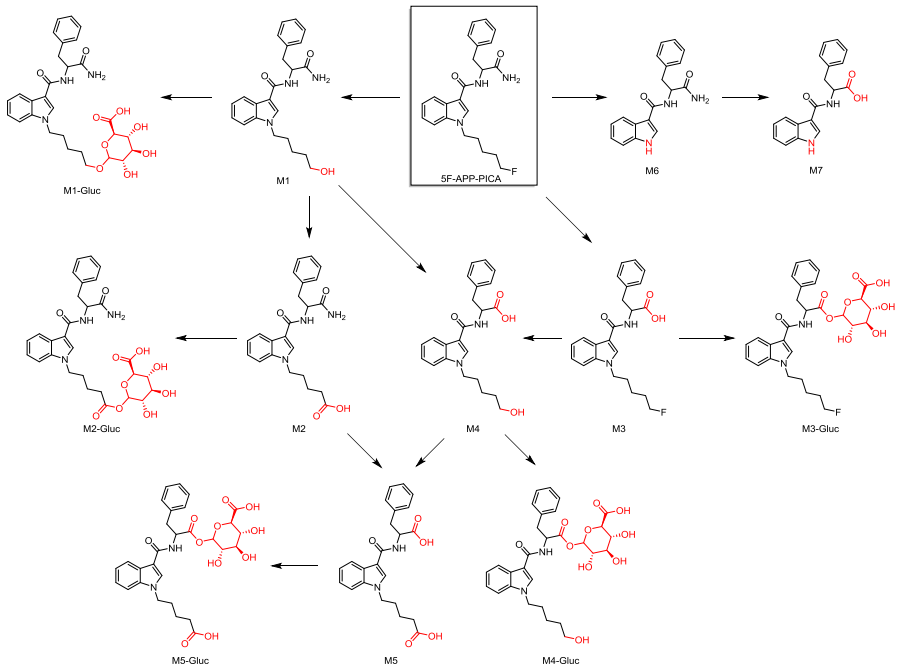


Figure 2. Proposed metabolic pathway for 5F-APP-PICA, based on the metabolites elucidated by UHPLC-HRMS.

Metabolic biotransformations seem to be focused on the removal of heteroatoms from both sides of the molecule. One of the most abundant biotransformations at 0 min was the oxidative defluorination (M1), together with the oxidation of the hydroxyl group in the alkylic chain after oxidative defluorination (M2). Both biotransformations were not observed in control incubations (without hepatocytes). These two biotransformations have been reported for other synthetic cannabinoids with an *N*-fluoropentyl moiety, such as MAM-2201 [46], 5F-AB-PINACA [47] and 5F-AMB [48]. The other most abundant biotransformation at 0 min, the oxidative deamination (M3), has also been widely reported in literature for synthetic cannabinoids with terminal amide moieties, such as AB-FUBINACA [24], AB-PINACA [47] and 5F-AB-PINACA [47]. As expected, combinations of these biotransformations were also found (M4 and M5). The *N*-dealkylation of the fluoropentyl moiety (M6) has also been reported for MAM-2201 [46]. Similarly to M4 and M5, the metabolite M7 was obtained after oxidative deamination from M6. Additional biotransformations described for these cannabinoids, such as *N*-alkyl hydroxylation/oxidation, were not found for 5F-APP-PICA [46,48].

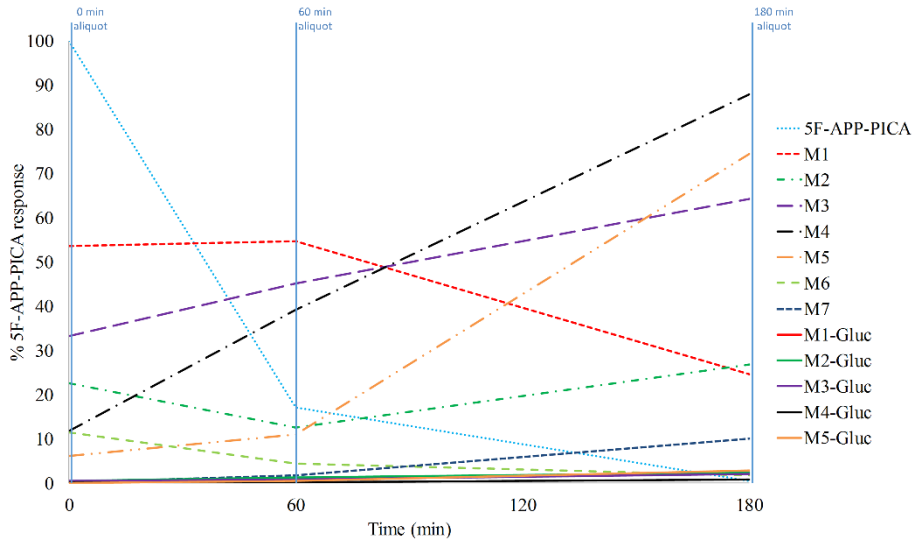


Figure 3. Prevalence of 5F-APP-PICA and its metabolites in incubation over time. Signals are relative to those obtained for 5F-APP-PICA at 0 min.

5F-APP-PICA was rapidly metabolised during incubation. At 60 min, only 20% of the initial response was present, with less than 0.5% remaining at 180 min, as depicted in **Figure 3**. M1 presented an important response at 0 min, but it started to decrease at 60 min. It is possible that the increment in the relative response of M2 after 60 min is related to the decrease of M1 at the same time, as M1 is transformed to M2, and also to M4. M2-M5 presented a continuous increase in response over time, with especially M5 increasing substantially after the 60 min time point. M6 presented a relative concentration of 11% at 0 min, while M7, which is derived from M6, was present at 10% at 180 min, with an increase in M7 being accompanied by a decrease in M6. Finally, glucuronide conjugates were not significantly important, all being below 3% of relative response. The most abundant phase II metabolite was M5-Gluc (2.85%), as expected after evaluation of phase I metabolites.

Based on these results, M3, M4 and M5 are the best detectable -and possibly the most abundant- metabolites at 180 min, and thus could be selected as potential biomarkers for the determination of 5F-APP-PICA consumption in forensic samples. As noted above, the signal response alone is not a good indicator to claim that a biotransformation is the most important one (e.g. owing to differences in ionisation efficiencies). However, due to the lack of reference standards for quantification, from a pragmatic point of view, it could be considered an adequate estimation. We are not aware of authentic cases that have been reported in literature via which these biomarkers could be validated.

3.4. AMB-FUBINACA metabolites

Six phase I metabolites and one phase II metabolite, corresponding to the glucuronide conjugate of the main phase I metabolite, were identified for AMB-FUBINACA. All the identified metabolites were detected in ESI^+. The fragmentation study performed for the identification of all metabolites can be found in hereunder.

Phase I metabolites

M1-M5 presented a common fragment at *m/z* 109 ($C_7H_6F^+$), also present in AMB-FUBINACA, which corresponds to the fluorotropylium fragment ion. The presence of this unaltered fragment indicated that no biotransformations were expected on the fluorobenzyl side-chain. The absence of biotransformations on this moiety has also been described for other synthetic cannabinoids with an *N*-fluorobenzyl, such as AB-FUBINACA and ADB-FUBINACA [25,42].

M1 $[M+H]^+$ was observed at *m/z* 370.1560 ($C_{20}H_{21}FN_3O_3$+, -0.30 ppm), corresponding to a demethylation. The presence of a water molecule loss (*m/z* 352.1455, $C_{20}H_{19}FN_3O_2^+$, -0.30 ppm) followed by a CO loss (*m/z* 324.1506, $C_{19}H_{19}FN_3O^+$, -0.29 ppm) indicated an *O*-demethylation in the terminal methyl ester moiety. This pathway is similar to AMB-FUBINACA, which presented a methanol loss (fragment at *m/z* 352) followed by a CO loss (fragment at *m/z* 324). Other fragments at *m/z* 271, 253 and 109 were shared with AMB-FUBINACA (see **Table S2**). The high response of M1 allowed the detection of an additional fragment at *m/z* 72.0815 ($C_4H_{10}N^+$, 10.43 ppm), corresponding to the nitrogen atom bonded to the isopropyl moiety. After that, a plausible fragmentation pathway was proposed (**Figure S25**) based on the MS/MS fragmentation at 10 and 30 eV (**Figure S24**).

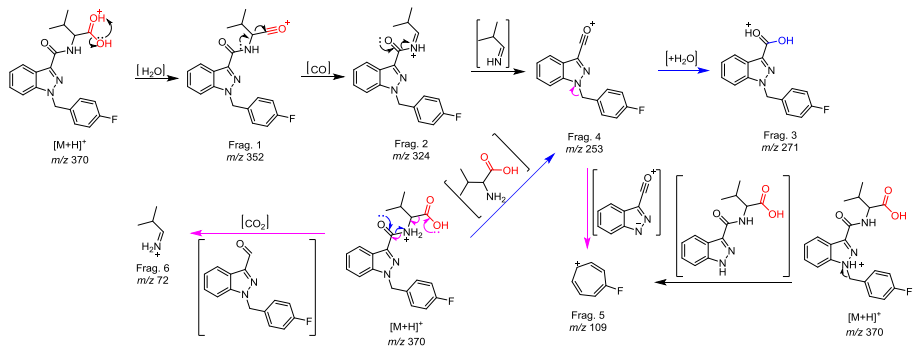


Figure S25. Proposed fragmentation pathway for AMB-FUBINACA M1 based on the observed MS/MS fragmentation.

M2, M3 and M4 were observed at *m/z* 386, all 3 corresponding to hydroxylations of M1 (for the information of their complete fragmentation, see **Table S2**). The position of the hydroxylation point was successfully determined after an accurate fragmentation study. The easiest one was M4 (*m/z* 386.1507, $C_{20}H_{21}FN_3O_4^+$, -0.95 ppm), rapidly identified after the evaluation of their fragments at *m/z* 269.0719 ($C_{15}H_{10}FN_2O_2^+$, -0.57 ppm) and *m/z* 109. The presence of the unaltered fluorotropylium ion (Fragment 3) indicated that the hydroxylation was on the indazole ring. The Fragment 2 (*m/z* 269) would correspond to the fragment ion 4 of AMB-FUBINACA (*m/z* 253). All fragments observed in MS/MS spectra (**Figure S26**) were successfully justified based on the proposed structure, and the fragmentation pathway was also proposed (**Figure S27**).

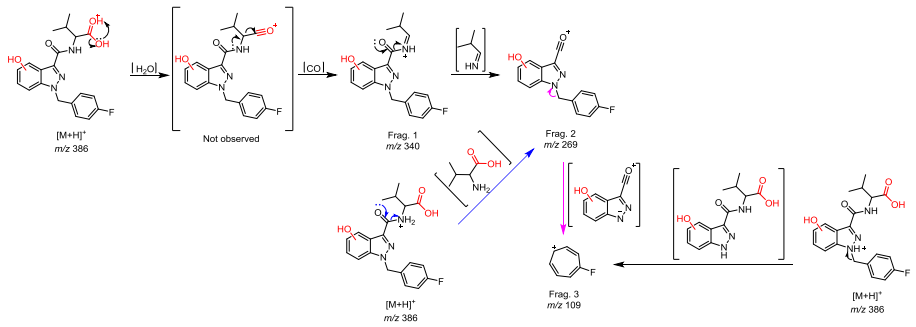


Figure S27. Proposed fragmentation pathway for AMB-FUBINACA M4 based on the observed MS/MS fragmentation.

M2 (*m/z* 386.1508, $C_{20}H_{21}FN_3O_4^+$, -0.63 ppm) and M3 (*m/z* 386.1513, $C_{20}H_{21}FN_3O_4^+$, 0.71 ppm) structures were not so directly identified as M4. The presence of a fragment ion at *m/z* 253 (shared with AMB-FUBINACA) revealed that the indazole and fluorophenyl moieties were not changed. Both compounds presented an initial loss of a water molecule (fragment at *m/z* 368); for M3, a second loss of water was observed (*m/z* 350) whereas for M2 the loss of water plus CO was found (fragment at *m/z* 322) (see **Table S2**). As AMB-FUBINACA presented also the loss of a water molecule followed by a CO loss, it was expected that M2 and M3 were hydroxylated on the isopropyl moiety, explaining the second water loss observed for these metabolites.

Nevertheless, M3 presented a characteristic fragment ion, which revealed the position of the hydroxyl group in this metabolite. The Fragment 3 of M3 (*m/z* 310.1349, $C_{18}H_{17}FN_3O^+$, -0.52 ppm) corresponded to a CH_2O + CO loss from Fragment 1. The CO loss was originated from the carbonyl moiety after the first water loss; the CH_2O loss could only be explained if the hydroxyl group was in one of the methyl groups of the isopropyl moiety. This CH_2O + CO loss was not observed for M2, and the fragmentation occurred through losses of water and CO molecules, indicating that the hydroxyl group should be on the tertiary carbon of the isopropyl group. All the fragment ions observed for M2 and M3 (see **Table S2**) in the MS/MS spectra at 10 and 30 eV (**Figure S28** and **Figure S30**, respectively) were successfully justified based on the proposed structure, and their fragmentation pathways could be proposed (**Figure S29** and **Figure S31**, respectively).

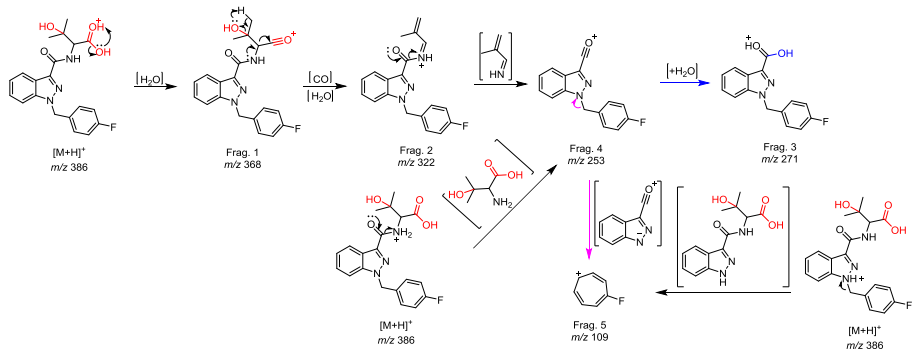


Figure S29. Proposed fragmentation pathway for AMB-FUBINACA M2 based on the observed MS/MS fragmentation.

M5 (*m/z* 400.1674, $C_{21}H_{23}FN_3O_4^+$, 1.69 ppm) was produced after the hydroxylation of AMB-FUBINACA. On the basis of the fragmentation observed for the parent compound and M2-M4, the position of the hydroxyl group could be directly stablished. As a fragment ion at *m/z* 253 was observed, the hydroxylation on the indazole ring was discarded. M5 fragmentation was similar to M3, keeping in mind that M3 was *O*-demethylated respect M5. Fragment at

m/z 350 (Fragment 2) was observed for M5 and M3, and the CH_2O loss observed for M2 was not present.

Based on these premises, M5 should have a hydroxyl on the tertiary carbon of the isopropyl. The proposed fragmentation pathway (**Figure S33**) could justify all the observed fragments in MS/MS spectra at 10 and 30 eV (**Figure S32**).

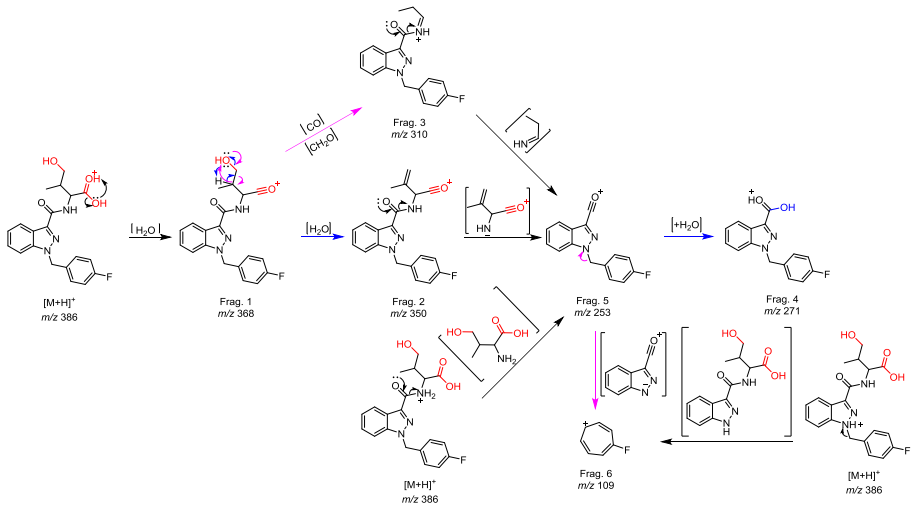


Figure S31. Proposed fragmentation pathway for AMB-FUBINACA M3 based on the observed MS/MS fragmentation.

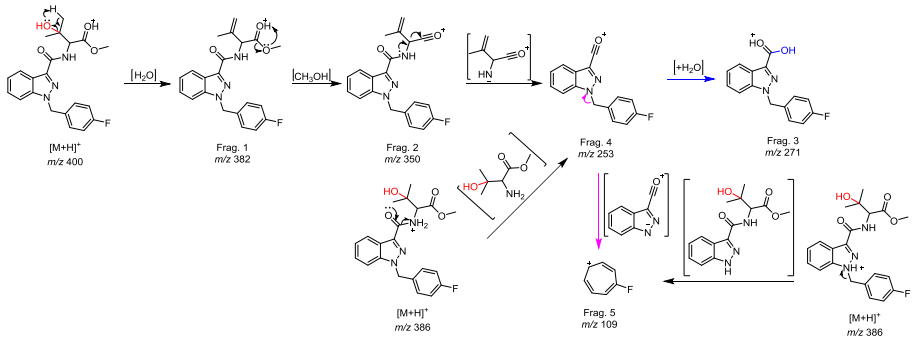


Figure S33. Proposed fragmentation pathway for AMB-FUBINACA M5 based on the observed MS/MS fragmentation.

M6 was observed at *m/z* 262.1185 ($C_{13}H_{16}N_3O_3$+, -0.57 ppm), and its elemental composition revealed that this metabolite should be produced after an *N*-dealkylation from M1, as the mass difference respect to the parent, corresponded to a fluorobenzyl loss. Fragment 3 (*m/z* 145.0396, $C_8H_5N_2O^+$, -0.52 ppm) and 2 (Fragment 3+H_2O, *m/z* 163.0501, $C_8H_7N_2O_2^+$, -0.48 ppm) confirmed the *N*-dealkylation of this metabolite. Moreover, the consecutive losses of H_2O and CO observed in Fragment 1 (*m/z* 216.1131, $C_{12}H_{14}N_3O^+$, 0.02 ppm) confirmed the presence of a carboxylic acid moiety, as these losses were also observed for M1. Thus, all the MS/MS fragments (**Figure S34**, **Table S2**) were justified based on the proposed structure for M6, and the fragmentation pathway was stablished (**Figure S35**).

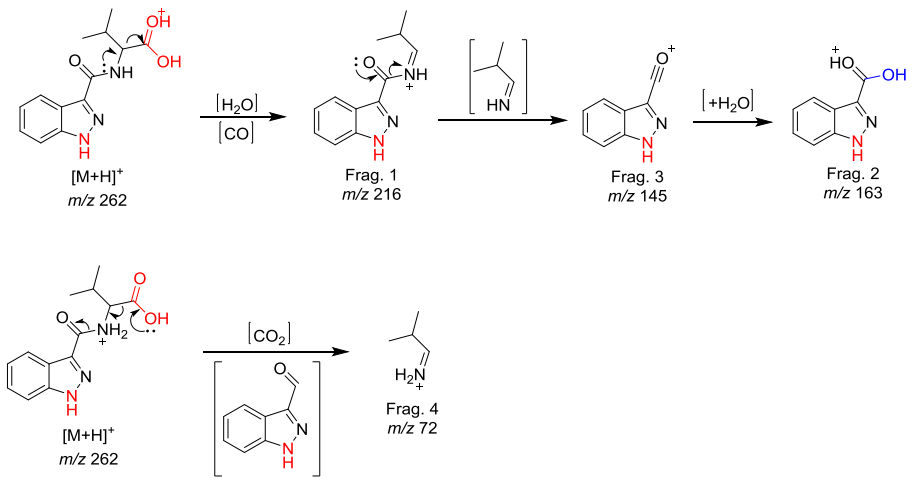


Figure S35. Proposed fragmentation pathway for AMB-FUBINACA M6 based on the observed MS/MS fragmentation.

M6-Methyl was the last phase I metabolite identified for AMB-FUBINACA, observed at *m/z* 276.1342 ($C_{14}H_{18}N_3O_3^+$, -0.23 ppm). Based on the elemental composition, it was expected that M6-Methyl was the *N*-dealkylation product of AMB-FUBINACA. Nevertheless, the fragmentation evaluation revealed that this preliminary evaluation was not the correct one. Similar to M6 and M1, the consecutive losses of H_2O and CO (Fragment 1, *m/z* 230.1288, $C_{13}H_{16}N_3O^+$, -0.15 ppm) indicated the presence of a carboxylic acid moiety on the molecule. Thus,

M6-Methyl should also correspond to an *O*-demethylation. The correct position of this extra methyl moiety was determined by Fragment 3 (*m/z* 159.0552, $C_9H_7N_2O^+$, -0.52 ppm), locating it in the nitrogen of the indazole ring after an *N*-methylation of M6. On this way, a tentative fragmentation pathway (**Figure S37**) was proposed for this structure, justifying all the MS/MS fragments (**Figure S36**, **Table S2**).

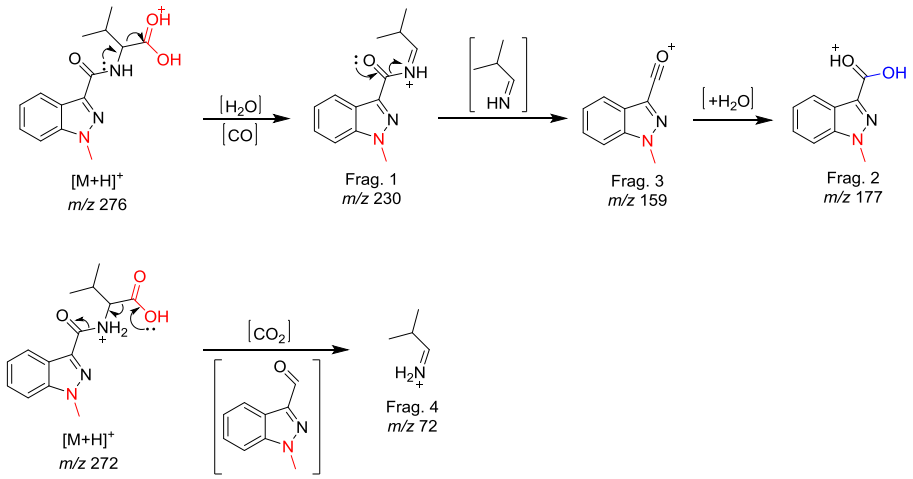


Figure S37. Proposed fragmentation pathway for AMB-FUBINACA M6-Methyl based on the observed MS/MS fragmentation.

Phase II metabolites

Only one phase II metabolite was identified in the DDA analyses in ESI+ and confirmed by MS/MS (**Table S2**) to be the glucuronide corresponding to M1 (M1-Gluc).

M1-Gluc presented exactly the same fragmentation than M1, including the fragment ion corresponding to the loss of the glucuronide, this is, to M1 (Fragment 1, *m/z* 370). The complete fragmentation of M1-Gluc can be checked in **Table S2**, and the MS/MS spectra in **Figure S38**.

3.4.1. Metabolic behaviour

Once all the AMB-FUBINACA metabolites were identified, the metabolic pathway for this SC was proposed (**Figure 4**). Similarly to 5F-APP-PICA, the prevalence of AMB-FUBINACA and its metabolites was studied, normalizing all the responses to the response obtained for the parent compound at 0 min (**Figure 5**). Also here, a limitation is that similar ionization and fragmentation efficiencies are assumed for the main compound and its metabolites.

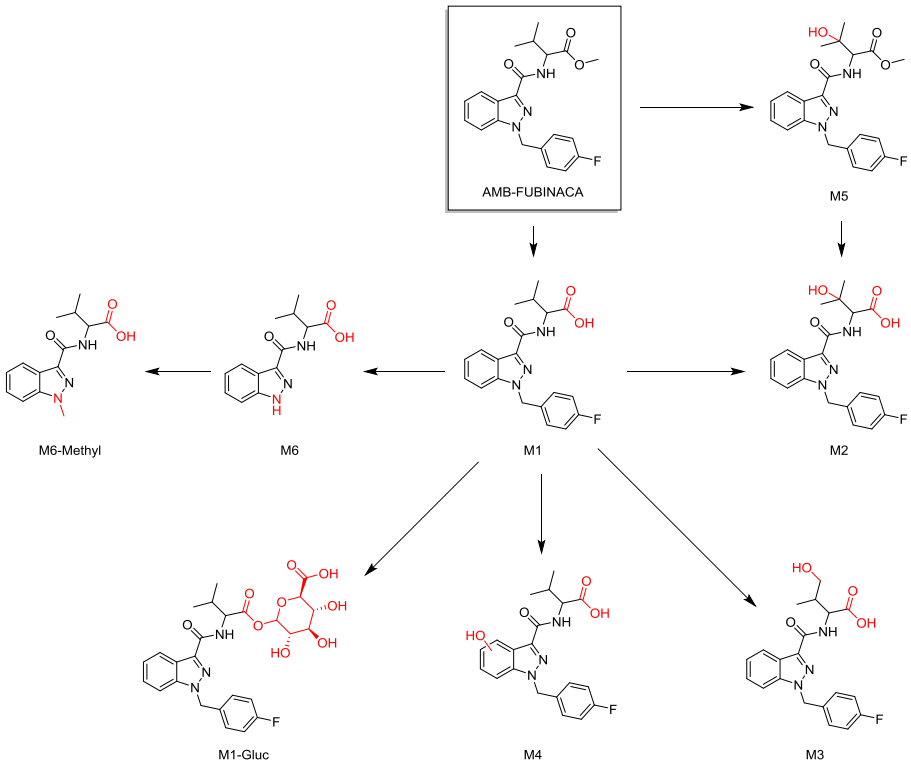


Figure 4. Proposed metabolic pathway for AMB-FUBINACA, based on the metabolites elucidated by UHPLC-HRMS.

Metabolic biotransformations were focused on the increment of polarity of the compound, by dealkylations and hydroxylations. The most abundant metabolite was M1, produced after *O*-demethylation of AMB-FUBINACA. The slight increase in relative response of M1 after 60 min could be owing to small variations in the response of the instrument. The *O*-dealkylation has already been

reported for other SCs with terminal ester moieties, such as MDMB-CHMICA [32]. M1 seems to be the precursor compound for the phase I hydroxylated metabolites M2-4. The hydroxylation after an *O*-demethylation has been described in literature, but only in the alkyl moiety (isopropyl for AMB-FUBINACA, and isobutyl for MDMB-CHMICA [32]) positioned between the amide and ester groups. For AMB-FUBINACA, three different positions for hydroxylation could be deduced, based on the observed fragmentation: hydroxylation in each one of the methyl groups (M3) and in the tertiary carbon (M2) of the isopropyl group; for MDMB-CHMICA, instead, the position of the hydroxyl group could not be established unequivocally by the authors [32]. In the case of M4, the hydroxylation took place on the indazole ring. M5 is the result of an indazole hydroxylation directly from the parent compound. So, M5 could also be metabolized into M2 following an *O*-demethylation process. Regarding M6, this metabolite was produced after *N*-dealkylation of M1. This biotransformation has been observed for SCs with an indazole ring and an *N*-fluorobenzyl moiety, such as ADB-FUBINACA and AB-FUBINACA [26,44]. Finally, an additional potential metabolite was obtained after N-methylation of M6 (M6-Methyl), as either an enzymatic reaction or artefact formation. No information about the *N*-methylation of related drugs has been found. *N*-methylation of amines are common artefacts when working with methanolic solutions [49], particularly at elevated temperatures. In the present study, the compounds were dissolved in methanol and later incubated at a low final methanolic concentration level of 0.3%. The quenching solution and mobile phases were acetonitrile-based which should not form *N*-methyl artefacts. In order to determine whether M6-Methyl is an artefact or a metabolite, the experiment should be repeated using CD_3OH as dissolving agent to observe if there is a corresponding accurate mass shift. Additional biotransformations described for other imidazole cannabinoids, such as amide *N*-dealkylation or polyhydroxylation, were not found for AMB-FUBINACA [26,44].

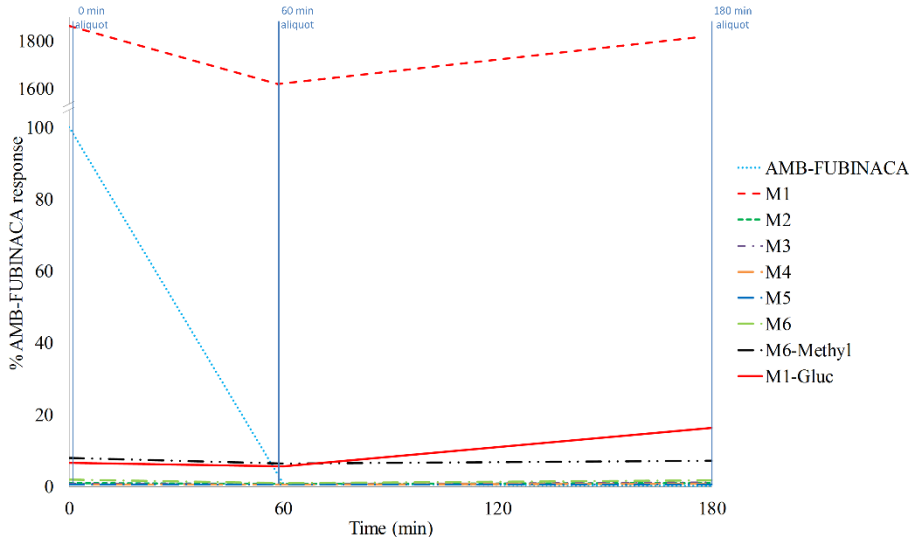


Figure 5. Prevalence of AMB-FUBINACA and its metabolites in incubation over time. Signals are relative to those obtained for AMB-FUBINACA at 0 min.

AMB-FUBINACA was metabolised extremely fast. At 0 min, M1 represented near 1900% of parent response, illustrating that the *O*-demethylation is the most important and rapid biotransformation. *O*-demethylated metabolite represented less than 1% in the biological blank (incubation without hepatocytes), indicating that this metabolite is not an impurity from the compound or a hydrolysis product. Two hypotheses could explain the huge relative response observed for M1 at 0 min. One reason could be related to the little delay (2-3 min) between the incubation starting and the aliquot collection times, which could be enough for the *O*-demethylation reaction occurring. The second one could be the much higher efficient ionisation of M1 respect to parent compound. At 60 min, only 0.5% of AMB-FUBINACA remained, as can be observed in **Figure 5**. M1-Gluc increased over time, until 16% at 180 min, as expected, given the fact that M1 is the most important compound present in the incubation mixture. The other metabolites (M2-M5) were below 1% at 180 min, and only M6 (1.6% at 180 min) and M6-Methyl (7.2% at 180 min) yielded a noteworthy response in the incubations. It could be possible that other reactions might be more prominent

but the resulting metabolites might not give a good MS response. Therefore, based on MS response, M1 would be the most suitable metabolite to be considered as a biomarker for the determination of AMB-FUBINACA consumption in real samples.

In a recent report where AMB-FUBINACA was associated with a massive intoxication [50], the parent compound was not detected in blood while the carboxylic metabolite was detected at high concentrations in the urine samples. During the processing of this manuscript, Xu and colleagues also reported the metabolic profiles of AMB-FUBINACA [51] using both human liver microsome and zebrafish models. Although in this work, a higher number of metabolites was found, in both cases the main metabolic pathways of the phase I metabolism included ester hydrolysis, hydroxylation and a combination of both processes, while glucuronidation served as the main metabolic pathway of the phase II metabolism.

3.5. Potency and efficacy of synthetic cannabinoids

The EC_{50} and E_{max} values were estimated for 5F-APP-PICA and AMB-FUBINACA. The EC_{50} value of 5F-APP-PICA at CB1 could not be estimated as it did not reach a plateau within the tested concentration range, however a maximal effect of 88.9% (relative to JWH-018) at 10 μM was attained, indicating that even very high concentrations of 5F-APP-PICA only result in a partial recruitment of βarr2 to CB1. This weak activity of 5F-APP-PICA at CB1 might be the reason why this SC has not been associated with overdose cases so far. However, the correlation between the signalling pathway (in our case: βarr2 recruitment to the receptor, induced by the agonist) and the effects observed in humans after intake has not been fully elucidated for any of both receptors [52]. On the other hand, 5F-APP-PICA did show activity at CB2, with an EC50 of 83.2 nM and showing an E_{max} similar to that observed for the control JWH-018 (i.e. 96.6% of the JWH-018 E_{max}) (**Table 1**).

Table 1. Half maximal effective concentration (EC_{50}) and efficacy (E_{max}) of 5F-APP-PICA, AMB-FUBINACA and JWH-018 (control) at CB1 and CB2. The range of concentrations between brackets indicates the 95% confidence interval (CI).

Compound	EC_{50} (95% CI) (nM)		E_{max} (95% CI) (%)	
	CB1	CB2	CB1	CB2
5F-APP-PICA	-	83.2 (52.6–132)	-	96.6 (86.1–107)
AMB-FUBINACA	9.84 (0.56–17.3)	2.40 (1.30-4.42)	265 (237–292)	153 (139–167)
JWH-018 (control)	41.0 (33.4–50.3)	12.3 (9.89–15.4)	99.3 (95.8–103)	104 (100–108)

To the best of the authors' knowledge, there are no reference concentrations of 5F-APP-PICA in blood, so it is not possible to extrapolate our EC_{50} value to an *in vivo* situation.

With EC_{50} values of 9.84 nM and 2.40 nM for CB1 and CB2, respectively, AMB-FUBINACA was much more potent than the control JWH-018 at both receptors, for which EC_{50} values of 41.0 and 12.3 were obtained 41. Interestingly, also the E_{max} values for this SC are substantially higher than that observed for JWH-018, especially at CB1 (approximately 2.5 fold). Therefore, AMB-FUBINACA is not only more potent, but it also generates a stronger response at CB1 than the reference compound JWH-018 at high concentrations. Although it is difficult to compare EC_{50} values from different assays (due to different experimental setups), our low nanomolar values are in line with those found in other reports investigating the *in vitro* an *in vivo* activity of AMB-FUBINACA [53,54]. More particularly, Banister et al. and Gamage et al. estimated the EC_{50} and the E_{max} values for AMB-FUBINACA via the FLIPR® assay 53 and via the [^{35}S]GTP binding assay and via the inhibition of forskolin-stimulated cAMP production 38. Also *in vivo* data is available for AMB-FUBINACA [55]. All these data indicate that AMB-FUBINACA is a potent SC, as also reflected by the reported "Zombie" outbreak caused by AMB-FUBINACA in New York in July 2016 [50] and supported by the very high potency of the closely related ADB-FUBINACA [55]. Future work should include potency testing of the major metabolites, to reveal whether these may contribute to the (toxic) effects observed in users.

4. Conclusions

In this work, a three-step strategy has been proposed for a comprehensive SC *in vitro* study: (a) elucidation of the metabolites obtained by pooled human hepatocytes, (b) proposal of consumption biomarkers based on the obtained metabolites and their stability during incubations, and (c) estimation of the potency and efficacy based on their activation of CB1 and CB2 receptors.

This strategy has been applied to two synthetic cannabinoids, 5F-APP-PICA and AMB-FUBINACA, with a detailed explanation of the experimental procedure, data interpretation and compound elucidation. The results obtained revealed that both SCs were extensively metabolised during incubations, explaining why these synthetic drugs may hardly be found in urine as unaltered compounds. A total of 7 phase I and 5 phase II metabolites were elucidated for 5F-APP-PICA, and 6 phase I and 1 phase I metabolites for AMB-FUBINACA. Three of these metabolites are proposed as consumption biomarkers for 5F-APP-PICA, and only 1 metabolite for AMB-FUBINACA, based on the responses obtained at the end of our *in vitro* incubation studies. The CB bioassays revealed that 5F-APP-PICA does not present a high activity at CB receptors (certainly not at CB1), while AMB-FUBINACA shows a high CB1 activation potential, both in terms of potency and efficacy. The above can be linked to intoxication cases reported for AMB-FUBINACA and the absence of cases for 5F-APP-PICA.

The proposed methodology can be applied to the study of other cannabinoids. The accurate and detailed explanation of compound elucidation, based on HRMS data, included in this work will be useful for forensic laboratories, as many cannabinoids present similar fragmentation pathways.

Acknowledgements

D. Fabregat-Safont, J.V. Sancho, F. Hernández and M. Ibáñez acknowledge financial support from the Ministerio de Economía y Competitividad in Spain (Project CTQ2015-65603-P) and from University Jaume I (UJI-B2018-19). D. Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (Grant FPU15/02033), and for the financial support received for his research stay at the University of Copenhagen (Grant EST17/00024). Carolina Noble gratefully appreciates the financial support from the National Research and Innovation Agency of Uruguay (ANII). Carolina Noble was sponsored by the Graduate School of Health and Medical Sciences of the University of Copenhagen. C. Stove acknowledges financial support from the Belgian Science Policy Office (NPSSAY) and from the Ghent University Research Council (grants n° 01N00814 and 01J15517).

Conflict of interest The authors declare that they have no competing interests.

References

1. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2018. EMCDDA Publ. (2018). doi:10.2810/88175

2. Lam, R. P. K. et al. Supraventricular tachycardia and acute confusion following ingestion of e-cigarette fluid containing AB-FUBINACA and ADB-FUBINACA: a case report with quantitative analysis of serum drug concentrations. Clin. Toxicol. 55, 662–667 (2017).

3. Fattore, L. & Fratta, W. Beyond THC: The New Generation of Cannabinoid Designer Drugs. Front. Behav. Neurosci. 5, 1–12 (2011).

4. Ibáñez, M. et al. Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends. J. Mass Spectrom. 48, 685–694 (2013).

5. Gottardo, R. et al. Direct screening of herbal blends for new synthetic cannabinoids by MALDI-TOF MS. J. Mass Spectrom. 141–146 (2012). doi:10.1002/jms.2036

6. Bijlsma, L. et al. Mass spectrometric identification and structural analysis of the third-generation synthetic cannabinoids on the UK market since the 2013 legislative ban. Forensic Toxicol. 35, 376–388 (2017).

7. Moore, K. N., Garvin, D., Thomas, B. F. & Grabenauer, M. Identification of Eight Synthetic Cannabinoids, Including 5F-AKB48 in Seized Herbal Products Using DART-TOF-MS and LC-QTOF-MS as Nontargeted Screening Methods. J. Forensic Sci. 1–8 (2017). doi:10.1111/1556-4029.13367

8. Liu, C., Jia, W., Hua, Z. & Qian, Z. Identification and analytical characterization of six synthetic cannabinoids NNL-3, 5F-NPB-22-7 N , 5F-AKB-48-7 N , 5F-EDMB-PINACA, EMB-FUBINACA, and EG-018. Drug Test. Anal. (2017). doi:10.1002/dta.2160

9. Lobo Vicente, J. et al. Systematic analytical characterization of new psychoactive substances: A case study. Forensic Sci. Int. 265, 107–115 (2016).

10. Liechti, M. E. Novel psychoactive substances (designer drugs): Overview and pharmacology of modulators of monoamine signalling. Swiss Med. Wkly. 145, 1–12 (2015).

11. Pertwee, R. G. The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ9-tetrahydrocannabinol, cannabidiol and Δ9-tetrahydrocannabivarin. Br. J. Pharmacol. 199–215 (2008).

12. Tournebize, J., Gibaja, V. & Kahn, J.-P. Acute effects of synthetic cannabinoids: Update 2015. Subst. Abus. 7077, 1–23 (2016).

13. Yamagishi, I. et al. A case of intoxication with a mixture of synthetic cannabinoids EAM-2201, AB-PINACA and AB-FUBINACA, and a synthetic cathinone α-PVP. Leg. Med. 35, 44–49 (2018).

14. Harris, C. R. & Brown, A. Synthetic Cannabinoid Intoxication: A Case Series and Review. J. Emerg. Med. 44, 360–366 (2013).

15. Kusano, M. et al. Fatal intoxication by 5F–ADB and diphenidine: Detection, quantification, and investigation of their main metabolic pathways in humans by LC/MS/MS and LC/Q-TOFMS. Drug Test. Anal. 10, 284–293 (2018).

16. Barceló, B. et al. Acute intoxication caused by synthetic cannabinoids 5F-ADB and MMB-2201: A case series. Forensic Sci. Int. 273, e10–e14 (2017).

17. Lovrecic, B. & Lovrecic, M. Novel psychoactive synthetic cannabinoids and synthetic cathinones: the never-ending story of potential clinical toxicity. Heroin Addict Relat Clin Probl 20, 13–24 (2018).

18. Zattera, L., Errasti, J. & Supervía, A. Intoxicación por el cannabinoide sintético 5-fluoro-ABD, adquirido como ketamina. Med. Clin. (Barc). 151, 168 (2018).

19. Ninnemann, A. L., Lechner, W. V., Borges, A. & Lejuez, C. W. Synthetic cannabinoids to avoid urine drug screens: Implications for contingency management and other treatments for drug dependence. Addict. Behav. 63, 72–73 (2016).

20. Diao, X. & Huestis, M. Approaches, Challenges, and Advances in Metabolism of New Synthetic Cannabinoids and Identification of Optimal Urinary Marker Metabolites. Clin. Pharmacol. Ther. 101, 239–253 (2017).

21. Jang, M., Shin, I., Kim, J. & Yang, W. Simultaneous quantification of 37 synthetic cannabinoid metabolites in human urine by liquid chromatography-tandem mass spectrometry. Forensic Toxicol. 33, 221–234 (2015).

22. Scheidweiler, K. B., Jarvis, M. J. Y. & Huestis, M. A. Nontargeted SWATH acquisition for identifying 47 synthetic cannabinoid metabolites in human urine by liquid chromatography-high-resolution tandem mass spectrometry. Anal. Bioanal. Chem. 407, 883–897 (2015).

23. Jang, M. et al. Determination of urinary metabolites of XLR-11 by liquid chromatography–quadrupole time-of-flight mass spectrometry. Anal. Bioanal. Chem. 408, 503–516 (2016).

24. Castaneto, M. S. et al. Identification of AB-FUBINACA metabolites in human hepatocytes and urine using high-resolution mass spectrometry. Forensic Toxicol. 33, 295–310 (2015).

25. Diao, X. et al. In vitro and in vivo human metabolism of a new synthetic cannabinoid NM-2201 (CBL-2201). Forensic Toxicol. 35, 20–32 (2017).

26. Vikingsson, S., Gréen, H., Brinkhagen, L., Mukhtar, S. & Josefsson, M. Identification of AB-FUBINACA metabolites in authentic urine samples suitable as urinary markers of drug intake using liquid chromatography quadrupole tandem time of flight mass spectrometry. Drug Test. Anal. 8, 950–956 (2016).

27. Fabregat-Safont, D. et al. Proposal of 5-methoxy-N-methyl-N-isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. J. Chromatogr. A 1508, (2017).

28. Savchuk, S. et al. In vivo metabolism of the new synthetic cannabinoid APINAC in rats by GC–MS and LC–QTOF-MS. Forensic Toxicol. 35, 359–368 (2017).

29. Richter, L. H. J., Maurer, H. H. & Meyer, M. R. Metabolic fate of the new synthetic cannabinoid 7'N-5F-ADB in rat, human, and pooled human S9 studied by means of hyphenated high-resolution mass spectrometry. Drug Test. Anal. 1–13 (2018). doi:10.1002/dta.2493

30. Kevin, R. C. et al. In vitro and in vivo pharmacokinetics and metabolism of synthetic cannabinoids CUMYL-PICA and 5F-CUMYL-PICA. Forensic Toxicol. 35, 333–347 (2017).

31. Dalvie, D. et al. Assessment of three human in vitro systems in the generation of major human excretory and circulating metabolites. Chem. Res. Toxicol. 22, 357–368 (2009).

32. Franz, F., Angerer, V., Moosmann, B. & Auwärter, V. Phase I metabolism of the highly potent synthetic cannabinoid MDMB-CHMICA and detection in human urine samples. Drug Test. Anal. 9, 744–753 (2017).

33. Diao, X., Wohlfarth, A., Pang, S., Scheidweiler, K. B. & Huestis, M. A. High-resolution mass spectrometry for characterizing the metabolism of synthetic cannabinoid THJ-018 and its 5-fluoro analog THJ-2201 after incubation in human hepatocytes. Clin. Chem. 62, 157–169 (2016).

34. Wohlfarth, A. et al. First metabolic profile of XLR-11, a novel synthetic cannabinoid, obtained by using human hepatocytes and high-resolution mass spectrometry. Clin. Chem. 59, 1638–1648 (2013).

35. Cannaert, A., Storme, J., Franz, F., Auwärter, V. & Stove, C. P. Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. Anal. Chem. 88, 11476–11485 (2016).

36. Wiley, J. L. et al. AB-CHMINACA, AB-PINACA, and FUBIMINA: Affinity and Potency of Novel Synthetic Cannabinoids in Producing 9-Tetrahydrocannabinol-Like Effects in Mice. J. Pharmacol. Exp. Ther. 354, 328–339 (2015).

37. Banister, S. D. et al. Effects of Bioisosteric Fluorine in Synthetic Cannabinoid Designer Drugs JWH-018, AM-2201, UR-144, XLR-11, PB-22, 5F-PB-22, APICA, and STS-135. ACS Chem. Neurosci. 6, 1445–1458 (2015).

38. Gamage, T. F. et al. Molecular and Behavioral Pharmacological Characterization of Abused Synthetic Cannabinoids MMB- and MDMB-FUBINACA, MN-18, NNEI, CUMYL-PICA, and 5-Fluoro-CUMYL-PICA. J. Pharmacol. Exp. Ther. 365, 437–446 (2018).

39. European Monitoring Centre for Drugs and Drug Addiction. Fentanils and synthetic cannabinoids : driving greater complexity into the drug situation. (2018). doi:10.2810/006358

40. Cannaert, A., Franz, F., Auwärter, V. & Stove, C. P. Activity-Based Detection of Consumption of Synthetic Cannabinoids in Authentic Urine Samples Using a Stable Cannabinoid Reporter System. Anal. Chem. 89, 9527–9536 (2017).

41. Noble, C., Cannaert, A., Linnet, K. & Stove, C. P. Application of an activity-based receptor bioassay to investigate the in vitro activity of selected indole- and indazole-3-carboxamide-based synthetic cannabinoids at CB1 and CB2 receptors. Drug Test. Anal. 1–11 (2018). doi:10.1002/dta.2517

42. Pozo, O. 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. Drug Metab. Dispos. 43, 248–257 (2014).

43. Ibáñez, M. et al. Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry. Anal. Bioanal. Chem. 408, 151–164 (2016).

44. Carlier, J., Diao, X., Wohlfarth, A., Scheidweiler, K. & Huestis, M. A. In Vitro Metabolite Profiling of ADB-FUBINACA, A New Synthetic Cannabinoid. Curr. Neuropharmacol. 15, 682–691 (2017).

45. Alechaga, É., Moyano, E. & Galceran, M. T. Ion-molecule adduct formation in tandem mass spectrometry. Anal. Bioanal. Chem. 408, 1269–1277 (2016).

46. Kim, J.-H. et al. Targeted and non-targeted metabolite identification of MAM-2201 in human, mouse, and rat hepatocytes. Drug Test. Anal. 10, 1328–1335 (2018).

47. Wohlfarth, A. et al. Pentylindole/Pentylindazole Synthetic Cannabinoids and Their 5-Fluoro Analogs Produce Different Primary Metabolites: Metabolite Profiling for AB-PINACA and 5F-AB-PINACA. AAPS J. 17, 660–677 (2015).

48. Andersson, M., Diao, X., Wohlfarth, A., Scheidweiler, K. B. & Huestis, M. A. Metabolic profiling of new synthetic cannabinoids AMB and 5F-AMB by human hepatocyte and liver microsome incubations and high-resolution mass spectrometry. Rapid Commun. Mass Spectrom. 30, 1067–1078 (2016).

49. Sauerschnig, C., Doppler, M., Bueschl, C. & Schuhmacher, R. Methanol Generates Numerous Artifacts during Sample Extraction and Storage of Extracts in Metabolomics Research. Metabolites 8, 1 (2017).

50. Adams, A. J. et al. "Zombie" Outbreak Caused by the Synthetic Cannabinoid AMB-FUBINACA in New York. N. Engl. J. Med. 376, 235–242 (2017).

51. Xu, D. et al. Analysis of AMB-FUBINACA Biotransformation Pathways in Human Liver Microsome and Zebrafish Systems by Liquid Chromatography-High Resolution Mass Spectrometry. Front. Chem. 7, 1–9 (2019).

52. Ibsen, M. S., Connor, M. & Glass, M. Cannabinoid CB1 and CB2 Receptor Signaling and Bias. Cannabis Cannabinoid Res. 2, 48–60 (2017).

53. Banister, S. D. et al. Pharmacology of Valinate and tert -Leucinate Synthetic Cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and Their Analogues. ACS Chem. Neurosci. 7, 1241–1254 (2016).

54. Gatch, M. B. & Forster, M. J. Cannabinoid-like effects of five novel carboxamide synthetic cannabinoids. Neurotoxicology 70, 72–79 (2019).

55. Wouters, E., Mogler, L., Cannaert, A., Auwärter, V. & Stove, C. Functional evaluation of carboxy metabolites of synthetic cannabinoid receptor agonists featuring scaffolds based on L- valine or L- tert - leucine. Drug Test. Anal. dta.2607 (2019). doi:10.1002/dta.2607

MS/MS spectra

5F-APP-PICA and its metabolites

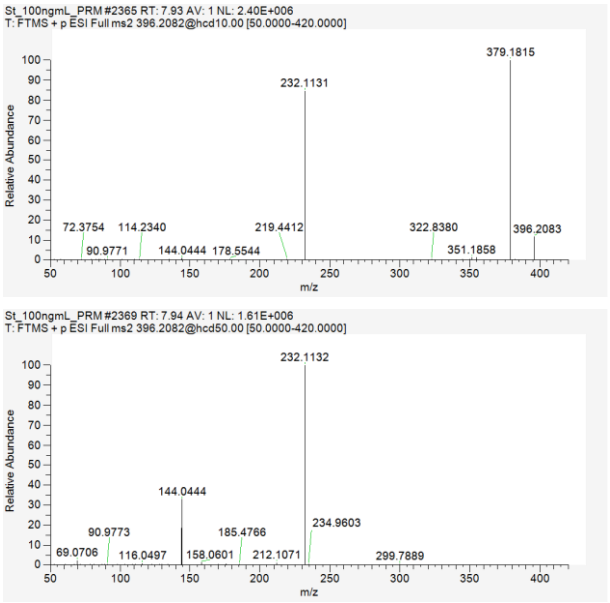


Figure S1. MS/MS spectra of 5F-APP-PICA at 10 eV (top) and 50 eV (bottom) collision energy.

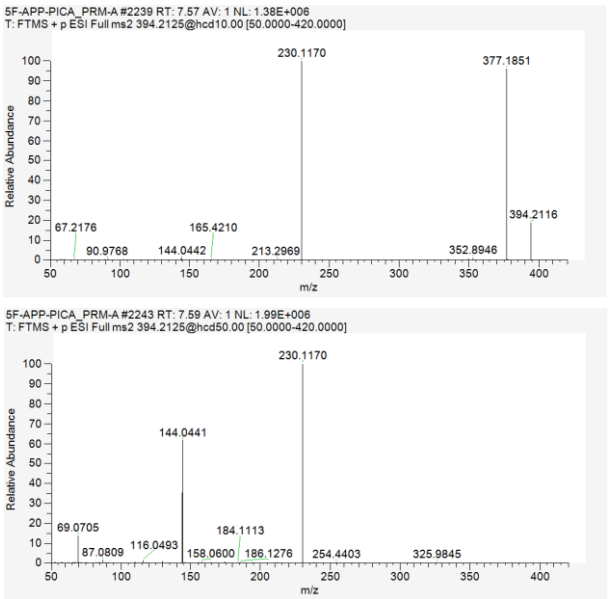


Figure S5. MS/MS spectra of 5F-APP-PICA M1 at 10 eV (top) and 50 eV (bottom) collision energy.

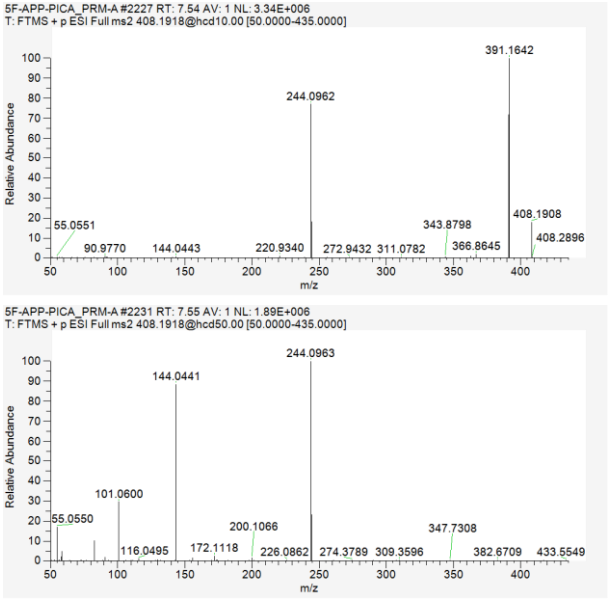


Figure S7. MS/MS spectra of 5F-APP-PICA M2 at 10 eV (top) and 50 eV (bottom) collision energy.

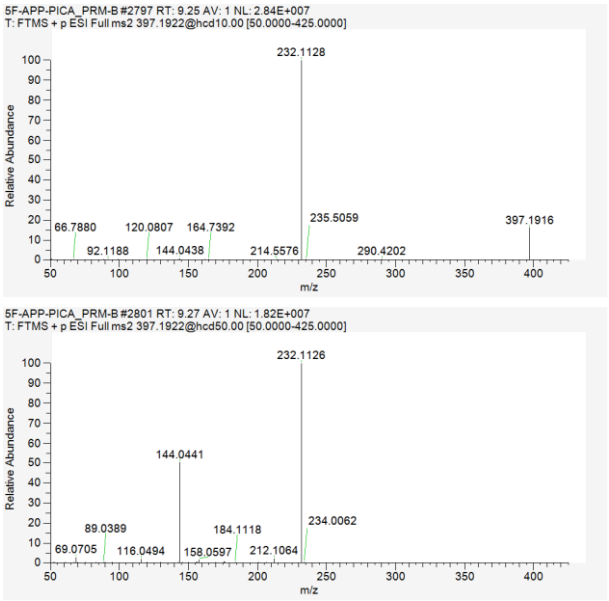


Figure S9. MS/MS spectra of 5F-APP-PICA M3 at 10 eV (top) and 50 eV (bottom) collision energy.

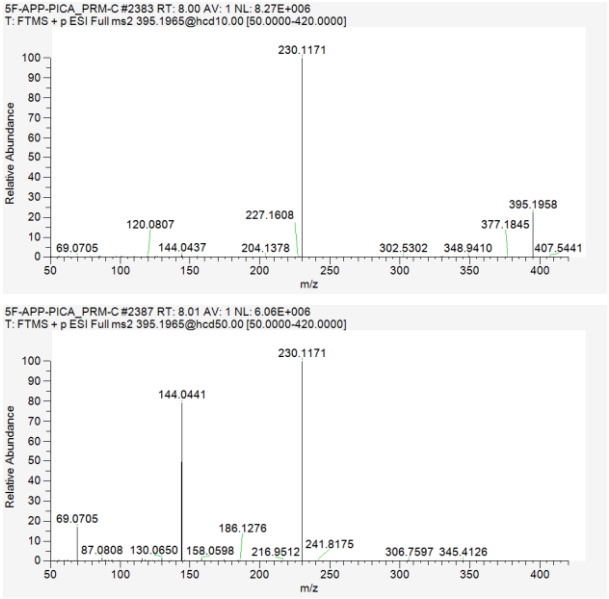


Figure S11. MS/MS spectra of 5F-APP-PICA M4 at 10 eV (top) and 50 eV (bottom) collision energy.

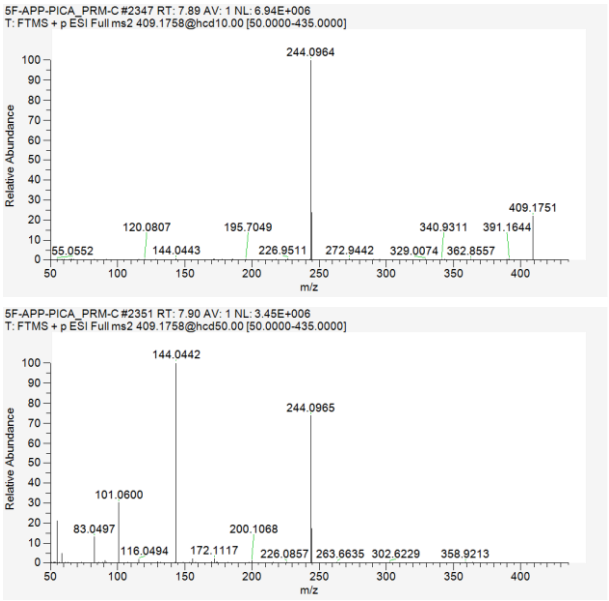


Figure S13. MS/MS spectra of 5F-APP-PICA M5 at 10 eV (top) and 50 eV (bottom) collision energy.

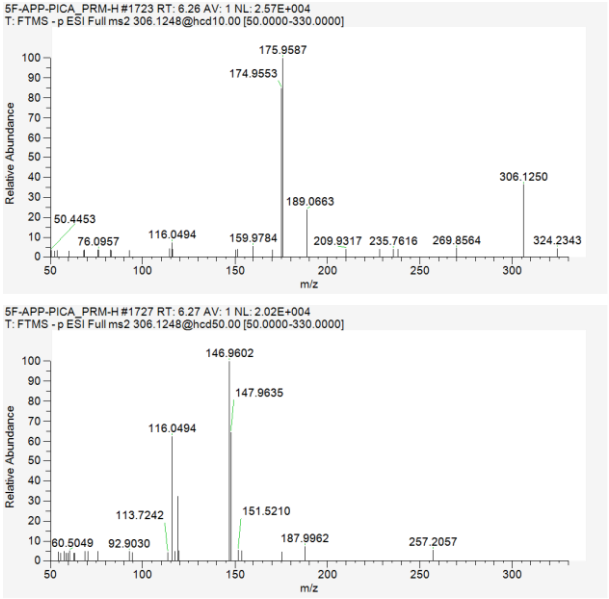


Figure S15. MS/MS spectra of 5F-APP-PICA M6 at 10 eV (top) and 50 eV (bottom) collision energy.

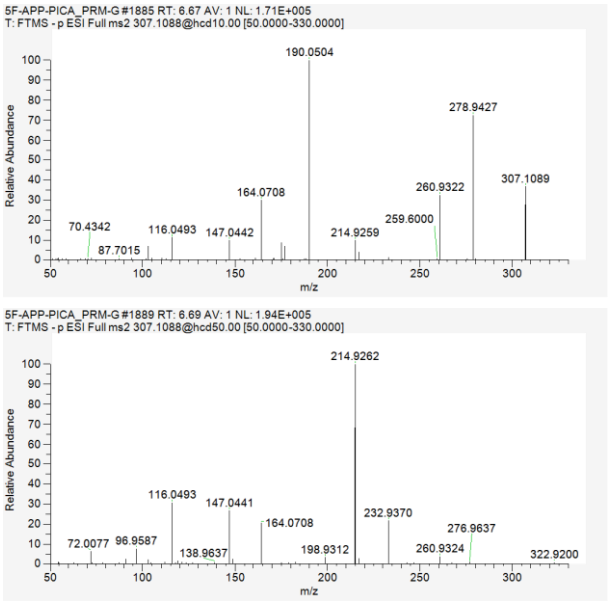


Figure S17. MS/MS spectra of 5F-APP-PICA M7 at 10 eV (top) and 50 eV (bottom) collision energy.

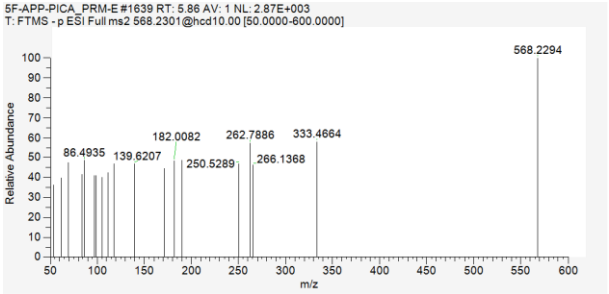


Figure S19. MS/MS spectrum of 5F-APP-PICA M1-Gluc at 10 eV collision energy.

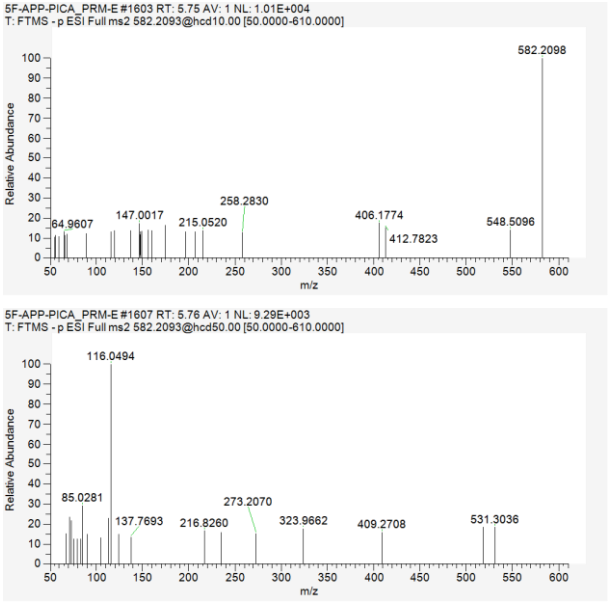


Figure S20. MS/MS spectra of 5F-APP-PICA M2-Gluc at 10 eV (top) and 50 eV (bottom) collision energy.

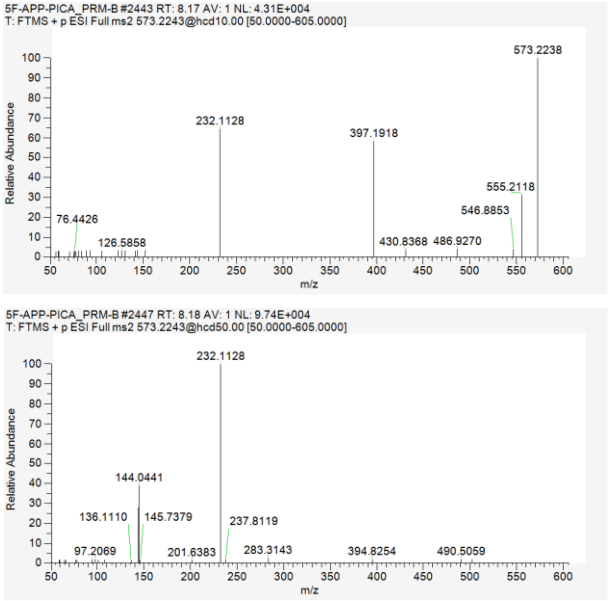


Figure S21. MS/MS spectra of 5F-APP-PICA M3-Gluc at 10 eV (top) and 50 eV (bottom) collision energy.

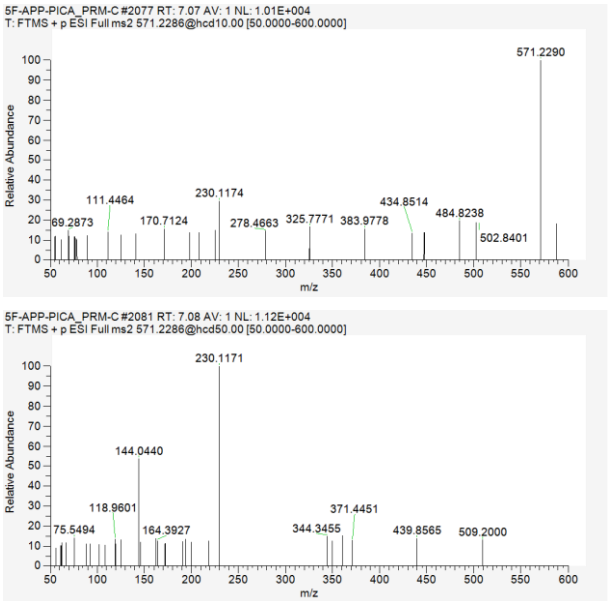


Figure S22. MS/MS spectra of 5F-APP-PICA M4-Gluc at 10 eV (top) and 50 eV (bottom) collision energy.

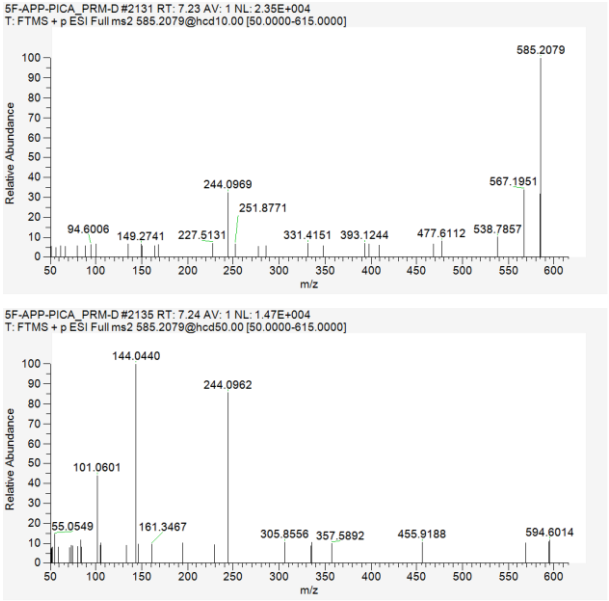


Figure S23. MS/MS spectra of 5F-APP-PICA M5-Gluc at 10 eV (top) and 50 eV (bottom) collision energy.

AMB-FUBINACA and its metabolites

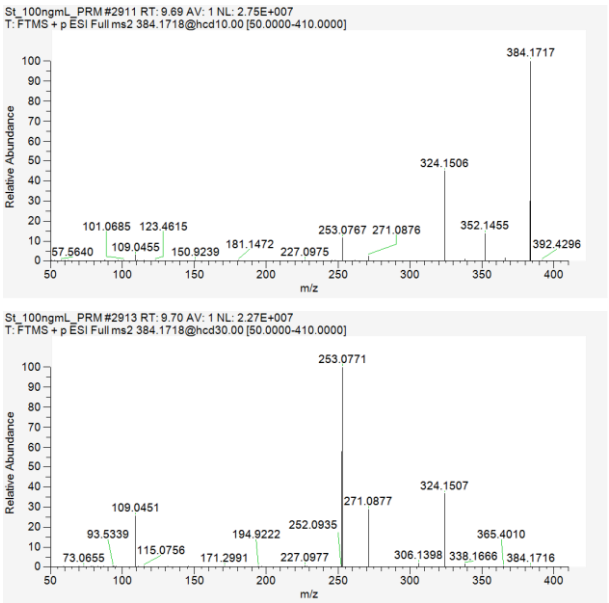


Figure S2. MS/MS spectra of AMB-FUBINACA at 10 eV (top) and 30 eV (bottom) collision energy.

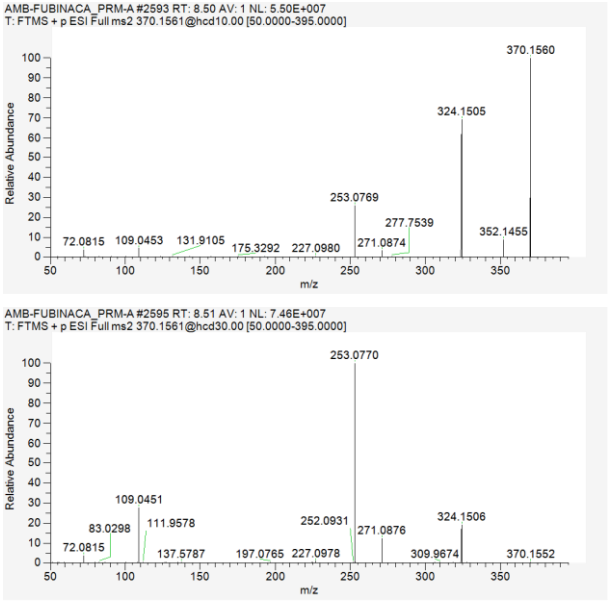


Figure S24. MS/MS spectra of AMB-FUBINACA M1 at 10 eV (top) and 30 eV (bottom) collision energy.

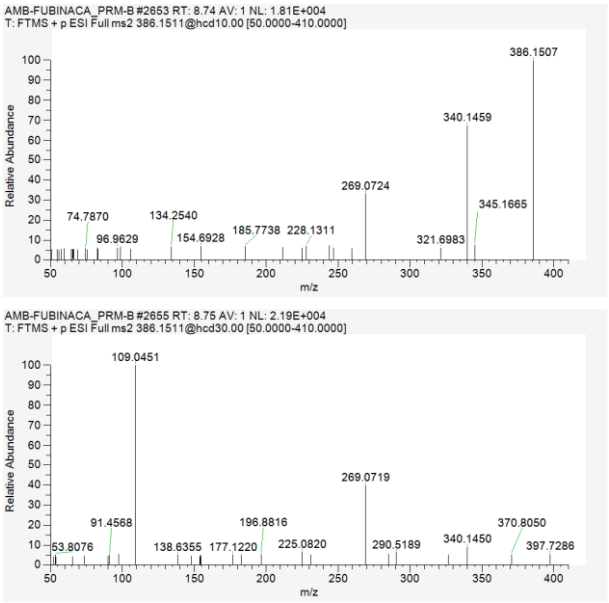


Figure S26. MS/MS spectra of AMB-FUBINACA M4 at 10 eV (top) and 30 eV (bottom) collision energy.

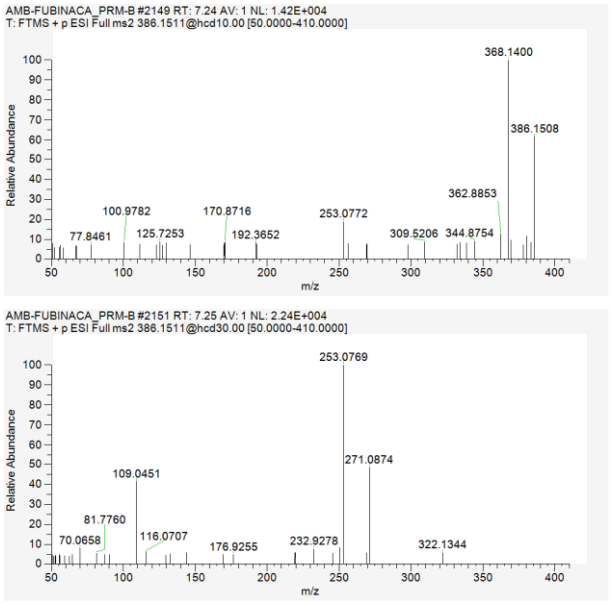


Figure S28. MS/MS spectra of AMB-FUBINACA M2 at 10 eV (top) and 30 eV (bottom) collision energy.

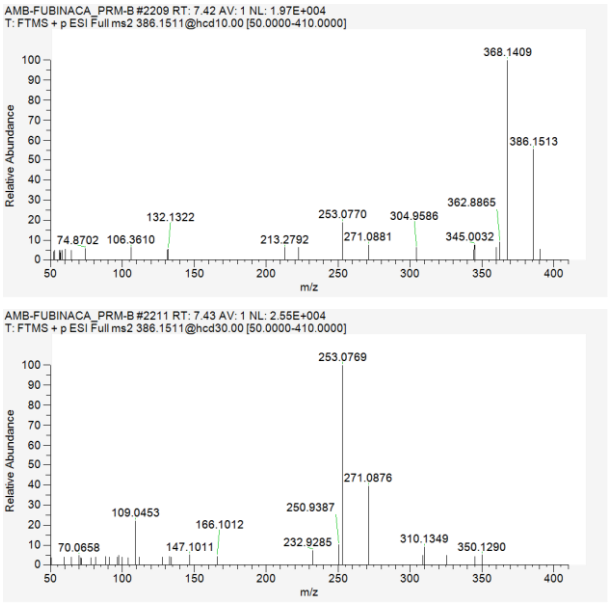


Figure S30. MS/MS spectra of AMB-FUBINACA M3 at 10 eV (top) and 30 eV (bottom) collision energy.

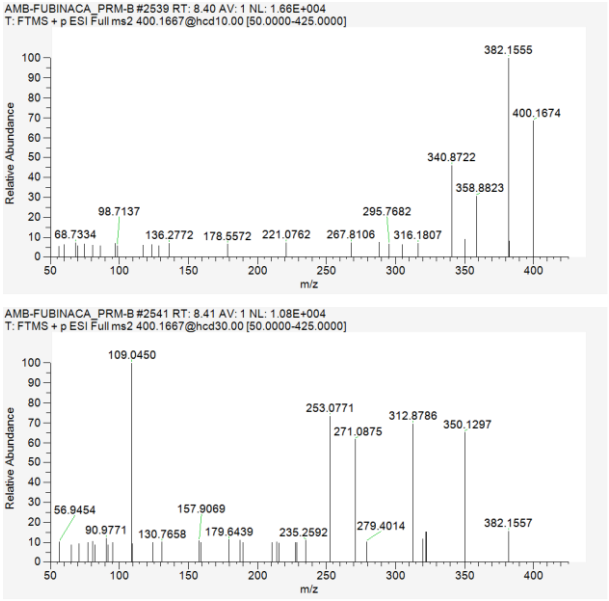


Figure S32. MS/MS spectra of AMB-FUBINACA M5 at 10 eV (top) and 30 eV (bottom) collision energy.

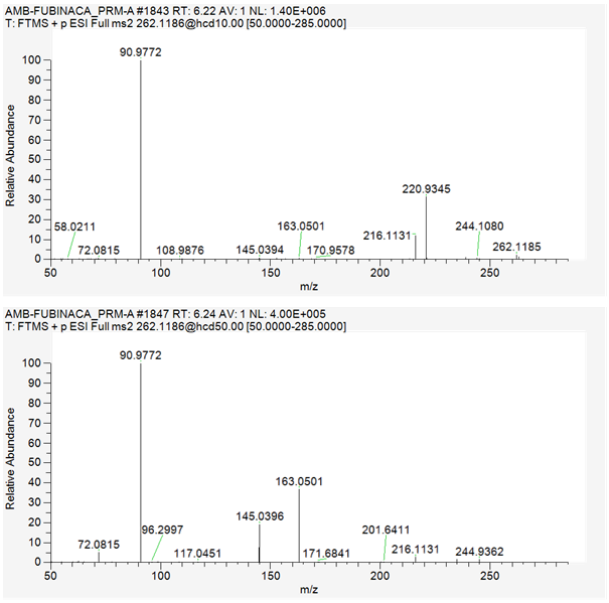


Figure S34. MS/MS spectra of AMB-FUBINACA M6 at 10 eV (top) and 50 eV (bottom) collision energy.

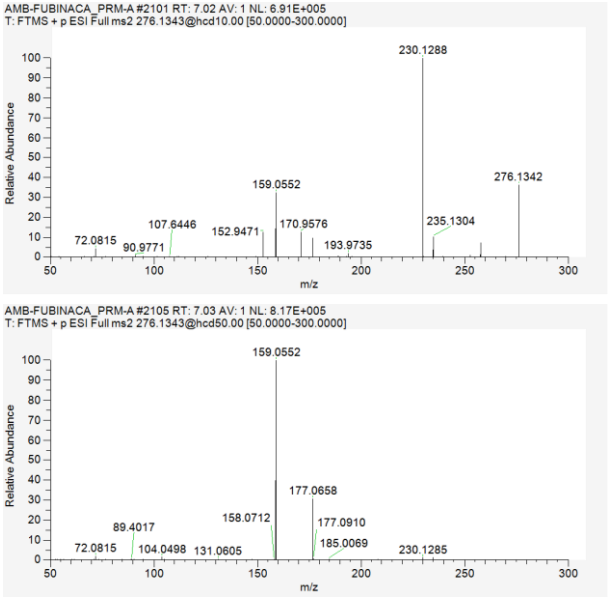


Figure S36. MS/MS spectra of AMB-FUBINACA M6-Methyl at 10 eV (top) and 50 eV (bottom) collision energy.

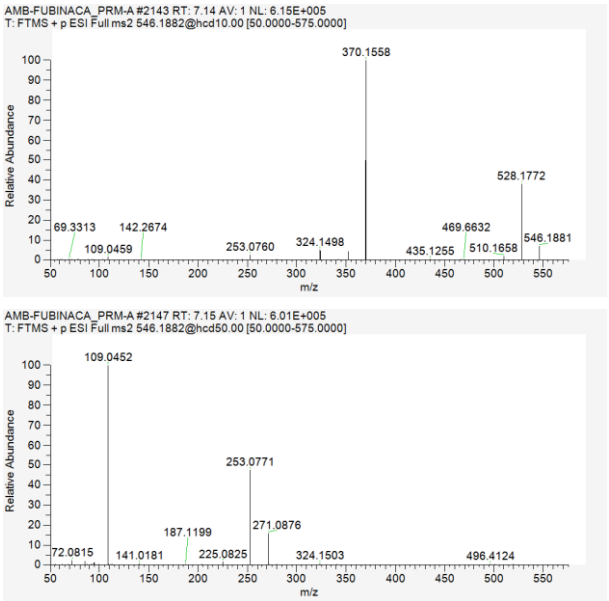


Figure S38. MS/MS spectra of AMB-FUBINACA M1-Gluc at 10 eV (top) and 50 eV (bottom) collision energy.

4.3. Research article VIII

Journal of Pharmaceutical Analysis 10 (2020) 147–156

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

journal homepage: www.elsevier.com/locate/jpa

Original article

Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation

David Fabregat-Safont [a], Marie Mardal [b, c], Juan V. Sancho [a], Félix Hernández [a], Kristian Linnet [b], María Ibáñez [a, *]

[a] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain
[b] Department of Forensic Medicine, Section of Forensic Chemistry, Faculty of Health and Medicinal Sciences, University of Copenhagen, Frederik V's vej 11, 2100, København Ø, Denmark
[c] Department of Occupational and Environmental Medicine, University Hospital of Northern Norway, Sykehusvegen, Tromsoe, Norway

ARTICLE INFO

Article history:
Received 2 July 2019
Received in revised form
23 December 2019
Accepted 27 December 2019
Available online 28 December 2019

Keywords:
Synthetic cathinones
5-PPDi
Metabolite identification
In vitro metabolism
Hepatocyte incubation
High resolution mass spectrometry

ABSTRACT

Synthetic cathinones are new psychoactive substances that represent a health risk worldwide. For most of the 130 reported compounds, information about toxicology and/or metabolism is not available, which hampers their detection (and subsequent medical treatment) in intoxication cases. The principles of forensic analytical chemistry and the use of powerful analytical techniques are indispensable for stablishing the most appropriate biomarkers for these substances. Human metabolic fate of synthetic cathinones can be assessed by the analysis of urine and blood obtained from authentic consumers; however, this type of samples is limited and difficult to access. In this work, the metabolic behaviour of three synthetic cathinones (4-CEC, 4-CPrC and 5-PPDi) and one amphetamine (3-FEA) has been evaluated by incubation with pooled human hepatocytes and metabolite identification has been performed by high-resolution mass spectrometry. This *in vitro* approach has previously shown its feasibility for obtaining excretory human metabolites. 4-CEC and 3-FEA were not metabolised, and for 4-CPrC only two minor metabolites were obtained. On the contrary, for the recently reported 5-PPDi, twelve phase I metabolites were elucidated. Up to our knowledge, this is the first metabolic study of an indanyl-cathinone. Data reported in this paper will allow the detection of these synthetic stimulants in intoxication cases, and will facilitate future research on the metabolic behaviour of other indanyl-based cathinones.

1. Introduction

Synthetic cathinones are one of the largest groups of new psychoactive substances (NPS), with around 130 compounds being monitored by the European Monitoring of Drug and Drug Addiction (EMCDDA) [1]. They constitute the second group most frequently seized in Europe (around 33% of seizures containing NPS), being α-PVP, 4-CMC, 3-CMC, 4-CEC and 3-MMC the most commonly found compounds [1]. In 2017, 12 novel synthetic cathinones were detected for the first time in Europe [1], which suggests the continuous expansion of this non-legal market.

The fast emerging of these NPS is evidenced by a number of research papers reporting novel cathinones [2–8]. This fact remarks the need of analytical strategies for monitoring the large number of cathinones already reported but also the constant apparition of new ones. Regarding consumption products, high-resolution mass spectrometry (HRMS) is one of the most powerful techniques for cathinones identification, as it allows the non-target and suspect analysis based on full-spectrum mass-accurate data provided by this technique [4,5,9,10]. However, facing the problem that synthetic cathinones consumption cannot be limited to only the analysis of legal highs and seizures, forensic and clinical analytical chemistry plays an important role in the monitoring of NPS in biological matrices. Several UHPLC-MS/MS and UHPLC-HRMS target methodologies have been applied for the determination of synthetic cathinones in hair [11], oral fluid [12], urine [13], and even wastewater [14]. Nevertheless, most current methodologies are focused on the determination of synthetic cathinones as unaltered compounds. The lack of information about the metabolic

Peer review under responsibility of Xi'an Jiaotong University.
* Corresponding author.
E-mail address: ibanezm@uji.es (M. Ibáñez).

https://doi.org/10.1016/j.jpha.2019.12.006

Journal of Pharmaceutical Analysis, 2020; 10:147

Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation

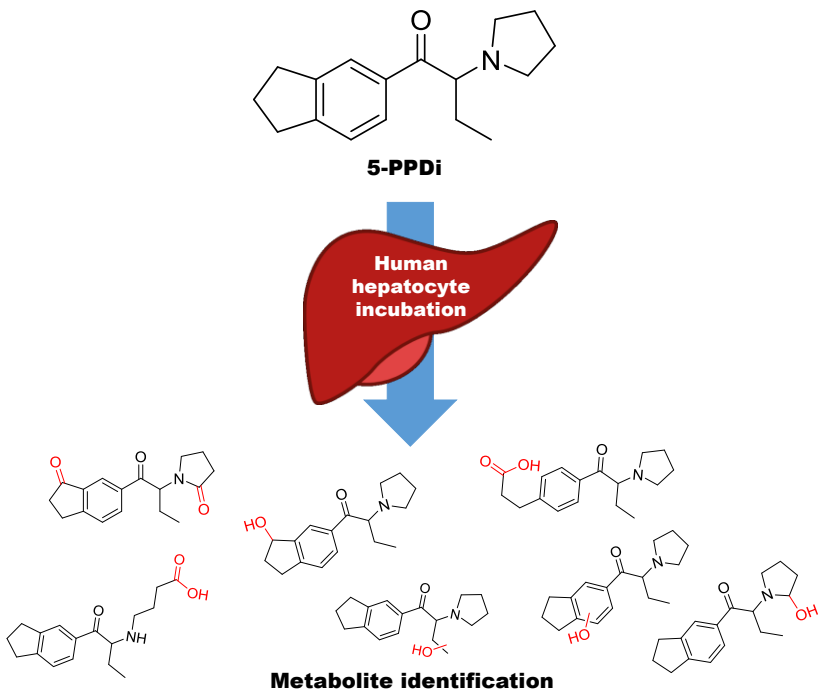
David Fabregat-Safont [a], Marie Mardal [b,c], Juan V. Sancho [a], Félix Hernández [a], Kristian Linnet [b], María Ibáñez [a,*]

[a] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain.

[b] Department of Forensic Medicine, Section of Forensic Chemistry, Faculty of Health and Medicinal Sciences, University of Copenhagen, Frederik V's vej 11, 2100 København Ø, Denmark.

[c] Department of Occupational and Environmental Medicine, University Hospital of Northen Norway, Sykehusvegen, Tromsoe, Norway.

Graphical abstract



Abstract

Synthetic cathinones are new psychoactive substances that represent a health risk worldwide. For most of the 130 reported compounds, information about toxicology and/or metabolism is not available, which hampers their detection (and subsequent medical treatment) in intoxication cases. The principles of forensic analytical chemistry and the use of powerful analytical techniques are indispensable for stablishing the most appropriate biomarkers for these substances. Human metabolic fate of synthetic cathinones can be assessed by the analysis of urine and blood obtained from authentic consumers; however, this type of samples is limited and difficult to access. In this work, the metabolic behaviour of three synthetic cathinones (4-CEC, 4-CPrC and 5-PPDi) and one amphetamine (3-FEA) has been evaluated by incubation with pooled human hepatocytes and metabolite identification has been performed by high-resolution mass spectrometry. This *in vitro* approach has previously shown its feasibility for obtaining excretory human metabolites. 4-CEC and 3-FEA were not metabolised, and for 4-CPrC only two minor metabolites were obtained. On the contrary, for the recently reported 5-PPDi, twelve phase I metabolites were elucidated. Up to our knowledge, this is the first metabolic study of an indanyl-cathinone. Data reported in this paper will allow the detection of these synthetic stimulants in intoxication cases, and will facilitate future research on the metabolic behaviour of other indanyl-based cathinones.

Keywords Synthetic cathinones; 5-PPDi; Metabolite identification; *In vitro* metabolism; Hepatocyte incubation; High resolution mass spectrometry.

1. Introduction

Synthetic cathinones is one of the largest groups of new psychoactive substances (NPS), with around 130 compounds being monitored by the European Monitoring of Drug and Drug Addiction (EMCDDA) [1]. It constitutes the second group most frequently seized in Europe (around 33% of seizures containing NPS), being α-PVP, 4-CMC, 3-CMC, 4-CEC and 3-MMC the most commonly found compounds [1]. In 2017, 12 novel synthetic cathinones were detected for the first time in Europe [1], which illustrates the continuous expansion of this non-legal market.

The fast emerging of these NPS is evidenced by the number of research papers reporting novel cathinones [2–8]. This fact remarks the need of analytical strategies for monitoring the high number of cathinones already reported but also the constant apparition of new ones. Regarding consumption products, high-resolution mass spectrometry (HRMS) is one of the most powerful techniques for cathinones identification, as it allows the non-target and suspect analysis based on full-spectrum mass-accurate data provided by this technique [4,5,9,10]. However, facing the problem of synthetic cathinones consumption cannot be limited to only the analysis of legal highs and seizures. Forensic and clinical analytical chemistry plays an important role on the monitoring of NPS in biological matrices. Several UHPLC-MS/MS and UHPLC-HRMS target methodologies have been applied for the determination of synthetic cathinones in hair [11], oral fluid [12], urine [13], and even wastewater [14]. Nevertheless, most current methodologies are focused on the determination of synthetic cathinones as unaltered compounds. The lack of information about the metabolic fate of these substances makes their detection difficult in intoxication cases, as they are commonly partially or totally metabolised. So, it is of utmost importance to study the pharmacology and metabolic behaviour of these compounds, trying to establish the most suitable biomarkers for their monitoring, especially for the most recent detected cathinones in the continuously moving "market" of NPS.

Ideally, human samples, mainly blood and urine, should be used but these samples are difficult to access and limited to authentic intoxication cases. [15–21]. As an alternative, the *in vitro* approach, using pooled human liver microsomes (pHLM), pooled human S9 fraction (pS9) or pooled human hepatocytes (pHH) can be applied to the study of human excretory metabolites [22,23]. Among them, pHH seem to provide the best realistic pattern of metabolites [24], with most of them being detected in authentic urine samples from intoxication cases [20,25,26].

In this work, incubation with pHH was applied for investigating the metabolic behaviour of three synthetic cathinones and one amphetamine: the recently reported 3,4-trimethylene-α-pyrrolidinobutiophenone (5-PPDi) [27], 4-chloro-α-pyrrolidinopropiophenone (4-CPrC or 4-chloro-PPP [3]), 4-chloroethcathinone (4-CEC [28]), and 3-fluoroethamphetamine (3-FEA [29]) (**Figure 1**). Metabolite identification was performed by UHPLC-HRMS. The results showed that 3-FEA and 4-CEC were not metabolised by pHH, while only 2 metabolites were detected for 4-CPrC. On the contrary, up to 12 phase I metabolite were elucidated for 5-PPDi. To our knowledge, this is the first study reporting the metabolic profile of an indanyl-cathinone. Data reported in this paper will allow to focus future analysis of urine from intoxication cases and hospital emergences associated to the consumption of cathinones and amphetamines.

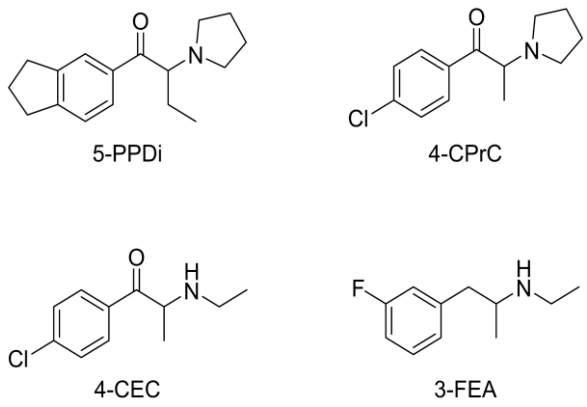


Figure 1. Structure of the three cathinones (5-PPDi, 4-CPrC and 4-CEC) and the amphetamine (3-FEA).

2. Material and methods

2.1. Reagents and chemicals

Research chemical samples containing 5-PPDi, 4-CPrC, 3-CEC and 4-FEA were kindly provided by Energy Control (Asociación Bienestar y Desarrollo, Barcelona, Spain), and compound purity was tested by nuclear magnetic resonance. MDMA-d_5 (used as internal standard) was purchased from Cerilliant (Round Rock, TX, USA). Diclofenac was purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents used in the incubation (0.4% trypan blue solution, fetal bovine serum, Leibovitz's L-15 medium, Gibco cryopreserved hepatocyte recovery medium), solvents (methanol, acetonitrile, ultrapure water), formic acid (LC–MS grade) as well as mass calibration solutions (Pierce® LTQ Velos ESI positive ion calibration solution, Pierce™ ESI negative ion calibration solution) were obtained from Fisher Scientific (Leicestershire, UK). The pooled human hepatocytes (pHH) were purchased from Lonza (Basel, Switzerland), and stored in liquid nitrogen until use.

2.2. Pooled human hepatocyte incubation

Cryopreserved pooled human hepatocytes were used in this work. Information about the incubation procedure applied can be found in literature [23].

The four tested compounds and diclofenac (used as positive control to test the feasibility of incubations) were added at a final concentration of 10 μM. Controls without the addition of hepatocytes were run simultaneously to identify hydrolysis products and artefacts. Aliquots of 20 μL were collected after 0, 60, and 180 min of incubation and mixed with 80 μL of an ice-cold acetonitrile solution containing 100 ng/mL of internal standard. The extracts were frozen until analysis.

2.3. Instrumentation

Extracts were analysed using a Dionex Ultimate 3000 UHPLC system from Thermo Scientific (Germering, Germany) coupled to a Q Exactive high resolution mass spectrometer from Thermo Scientific (Bremen, Germany) equipped with a hybrid quadrupole-Orbitrap mass analyser.

Chromatographic separation was performed using an Acquity HSS C18 1.8 μm, 2.1×150 mm analytical column from Waters (Wexford, Ireland). The mobile phase consisted of water with 5 mM ammonium formiate and 0.1% v/v formic acid (solvent A), and acetonitrile with 0.1% v/v formic acid (solvent B). Flow rate was set to 0.25 mL/min, maintaining the column at 40 °C. A 14 min gradient was used, changing the composition as follows: 5% B (0–0.5 min), 70% B (0.5–9.95 min), 99% B (9.9-10.0), where it remained isocratic for 2 min and followed by re-equilibration for 2 min. Samples were kept at 5 °C, and the injection volume was 3 μL.

HRMS was equipped with a heated electrospray ionization source (HESI-II) working in positive (ESI^+) and negative (ESI^-) ionization modes. The capillary temperature was 350 °C, and the spray voltage was 4.0 kV in ESI^+ and -4.0 kV in ESI^-. Data were acquired using data-dependent acquisition (dd-MS2, DDA) and parallel reaction monitoring (PRM, MS/MS). Full-scan data (FTMS) were collected in a scan-range of *m/z* 200–900 using a resolution of 70,000 FWHM. DDA MS/MS was acquired at a resolution of 35,000 FWHM, while PRM were acquired at 17,500 FWHM. Nitrogen was used as the collision gas at normalized collision energy (NCE) of 10, 30 or 50 eV. Data processing was performed using Compound Discoverer 2.0 software (Thermo Scientific) for a preliminary compound identification, and FreeStyle 1.3 (Thermo Scientific) for working with raw data. Further details about HRMS instrumentation can be found in literature [23].

3. Results and discussion

3.1. Analytical strategy for metabolite identification

Compound Discoverer 2.0 software was selected for UHPLC-HRMS DDA data processing. Based on the structure of the selected compounds, a list of expected biotransformations (and their combinations) (**Table S1**) were selected for obtaining potential metabolites. Extracted ion chromatograms (EIC) with a mass window of ±5 ppm were automatically performed for searching the (de)protonated molecule of the expected metabolites in the full-scan function of the DDA acquisition. If a chromatographic peak was detected, it was compared with the blank and stability incubations, in order to discard false positives. Additionally, a binary comparison between blank incubations and compound incubations was performed for unexpected metabolites detection. Once the potential metabolites were selected, MS/MS spectra acquired during DDA acquisition were manually processed using FreeStyle 1.3 software. When a potential metabolite presented a fragmentation which fits with its expected biotransformation, it was re-analysed using parallel reaction monitoring (PRM) acquisition at three different collision energies (10, 30 and 50 eV). The PRM fragmentation of the metabolite was compared with the fragmentation pathway of parent compound. Based on this information, the position, where the biotransformation occurred, was determined, and the structure of the metabolite was suggested. In order to enhance the confidence in the structure elucidation, fragmentation pathways were proposed for all metabolites. The present work includes an (extremely) detailed explanation of the identification of metabolites based on the observed PRM fragmentation.

For the studied compounds, no additional metabolites in ESI^- were observed.

Table S1. Expected biotransformations used for searching potential metabolites.

Biotransformation	Delta mass (Da)	Formula	Type
Deethylation	-28.0313	$-C_2H_4$	Phase I
Demethylation	-14.0157	$-CH_2$	Phase I
Desaturation	-2.0157	$-H_2$	Phase I
Dihydrodiol formation	34.0055	$+H_2O_2$	Phase I
Hydration	18.0106	$+H_2O$	Phase I
Oxidation	15.9949	+O	Phase I
Oxidation + desaturation	13.9793	$+O-H_2$	Phase I
Oxidative deamination to alcohol	0.984	-NH+O	Phase I
Oxidative deamination to ketone	-1.0316	$-NH_3+O$	Phase I
Oxidative dechlorination	-17.9661	-Cl+OH	Phase I
Oxidative defluorination	-1.9957	-F+OH	Phase I
Reduction	2.0157	$+H_2$	Phase I
Acetyl cysteine conjugation	161.0147	$+C_5H_7NO_3S$	Phase II
Acetylation	42.0106	$+C_2H_2O$	Phase II
Carnitine conjugation	144.1025	$+C_7H_{14}O_2N$	Phase II
CysGly S adduction	176.0256	$+C_5H_8N_2O_3S$	Phase II
Cysteine conjugation	103.0092	$+C_3H_5NOS$	Phase II
Cysteine S-conjugation	119.0041	$+C_3H_5NO_2S$	Phase II
Glucosylation	162.0528	$+C_6H_{10}O_5$	Phase II
Glucuronidation	176.0321	$+C_6H_8O_6$	Phase II
Glutamine conjugation	129.0426	$+C_5H_7O_3N$	Phase II
Glutathione conjugation	289.0732	$+C_{10}H_{15}N_3O_5S$	Phase II
Glutathione S-conjugation	305.0682	$+C_{10}H_{15}N_3O_6S$	Phase II
Glycine conjugation	57.0215	$+C_2H_3NO$	Phase II
Methylation	14.0157	$+CH_2$	Phase II
Phosphorylation	79.9663	$+HPO_3$	Phase II
Proprionyl conjugation	56.0262	$+C_3H_4O$	Phase II
Sulfation	79.9568	$+SO_3$	Phase II
Taurine conjugation	107.0041	$+C_2H_5NO_2S$	Phase II

3.2. Fragmentation pathway of 5-PPDi

The first step in the metabolite elucidation strategy applied was the in-depth study of the mass fragmentation behaviour of the parent compound ($C_{17}H_{24}NO^+$, *m/z* 258.1851, -0.9 ppm). **Table 1** shows the information obtained on the 5-PPDi fragmentation, including accurate mass, elemental composition and mass error. **Figure 2B** shows the MS/MS fragmentation spectrum at 50 eV (MS/MS spectra at 10 and 30 eV can be found in **Figure S1**).

Figure 2A shows the proposed fragmentation pathway for 5-PPDi. The fragmentation starts with the disconnection of the different bonds of the α-carbon atom. On the one hand, the minor product ion at *m/z* 229 corresponded to the homolytic dealkylation from parent compound. On the other hand, a product ion at *m/z* 187 was obtained after the loss of the pyrrolidine moiety. A subsequent CO loss and structure rearrangement led to the ion *m/z* 159. Product ions at *m/z* 117 and 131 were obtained after an alkylic disconnection from *m/z* 159, depending on the C-C bond disconnected. Product ion *m/z* 145 corresponded to the disconnection of the bond between the carbonyl and the α-carbon atom, releasing the alkylic chain and pyrrolidine moiety. Alternatively, product ion *m/z* 117 could be also obtained after a CO loss from product ion *m/z* 145. The pyrrolidine moiety ion (product ion *m/z* 72) was also observed as a product ion, similarly to the ion corresponding to the alkylic chain and pyrrolidine moiety (product ion *m/z* 112). In this case, *m/z* 112 produced also the product ions at *m/z* 84 and 70, corresponding the first one to the alkylic chain C-C bond disconnection and the second one to the *N*-dealkylation.

3.3. 5-PPDi metabolites elucidation

The proposed fragmentation pathway for the studied compounds and elucidated metabolites are included in the text, while the MS/MS spectra for these compounds can be found at the end of this research article.

12 phase I metabolites were found after incubation. 6 of these metabolites corresponded to hydroxylated compounds (M1-M6). Similarly, 2 metabolites corresponding to hydroxylation plus oxidation (M10 and M11), and 2 with ring opening and carboxylation (M8 and M9) were detected. The correct position of the biotransformation was determined after an accurate study of the observed PRM fragmentation. Additionally, metabolites corresponding to oxidation (M7) and double oxidation (M12) were also found. **Table 1** shows the accurate mass, elemental composition, observed fragmentation, and mass error for the 12 identified metabolites.

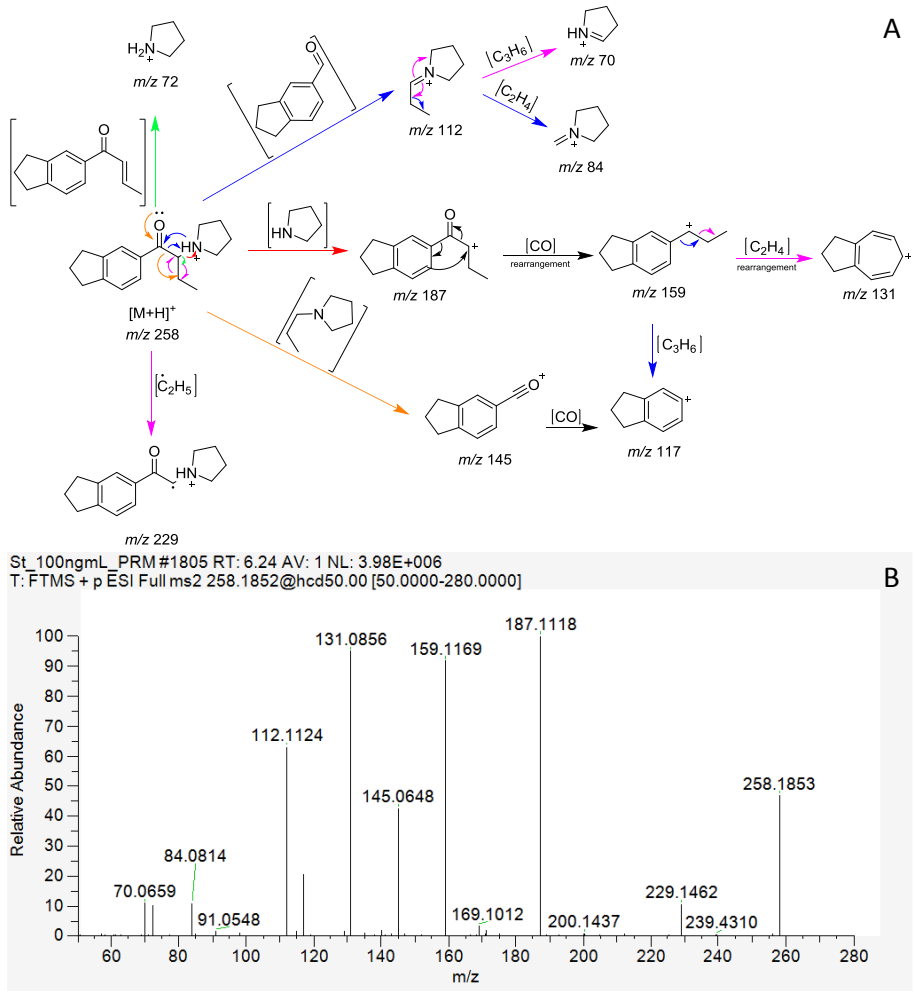


Figure 2. Mass spectrometric behaviour of 5-PPDi. (A) Proposed fragmentation pathway. (B) PRM spectrum at 50 eV.

Table 1. Mass spectrometric detection of 5-PPDi and its metabolites. Data obtained from PRM spectra.

Compound	Retention time (min)	$[M+H]^+$ (*m/z*)	Elemental composition	Mass error (ppm)	Product ion (*m/z*)	Elemental composition	Mass error (ppm)*
5-PPDi	7.50	258.1851	$C_{17}H_{24}NO^+$	-0.9	229.1462	$C_{15}H_{19}NO^{+\bullet}$	0.3
					187.1118	$C_{13}H_{15}O^+$	0.2
					159.1169	$C_{12}H_{15}^+$	0.2
					145.0648	$C_{10}H_9O^+$	0.2
					131.0856	$C_{10}H_{11}^+$	0.9
					117.0702	$C_9H_9^+$	2
					112.1124	$C_7H_{14}N^+$	3
					84.0814	$C_5H_{10}N^+$	8
					72.0815	$C_4H_{10}N^+$	10
					70.0659	$C_4H_8N^+$	11
M1	4.68	274.1799	$C_{17}H_{24}NO_2^+$	-0.9	245.1410	$C_{15}H_{19}NO_2^{+\bullet}$	0
					185.0961	$C_{13}H_{13}O^+$	0.1
					161.0597	$C_{10}H_9O_2^+$	-0.2
					157.1012	$C_{12}H_{13}^+$	0.2
					129.0700	$C_{10}H_9^+$	0.9
					112.1124	$C_7H_{14}N^+$	3
					84.0814	$C_5H_{10}N^+$	7
					72.0815	$C_4H_{10}N^+$	10
					70.0659	$C_4H_8N^+$	11
M2	4.96	274.1800	$C_{17}H_{24}NO_2^+$	-0.7	256.1695	$C_{17}H_{22}NO^+$	-0.5
					227.1305	$C_{15}H_{17}NO^{+\bullet}$	0.1
					158.0727	$C_{11}H_{10}O^{+\bullet}$	0.6
					143.0856	$C_{11}H_{11}^+$	0.5
					112.1125	$C_7H_{14}N^+$	4
					84.0815	$C_5H_{10}N^+$	8
					72.0816	$C_4H_{10}N^+$	11
					70.0651	$C_4H_8N^+$	11
M3	5.23	274.1798	$C_{17}H_{24}NO_2^+$	-1.1	185.0960	$C_{13}H_{13}O^+$	-0.6
					159.0804	$C_{11}H_{11}O^+$	-0.5
					131.0856	$C_{10}H_{11}^+$	0.7
					112.1124	$C_7H_{14}N^+$	3
					84.0814	$C_5H_{10}N^+$	7
					72.0815	$C_4H_{10}N^+$	10
					70.0659	$C_4H_8N^+$	10

*mass error (ppm) is shown with only one significant figure.

Table 1. Mass spectrometric detection of 5-PPDi and its metabolites. Data obtained from PRM spectra (continuation).

Compound	Retention time (min)	$[M+H]^+$ (*m/z*)	Elemental composition	Mass error (ppm)	Product ion (*m/z*)	Elemental composition	Mass error (ppm)*
M4	4.83	247.1799	$C_{17}H_{24}NO_2^+$	-0.9	203.1065	$C_{13}H_{15}O_2^+$	-0.8
					185.0961	$C_{13}H_{13}O^+$	-0.04
					159.0804	$C_{11}H_{11}O^+$	-0.3
					131.0856	$C_{10}H_{11}^+$	-0.6
					112.1124	$C_7H_{14}N^+$	3
					84.0814	$C_5H_{10}N^+$	7
					72.0815	$C_4H_{10}N^+$	10
					70.0659	$C_4H_8N^+$	11
M5	6.93	274.1800	$C_{17}H_{24}NO_2^+$	-0.7	256.1696	$C_{17}H_{22}NO^+$	-0.1
					245.1412	$C_{15}H_{19}NO_2^{+\bullet}$	0.5
					228.1747	$C_{16}H_{22}N^+$	0.3
					187.1117	$C_{13}H_{15}O^+$	0.2
					159.1169	$C_{12}H_{15}^+$	0.2
					145.0647	$C_{10}H_9O^+$	0.06
					131.0856	$C_{10}H_{11}^+$	0.9
					128.1071	$C_7H_{14}NO^+$	1
					117.0702	$C_9H_9^+$	3
					100.0762	$C_5H_{10}NO^+$	5
					85.0290	$C_4H_5O_2^+$	7
					70.0659	$C_4H_8N^+$	11
M6	7.82	274.1801	$C_{17}H_{24}NO_2^+$	-0.3	145.0648	$C_{10}H_9O^+$	0.2
					112.1124	$C_7H_{14}N^+$	3
					88.0762	$C_4H_{10}NO^+$	6
					86.0607	$C_4H_8NO^+$	7
					70.0658	$C_4H_8N^+$	10
M7	5.16	272.1644	$C_{17}H_{22}NO_2^+$	-0.3	243.1254	$C_{15}H_{17}NO_2^{+\bullet}$	0.3
					201.0907	$C_{13}H_{13}O_2^+$	-1
					173.0961	$C_{12}H_{13}O^+$	0.3
					159.0804	$C_{11}H_{11}O^+$	0.02
					145.0648	$C_{10}H_9O^+$	-0.02
					131.0857	$C_{10}H_{11}^+$	1
					112.1124	$C_7H_{14}N^+$	3
					84.0814	$C_5H_{10}N^+$	8
					72.0815	$C_4H_{10}N^+$	10
					70.0659	$C_4H_8N^+$	11

*mass error (ppm) is shown with only one significant figure.

Table 1. Mass spectrometric detection of 5-PPDi and its metabolites. Data obtained from PRM spectra (continuation).

Compound	Retention time (min)	$[M+H]^+$ (*m/z*)	Elemental composition	Mass error (ppm)	Product ion (*m/z*)	Elemental composition	Mass error (ppm)*
M8	5.05	290.1750	$C_{17}H_{24}NO_3^+$	-0.2	261.1361	$C_{15}H_{19}NO_3^{+\bullet}$	0.7
					201.0912	$C_{13}H_{13}O_2^+$	0.9
					191.1096	$C_{12}H_{15}O_2^+$	1
					177.0546	$C_{10}H_9O_3^+$	0.1
					175.1118	$C_{12}H_{15}O^+$	0.6
					163.0754	$C_{10}H_{11}O_2^+$	0.1
					147.1168	$C_{11}H_{15}^+$	-0.04
					119.0858	$C_9H_{11}^+$	2
					112.1124	$C_7H_{14}N^+$	3
					91.0548	$C_7H_7^+$	6
					84.0814	$C_5H_{10}N^+$	8
					72.0815	$C_4H_{10}N^+$	10
					70.0659	$C_4H_8N^+$	11
M9	7.09	290.1749	$C_{17}H_{24}NO_3^+$	-0.3	272.1644	$C_{17}H_{22}NO_2^+$	-0.5
					254.1539	$C_{17}H_{20}NO^+$	-0.2
					236.1433	$C_{17}H_{18}N^+$	-0.5
					226.1225	$C_{15}H_{16}NO^+$	-0.4
					198.1277	$C_{14}H_{16}N^+$	-0.2
					187.1118	$C_{13}H_{15}O^+$	0.07
					186.1277	$C_{13}H_{16}N^+$	-0.05
					170.0963	$C_{12}H_{12}N^+$	-0.5
					159.1168	$C_{12}H_{15}^+$	-0.2
					145.0648	$C_{10}H_9O^+$	0.2
					131.0856	$C_{10}H_{11}^+$	0.6
					126.0915	$C_7H_{12}NO^+$	1
					117.0701	$C_9H_9^+$	2
					87.0447	$C_4H_7O_2^+$	1
					86.0606	$C_4H_8NO^+$	7
					69.0342	$C_4H_5O^+$	4
M10	6.63	288.1593	$C_{17}H_{22}NO_3^+$	-0.3	270.1487	$C_{17}H_{20}NO_2^+$	-0.5
					185.0961	$C_{13}H_{13}O^+$	0.4
					157.1011	$C_{12}H_{13}^+$	-0.4
					143.0855	$C_{11}H_{11}^+$	-0.3
					129.0698	$C_{10}H_9^+$	-0.3
					126.0915	$C_7H_{12}NO^+$	0.9
					98.0605	$C_5H_8NO^+$	5
					86.0606	$C_4H_8NO^+$	7

*mass error (ppm) is shown with only one significant figure.

Table 1. Mass spectrometric detection of 5-PPDi and its metabolites. Data obtained from PRM spectra (continuation).

Compound	Retention time (min)	$[M+H]^+$ (*m/z*)	Elemental composition	Mass error (ppm)	Product ion (*m/z*)	Elemental composition	Mass error (ppm)*
M11	4.76	288.1594	$C_{17}H_{22}NO_3^+$	0.01	173.0965	$C_{12}H_{13}O^+$	2
					159.0803	$C_{11}H_{11}O^+$	-0.9
					128.1073	$C_7H_{14}NO^+$	3
					70.0658	$C_4H_8N^+$	9
M12	8.20	286.1434	$C_{17}H_{20}NO_3^+$	-1.4	159.0803	$C_{11}H_{11}O^+$	-0.6
					126.0916	$C_7H_{12}NO^+$	2

*mass error (ppm) is shown with only one significant figure.

3.3.1. Metabolite M1

The molecular formula obtained for metabolite M1 (*m/z* 274.1799, $C_{17}H_{24}NO_2^+$, -0.9 ppm) corresponded to a hydroxylation. Product ion at *m/z* 161 suggested the hydroxyl group to be in the aromatic moiety, based on the 16 Da shift respect the corresponding product ion of 5-PPDi (*m/z* 145). Moreover, the product ion at *m/z* 185 ($C_{13}H_{13}O^+$) revealed that this product ion corresponds to the 187 observed for the parent compound ($C_{13}H_{15}O^+$) but with a -2 Da shift, this is, with an additional insaturation. This would point out where the water loss had occurred. Based on both premises, the hydroxyl group was located on the trimethylene moiety (if it was in the aromatic ring, a water loss would not be possible). The other observed product ions could be satisfactorily justified based on the proposed structure. **Figure S2** shows the PRM spectra for M1, and **Figure S3** the proposed fragmentation pathway.

3.3.2. Metabolite M2

M2 was detected at *m/z* 274.1800 ($C_{17}H_{24}NO_2^+$, -0.7 ppm). In this case, the observed fragmentation (**Table 1**, **Figure S4**) indicated the hydroxyl group to be on the aromatic ring. Thus, the product ion at *m/z* 158 ($C_{11}H_{10}O^+$) could only be justified if the hydroxyl group was in the aromatic ring (**Figure S5**). This allowed the loss of a water molecule from the carbonyl group (*m/z* 256), as the subsequent insaturation could be stabilised by the aromatic ring. After that, product ions at

m/z 227 and 143 were produced, depending on which bond was disconnected. With this assumption, the fragmentation pathway was proposed, fitting the observed fragmentation with the proposed structure. In order to facilitate the visualisation of the electronic rearrangement during fragmentation pathway, the hydroxylation has been placed in a certain atom of the aromatic ring.

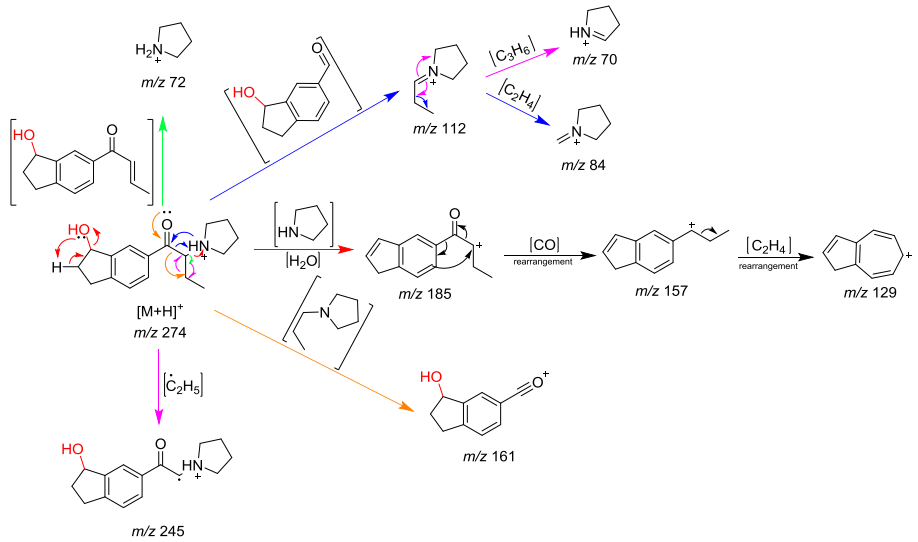


Figure S3. Proposed fragmentation pathway for 5-PPDi M1.

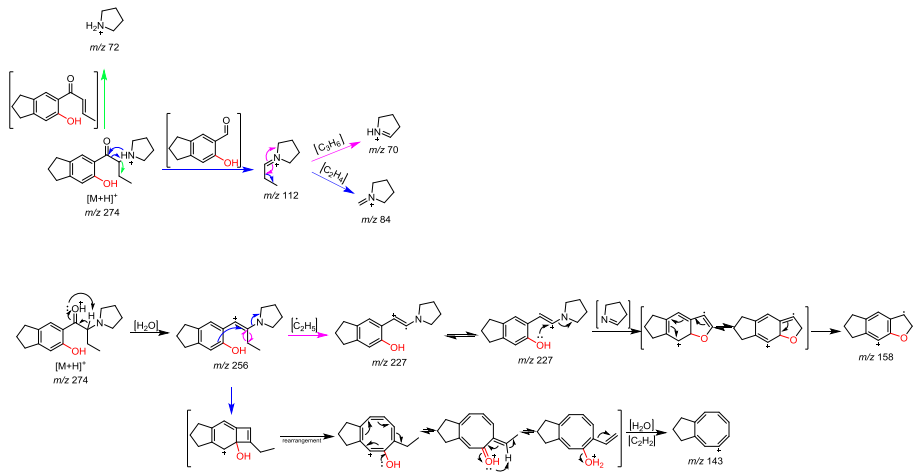


Figure S5. Proposed fragmentation pathway for 5-PPDi M2.

3.3.3. Metabolites M3 and M4

M3 and M4 were observed at *m/z* 274.1798 and 274.1799 corresponding to $C_{17}H_{24}NO_2^+$ (-1.1 and -0.9 ppm, respectively). Both metabolites presented exactly the same PRM fragmentation (available in **Figure S6** and **S8**). The productions at *m/z* 72 ($C_4H_{10}N^+$) and 70 ($C_4H_8N^+$) indicated that the hydroxylation did not occur in the pyrrolidine moiety. The product ion *m/z* 203 was the equivalent to the 187 of 5-PPDi, but with a chemical shift of 16 Da. This would locate the hydroxyl group to be on the aromatic moiety or the alkylic chain. Product ion at *m/z* 131 excluded the possibility of hydroxylation in the aromatic moiety whereas product ion at *m/z* 112 suggested that the alkylic chain remained unaltered. Nevertheless, if the hydroxylation point was located in the alkylic chain, it was possible to obtain the product ion *m/z* 112 after an intramolecular nucleophilic substitution (type SN_2). Thus, M3 would correspond to the hydroxylation in the methyl group of the alkylic chain and M4 in the methylene (or vice-versa). Finally, fragmentation pathways of M3 (**Figure S7**) and M4 (**Figure S9**) were proposed (similarly to M2, the hydroxylation is placed in a certain atom of the alkylic chain in order to facilitate the visualisation of the electronic rearrangement).

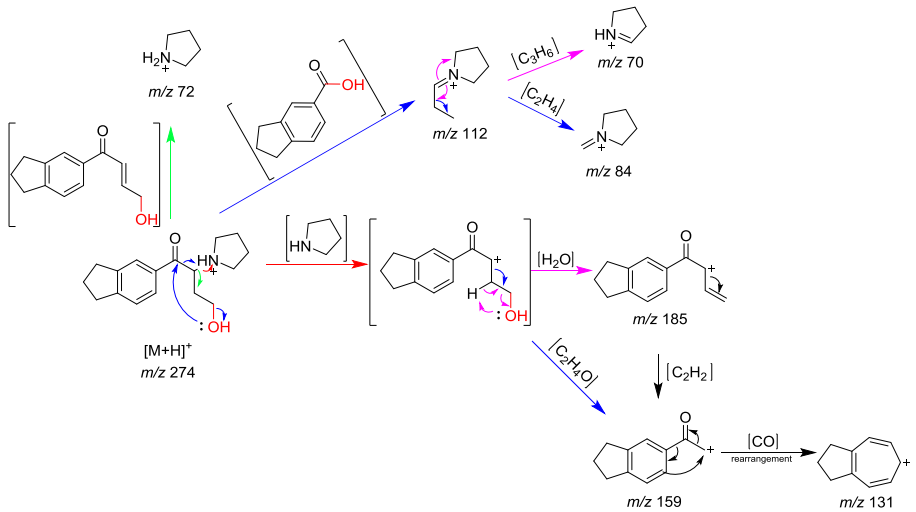


Figure S7. Proposed fragmentation pathway for 5-PPDi M3.

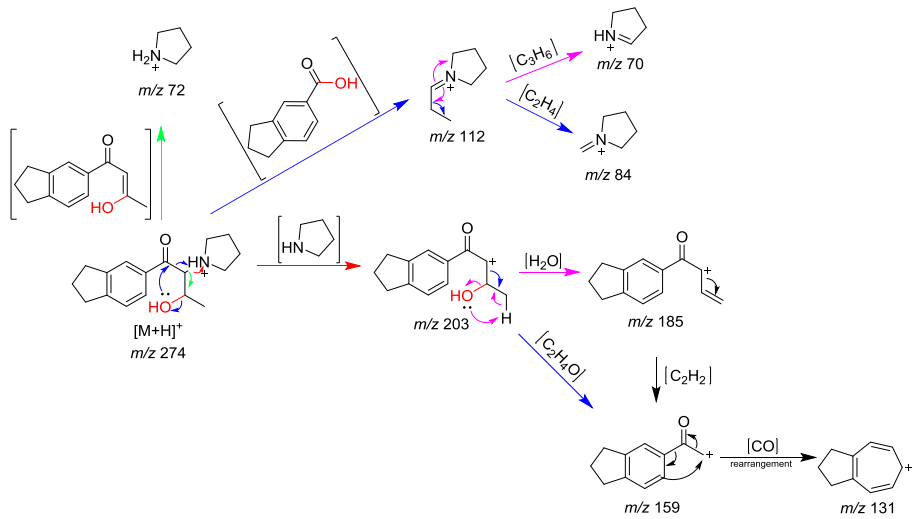


Figure S9. Proposed fragmentation pathway for 5-PPDi M4.

3.3.4. Metabolite M5

The molecular formula obtained for metabolite 5 corresponded, again, to a hydroxylated metabolite (*m/z* 274.1799, $C_{17}H_{24}NO_2^+$, -0.7 ppm). In this case, the PRM at 50 eV showed a complex fragmentation spectrum, with up to 12 product ions (**Table 1**). As some of the product ions were also observed for 5-PPDi (*m/z* 159, 145, 131, 117), the hydroxylation in the aromatic ring was discarded. Product ions *m/z* 245, 128 and 100 (all of them with a 16 Da shift from 5-PPDi product ions) were crucial for locating the correct position of the biotransformation. Thus, the hydroxylation was situated in the α-carbon atom. The product ion at *m/z* 85 ($C_4H_5O_2^+$), corresponding to the alkylic chain and the carbonyl, enhanced the confidence on the metabolite structure. The proposed structure for M5 could justify all the observed PRM product ions (**Figure S10**), and its fragmentation pathway (**Figure S11**) was proposed.

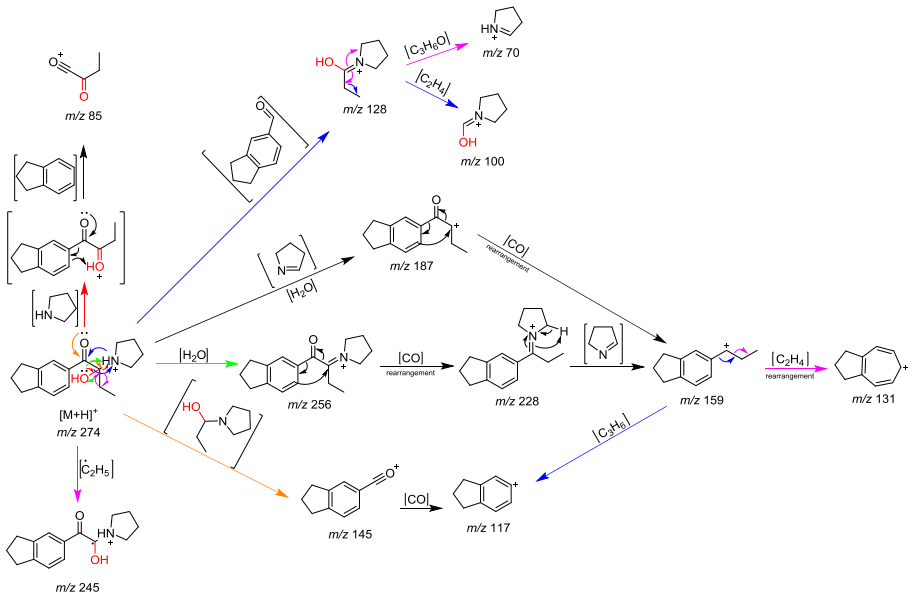


Figure S11. Proposed fragmentation pathway for 5-PPDi M5.

3.3.5. Metabolite M6

M6 was the last hydroxylated metabolite, observed at *m/z* 274.1801 ($C_{17}H_{24}NO_2^+$, -0.3 ppm). The position of the hydroxylation was easily assigned to be in the pyrrolidine moiety based on the product ions observed at *m/z* 88 and 86 (16 Da shifted from 5-PPDi product ions at *m/z* 72 and 70, respectively). In this case, only 5 product ions were observed in PRM spectra (**Figure S12**), being all of them justified based on the proposed structure and fragmentation pathway (**Figure S13**).

3.3.6. Metabolite M7

M7 corresponded to an oxidized metabolite, based on its elemental composition (*m/z* 272.1644, $C_{17}H_{22}NO_2^+$, -0.3 ppm). This metabolite presented a similar fragmentation to M1 (hydroxylation in the trimethylene moiety of the indanyl), but with some product ions shifted 16 Da (*m/z* 201 vs 185, 173 vs 157, and 145 vs 129). It is not surprising that some hydroxylated metabolites become oxidized, generating a ketone group which cannot be lost as water molecule. With this

premise, the ketone group was placed on the indanyl ring, trying to justify all the observed PRM product ions (**Table 1**, **Figure S14**). Product ions at *m/z* 173 and 159 guarantee the biotransformation location. The proposed fragmentation pathway (**Figure S15**) fitted perfectly with this biotransformation.

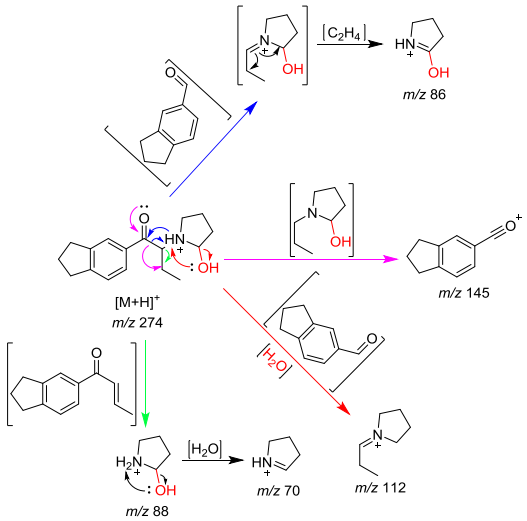


Figure S13. Proposed fragmentation pathway for 5-PPDi M6.

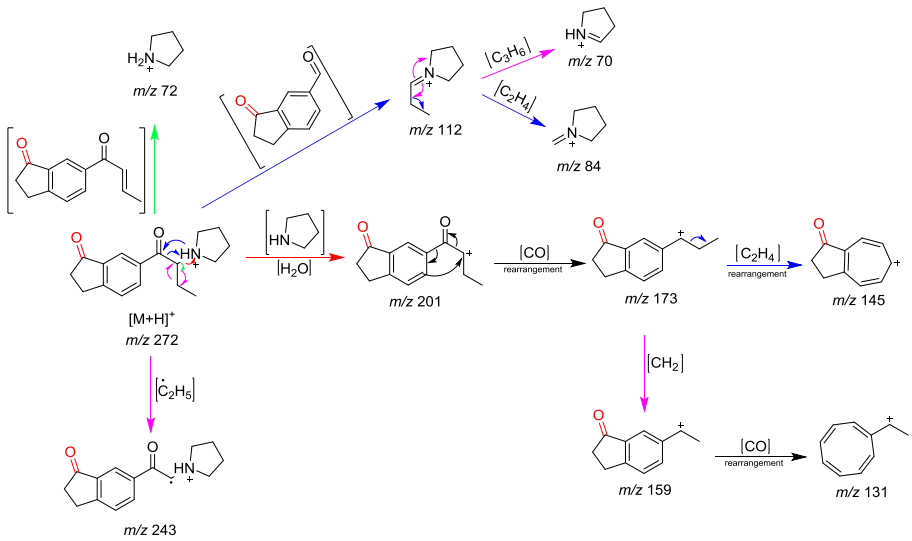


Figure S15. Proposed fragmentation pathway for 5-PPDi M7.

3.3.7. Metabolite M8

M8 exhibited a molecular formula containing 2 oxygen atoms more than parent compound (*m/z* 290.1750, $C_{17}H_{24}NO_3^+$, -0.2 ppm), which could fit with a double hydroxylation or with a ring opening followed by a carboxylation. Product ion *m/z* 177, with a 32 Da chemical shift respect the *m/z* 145 of 5-PPDi, revealed a possible double hydroxylation on the indanyl moiety. Nevertheless, it is hardly difficult to observe double hydroxylation in cathinones after pHH incubation [24], and the most common biotransformation described in literature for these compounds is the pyrrolidine ring opening followed by carboxylation [19,21,24]. So, it was proposed a ring-opening and carboxylation in the indanyl moiety, which perfectly fit with the observed PRM fragmentation (**Table 1**, **Figure S16**). The fragmentation pathway (**Figure 3A**) was finally proposed, taking into account the fragmentation pathway described in literature for MDPV [19]. Product ion at *m/z* 119 was determining, as it is more feasible to produce this fragmentation with a ring opening carboxylation, instead of double hydroxylation. Nevertheless, it is not possible to completely rule out the double hydroxylation based only on the presence of product ion *m/z* 119. For an unequivocal identification, analytical standard of both compounds should be needed for retention time and fragmentation comparison, or the use of additional analytical techniques such as nuclear magnetic resonance.

3.3.8. Metabolite M9

M9 presented the same elemental composition than M8 (*m/z* 290.1749, $C_{17}H_{24}NO_3^+$, -0.3 ppm). In this case, the rich PRM fragmentation spectra obtained (**Figure S17**), and more concretely the product ions at *m/z* 272 ($C_{17}H_{22}NO_2^+$), 187 ($C_{13}H_{15}O^+$) and 87 ($C_4H_7O_2^+$), suggested the pyrrolidine ring opening and carboxylation of the alkylic chain obtained [19,21,24]. The 16 product ions observed in PRM spectra were satisfactorily justified using the fragmentation pathway reported for MDPV-COOH metabolite [19], and the fragmentation pathway of M9 was established (**Figure S18**).

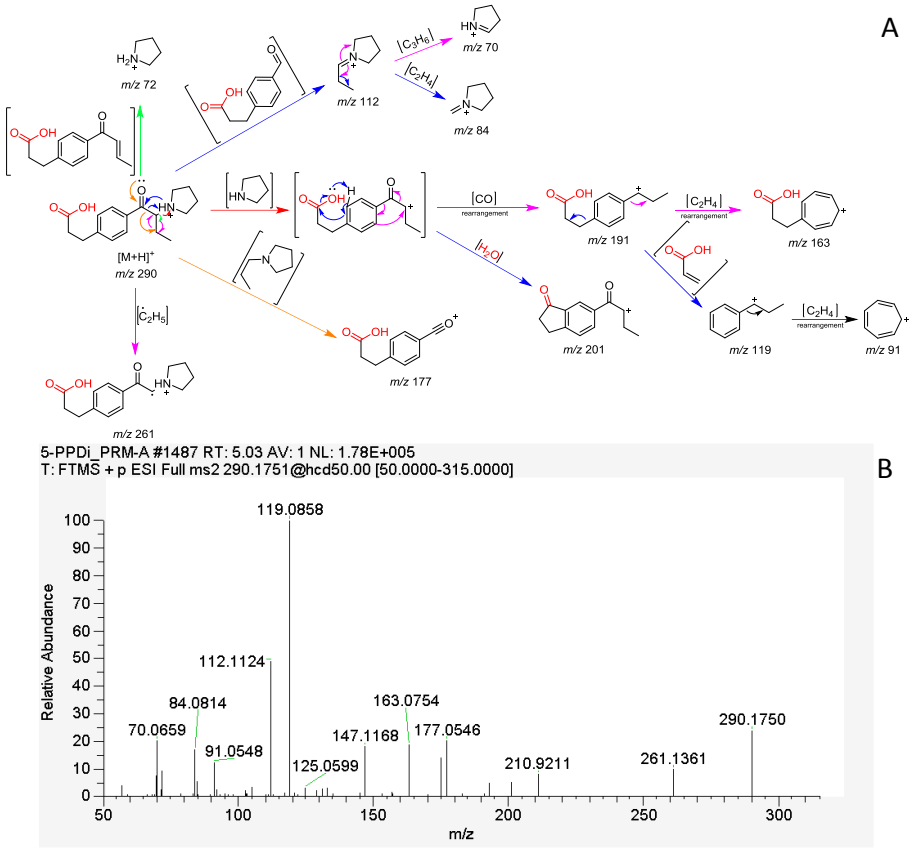


Figure 3. Mass spectrometric behaviour of M8. (A) Proposed fragmentation pathway. (B) PRM spectrum at 50 eV.

3.3.9. Metabolites M10 and M11

M10 and M11 presented the same proposed elemental composition (*m/z* 288.1593 and 288.1594, $C_{17}H_{22}NO_3^+$, -0.3 and 0.01 ppm, respectively), which could match with a hydroxylation plus oxidation (carbonyl formation). In both cases, the observed PRM fragmentation (**Table 1**, **Figure S19** and **S21**) revealed that both indanyl and pyrrolidine moieties presented biotransformations. So, it was assumed that M10 and M11 should have a hydroxyl in the indanyl group and a ketone in the pyrrolidine, and vice-versa.

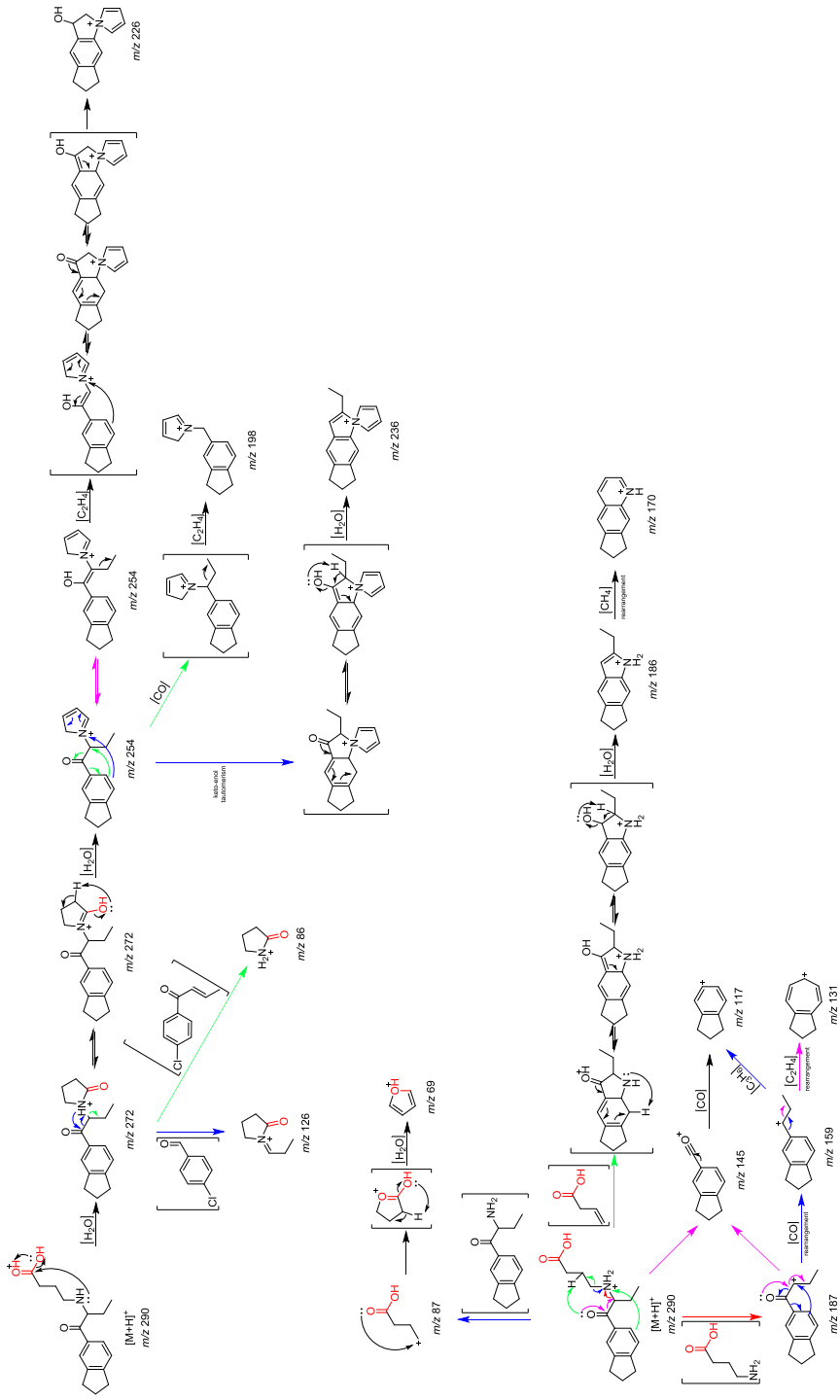


Figure S18. Proposed fragmentation pathway for 5-PPDi M9.

The product ions at *m/z* 86 ($C_4H_8NO^+$, common to M6 and M9), 98 ($C_5H_8NO^+$), 126 ($C_7H_{12}NO^+$, also shared with M9) confirmed the ketone group to be in the pyrrolidine ring; the one at *m/z* 157 (shared with M1) confirmed the structure of M10. In a similar way, M11 was elucidated based on product ions at *m/z* 173 ($C_{12}H_{13}O^+$) and 159 ($C_{11}H_{11}O^+$, shared with M4 and M7) and 128 ($C_7H_{14}NO^+$, common to M5), which indicated that this metabolite presented a ketone group in the pyrrolidine moiety, and a hydroxyl in the indanyl, respectively. The structures of M10 and M11 were confirmed after proposing the fragmentation pathway for both compounds (**Figure S20** and **S22**, respectively).

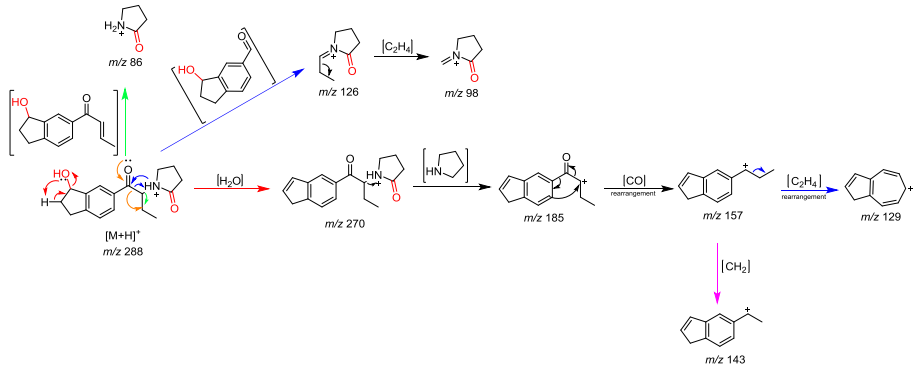


Figure S20. Proposed fragmentation pathway for 5-PPDi M10.

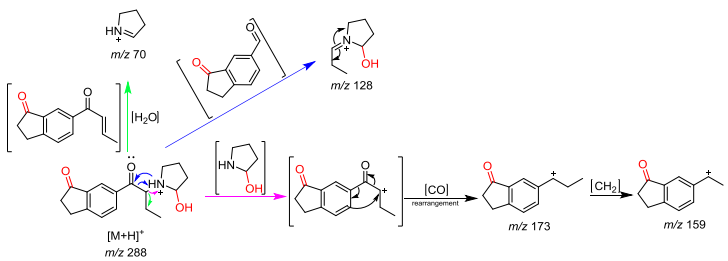


Figure S22. Proposed fragmentation pathway for 5-PPDi M11.

3.3.10. Metabolite M12

M12 was detected at *m/z* 286.1434 ($C_{17}H_{22}NO_3^+$, -1.4 ppm), being the compound that presented the lowest response. In this case, only two product ions were observed (**Figure S23**) at *m/z* 159 (shared with M7 and M11) and *m/z* 126 (shared

with M10). M12 was then proposed as a compound with two ketone groups, one in the indanyl moiety and the other in the pyrrolidine ring. The proposed fragmentation pathway (**Figure S24**) fitted with the structure and fragmentation observed.

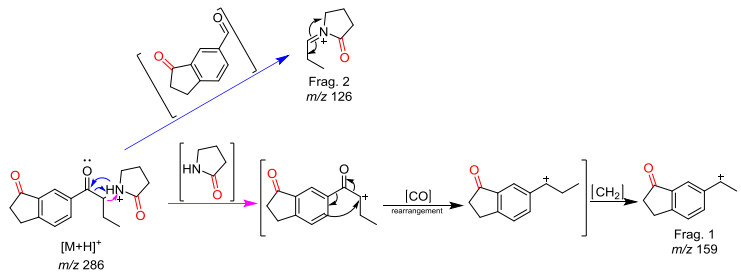


Figure S24. Proposed fragmentation pathway for 5-PPDi M12.

3.4. 5-PPDi metabolic fate

Once elucidated the 12 5-PPDi metabolites formed after pHH incubation, the metabolic pathway of this compound was proposed (**Figure 4).** This indanyl-cathinone is hepatically metabolised mainly through hydroxylation and oxidation processes.

In the case of the pyrrolidine moiety, hydroxylation (M6), oxidation (ketone formation, M10 and M12) and oxidative opening (M9) occurred. These biotransformations have been reported for pyrrolidine-cathinones, such as MDPV [19], α-PHP [21], α-PBP [17] or 4-methoxy-α-PVP [25]. M3 and M4 corresponded to hydroxylations in the methyl or methylene groups of the alkylic chain, biotransformations also observed for MDPV [19], α-PVT [26], PV8 [20] and α-PHP [21]. Nevertheless, no information about hydroxylations in the α-carbon of cathinones (M5) has been found in literature. Regarding the indanyl moiety, hydroxylation (M1, M10), ketone formation (M7, M11, M12) and oxidative ring-opening (M8) were observed. These biotransformations are quite similar to the observed for pyrrolidine moieties in literature. In some cases, more than one biotransformation was found, such as hydroxylation plus ketone formation (M10, M11) or formation of two ketones (M12).

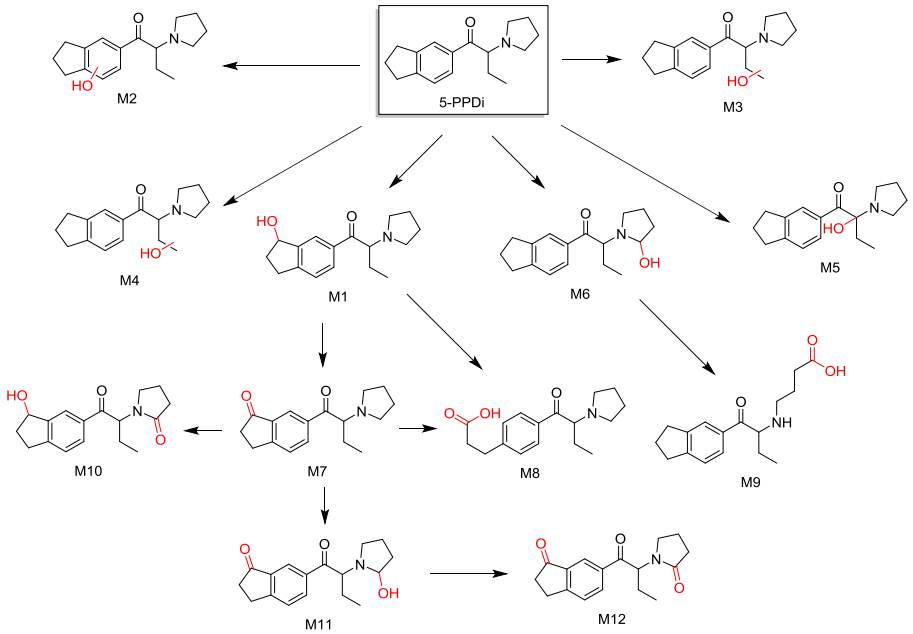


Figure 4. Proposed metabolic pathway for 5-PPDi.

At the end of the pHH incubation, the presence of 5-PPDi in media was around 67% respect to its initial response, illustrating a relative high hepatic stability. In the case of the metabolites, M9 presented the higher response after 180 min (around 3.3% respect to 5-PPDi initial response); together with M8 (3.2%), M4 (3.1%), M2 (1.6%) and M3 (1.4%). The remaining metabolites were found at responses lower than 0.4%, the lowest one being M12 with a relative response of 0.001%. These results illustrate that the metabolic fate of 5-PPDi goes to the generation of carboxylic acid that involve ring-opening reactions (in this case, indanyl and pyrrolidine). Based on these results, the potential biomarkers proposed for detecting the consumption of 5-PPDi in intoxication cases by the analysis of urine and blood samples are 5-PPDi itself and the metabolites M8 and M9. Both compounds are the major metabolites after pHH incubation, and may be expected to be major metabolites of 5-PPDi in urine as well. Nevertheless, for analysis of urine samples, a previous enzymatic hydrolysis with β-glucuronidase should be performed, in order to de-conjugate M8/M9 glucuronide and detect the phase I metabolites.

3.5. Metabolic behaviour of 3-FEA, 4-CEC and 4-CPrC

Data obtained in this work revealed that amphetamine 3-FEA and the cathinone 3-CEC were not metabolised by the hepatocytes, probably due to the high polarity of both compounds. Different *in vitro* experiments performed with similar compounds also obtained few metabolites, and at low concentrations. As an example, 3-FEA was compared with the amphetamines 5-APDB [30] and 2-AEPB [31] using pHLM, which usually present higher metabolic activity than pHH, identifying3 metabolites per compound. In the case of 3-CEC, analogous compounds, such as 4-MEC [32], 3-BMC and 3-FMC [33], have been studied, showing they are highly metabolised using pHLM (between 4-7 metabolites). Nevertheless, these incubations were performed at high concentration levels: 50 μM for 4-MEC [32] and 250 μM for 3-BMC and 3-FMC [33]. In our work, the concentration tested (10 μM) was selected after considering the typical dose used by authentic consumers. Moreover, pHH were used in order to simulate the human hepatic metabolic fate for the studied compounds. The absence of metabolites for 3-FEA and 3-CEC suggest they are excreted as unaltered compounds in human urine, and so, the consumption biomarker proposed is the parent compound.

For the last compound studied, 4-CPrC, only two minor metabolites were found, each corresponding to less than 0.5% of the initial parent response at 180 min. The metabolite M1 corresponded to the pyrrolidine moiety-hydroxylated metabolite, and M2 to the pyrrolidine-ring opening followed by the formation of carboxylic acid. Both biotransformations have been described in literature for pyrrolidinophenone-derived cathinones, such as α-pyrrolidinohexiophenone (α-PHP) [21], or 3,4-methylenedioxypyrovalerone (MDPV) [19]. Here below the complete information about the 4-CPrC metabolism study, including the proposed metabolic pathway (**Figure S25**), PRM fragmentation for parent compound and metabolites (**Table S2**, **Figure S26-28**), and the proposed fragmentation pathways (**Figure S29-31**), is presented.

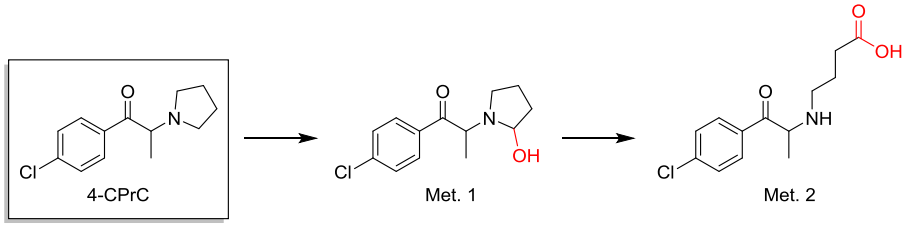


Figure S25. Proposed metabolic pathway for 4-CPrC.

Table S2. Mass spectrometric detection of 4-CPrCand its metabolites. Data obtained from PRM spectra.

Compound	Retention time (min)	$[M+H]^+$ (*m/z*)	Elemental composition	Mass error (ppm)	Product ion (*m/z*)	Elemental composition	Mass error (ppm)
4-CPrC	6.16	238.0993	$C_{13}H_{17}ClNO^+$	-0.1	223.0758	$C_{12}H_{14}ClNO^{+\cdot}$	-0.2
					185.1200	$C_{13}H_{12}N^{+*}$	0.8
					167.0259	$C_9H_8ClO^+$	0.5
					139.0310	$C_8H_8Cl^+$	0.4
					103.0547	$C_8H_7^+$	4
					98.0970	$C_6H_{12}N^+$	5
					84.0815	$C_5H_{10}N^+$	8
					72.0816	$C_4H_{10}N^+$	11
					70.0659	$C_4H_8N^+$	11
M1	6.43	254.0937	$C_{13}H_{17}ClNO_2^+$	-2	236.0833	$C_{13}H_{15}ClNO^+$	-1
					139.0308	$C_8H_8Cl^+$	-0.9
					98.0968	$C_6H_{12}N^+$	4
					86.0606	$C_4H_8NO^+$	6
					56.0503	$C_3H_6N^+$	14
M2	5.87	270.0887	$C_{13}H_{17}ClNO_3^+$	-2	252.0783	$C_{13}H_{15}ClNO_2^+$	-1
					234.0676	$C_{13}H_{13}ClNO^+$	2
					199.0989	$C_{13}H_{13}ClNO^{+\cdot}$	-1
					184.0521	$C_9H_{11}ClNO^+$	-2
					167.0255	$C_9H_8ClO^+$	-2
					166.0416	$C_9H_9ClN^+$	-1
					139.0307	$C_8H_8Cl^+$	-1
					131.0729	$C_9H_9N^{+\cdot}$	-0.3
					112.0759	$C_6H_{10}NO^+$	2
					103.0549	$C_8H_7^+$	3
					87.0445	$C_4H_7O_2^+$	5
					86.0605	$C_5H_8NO^+$	5
					69.0342	$C_3H_5O^+$	10

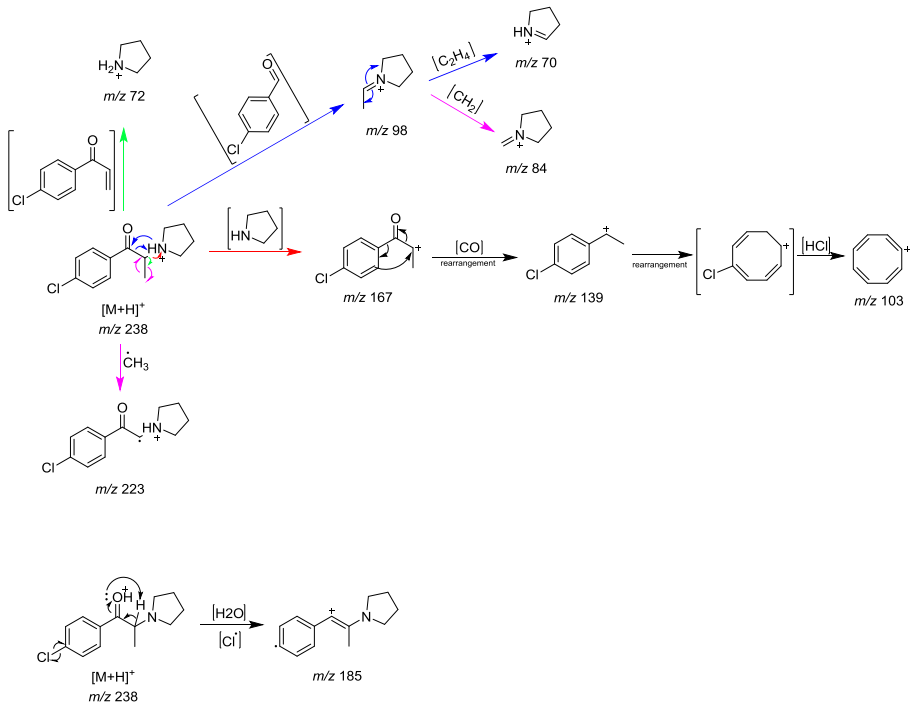


Figure S29. Proposed fragmentation pathway for 4-CPrC.

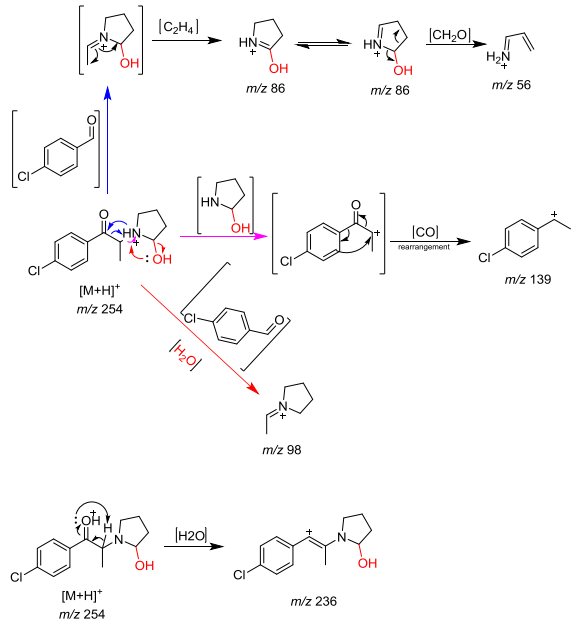


Figure S30. Proposed fragmentation pathway for 4-CPrC M1.

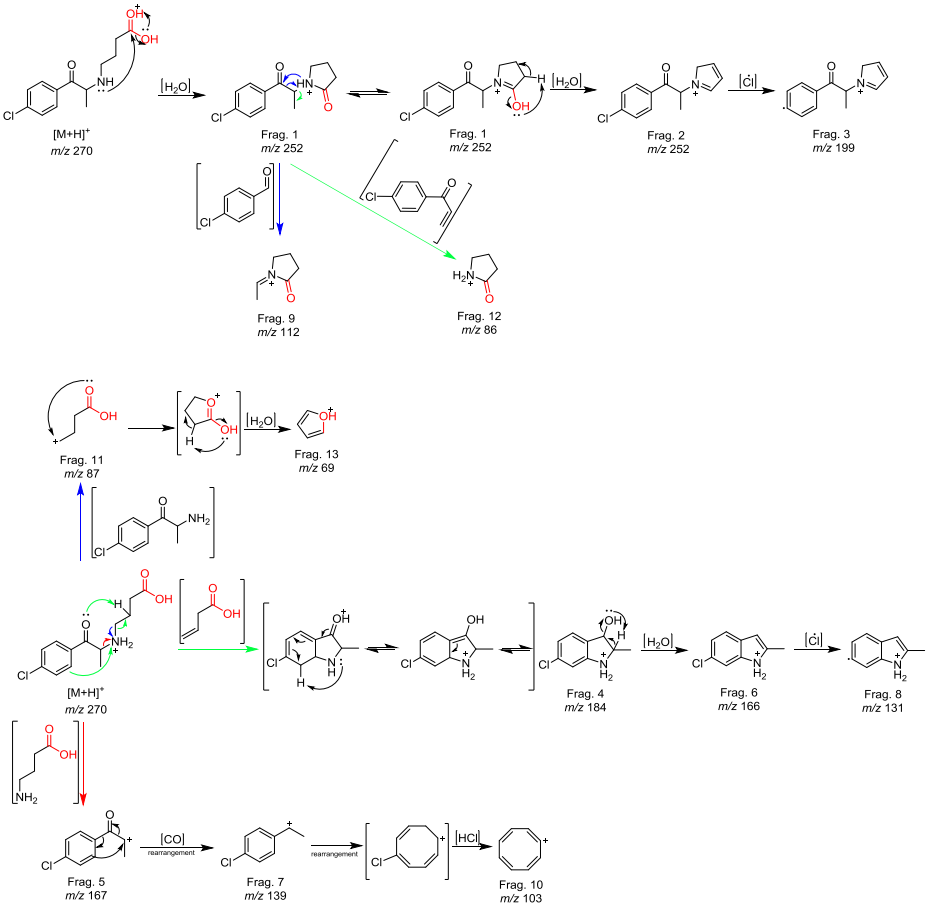


Figure S31. Proposed fragmentation pathway for 4-CPrC M2.

4. Conclusion

This work reports the metabolic behaviour of four new psychoactive substances -3 synthetic cathinones and 1 amphetamine- after incubation with pooled human hepatocytes. The stimulants 3-FEA and 4-CEC were not metabolised, and therefore the parent compounds are proposed as biomarkers for monitoring the consumption of these compounds. 4-CPrC also presented high hepatic stability, and only 2 phase I metabolites were detected. The parent compound and the oxidative ring-opening metabolite M2 seem to be the most suitable consumption biomarkers. In relation to the recently reported cathinone 5-PPDi, up to 12 phase I metabolites were identified, all of them resulting from hydroxylation and

oxidation processes. The oxidative ring-opening metabolites M8 (indanyl ring-opening) and M9 (pyrrolidine ring-opening) were the major metabolites detected in terms of response. Nevertheless, at the end of the incubation 5-PPDi gave around 67% of the initial response, illustrating again a high resistance of synthetic cathinones to hepatic metabolism. For monitoring the use of 5-PPDi, the parent compound and the metabolites M8 and M9 are proposed as consumption biomarkers. It is worth to noticing that 5-PPDi presents an indanyl moiety, and to our knowledge this is the first metabolic profile reported for an indanyl-based cathinone. The information provided in this work will be useful in forensic and clinical chemistry to focus the analytical determination of cathinones in biofluids, and also in other areas related to metabolic pathways of new psychoactive substances, such as toxicology and pharmacology.

Acknowledgements

D. Fabregat-Safont, J.V. Sancho, F. Hernández and M. Ibáñez acknowledge financial support from the Ministerio de Economía y Competitividad in Spain (Project CTQ2015-65603-P) and from University Jaume I (UJI-B2018-19). D. Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (FPU15/02033) and for the financial support received for his research stay at the University of Copenhagen (EST17/00024).

Competing Interests The authors declare that they have no competing interests.

References

[1] European Monitoring Centre for Drugs and Drug Addiction, European Drug Report 2018, EMCDDA Publ. (2018). doi:10.2810/88175.

[2] S.D. Brandt, P.F. Daley, N. V. Cozzi, Analytical characterization of three trifluoromethyl-substituted methcathinone isomers, Drug Test. Anal. 4 (2012) 525–529. doi:10.1002/dta.382.

[3] Z. Qian, W. Jia, T. Li, et al., Identification of five pyrrolidinyl substituted cathinones and the collision-induced dissociation of electrospray-generated pyrrolidinyl substituted cathinones, Drug Test. Anal. 9 (2017) 778–787. doi:10.1002/dta.2035.

[4] C. Liu, W. Jia, T. Li, et al., Identification and analytical characterization of nine synthetic cathinone derivatives N -ethylhexedrone, 4-Cl-pentedrone, 4-Cl- α -EAPP, propylone, N -ethylnorpentylone, 6-MeO-bk-MDMA, α -PiHP, 4-Cl- α -PHP, and 4-F- α -PHP, Drug Test. Anal. 9 (2017) 1162–1171. doi:10.1002/dta.2136.

[5] E. Fornal, Identification of substituted cathinones: 3,4-Methylenedioxy derivatives by high performance liquid chromatography–quadrupole time of flight mass spectrometry, J. Pharm. Biomed. Anal. 81–82 (2013) 13–19. doi:10.1016/j.jpba.2013.03.016.

[6] M. Majchrzak, M. Rojkiewicz, R. Celiński, et al., Identification and characterization of new designer drug 4-fluoro-PV9 and α-PHP in the seized materials, Forensic Toxicol. 34 (2016) 115–124. doi:10.1007/s11419-015-0295-4.

[7] Z. Qian, W. Jia, T. Li, et al., Identification and analytical characterization of four synthetic cathinone derivatives iso-4-BMC, β -TH-naphyrone, mexedrone, and 4-MDMC, Drug Test. Anal. 9 (2017) 274–281. doi:10.1002/dta.1983.

[8] T. Doi, B. Akiko Asada, B. Akihiro Takeda, et al., Identification and characterization of a-PVT, a-PBT, and their bromothienyl analogs found in illicit drug products, Forensic Toxicol. 34 (2016) 76–93. doi:10.1007/s11419-015-0288-3.

[9] M. Ibañez, J. V. Sancho, L. Bijlsma, et al., Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances, TrAC - Trends Anal. Chem. 57 (2014) 107–117. doi:10.1016/j.trac.2014.02.009.

[10] P. Kuś, J. Kusz, M. Książek, et al., Spectroscopic characterization and crystal structures of two cathinone derivatives: N-ethyl-2-amino-1-phenylpropan-1-one (ethcathinone) hydrochloride and N-ethyl-2-amino-1-(4-chlorophenyl)propan-1-one (4-CEC) hydrochloride, Forensic Toxicol. 35 (2017) 114–124. doi:10.1007/s11419-016-0345-6.

[11] V.A. Boumba, M. Di Rago, M. Peka, et al., The analysis of 132 novel psychoactive substances in human hair using a single step extraction by tandem LC/MS, Forensic Sci. Int. 279 (2017) 192–202. doi:10.1016/j.forsciint.2017.08.031.

[12] C. Richeval, S.M.R. Wille, M. Nachon-Phanithavong, et al., New psychoactive substances in oral fluid of French and Belgian drivers in 2016, Int. J. Drug Policy. 57 (2018) 1–3. doi:10.1016/j.drugpo.2018.03.013.

[13] M. Concheiro, M. Castaneto, R. Kronstrand, et al., Simultaneous determination of 40 novel psychoactive stimulants in urine by liquid chromatography–high resolution mass spectrometry and library matching, J. Chromatogr. A. 1397 (2015) 32–42. doi:10.1016/j.chroma.2015.04.002.

[14] A. Celma, J. V. Sancho, N. Salgueiro-González, et al., Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology, J. Chromatogr. A. (2019) 10–14. doi:10.1016/j.chroma.2019.05.051.

[15] O.J. Pozo, M. Ibáñez, J. V. Sancho, et al., Mass Spectrometric Evaluation of Mephedrone In Vivo Human Metabolism: Identification of Phase I and Phase II Metabolites, Including a Novel Succinyl Conjugate, Drug Metab. Dispos. 43 (2014) 248–257. doi:10.1124/dmd.114.061416.

[16] V. Uralets, S. Rana, S. Morgan, et al., Testing for designer stimulants: Metabolic profiles of 16 synthetic cathinones excreted free in human urine, J. Anal. Toxicol. 38 (2014) 233–241. doi:10.1093/jat/bku021.

[17] S. Matsuta, N. Shima, H. Kamata, et al., Metabolism of the designer drug α-pyrrolidinobutiophenone (α-PBP) in humans: Identification and quantification of the phase I metabolites in urine, Forensic Sci. Int. 249 (2015) 181–188. doi:10.1016/j.forsciint.2015.02.004.

[18] N. Shima, H. Kakehashi, S. Matsuta, et al., Urinary excretion and metabolism of the α-pyrrolidinophenone designer drug 1-phenyl-2-(pyrrolidin-1-yl)octan-1-one (PV9) in humans, Forensic Toxicol. 33 (2015) 279–294. doi:10.1007/s11419-015-0274-9.

[19] M. Ibáñez, Ó.J. Pozo, J. V. Sancho, et al., Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 151–164. doi:10.1007/s00216-015-9088-1.

[20] M.J. Swortwood, K.N. Ellefsen, A. Wohlfarth, et al., First metabolic profile of PV8, a novel synthetic cathinone, in human hepatocytes and urine by high-resolution mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 4845–4856. doi:10.1007/s00216-016-9599-4.

[21] M. Paul, S. Bleicher, S. Guber, et al., Identification of phase I and II metabolites of the new designer drug α -pyrrolidinohexiophenone (α -PHP) in human urine by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), J. Mass Spectrom. 50 (2015) 1305–1317. doi:10.1002/jms.3642.

[22] D. Dalvie, R.S. Obach, P. Kang, et al., Assessment of three human in vitro systems in the generation of major human excretory and circulating metabolites, Chem. Res. Toxicol. 22 (2009) 357–368. doi:10.1021/tx8004357 [doi].

[23] D. Fabregat- Safont, M. Mardal, C. Noble, et al., Comprehensive investigation on synthetic cannabinoids: metabolic behaviour and potency testing, using 5F- APP- PICA and AMB- FUBINACA as model compounds, Drug Test. Anal. (2019) dta.2659. doi:10.1002/dta.2659.

[24] S.K. Manier, L.H.J. Richter, J. Schäper, et al., Different in vitro and in vivo tools for elucidating the human metabolism of alpha-cathinone-derived drugs of abuse, Drug Test. Anal. 10 (2018) 1119–1130. doi:10.1002/dta.2355.

[25] L.H.J. Richter, H.H. Maurer, M.R. Meyer, New psychoactive substances: Studies on the metabolism of XLR-11, AB-PINACA, FUB-PB-22, 4-methoxy-α-PVP, 25-I-NBOMe, and meclonazepam using human liver preparations in comparison to primary human hepatocytes, and human urine, Toxicol. Lett. 280 (2017) 142–150. doi:10.1016/j.toxlet.2017.07.901.

[26] M.J. Swortwood, J. Carlier, K.N. Ellefsen, et al., In vitro, in vivo and in silico metabolic profiling of α-pyrrolidinopentiothiophenone, a novel thiophene stimulant, Bioanalysis. 8 (2016) 65–82. doi:10.4155/bio.15.237.

[27] D. Fabregat-Safont, X. Carbón, C. Gil, et al., Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance, Forensic Toxicol. 36 (2018) 447–457. doi:10.1007/s11419-018-0422-0.

[28] M. Grifell, M. Ventura, X. Carbón, et al., Patterns of use and toxicity of new para-halogenated substituted cathinones: 4-CMC (clephedrone), 4-CEC (4-chloroethcatinone) and 4-BMC (brephedrone), Hum. Psychopharmacol. Clin. Exp. 32 (2017) e2621. doi:10.1002/hup.2621.

[29] Á. Yanini, S. Armenta, F.A. Esteve-Turrillas, et al., Identification and characterization of the new psychoactive substance 3-fluoroethamphetamine in seized material, Forensic Toxicol. 36 (2018) 404–414. doi:10.1007/s11419-018-0416-y.

[30] M. Kobayashi, A. Pelander, R.A. Ketola, et al., Targeting misuse of 2-amino- N -ethyl-1-phenylbutane in urine samples: in vitro-in vivo correlation of metabolic profiles and development of LC-TOF-MS method, Drug Test. Anal. 7 (2015) 89–94. doi:10.1002/dta.1642.

[31] J.S. Park, S.U. Rehman, I.S. Kim, et al., Tentative identification of in vitro metabolites of 5-APDB, a synthetic benzofuran, by LC-Q/TOF-MS, J. Chromatogr. B. 1033–1034 (2016) 296–300. doi:10.1016/j.jchromb.2016.08.043.

[32] A.G. Helfer, A. Turcant, D. Boels, et al., Elucidation of the metabolites of the novel psychoactive substance 4-methyl- N -ethyl-cathinone (4-MEC) in human urine and pooled liver microsomes by GC-MS and LC-HR-MS/MS techniques and of its detectability by GC-MS or LC-MS n standard screening approach, Drug Test. Anal. 7 (2015) 368–375. doi:10.1002/dta.1682.

[33] M.R. Meyer, C. Vollmar, A.E. Schwaninger, et al., New cathinone-derived designer drugs 3-bromomethcathinone and 3-fluoromethcathinone: studies on their metabolism in rat urine and human liver microsomes using GC-MS and LC-high-resolution MS and their detectability in urine, J. Mass Spectrom. 47 (2012) 253–262. doi:10.1002/jms.2960.

MS/MS spectra

5-PPDi and its metabolites

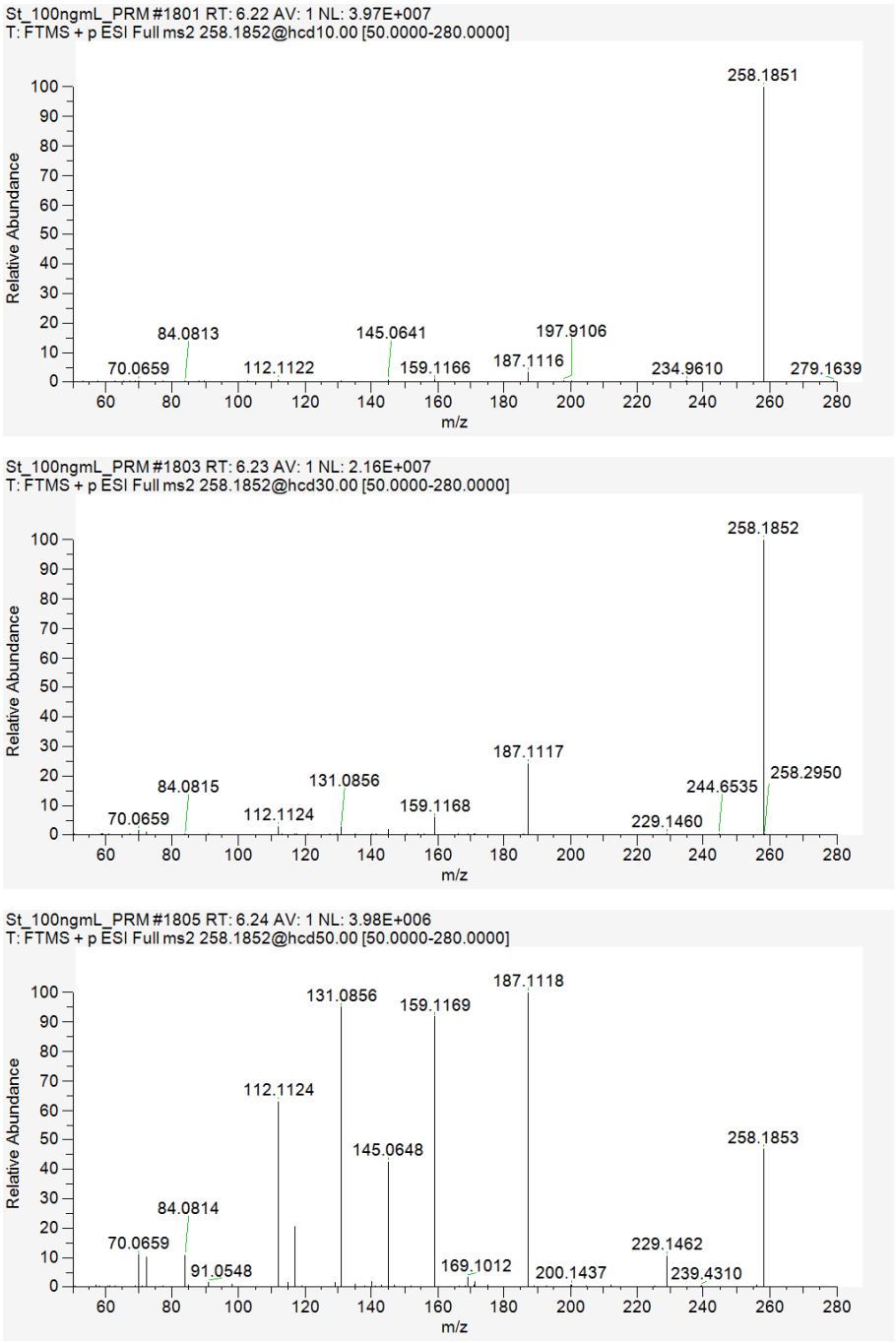


Figure S1. PRM spectra of 5-PPDi at 10, 30 and 50 eV.

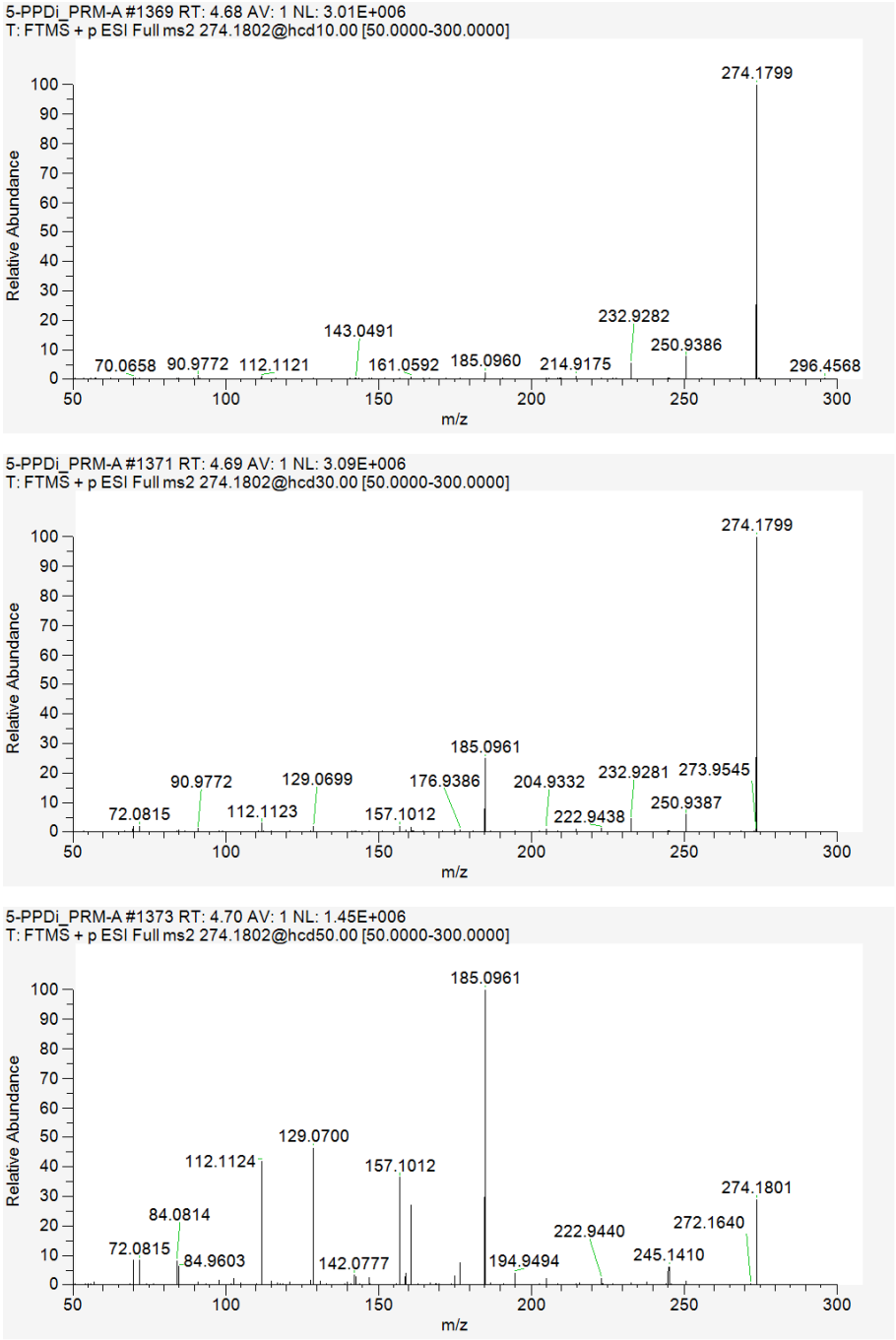


Figure S2. PRM spectra of 5-PPDi M1 at 10, 30 and 50 eV.

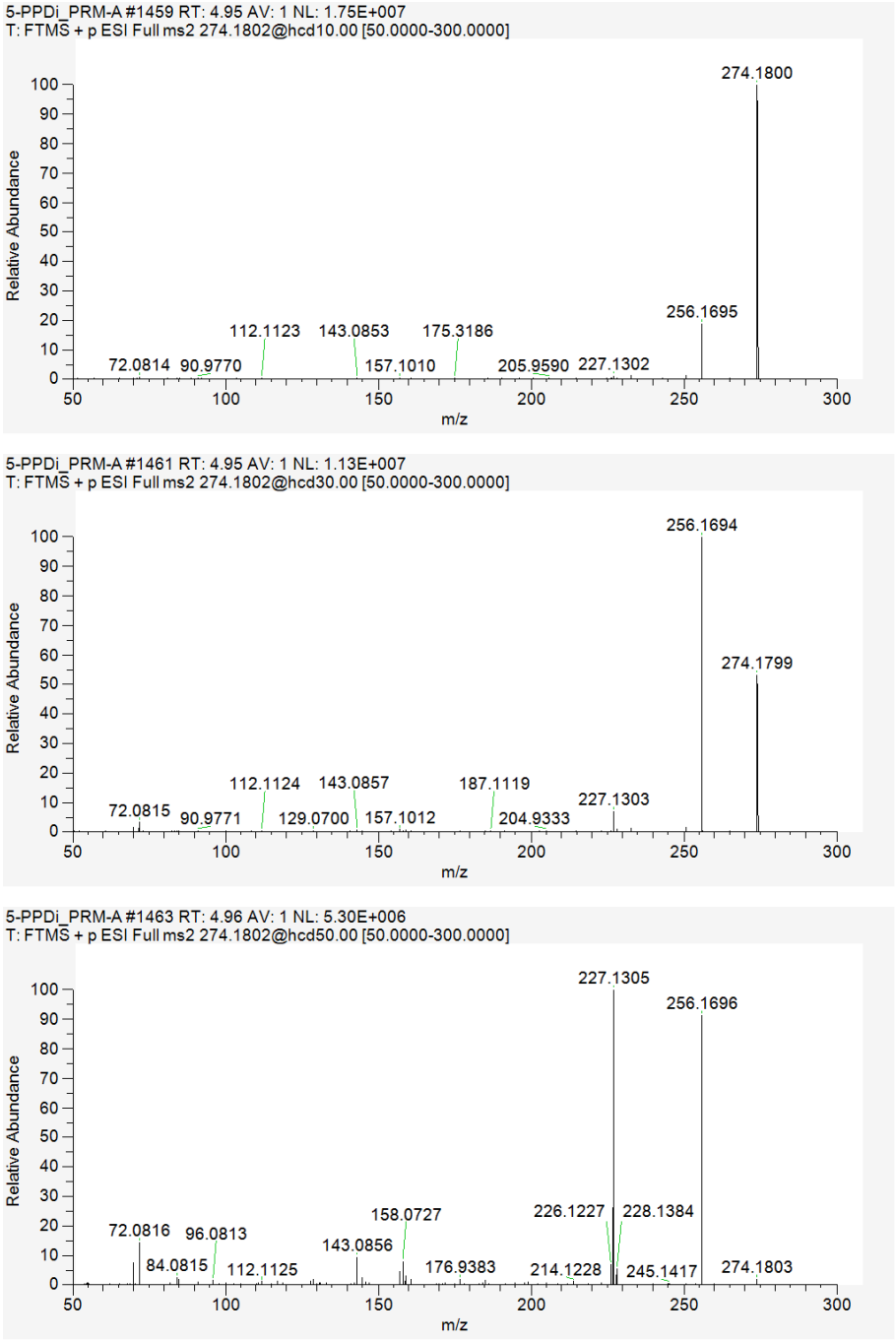


Figure S4. PRM spectra of 5-PPDi M2 at 10, 30 and 50 eV.

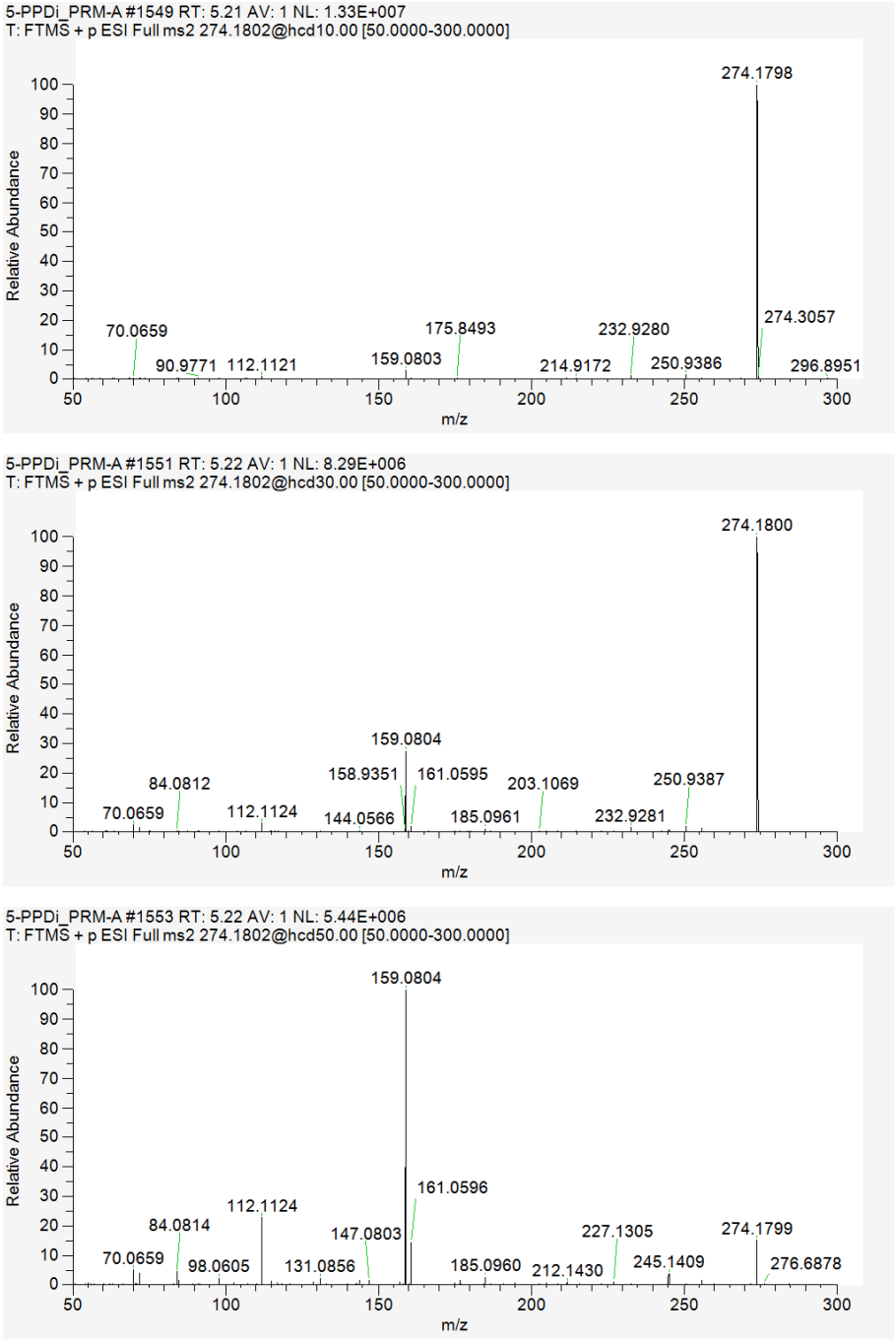


Figure S6. PRM spectra of 5-PPDi M3 at 10, 30 and 50 eV.

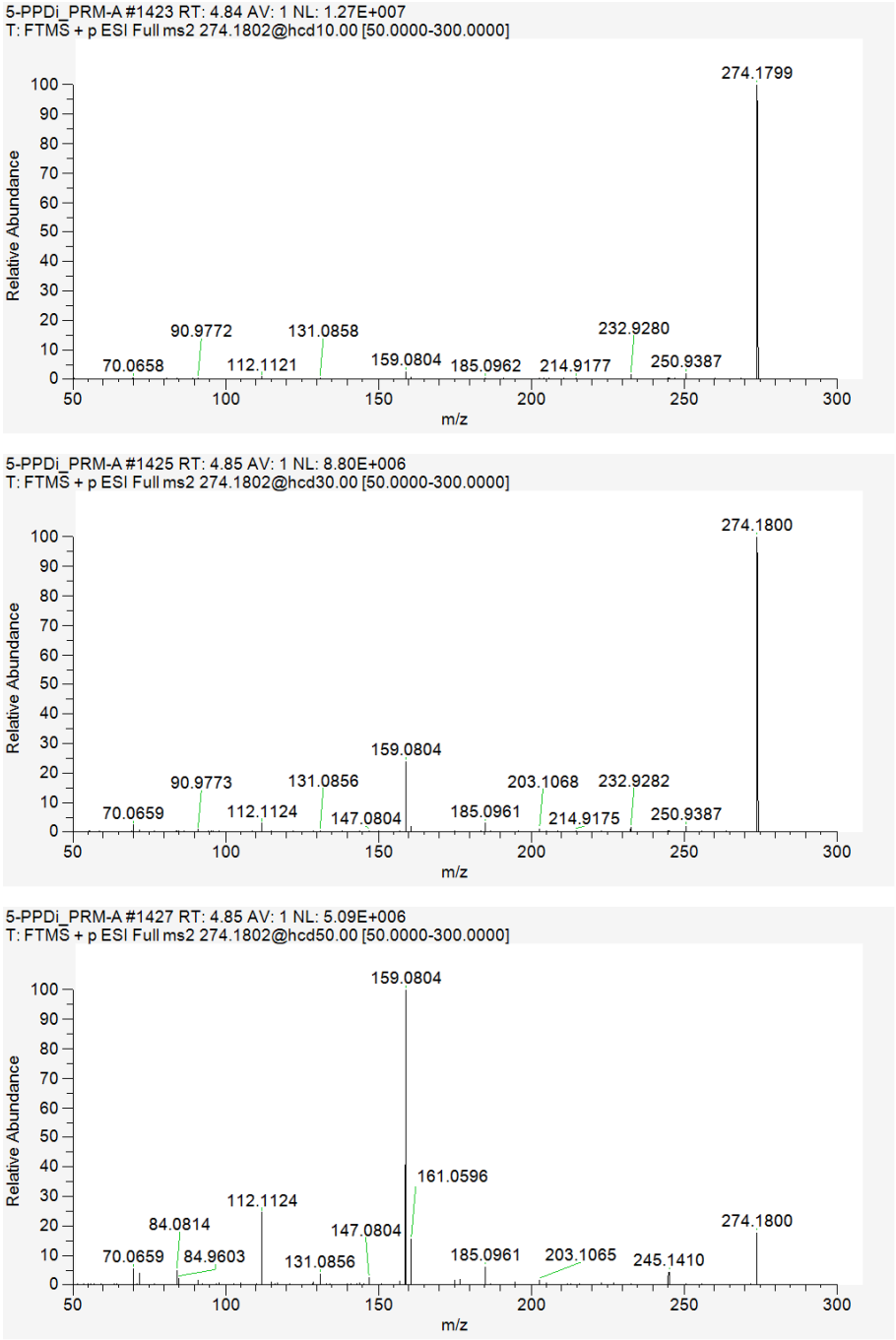


Figure S8. PRM spectra of 5-PPDi M4 at 10, 30 and 50 eV.

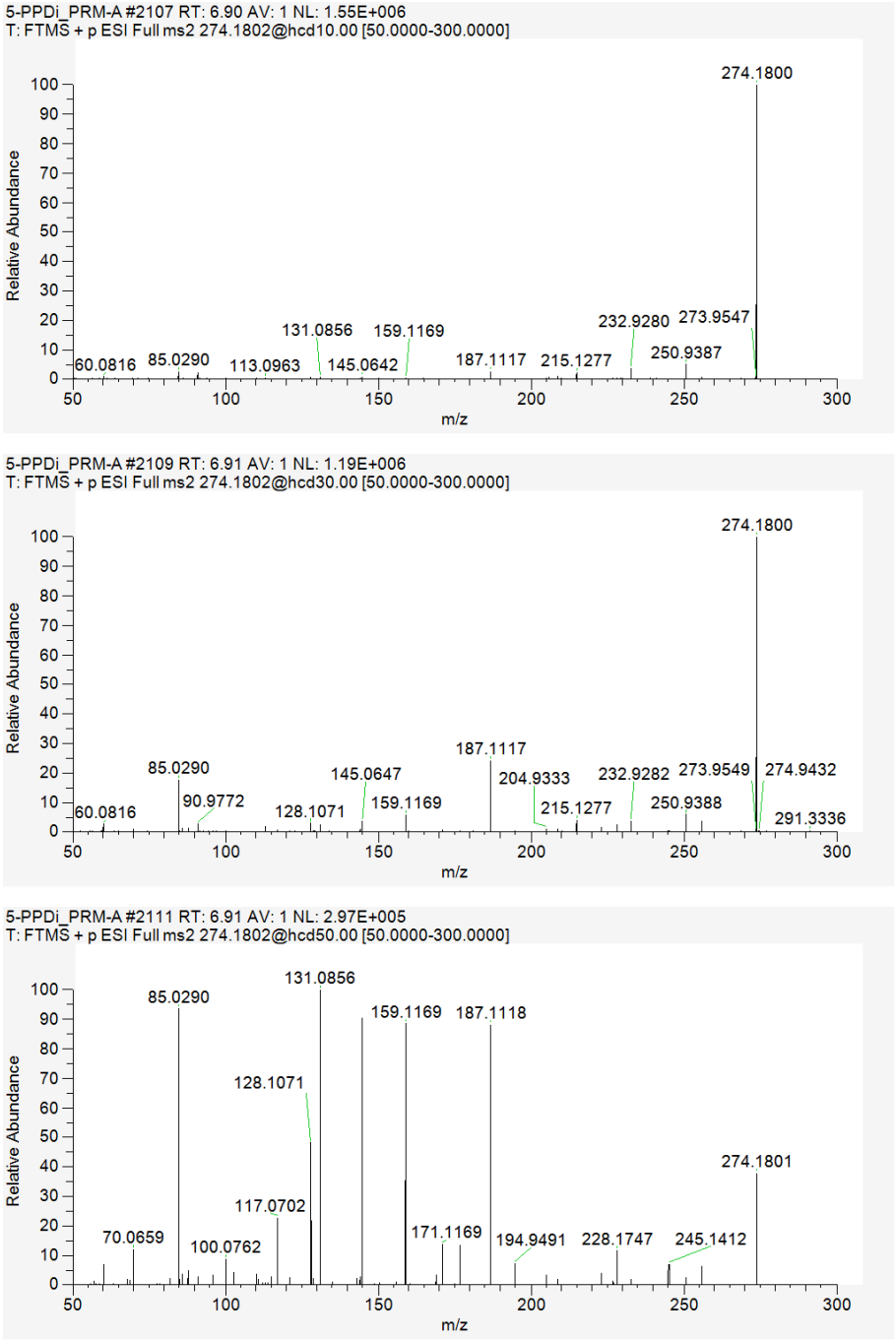


Figure S10. PRM spectra of 5-PPDi M5 at 10, 30 and 50 eV.

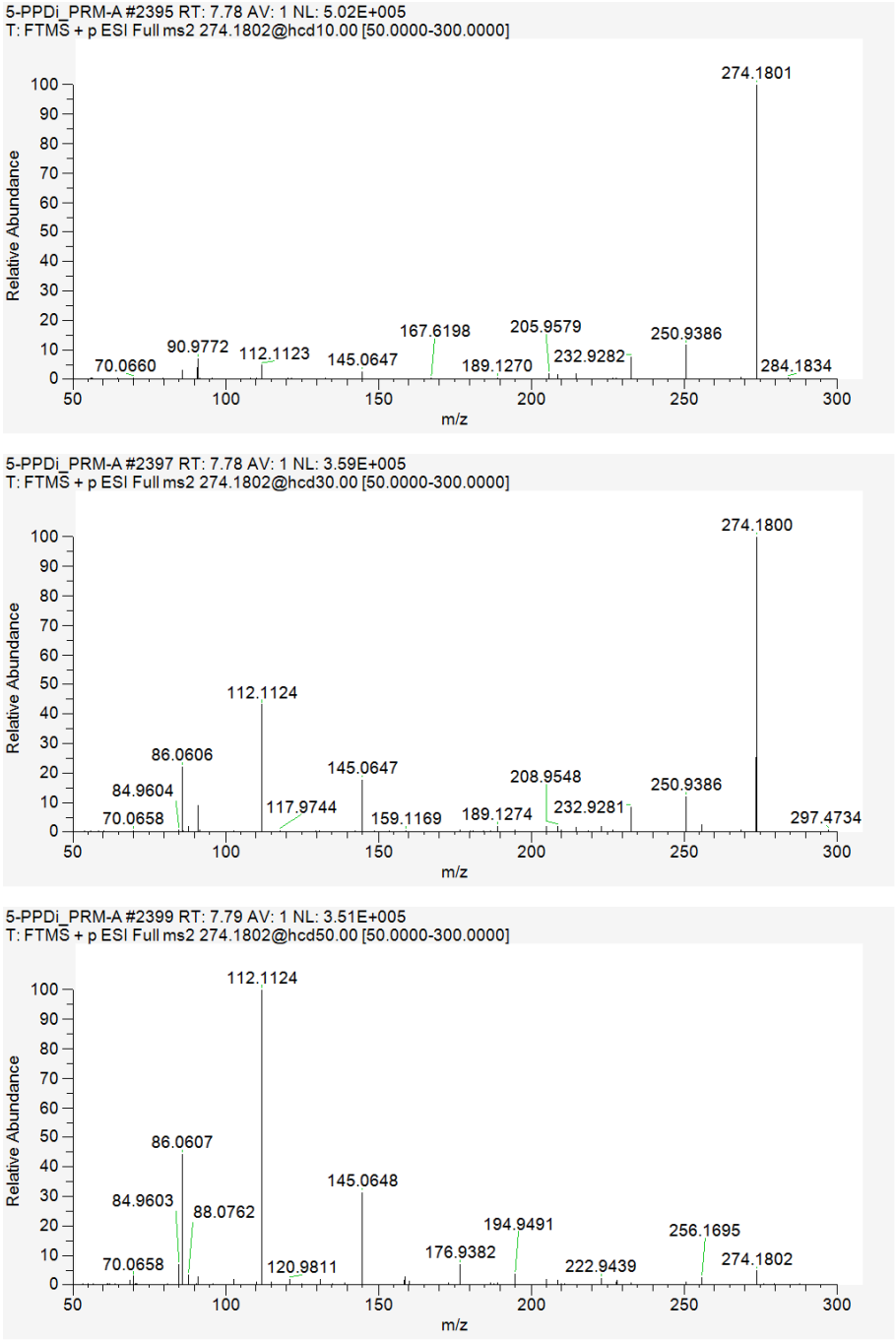


Figure S12. PRM spectra of 5-PPDi M6 at 10, 30 and 50 eV.

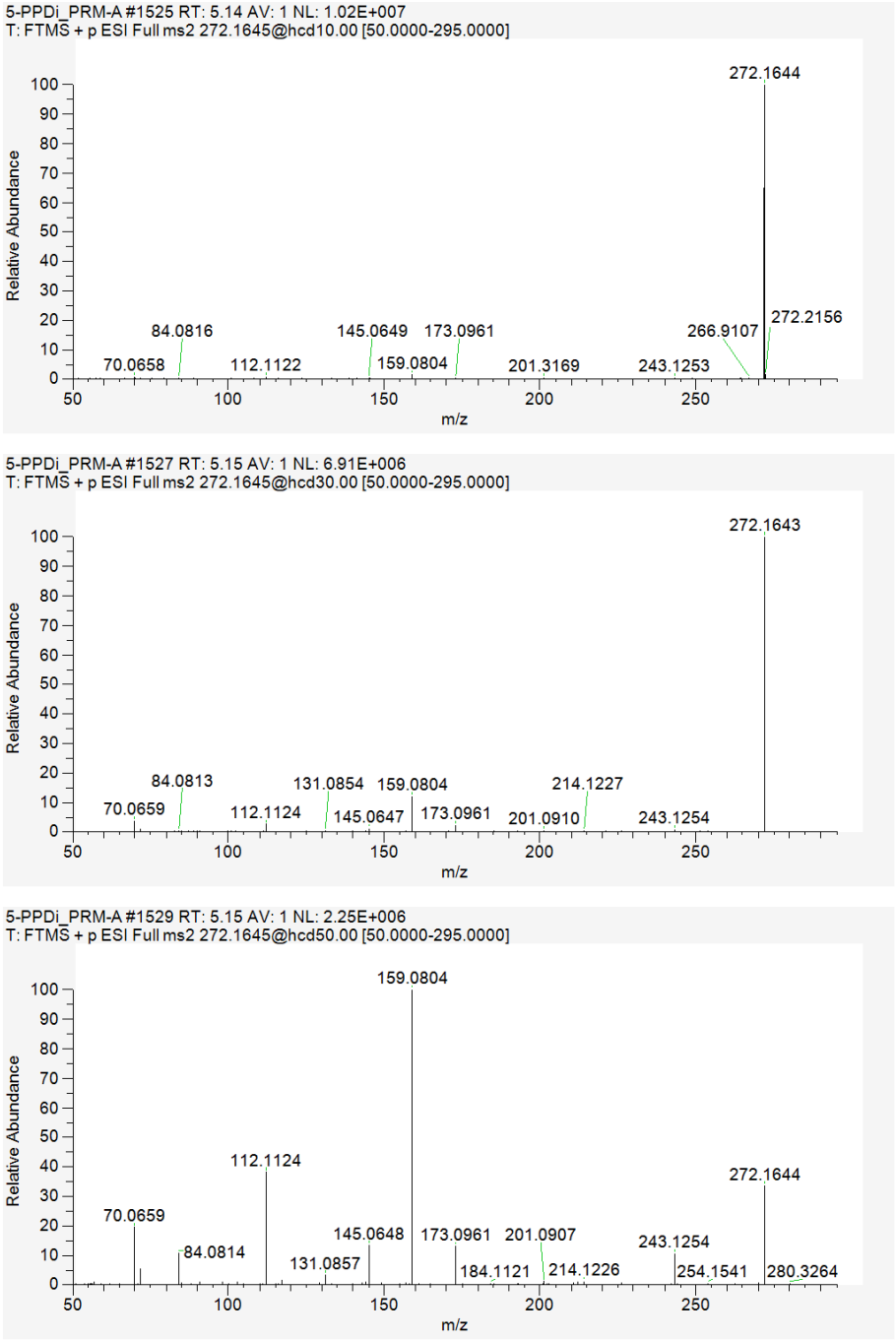


Figure S14. PRM spectra of 5-PPDi M7 at 10, 30 and 50 eV.

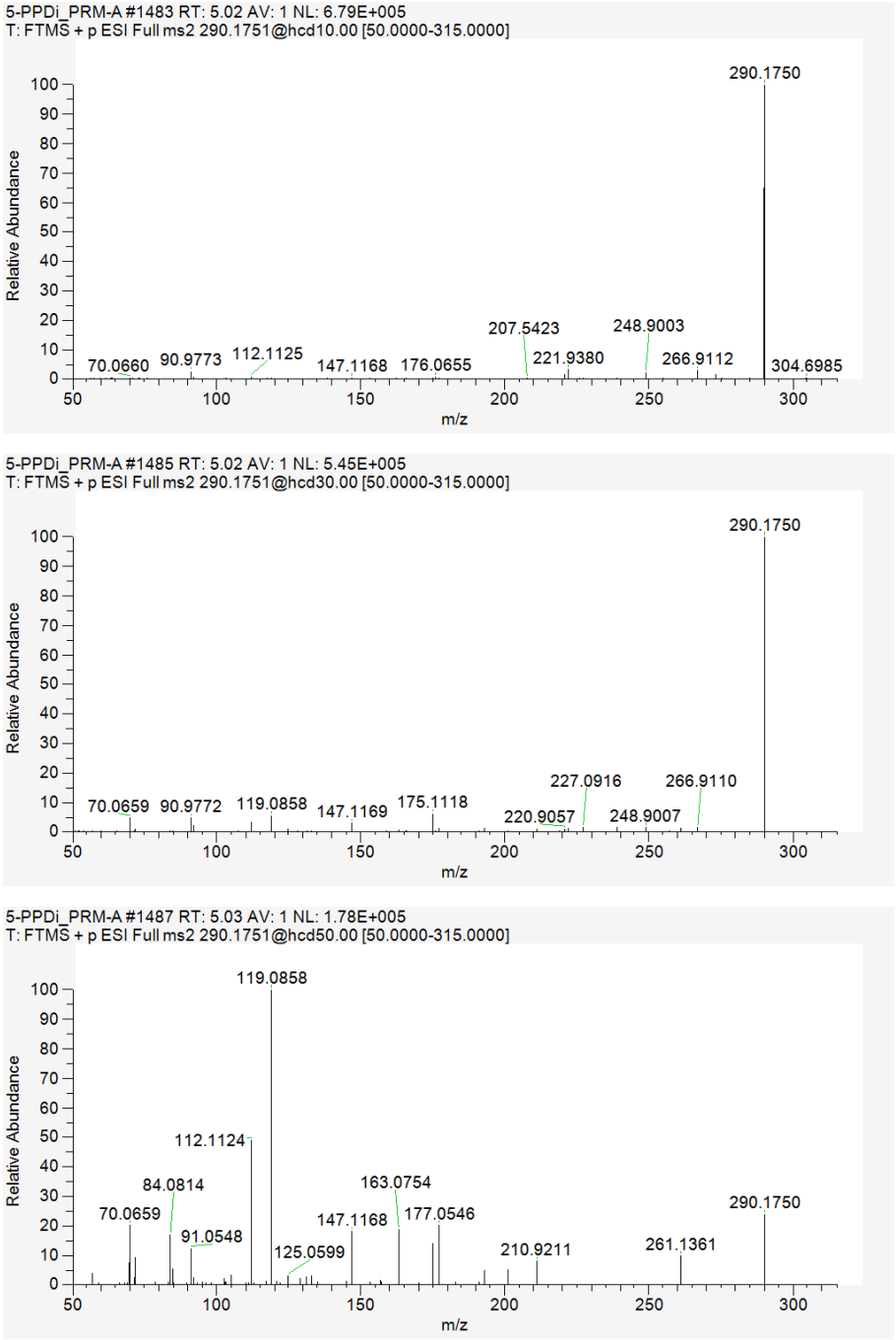


Figure S16. PRM spectra of 5-PPDi M8 at 10, 30 and 50 eV.

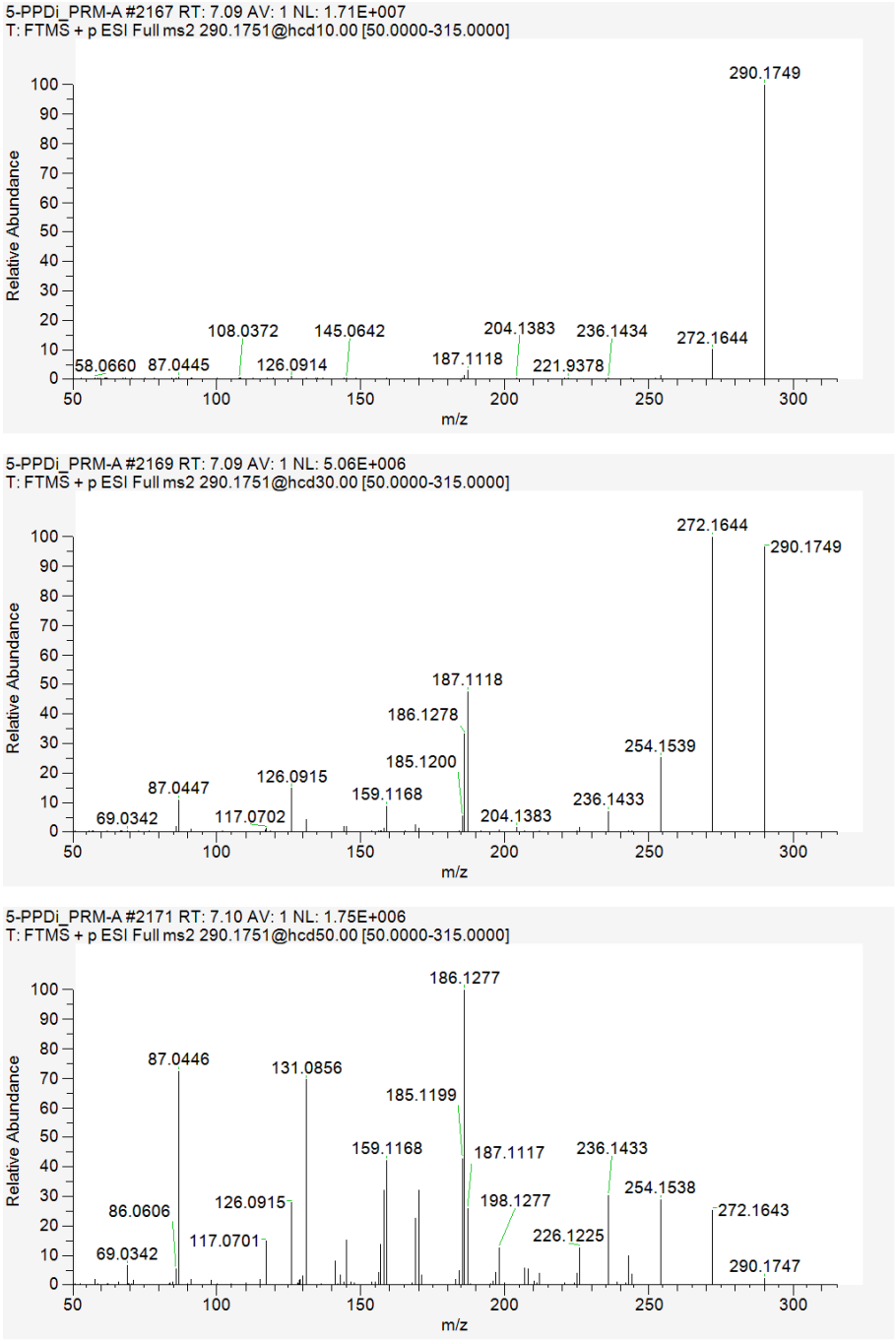


Figure S17. PRM spectra of 5-PPDi M9 at 10, 30 and 50 eV.

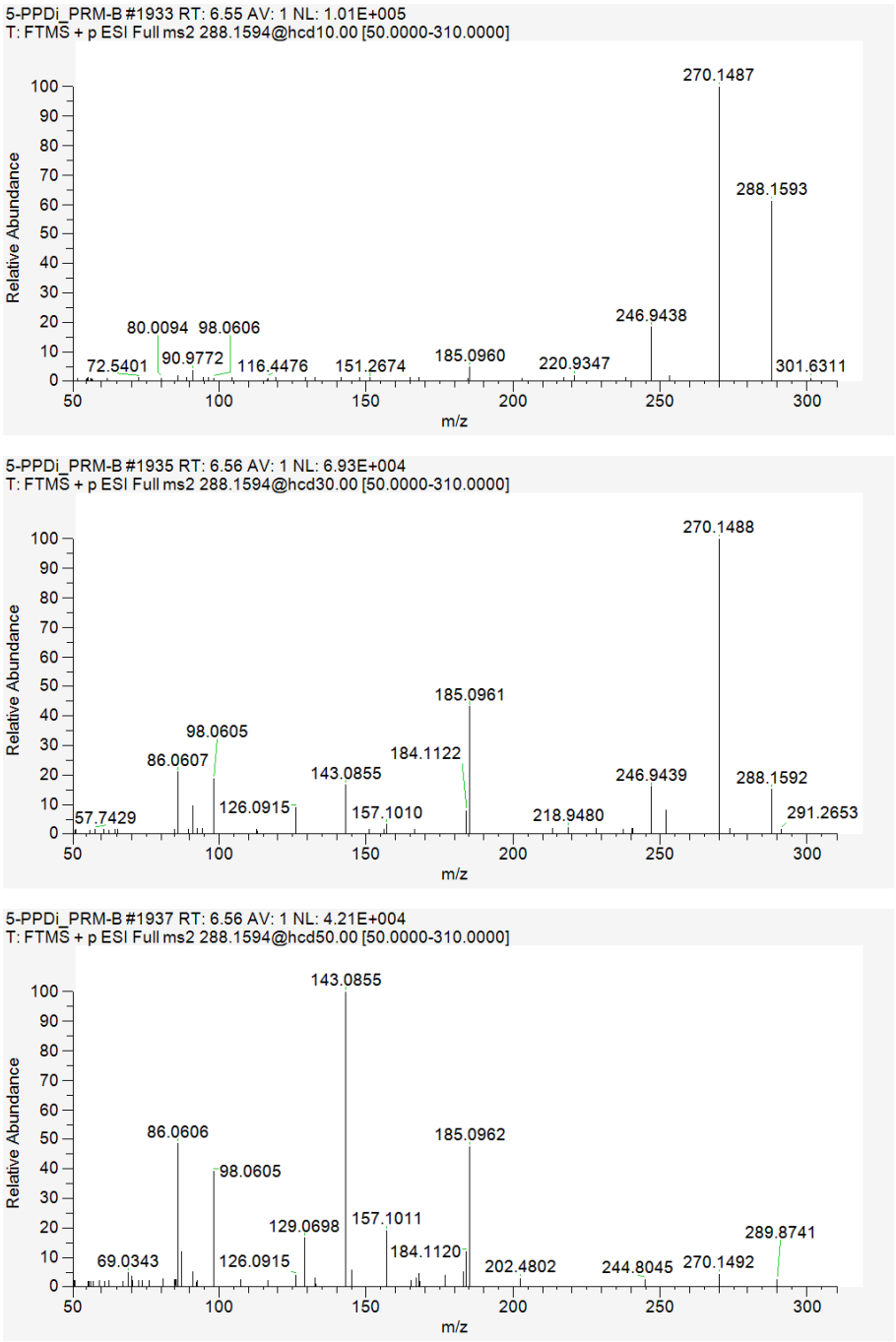


Figure S19. PRM spectra of 5-PPDi M10 at 10, 30 and 50 eV.

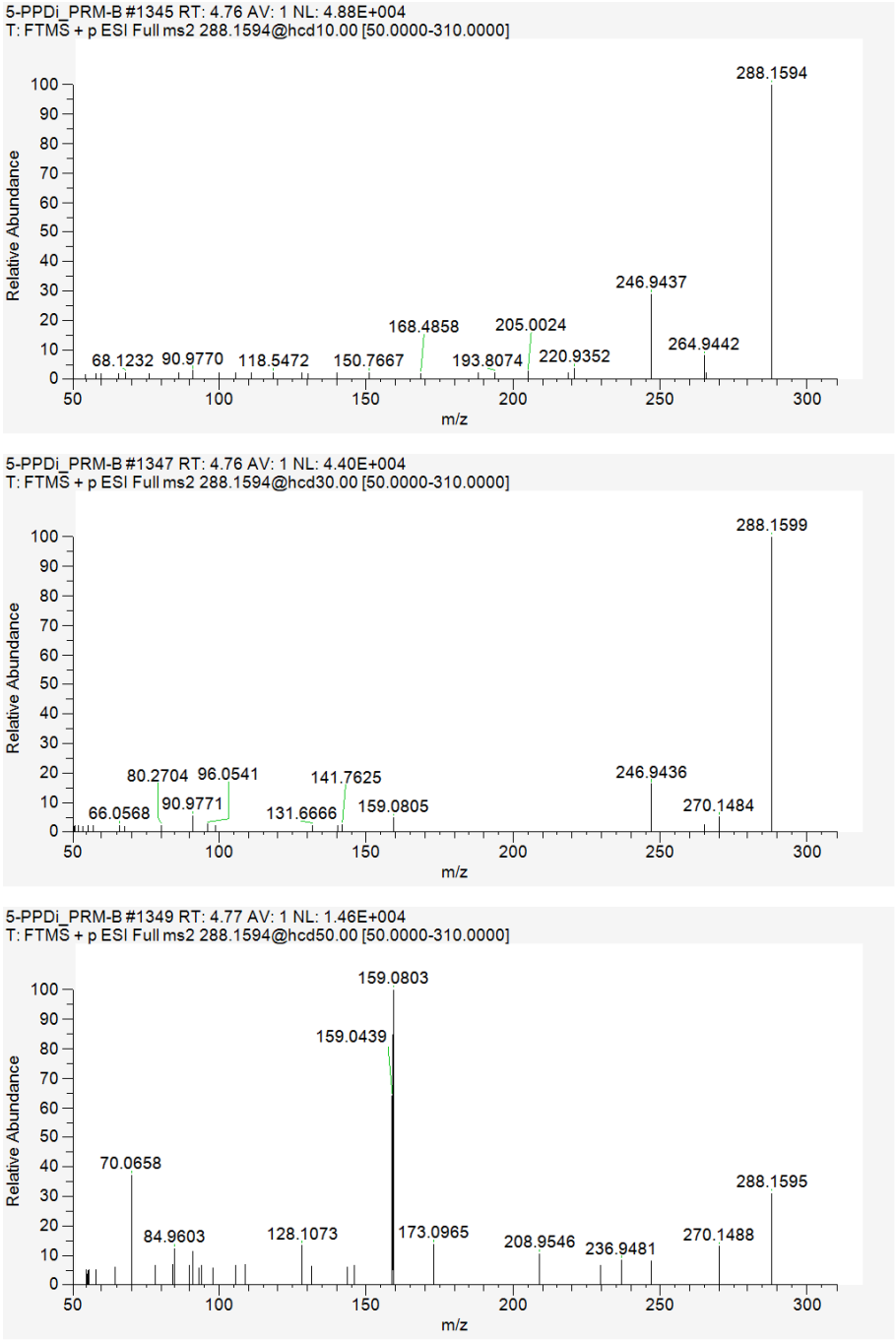


Figure S21. PRM spectra of 5-PPDi M11 at 10, 30 and 50 eV.

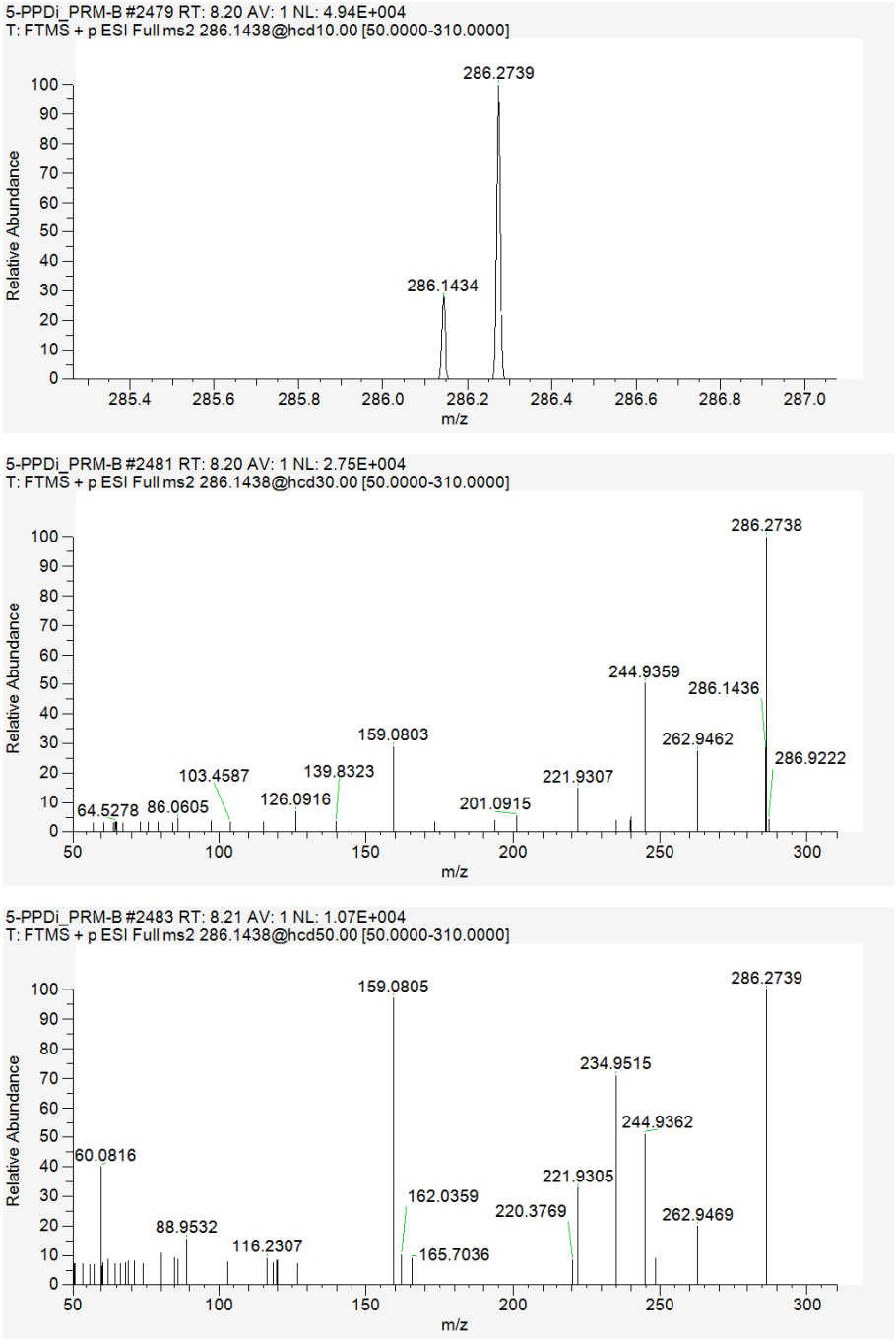


Figure S23. PRM spectra of 5-PPDi M12 at 10, 30 and 50 eV.

4-CPrC and its metabolites

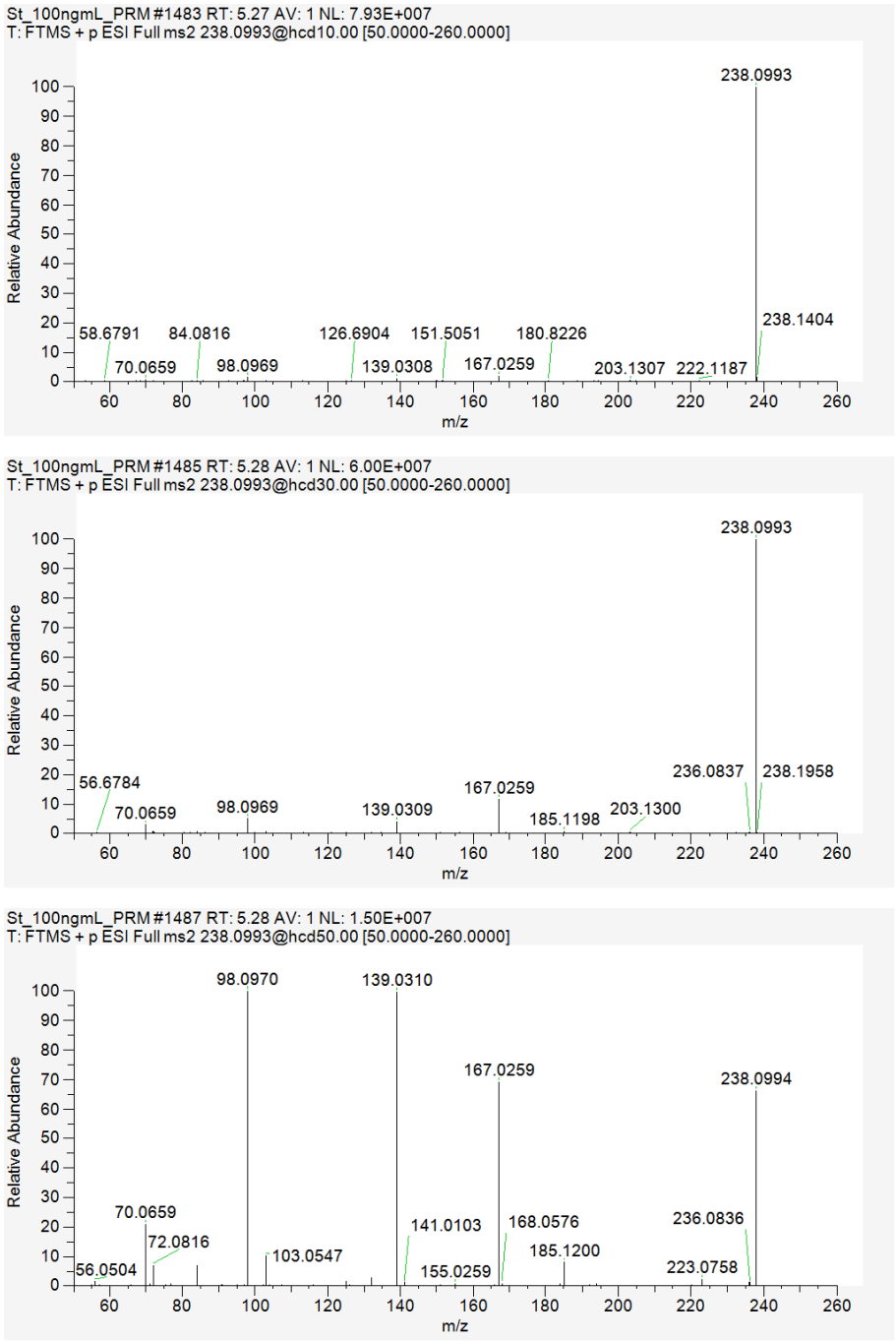


Figure S26. PRM spectra of 4-CPrC at 10, 30 and 50 eV.

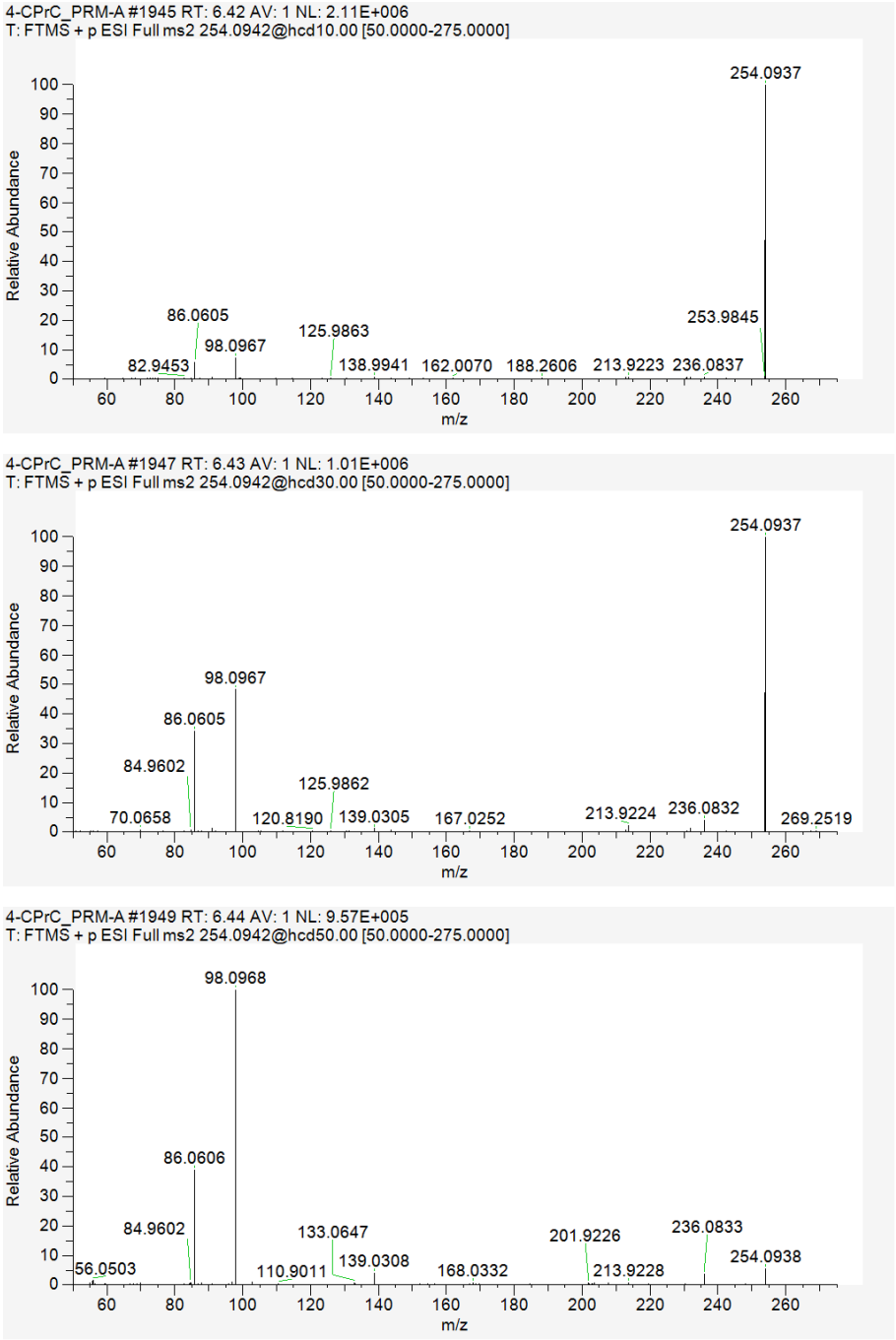


Figure S27. PRM spectra of 4-CPrC M1 at 10, 30 and 50 eV.

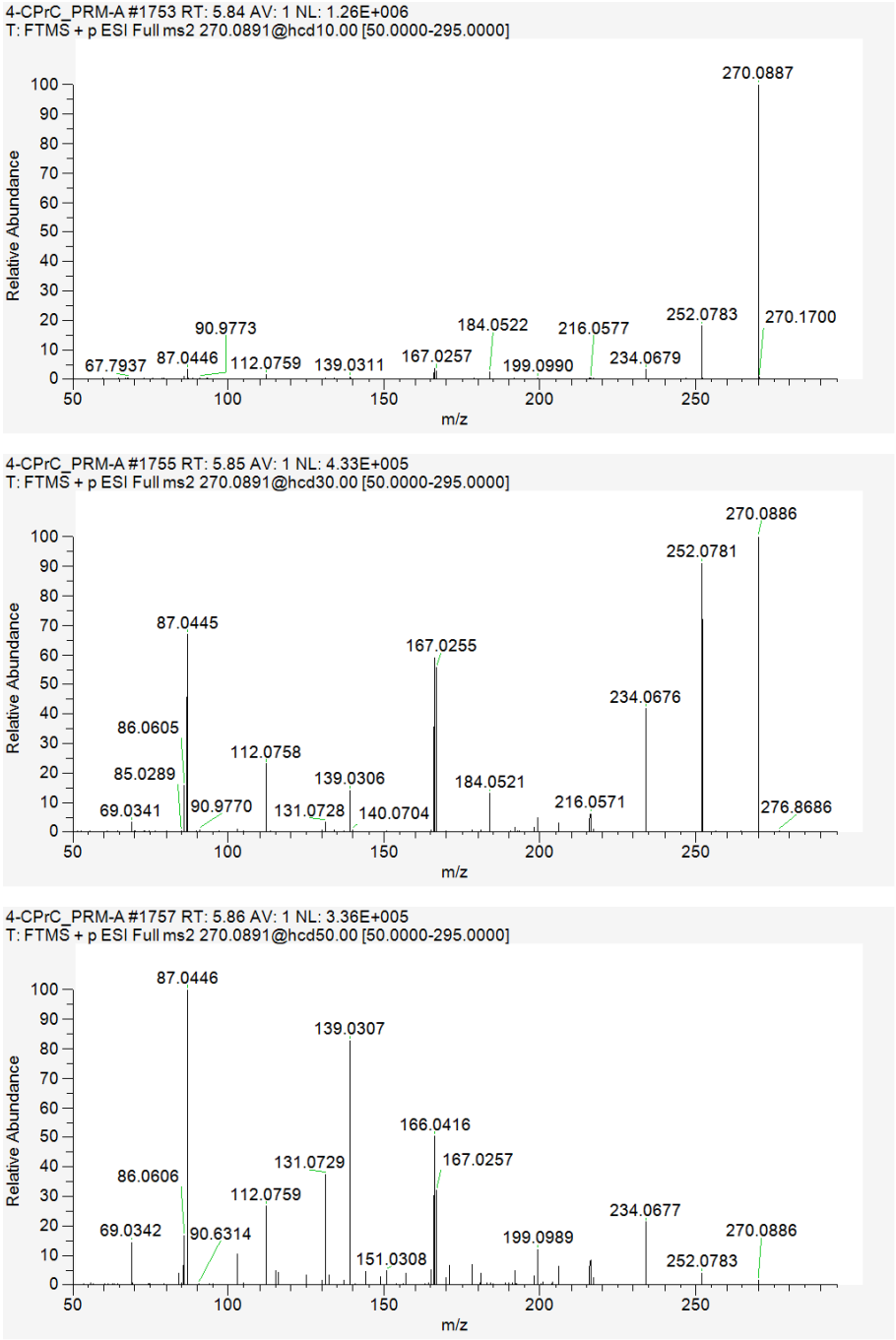


Figure S28. PRM spectra of 4-CPrC M2 at 10, 30 and 50 eV.

4.4. Discussion of the results obtained

In vitro approaches for performing NPS metabolite profiling have been widely used, in order to identify the biotransformation products of selected NPS, as well as to propose the most suitable consumption biomarkers. As shown in the two scientific articles included in this chapter, as well as in the introduction section of this chapter, the use of pHH is one of the most adequate *in vitro* models for metabolic profiling of NPS, as phase I and phase II biotransformations are directly obtained.

On the one hand, **research article VII** illustrates that the two SCRAs (5F-APP-PICA and AMB-FUBINACA) presented a fast-metabolic transformation, being both of them undetected after 180 min of incubation. Nevertheless, several phase I and phase II metabolites were elucidated by UHPLC-HRMS, proposing the most suitable consumption biomarkers for these compounds. The fast-metabolic behaviour of SCRAs is also discussed in **Chapter 6**, which deals with the screening of these compounds in urine samples in order to determine their consumption. On the other hand, **research article VIII** shows that synthetic cathinones are less biotransformed than SCRAs. For the cathinone 5-PPDi, up to 12 phase I metabolites (all of them corresponding to oxidation products) were identified after 180 min of incubation, but the parent compound was still the major compound present in the incubation media. For the remaining studied stimulants, only two minor metabolites were detected for 4-CPrC, while no metabolites were identified for the cathinone 4-CEC and the amphetamine 3-FEA.

Regarding the results obtained in **research article VII**, 12 metabolites were identified for 5F-APP-PICA, 7 produced by phase I biotransformation and 5 by phase II. The phase I reactions observed were oxidative defluorination, oxidation to carboxylic acid, amide to carboxylic acid, *N*-dealkylation, and combinations of all them, while the phase II metabolites were all conjugations with glucuronic acid. One month after the publication of **research article VII**, the *in vitro*

metabolism of 5F-APP-PICA, together with 5F-APP-PINACA (or PX-2) and APP-CHMINACA (or PX-3), using pHLM was published by Travon Cooman and Suzanne Bell [30], using also a UHPLC-HRMS system with a Q-Orbitrap (Q Exactive) mass spectrometer. In that study, only four phase I metabolites were identified, corresponding to oxidative defluorination (M1 in **research article VII**), amide to carboxylic acid (M3), *N*-dealkylation (M6), and oxidative defluorination plus amide to carboxylic acid conversion (M4). These authors propose the use of the oxidative defluorinated metabolite as the most suitable biomarker, followed by the *N*-dealkylated metabolite, based on the observed compound response [30]. Nevertheless, in this study the incubations with pHLM were performed up to 60 min, while our incubations with pHH were up to 180 min. Our results illustrate that M1 was the major metabolite at 60 min, agreeing with Cooman & Bell. However, at 180 min the presence of M1 decreased significantly, being then the major metabolites M4 (also reported by Cooman & Bell), M5 and M3 (also reported by Cooman & Bell). This fact illustrates the benefits obtained using pHH instead of pHLM, as the prevalence of the obtained metabolites can be assessed with longer times and then, establishing most adequate consumption biomarkers.

A second research about the *in vitro* metabolism of 5F-APP-PICA using pHLM and UHPLC-HRMS (QTOF instrument) was published by Brandon Presley and colleagues [31] six months after **research article VII** publication. In this case, incubations with pHLM were performed up to 120 min, identifying 10 phase I metabolites produced after oxidative defluorination, hydroxylation, oxidation to carboxylic acid, amide to carboxylic acid, *N*-dealkylation, and combinations of all them [31]. Several metabolites identified by Presley and colleagues were in concordance with our results, with the exception of hydroxylated metabolites. In our study, no hydroxylated metabolites were reported, whereas Presley and colleagues described the presence of *N*-alkyl hydroxylation (keeping also the fluorine atom), phenyl hydroxylation, and indole ring hydroxylation [31]. Although these biotransformations have been reported for similar SCRAs using human,

mouse and rat hepatocytes [32], and pHH and pHLM [32], these biotransformations were not found for 5F-APP-PICA in our study. Presley and colleagues performed an accurate discussion of their findings compared with the metabolites described in **research article VII** [31], indicating that both studies are complementary and thus, demonstrating the adequation of using pHLM and pHH for NPS metabolite profiling [31]. As well-stated by Presley and colleagues, pHLM are a more cost-effective option for *in vitro* metabolism studies compared with pHH [31]. Nevertheless, the results obtained by pHLM should also be contrasted with an additional *in vitro* or *in vivo* model in order to discard possible artefacts produced by the high-metabolic rate of pHLM [11].

For the second SCRA studied in **research article VII**, the AMB-FUBINACA, up to 7 phase I metabolites and 1 phase II were found after pHH incubations. The phase I reactions observed were hydroxylation (alkyl and indazole ring), *O*-demethylation, *N*-dealkylation, a putative *N*-methylation, and combinations of them, while the phase II metabolite was produced by glucuronidation. During the publication process of our study, Duo-qi Xu and colleagues published the metabolic behaviour of AMB-FUBINACA, using pHLM and zebrafish as *in vitro* and *in vivo* models, respectively, and UHPLC-HRMS Q-Orbitrap (Q Exactive) [34]. In this case, up to 14 phase I and 3 phase II (glucuronide conjugation) metabolites were obtained after 120 min of incubation with pHLM [34]. The main metabolic pathway obtained in our study was consistent with the results reposted by Xu and colleagues, with some differences that will be discussed hereafter. Xu and colleagues indicated the presence of 2 *O*-ethylated metabolites produced after *O*-demethylation of AMB-FUBINACA. This behaviour was also observed in our study, and as explained in **research article VII**, the origin of the M6-methyl metabolite could not be assured to be produced by metabolic reactions. The major metabolites proposed as consumption biomarkers by Xu and co-authors were the *O*-demethylated (M1 in our study) and its glucuronide conjugated (M1-Gluc) metabolites [34], which is in agreement with our proposal. So again, the adequation of pHLM and pHH was demonstrated for NPS metabolite profiling studies,

keeping in mind that the results obtained by pHLM should be contrasted, as performed by Xu using zebrafish as *in vivo* model [34].

The adequation of M1 as consumption biomarker was demonstrated by Rui Shen Ong and colleagues, who validated an LC-MS/MS methodology for the determination of 29 SCRA and metabolites in human whole blood [35]. This study showed that M1 presented higher prevalence than AMB-FUBINACA in 564 blood samples analysed, being M1 identified in 81 samples whereas AMB-FUBINACA only in 30 [35]. Moreover, a recent study performed by Florian Franz demonstrated that M1, the major metabolite of AMB-FUBINACA and AB-FUBINACA, can be detected 311 h (13 days) after a single oral ingestion of AB-FUBINACA [36]. These results suggest that M1 could also be the most adequate long-term consumption biomarker for AMB-FUBINACA. Indeed, in a massive intoxication related to AMB-FUBINACA in New York on the 12th July 2016 (known as "zombie" outbreak), the M1 was detected in blood and urine samples from 8 people, whereas the parent compound was not detected [37].

Finally, the results obtained for the synthetic cathinone 5-PPDi in **research article VIII** show that the metabolic transformation of this compound occurs mainly by hydroxylation/oxidation processes, identifying up to 12 phase I metabolites. Two months after the publication of this study, the metabolic behaviour of 5-PPDi in human urine was reported by Ayumu Ishii and colleagues, using a UHPLC-HRMS Q-Orbitrap (Q Exactive) system [38]. The results obtained in human urine showed the presence of four phase I metabolites produced by hydroxylation, oxidation and reduction processes, being the indanyl hydroxylated compound (M1 in **research article VIII**) the major metabolite found in the urine sample, together with parent compound [38]. The remaining three metabolites reported by Ishii and colleagues were not found during pHH incubation. The unaltered synthetic cathinones are usually found in consumers urine samples at high concentration [39,40], probably due to their high polarity that allows a fast excretion after intake. This fact was also observed for 5-PPDi by Ishii and colleagues, as the major consumption biomarkers for this cathinone were the

unaltered compound and M1 [38]. No information about the dosing method, the route of acquisition, and the time from administration to urine sampling was available in this study; consequently, no information about pharmacokinetics, excretion rate and long-term metabolites (including additional metabolites that could be excreted at higher time) was provided [38]. It is possible that 180 min was too much incubation time when using pHH and synthetic cathinones due to the high polarity and excretion of these compounds, or maybe additional metabolites (such as those reported in **research article VIII**) could be found in urine samples at different times from administration. In order to verify this fact, and to study the pharmacokinetic behaviour of 5-PPDi and its metabolites, *in vivo* experiments using mice or rats must be performed, collecting blood and urine samples at different times, and comparing the results with the information available in **research article VIII** and data from Ishii and colleagues. In this way, an accurate metabolic fate of 5-PPDi would be obtained.

The information presented in **Chapter 4** showed that pHH is an adequate *in vitro* model for assessing the metabolic behaviour of NPS, but in some cases, additional *in vivo* experiments should be performed for obtaining a complete and accurate description of the metabolism of these compounds.

4.5. Literature

1. Wrighton, S. A., Ring, B. J. & Vandenbranden, M. The Use of In Vitro Metabolism Techniques in the Planning and Interpretation of Drug Safety Studies. Toxicol. Pathol. 23, 199–208 (1995).

2. Noble, C., Mardal, M., Bjerre Holm, N., Stybe Johansen, S. & Linnet, K. In vitro studies on flubromazolam metabolism and detection of its metabolites in authentic forensic samples. Drug Test. Anal. 9, 1182–1191 (2017).

3. Silva, J. P., Araújo, A. M., de Pinho, P. G., Carmo, H. & Carvalho, F. Synthetic Cannabinoids JWH-122 and THJ-2201 Disrupt Endocannabinoid-Regulated Mitochondrial Function and Activate Apoptotic Pathways as a Primary Mechanism of In Vitro Nephrotoxicity at In Vivo Relevant Concentrations. Toxicol. Sci. 169, 422–435 (2019).

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

5. Mardal, M., Annaert, P., Noble, C., Oorts, M. & Linnet, K. Metabolism of the synthetic cannabinoid 5F-PY-PICA by human and rat hepatocytes and identification of biliary analytical targets by directional efflux in sandwich-cultured rat hepatocytes using UHPLC-HR-MS/MS. J. Pharm. Biomed. Anal. 149, 296–307 (2018).

6. Cannaert, A., Storme, J., Franz, F., Auwärter, V. & Stove, C. P. Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. Anal. Chem. 88, 11476–11485 (2016).

7. Rostami-Hodjegan, A. & Tucker, G. T. Simulation and prediction of in vivo drug metabolism in human populations from in vitro data. Nat. Rev. Drug Discov. 6, 140–148 (2007).

8. Maurer, H. H. & Brandt, S. D. New Psychoactive Substances. Pharmacology, Clinical, Forensic and Analytical Toxicology. Handbook of Experimental Pharmacology 252, (Springer International Publishing, 2018).

9. Jakobyz, W. B. & Ziegler, D. M. The Enzymes of Detoxication. J. Biol. Chem. 265, 20715–20718 (1990).

10. Richter, L. H. J., Maurer, H. H. & Meyer, M. R. New psychoactive substances: Studies on the metabolism of XLR-11, AB-PINACA, FUB-PB-22, 4-methoxy-α-PVP, 25-I-NBOMe, and meclonazepam using human liver preparations in comparison to primary human hepatocytes, and human urine. Toxicol. Lett. 280, 142–150 (2017).

11. Manier, S. K., Richter, L. H. J., Schäper, J., Maurer, H. H. & Meyer, M. R. Different in vitro and in vivo tools for elucidating the human metabolism of alpha-cathinone-derived drugs of abuse. Drug Test. Anal. 10, 1119–1130 (2018).

12. Richter, L. H. J. et al. Tools for studying the metabolism of new psychoactive substances for toxicological screening purposes – A comparative study using pooled human liver S9, HepaRG cells, and zebrafish larvae. Toxicol. Lett. 305, 73–80 (2019).

13. Jia, L. & Liu, X. The Conduct of Drug Metabolism Studies Considered Good Practice (II): In Vitro Experiments. Curr. Drug Metab. 8, 822–829 (2007).

14. Gerets, H. H. J. et al. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol. Toxicol. 28, 69–87 (2012).

15. Li, A. P., Maurel, P., Gomez-Lechon, M. J., Cheng, L. C. & Jurima-Romet, M. Preclinical evaluation of drug—drug interaction potential: present status of the application of primary human hepatocytes in the evaluation of cytochrome P450 induction. Chem. Biol. Interact. 107, 5–16 (1997).

16. Wohlfarth, A. et al. First metabolic profile of XLR-11, a novel synthetic cannabinoid, obtained by using human hepatocytes and high-resolution mass spectrometry. Clin. Chem. 59, 1638–1648 (2013).

17. Maurer, H. H. & Meyer, M. R. High-resolution mass spectrometry in toxicology: current status and future perspectives. Arch. Toxicol. 90, 2161–2172 (2016).

18. Diao, X. et al. In Vitro and In Vivo Human Metabolism of Synthetic Cannabinoids FDU-PB-22 and FUB-PB-22. AAPS J. 18, 455–464 (2016).

19. Wohlfarth, A. et al. Pentylindole/Pentylindazole Synthetic Cannabinoids and Their 5-Fluoro Analogs Produce Different Primary Metabolites: Metabolite Profiling for AB-PINACA and 5F-AB-PINACA. AAPS J. 17, 660–677 (2015).

20. Nordmeier, F., Richter, L. H. J., Schmidt, P. H., Schaefer, N. & Meyer, M. R. Studies on the in vitro and in vivo metabolism of the synthetic opioids U-51754, U-47931E, and methoxyacetylfentanyl using hyphenated high-resolution mass spectrometry. Sci. Rep. 9, 13774 (2019).

21. Castaneto, M. S. et al. Identification of AB-FUBINACA metabolites in human hepatocytes and urine using high-resolution mass spectrometry. Forensic Toxicol. 33, 295–310 (2015).

22. Castell, J. V, Jover, R., Martnez-Jimnez, C. P. & Gmez-Lechn, M. J. Hepatocyte cell lines: their use, scope and limitations in drug metabolism studies. Expert Opin. Drug Metab. Toxicol. 2, 183–212 (2006).

23. Anthérieu, S., Chesné, C., Li, R., Guguen-Guillouzo, C. & Guillouzo, A. Optimization of the HepaRG cell model for drug metabolism and toxicity studies. Toxicol. Vitr. 26, 1278–1285 (2012).

24. Noble, C., Cannaert, A., Linnet, K. & Stove, C. P. Application of an activity-based receptor bioassay to investigate the in vitro activity of selected indole- and indazole-3-carboxamide-based synthetic cannabinoids at CB1 and CB2 receptors. Drug Test. Anal. 1–11 (2018). doi:10.1002/dta.2517

25. Glennon, R. A. & Dukat, M. Structure-Activity Relationships of Synthetic Cathinones. in Neuropharmacology of New Psychoactive Substances (NPS): The Science Behind the Headlines (eds. Baumann, M. H., Glennon, R. A. & Wiley, J. L.) 19–47 (Springer International Publishing, 2017). doi:10.1007/7854_2016_41

26. Wiley, J. L. et al. AB-CHMINACA, AB-PINACA, and FUBIMINA: Affinity and Potency of Novel Synthetic Cannabinoids in Producing 9-Tetrahydrocannabinol-Like Effects in Mice. J. Pharmacol. Exp. Ther. 354, 328–339 (2015).

27. Cannaert, A., Franz, F., Auwärter, V. & Stove, C. P. Activity-Based Detection of Consumption of Synthetic Cannabinoids in Authentic Urine Samples Using a Stable Cannabinoid Reporter System. Anal. Chem. 89, 9527–9536 (2017).

28. Cannaert, A. et al. Activity-Based Concept to Screen Biological Matrices for Opiates and (Synthetic) Opioids. Clin. Chem. 64, 1221–1229 (2018).

29. Vasudevan, L. et al. Assessment of structure-activity relationships and biased agonism at the Mu opioid receptor of novel synthetic opioids using a novel, stable bio-assay platform. Biochem. Pharmacol. 113910 (2020). doi:10.1016/j.bcp.2020.113910

30. Cooman, T. & Bell, S. In vitro metabolism of the synthetic cannabinoids PX- 1, PX- 2, and PX- 3 by high- resolution mass spectrometry and their clearance rates in human liver microsomes. Rapid Commun. Mass Spectrom. 33, 1816–1825 (2019).

31. Presley, B. C., Logan, B. K. & Jansen- Varnum, S. A. Phase I metabolism of synthetic cannabinoid receptor agonist PX- 1 (5F- APP- PICA) via incubation with human liver microsomes and UHPLC–HRMS. Biomed. Chromatogr. 34, 0–2 (2020).

32. Kim, J.-H. et al. Targeted and non-targeted metabolite identification of MAM-2201 in human, mouse, and rat hepatocytes. Drug Test. Anal. 10, 1328–1335 (2018).

33. Andersson, M., Diao, X., Wohlfarth, A., Scheidweiler, K. B. & Huestis, M. A. Metabolic profiling of new synthetic cannabinoids AMB and 5F-AMB by human hepatocyte and liver microsome incubations and high-resolution mass spectrometry. Rapid Commun. Mass Spectrom. 30, 1067–1078 (2016).

34. Xu, D. et al. Analysis of AMB-FUBINACA Biotransformation Pathways in Human Liver Microsome and Zebrafish Systems by Liquid Chromatography-High Resolution Mass Spectrometry. Front. Chem. 7, 1–9 (2019).

35. Ong, R. S. et al. Simultaneous analysis of 29 synthetic cannabinoids and metabolites, amphetamines, and cannabinoids in human whole blood by liquid chromatography–tandem mass spectrometry – A New Zealand perspective of use in 2018. Drug Test. Anal. 12, 195–214 (2020).

36. Franz, F., Haschimi, B., King, L. A. & Auwärter, V. Extraordinary long detection window of a synthetic cannabinoid metabolite in human urine – Potential impact on therapeutic decisions. Drug Test. Anal. 12, 391–396 (2020).

37. Adams, A. J. et al. “Zombie” Outbreak Caused by the Synthetic Cannabinoid AMB-FUBINACA in New York. N. Engl. J. Med. 376, 235–242 (2017).

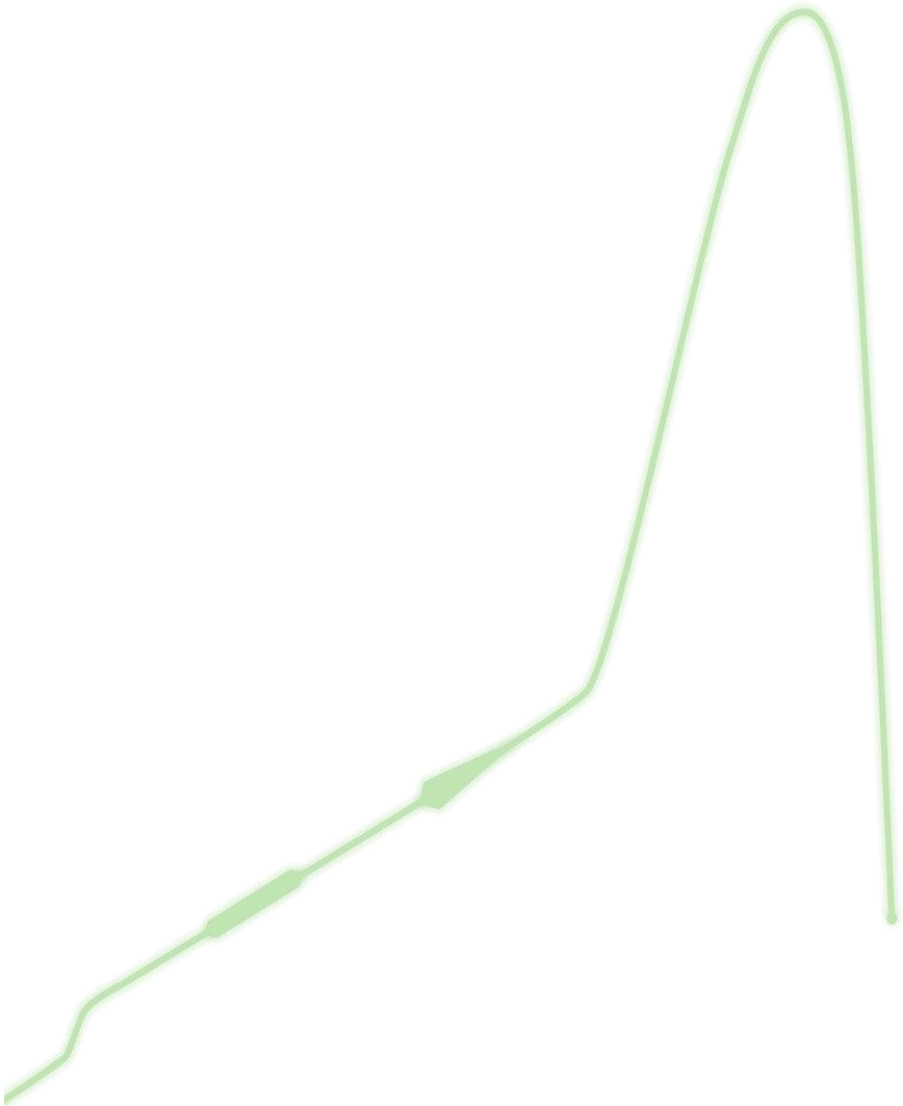
38. Ishii, A. et al. Studies on the phase I metabolites of the new designer drug 1-(2,3-dihydro-1H-inden-5-yl)-2-(pyrrolidine-1-yl)butan-1-one (5-PPDI) in human urine. Forensic Sci. Int. 310, 110214 (2020).

39. Pozo, O. 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. Drug Metab. Dispos. 43, 248–257 (2014).

40. Ibáñez, M. et al. Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry. Anal. Bioanal. Chem. 408, 151–164 (2016).

CHAPTER 5

THE *IN VIVO* APPROACH: METABOLISM AND PHARMACOKINETICS



CHAPTER 5
THE *IN VIVO* APPROACH: METABOLISM AND PHARMACOKINETICS

5.1. Introduction

5.2. Research article IX

> "Proposal of 5-methoxy-*N*-methyl-*N*-isopropyltryptamine consumption biomarkers through identification of *in vivo* metabolites from mice". *Journal of Chromatography A, 2017; 1508:95.*

5.3. Research article X

> "Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats". *Accepted in Addiction Biology.*

5.4. Discussion of the results obtained

5.5. Literature

5.1. Introduction

In **Chapter 4**, two applications of the *in vitro* models have been used and discussed: on the one hand, the study of metabolic behaviour of NPS, including the proposal of potential consumption biomarkers, and, on the other hand, the estimation of their potency by the use of specific receptors. The *in vivo* approaches offer a new dimension for assessing the pharmacology of NPS, as complete organisms can be used (already explained in **Table 1.1** of the present Thesis). They allow the so-called ADME (Absorption, Distribution, Metabolism, and Excretion) studies [1], being possible to evaluate all the steps involved in the pharmacology study of a certain substance. These studies include the testing of the oral bioavailability, distribution (transporters and protein binding), absorption, mechanisms of permeability (including active transport, for example though the blood-brain barrier or BBB), metabolism, and (biliary and renal) excretion [1].

The *in vivo* experiments providing the most accurate ADME information are those performed with healthy volunteers; however, they are also the most difficult due to the limited accessibility to these samples by research laboratories, as well as to the need of regulatory approval and information about toxicology and formulation (**Table 1.1**) [1], among other requirements. Some NPS ADME studies have already been reported in literature, such as the metabolism and pharmacokinetics of the synthetic cathinone mephedrone [2,3], or the excretion rate of the main metabolite of the AB-FUBINACA [4]. Nevertheless, most of the NPS studies reported with humans are related with intoxication cases, as for example the identification of the urinary metabolites of the cathinone MDPV [5] and the SCRA XLR-11 [6], the confirmation in human blood and oral fluid of previously identified *in vitro* metabolites of the cathinone ephylone [7], and the identification of the metabolites of the opioid U-47700 in urine samples [8].

At this point, *in vivo* experiments using animals provide an alternative for performing NPS ADME studies, being also a little bit controversial [1] due to the unknown toxicology and adverse effects of most of the tested NPS. Different animal models have been used, such as mice [9,10], rats [11,12], zebrafish [13,14], pigs [15], and also microbial models [16]. Nevertheless, mice and rat are the most commonly used [17], and a wide variety of studies have been reported in literature [18], including metabolite profiling [11,12,19,20], pharmacokinetics [21–23], detection in brain tissue [24], quantification of neurotransmitters related to NPS in different matrices [25,26], and permeability/transport through membranes [27,28].

In this chapter, two application of the *in vivo* models for the study of different pharmacological aspects of NPS have been developed. The **research article IX** presents the metabolic profiling, pharmacokinetics and urinary excretion of the synthetic tryptamine 5-MeO-MiPT. This compound was selected after being found in two pill samples available in a local smartshop (as explained in **Chapter 2**), together with the Spanish Early Warning System (EWS) communicated its presence in additional seized samples. For that, C57BL/J6 adult male mice was used as animal model, collecting blood and urine samples at different times for metabolite profiling and pharmacokinetic purposes. In **research article X**, the relationship between the structure of synthetic cathinones and their permeability through the blood-brain barrier was assessed. In this case, Sprague-Dawley adult female rats were used and the permeability of 13 selected cathinones with slight modifications in their functional moieties (regarding *N*-functionalisation, α alkyl chain length and aromatic substitution) was evaluated. Prior to the analysis of samples, an analytical methodology based on UHPLC-MS/MS QqQ was developed and validated for the quantification of these compounds in rat cerebrum tissue.

The studies included in this chapter were performed in collaboration with Dr. Manuela Barneo-Muñoz and Prof. Dr. Ferran Martinez-Garcia, from the Predepartamental Unit of Medicine of University Jaume I.

5.2. Research article IX

Journal of Chromatography A, 1508 (2017) 95–105

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Proposal of 5-methoxy-*N*-methyl-*N*-isopropyltryptamine consumption biomarkers through identification of *in vivo* metabolites from mice☆

D. Fabregat-Safont[a], M. Barneo-Muñoz[b], F. Martinez-Garcia[b], J.V. Sancho[a], F. Hernández[a], M. Ibáñez[a,*]

[a] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain
[b] Predepartmental Unit of Medicine, University Jaume I. Unitat Mixta de Neuroanatomia Funcional NeuroFun-UVEG-UJI, Avda. Sos Baynat s/n, 12071, Castellón, Spain

ARTICLE INFO

Article history:
Received 12 April 2017
Received in revised form 31 May 2017
Accepted 3 June 2017
Available online 5 June 2017

Keywords:
5-MeO-MiPT
Tryptamines
In vivo studies
Metabolite identification
High resolution mass spectrometry
New psychoactive substances

ABSTRACT

New psychoactive substances (NPS) are a new breed of synthetically produced substances designed to mimic the effects of traditional illegal drugs. Synthetic cannabinoids and synthetic cathinones are the two most common groups, which try to mimic the effects of the natural compounds $^{9}\Delta$-tetrahydrocannabinol and cathinone, respectively. Similarly, synthetic tryptamines are designer compounds which are based on the compounds psilocin, *N*,*N*-dimethyltryptamine and 5-methoxy-*N*,*N*-dimethyltryptamine found in some mushrooms. One of the most important tryptamine compounds found in seizures is 5-methoxy-*N*,*N*-diisopropyltryptamine, which has been placed as controlled substance in USA and some European countries. The control of this compound has promoted the rising of another tryptamine, the 5-methoxy-*N*-methyl-*N*-isopropyltryptamine, which at the time of writing this article has not been banned yet. So, it is undeniable that this new substance should be monitored.

5-methoxy-*N*-methyl-*N*-isopropyltryptamine has been reported by the Spanish Early Warning System and detected in our laboratory in two pill samples purchased in a local smart shop. This has promoted the need of stablishing consumption markers for this compound in consumers' urine. In the present work, the metabolism and pharmacokinetic of 5-methoxy-*N*-methyl-*N*-isopropyltryptamine has been studied by an *in vivo* approach, using adult male mice of the inbred strain C57BLJ/6. The use of ultra-high performance liquid chromatography coupled to high resolution mass spectrometry allowed the identification of four metabolites. After the pharmacokinetic study in serum and urine, the *O*-demethylated metabolite and the non-metabolised parent compound are proposed as consumption markers in hydrolysed urine. Data reported in this work will help hospitals and forensic laboratories to monitor the consumption and potential intoxication cases related to this tryptamine.

1. Introduction

The term "new psychoactive substances" (NPS) includes synthetic compounds, but also plant or fungal substances that are used as recreational drugs by elicitation of a psychoactive response, and that are not classified as illegal substances [1]. Synthetic cannabinoids and synthetic cathinones make up most of the NPS described for the EMCDDA in its 2016 report [2]. These compounds try to produce the same psychoactive effects than $^{9}\Delta$-tetrahydrocannabinol (THC), a natural compound found in cannabis (*Cannabis sativa*), or the natural alkaloid cathinone found in khat (*Catha edulis*) [3], respectively. Other widely consumed natural products are the "magic mushrooms" belonging to the genera *Psilocybe*, *Conocybe* and *Hygrocybe* [1]. These fungi, especially *Psilocybe cubensis*, contain high concentrations of psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine) and psilocin (4-hydroxy-*N*,*N*-dimethyltriptamine), natural tryptamines with hallucinogenic and sedative effects [4]. Other natural tryptamines currently consumed are DMT (*N*,*N*-dimethyltryptamine) and 5-MeO-DMT (5-methoxy-*N*,*N*-dimethyltryptamine), both of them present in the popular "ayahuasca" [4,5]. Several new tryptamines, structurally similar to natural ones, have been detected in the last few years. One of the most popular synthetic tryptamine is 5-MeO-

☆ Selected paper from the XVI Scientific Meeting of the Spanish Society of Chromatography and Related Techniques (SECyTA 2016), 2–4 November 2016, Seville, Spain.

* Corresponding author.
E-mail address: ibanezm@uji.es (M. Ibáñez).

http://dx.doi.org/10.1016/j.chroma.2017.06.010
0021-9673/

Journal of Chromatography A, 2017; 1508:95

Proposal of 5-methoxy-*N*-methyl-*N*-isopropyltryptamine consumption biomarkers through identification of *in vivo* metabolites from mice

D. Fabregat-Safont [a], M. Barneo-Muñoz [b], F. Martinez-Garcia [b], J.V. Sancho [a], F. Hernández [a,] M. Ibáñez [a*]

[a] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain.

[b] Predepartamental Unit of Medicine, University Jaume I. Unitat Mixta de Neuroanatomia Funcional NeuroFun-UVEG-UJI, Avda. Sos Baynat s/n, 12071, Castellón, Spain.

Abstract

New psychoactive substances (NPS) are a new breed of synthetically produced substances designed to mimic the effects of traditional illegal drugs. Synthetic cannabinoids and synthetic cathinones are the two most common groups, which try to mimic the effects of the natural compounds 9Δ-tetrahydrocannabinol and cathinone, respectively. Similarly, synthetic tryptamines are designer compounds which are based on the compounds psilocin, *N*,*N*-dimethyltryptamine and 5-methoxy-N,N-dimethyltryptamine found in some mushrooms. One of the most important tryptamine compounds found in seizures is 5-methoxy-*N*,*N*-diisopropyltryptamine, which has been placed as controlled substance in USA and some European countries. The control of this compound has promoted the rising of another tryptamine, the 5-methoxy-N-methyl-*N*-isopropyltryptamine, which at the time of writing this article has not been banned yet. So, it is undeniable that this new substance should be monitored.

5-methoxy-*N*-methyl-*N*-isopropyltryptamine has been reported by the Spanish Early Warning System and detected in our laboratory in two pill samples purchased in a local smart shop. This has promoted the need of stablishing consumption markers for this compound in consumers' urine. In the present work, the metabolism and pharmacokinetic of 5-methoxy-*N*-methyl-*N*-isopropyltryptamine has been studied by an *in vivo* approach, using adult male mice of the inbred strain C57BLJ/6. The use of ultra-high performance liquid chromatography coupled to high resolution mass spectrometry allowed the identification of four metabolites. After the pharmacokinetic study in serum and urine, the *O*-demethylated metabolite and the non-metabolised parent compound are proposed as consumption markers in hydrolysed urine. Data reported in this work will help hospitals and forensic laboratories to monitor the consumption and potential intoxication cases related to this tryptamine.

Keywords 5-MeO-MiPT; Tryptamines; *In vivo* studies; Metabolite identification; High resolution mass spectrometry; New psychoactive substances.

1. Introduction

The term "new psychoactive substances" (NPS) includes synthetic compounds, but also plant or fungal substances that are used as recreational drugs by elicitation of a psychoactive response, and that are not classified as illegal substances [1]. Synthetic cannabinoids and synthetic cathinones make up most of the NPS described for the EMCDDA in its 2016 report [2]. These compounds try to produce the same psychoactive effects than $^{9}\Delta$-tetrahydrocannabinol (THC), a natural compound found in cannabis (*Cannabis sativa*), or the natural alkaloid cathinone found in khat (*Catha edulis*) [3], respectively. Other widely consumed natural products are the "magic mushrooms" belonging to the genera *Psilocybe*, *Conocybe* and *Hygrocybe* [1]. These fungi, especially *Psilocybe cubensis*, contain high concentrations of psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine) and psilocin (4-hydroxy-*N*,*N*-dimethyltriptamine), natural tryptamines with hallucinogenic and sedative effects [4]. Other natural tryptamines currently consumed are DMT (*N*,*N*-dimethyltryptamine) and 5-MeO-DMT (5-methoxy-*N*,*N*-dimethyltryptamine), both of them present in the popular "ayahuasca" [4,5]. Several new tryptamines, structurally similar to natural ones, have been detected in the last few years. One of the most popular synthetic tryptamine is 5-MeO-DiPT (5-methoxy-*N*,*N*-diisopropyltryptamine), and its analogues DiPT (*N*,*N*-diisopropyltryptamine) and 4-OH-DiPT (4-hydroxy-*N*,*N*-diisopropyltryptamine).

The compound 5-MeO-DiPT (also known as "Foxy" or "Foxy Methoxy") was reported for the first time in 1999, and in 2003 the DEA reported law enforcements seizures for this compound [6]. Some studies revealed that this tryptamine is a high affinity inhibitor of serotonin, dopamine and norepinephrine transporters [7], but it also acts as a toxin of the serotonergic cells in the brain [8–11]. After the inclusion of 5-MeO-DiPT and its natural analogue 5-MeO-DMT in the list of controlled substances in USA and some European countries [12,13], another tryptamine has been recently identified, 5-MeO-MiPT (5-methoxy-*N*-methyl-*N*-isopropyltryptamine), which is a 5-MeO-DMT

derivative that changes a *N*-methyl group by *N*-isopropyl. This compound, as well as its derivatives MiPT and 4-OH-MiPT, were already described by Alexander Shulgin in his book [14]. **Figure 1** shows the structure of natural tryptamines and their synthetic analogues, including 5-MeO-DiPT and 5-MeO-MiPT. The last one has been reported in intoxication episodes [15,16]. In Spain, it was reported by the Early Warning System (EWS) in 2016, with its first detection taking place in April 2015 [17].

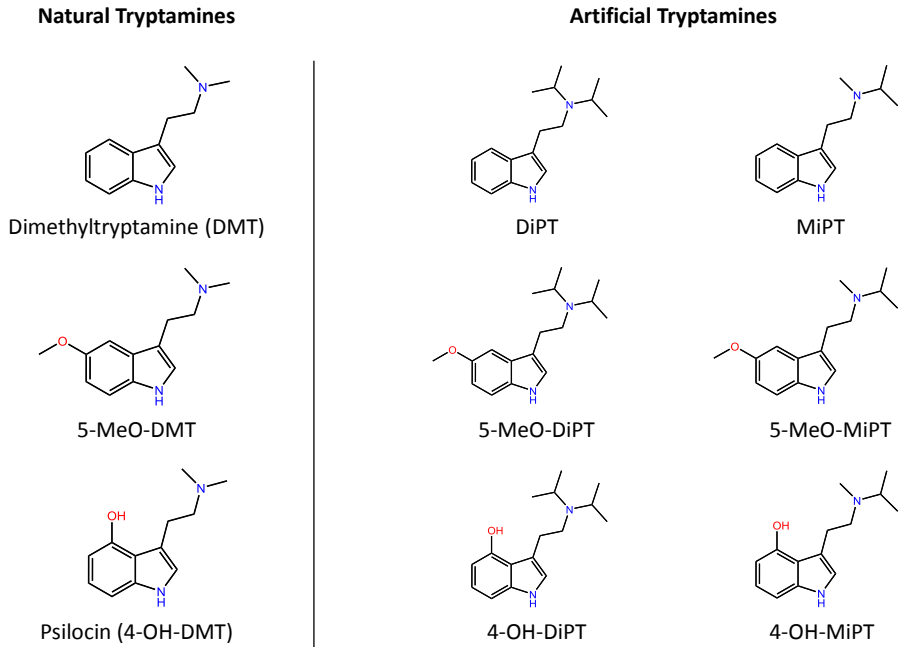


Figure 1. Chemical structure of the natural tryptamines found in plants and mushrooms, and their synthetic analogues.

It seems evident that 5-MeO-MiPT should be monitored in seizures and possible intoxication cases. Identification of NPS is commonly an analytical challenge because NPS structures are continuously changing, needing complex and complementary techniques for their structural elucidation [18,19]. In addition, the detection of these compounds in biological samples with the aim of determining the origin of an intoxication/consumption is troublesome. The main handicap lies on the establishment of the target compound to be monitored, which

in many cases is unknown to the analyst. Several works developing methodologies for the determination of NPS in blood and urine have been published, searching for the parent compound [21–24]. However, prior to monitoring NPS in biological fluids, drug metabolism studies are commonly required. These studies allow to identify the major metabolites and the most specific ones, thus establishing the potential consumption biomarkers.

Undoubtedly, the most useful experiments would be with humans, but these are in most cases rather problematic, as they are limited to intoxication cases [25] and/or require the participation of healthy volunteers [26], with the subsequent risk. At this point, *in vitro* experiments, using microsomes or cell cultures, and *in vivo* experiments, using animal models, are the most common way of establishing potential consumption markers. The *in vivo* approach has been successfully used in different NPS metabolite identification studies [27–31]. For the tryptamine derivative 5-MeO-DiPT, its metabolites have been studied using the *in vitro* approach with rat liver microsomes [32] and rat and human hepatocytes [33], as well as using in vivo experiments with urine of rats [34] and human volunteers [35]. These experiments have allowed monitoring the consumption of this substance throughout the analysis of biological samples, searching for the markers previously established [36].

Our research started when the 5-MeO-MiPT was identified in two pill samples purchased in a local smart shop, at the same time than the Spanish EWS reported its presence, which encourage us to study its metabolism (no data was found in literature). *In vivo* experiments were carried out with adult male mice of the inbred strain C57BLJ/6. Ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) was used for metabolite structural elucidation. Using different approaches for metabolite identification, two phase I and two phase II metabolites were tentatively identified. The identified phase I metabolites resulted from demethylation of the methoxy group and the putative formation of the *N*-oxide, while the phase II were the glucuronide

conjugation of the demethylated metabolite, and the glucuronide of the hydroxyindole metabolite.

2. Experimental procedures

2.1. Reagents and chemicals

5-MeO-MiPT reference standard was purchased from Cayman Chemical (Ann Arbor, MI, USA). HPLC-grade water was obtained by purifying demineralised water using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), acetone, sodium chloride (NaCl), dimethyl sulfoxide (DMSO), formic acid (HCOOH), hydrochloric acid (HCl 37%) and sodium hydroxide (NaOH) were purchased from Scharlau (Scharlab, Barcelona, Spain). Diamino hydrogen phosphate ($(NH_4)_2HPO_4$) was purchased from Merck (Darmstadt, Germany). β-glucuronidase from *E. Coli K12* (80 U/mg at 25 ºC) was purchased from Roche (Indianapolis, IN, USA). Leucine enkephalin was acquired from Sigma-Aldrich (St. Louis, MO, USA).

1 M $H_2PO_4^-/HPO_4^{2-}$ buffer was prepared by dissolving the corresponding amount of $(NH_4)_2HPO_4$ in Milli-Q water and adjusting the pH to 7 with HCl.

2.2. Legal high samples containing 5-MeO-MiPT

Two different pills, *Estrella* (which means "Star" in Spanish) and *Corazon* ("Heart") (**Figure 2**) were bought in a local smart shop through its webpage. Both samples were analysed in order to identify the NPS present in their composition. The tryptamine 5-MeO-MiPT was tentatively identified in both samples and unequivocally identified after purchasing its reference standard.

2.3. Animal experiments

In vivo experiments were performed using C57BLJ6 adult male mice (Janvier, France). The study protocol was approved by the ethical committee of Generalitat Valenciana (Ref. 2015/VSC/PEAI00055). Animals were caged in groups of 3-4 mice and they received intra-peritoneal (i.p.) injections of the drug solution or the

vehicle (control). Different groups of animals were used to obtain blood and urine samples.

Figure 2. Front of the packaging of two pill samples containing the 5-MeO-MiPT: *Corazon* ("Heart", left) and *Estrella* ("Star", right).

150 µL of blank solution (0.9% of NaCl and 1% of DMSO) were injected to control group, while for metabolism experiments, 5-MeO-MiPT was added to blank solution in order to obtain a dose of 0.27 mg/kg in a 150 µL injection. This dose was estimated based on a rough quantification of the 5-MeO-MiPT in pill samples by area comparison with the 5-MeO-MiPT standard, considering a standard human consumer of 75 kg.

For obtaining blood samples, four groups of three animals were injected with the 5-MeO-MiPT solution. 10, 20, 40 and 60 min after drug administration, animals were quickly sacrificed by cervical dislocation and decapitated to obtain large volumes of blood. For the control group (three animals), blood was collected in the same way, 60 min after vehicle injections. Blood of each animal was allowed to coagulate and centrifuged. The serum from the animals of each group (either experimental or control) was mixed, and aliquots were frozen for subsequent use. In conclusion, a total of five serum samples (3 animals per sample) was obtained, one control and four obtained at different times after 5-MeO-MiPT administration.

For urine samples, four groups of three animals were injected with 5-MeO-MiPT solution, and four groups of three animals with the blank solution. Urine from control and 5-MeO-MiPT groups were collected at 1, 2, 3, 4, 5, 6 and 24 h after the administration of the drug or the vehicle solution. To do so, the three mice were put together in a previously sterilized, regular home cage with a meshwire platform that allowed safely collecting urine and avoiding those areas were excrements were present. The sample obtained at a given time point after drug or vehicle injection, was centrifuged, and the aliquots of the urine were frozen for its subsequent use. In summary, seven control samples and seven 5-MeO-MiPT samples, obtained at different times after vehicle/drug administration (respectively), were collected.

2.4. Sample treatment

For the pill samples, the same extraction procedure described in literature for extracting NPS from legal highs samples was used [18]. Approximately 50 mg of sample was weighted in 2 mL propylene tubes and 1 mL of acetone was added. Extraction was performed under sonication during 15 min. After centrifugation during 15 min at 12000 rpm, supernatant was 10^4-fold diluted with H_2O:MeOH 90:10 and injected into the UHPLC-HRMS system.

For the blood samples, 300 µL of ACN were added to 100 µL of serum in 2 mL propylene tubes. Extracts were shaken during 1 min in a vortex in order to insolubilize the proteins and centrifuged at 12000 rpm during 10 min. Supernatant was collected and evaporated until dryness under gentle nitrogen stream at 40 ºC. Solid residue was dissolved with 100 µL H_2O:MeOH (90:10) and 20 µL were injected into the UHPLC-HRMS.

For urine samples, two different procedures were used in order to evaluate phase I and phase II metabolites. For phase I studies, the procedure used was adapted from the literature [35–37]. Briefly, 200 µL of mice urine were hydrolysed with of 10 µL of β-glucuronidase, buffering the sample with 100 µL of a phosphate buffer adjusted to pH = 7. After incubating for 1 hour at 55 ±2 ºC, samples were

frozen for 3 hours in order to remove proteins and lipids by precipitation. Finally, samples were centrifuged at 12000 rpm during 15 min and 20 µL of supernatant were injected into the UHPLC-HRMS system. For phase II metabolites identification, 100 µL of mice urine was diluted with 100 µL Milli-Q water and frozen in order to remove lipids and proteins. After that, sample was centrifuged at 12000 rpm during 15 min and 20 µL were injected.

2.5. Instrumentation

Sample analysis were performed using an Acquity UPLC liquid chromatography system (Waters, Mildford, MA, USA) interfaced to a XEVO G2 QTOF hybrid quadrupole-time of flight (QTOF) mass spectrometer (Waters Micromass, Manchester, UK) with an orthogonal Z-spray electrospray (ESI) operating in positive and negative ionisation mode. A CORTECS C18 100 x 2.1 mm 2.7 µm particle size analytical column (Waters) was used to perform chromatographic separation, with a flow rate of 0.3 mL/min. Mobile phases were H_2O with 0.01% HCOOH (A) and MeOH with 0.01% HCOOH (B). The mobile phase gradient was performed as follows: 10% of B at 0 min, 90% of B at 14 min linearly increased, 90% of B at 16 min, and finally 10% B at 18 min in order to return to initial conditions. The injection volume was 5 µL for MS^E acquisition, and 20 µL for MS/MS experiments. The column temperature was set to 40 ºC. The TOF resolution was ~20000 at FWHM at *m/z* 556 in positive ionisation mode. The range acquired by the MS system was from *m/z* 50 to 1000. A capillary voltage of 0.7 kV and a cone voltage of 20 V for positive ionisation, and a capillary voltage of -1.5 kV and a cone voltage of 20 V for negative ionisation were used during all the chromatographic run. Nitrogen (Praxair, Valencia, Spain) was used as desolvation and nebulizing gas. The desolvation gas flow was set at 1000 L/h. Argon 99.995% (Praxair) was used as a collision gas. The interface temperature was set to 600 ºC and the source temperature to 120 ºC. For MS^E experiments, two acquisition functions with different collision energy were created. The low energy function (LE) used a collision energy of 4 eV in order to obtain information about the (de)protonated molecule and adducts (if exist), while the

high energy function (HE) applied a collision energy ramp from 15 to 40 eV, in order to promote collision-induced fragmentation of the compounds. Calibration of the mass-axis was performed daily from *m/z* 50 to 1000 using a 1:1 mixture of 0.05 M NaOH:5% HCOOH, diluted 1:25 with ACN:H_2O 80:20 mixture. For accurate mass measurement, a 2 µg/mL leucine enkephalin solution in ACN:H_2O 50:50 with 0.1% HCOOH was used as lock-mass, pumped at a flow rate of 20 µL/min. The leucine enkephalin (de)protonated molecule (*m/z* 556.2771 for positive ionisation, and *m/z* 554.2515 for negative) were used for recalibrating the mass axis and ensure an accurate mass during all the chromatographic run. MS data were acquired in continuum mode using MassLynx data station operation software version 4.1 (Waters), and processed with UNIFI scientific information system version 1.8 (Waters).

3. Results and discussion

3.1. Identification of 5-MeO-MiPT in legal highs samples

The suspect screening of the legal high samples (*Estrella* and *Corazon*) retrieved the 5-MeO-MiPT as a potential candidate to be present in both pills. After an accurate fragment evaluation, the compound was tentatively identified in the two samples. **Figure 3A** shows the LE and HE spectra (**Left**) of the chromatographic peak at 3.24 min observed after analysis of *Estrella* pill. The extracted ion chromatograms (EIC) of the protonated molecule and the collision induced dissociation (CID) fragments are also showed (**Right**). The tentative identification of the compound was based on the fragmentation observed. After this careful evaluation, the analytical reference standard of 5-MeO-MiPT was bought to unequivocally confirm the identity of the compound based on retention time and fragmentation (**Figure 3B**).

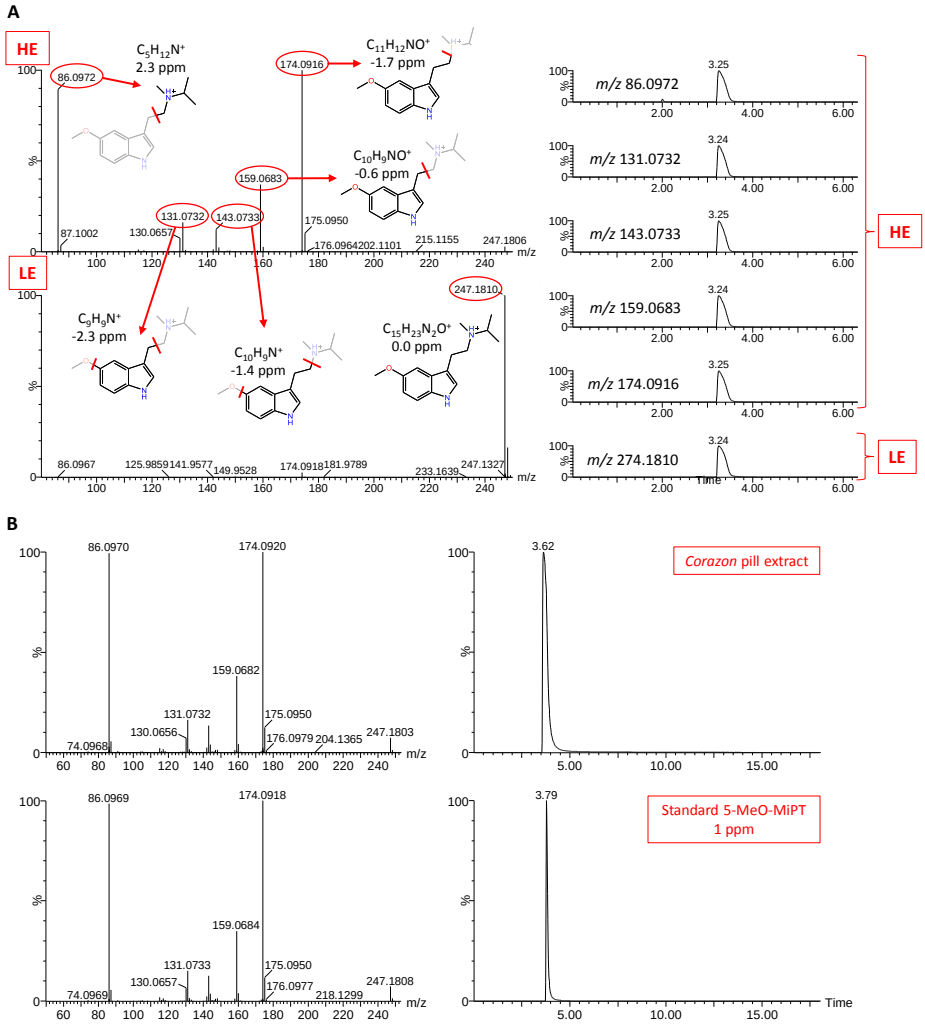


Figure 3. (**A**) Tentative identification of 5-MeO-MiPT in *Estrella* pill. LE and HE spectra of suspect compound (left) and XICs with a ±20 mDa mass window (right). (**B**) Identification of 5-MeO-MiPT in *Corazon* pill. HE spectra (left) and chromatographic peak (right) obtained for the suspect compound in the sample extract (top) and for 5-MeO-MiPT reference standard (bottom).

The fragmentation spectrum showed five fragment ions (**Figure 3A**). The most intense fragment ion was observed at *m/z* 174.0916 ($C_{11}H_{12}NO^+$), corresponding to the neutral loss of the *N*-isopropylmethylamine (73.0892 Da). The second most intense fragment, at *m/z* 86.0972 ($C_5H_{12}N^+$), corresponded to the *N*-isopropylmethylamine with an additional *N*-methylene coming from the alkyl

chain bonded to the amine with the indole ring. This fragment has its complementary ion fragment corresponding to the molecule without the functionalised amine at *m/z* 159.0683 ($C_{10}H_9NO^+$). In this case, the fragment corresponded to a radical ion originated by a homolytic cleavage. This fragment is the precursor ion of another radical fragment at *m/z* 131.0732 ($C_9H_9N^+$), originated by a 27.9949 Da neutral loss (corresponding to a CO molecule). Finally, fragment at *m/z* 143.0733 ($C_{10}H_9N^+$) would come from fragment ion at *m/z* 174 after a methoxide radical loss (CH_3O). It can be observed than fragments involving radicals are quite less intense that full-paired electron fragments. The complete fragmentation pathway of 5-MeO-MiPT can be found in **Figure S1**.

The accurate fragmentation study revealed that the protonation centre would be the *N*-isopropylmethylamine instead of the indole ring, as the fragmentation of the molecule starts on the functionalised amine.

3.3. Analytical strategy for detecting 5-MeO-MiPT metabolites in mice serum and urine

The detection of 5-MeO-MiPT metabolites was performed by 3 different approaches, using UNIFI scientific information system for data processing and compound elucidation. The first approach consisted on the comparison between the blood and urine samples collected after injection with 5-MeO-MiPT, and the control samples, obtained from mice injected with blank solution. Only the compounds which were present in 5-MeO-MiPT samples in a 5:1 ratio with respect to blank samples were considered as potential metabolites. This ratio was experimentally stablished in order to avoid false-positives (endogenic compounds that could be proposed as potential metabolites) or false-negatives (no detection of a potential metabolite due to its confusion with an endogenic compound present in blanks). For urine samples, each 5-MeO-MiPT sample had its corresponding control sample collected at the same time. In the case of blood samples, blank sample collected at 60 min was used as reference for compound discrimination in all blood samples. Obviously, control samples were processed

with the same sample treatment than drug samples. Once identified the potential metabolites, metabolite structure was determined based on the observed fragmentation.

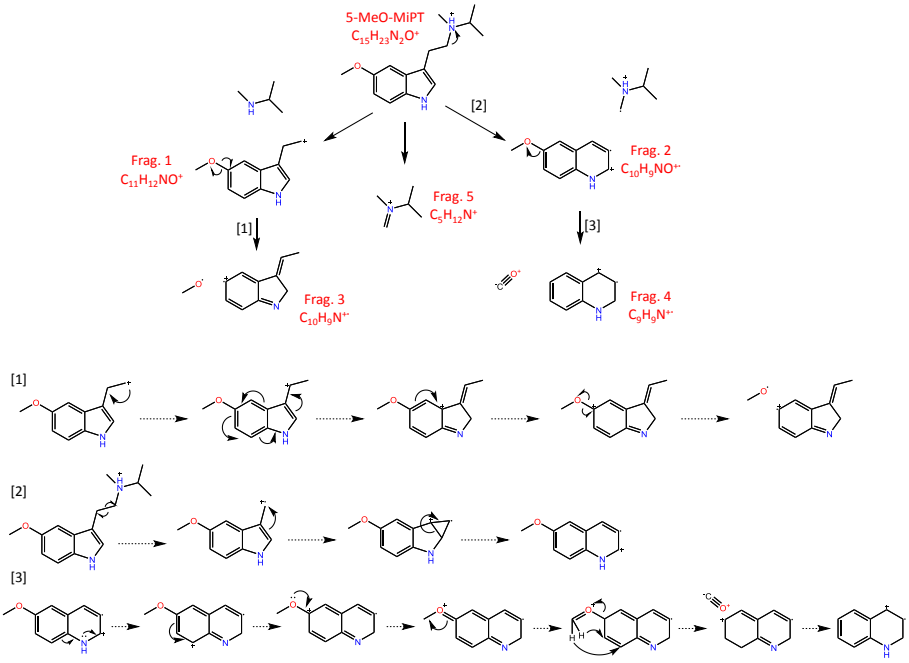


Figure S1. Proposed fragmentation pathway for 5-MeO-MiPT.

In the second approach, 5-MeO-MiPT metabolites were searched based on expected biotransformations in a two-step strategy for urine samples. The first step was to search for phase I metabolites in the hydrolysed urine. In this step, potential phase I metabolites were searched by applying phase I biotransformations (such as cleavages, oxidations, reductions…) to the elemental composition of 5-MeO-MiPT. Once identified all the phase I metabolites, phase II metabolites were searched in the diluted non-hydrolysed urine. Now, conjugations with glucuronides or sulphates of the elemental composition of the parent compound and the elucidated phase I metabolites were searched.

Finally, in the third approach, the common fragmentation pathway and neutral loss search strategies were applied, assuming that some metabolites would share common fragments and/or neutral losses with the parent compound.

In the case of serum, due to the low amount obtained, the hydrolysis step could not be performed.

The combination of the three different approaches allowed the identification of two phase I and two phase II metabolites. The four identified metabolites were detected only in positive ionisation mode. In negative ionisation mode, no additional metabolites were identified.

3.4. 5-MeO-MiPT Phase I metabolites

Demethylated metabolite (Metabolite 1)

The Metabolite 1 was detected at *m/z* 233.1649 ($[M+H]^+$, $C_{14}H_{21}N_2O^+$, at chromatographic retention time (rt) of 2.49 min), which was the result of the loss of a methyl group respect the 5-MeO-MiPT. The fragments observed (**Figure 4A**, **Table 1**) were similar to the observed for 5-MeO-MiPT. The fragments at *m/z* 86.0961 ($C_5H_{12}N^+$) and *m/z* 160.0751 ($C_{10}H_{10}NO^+$), and the corresponding neutral loss of the *N*-isopropylmethylamine (73.0892 Da), were also observed for the 5-MeO-MiPT. This fragmentation would suggest that the demethylation point was not located in the functionalised amine but in the methoxy group. Additionally, the fragment at *m/z* 142.0648 ($C_{10}H_8N^+$) was also observed for the 5-MeO-MiPT, with a difference of 1 Da. This fragment ion was obtained after the homolytic fragmentation of a methoxy loss instead of the heterolytic fragmentation of a water loss observed for the parent compound. Fragment at *m/z* 132.0799 ($C_9H_{10}N^+$) has also the same 1 Da difference respect to the corresponding 5-MeO-MiPT fragment, produced by heterolytic/homolytic fragmentation. The other two minor fragments at *m/z* 117.0573 ($C_8H_7N^+$) and *m/z* 115.0536 (C_9H7^+) were not observed for 5-MeO-MiPT.

After this accurate fragmentation analysis, a plausible fragmentation pathway for the Metabolite 1 was proposed (**Figure 4B**). The aromaticity of the indole ring, and the multiple resonance structures that could be formed allow the fragmentation of the molecule by the groups linked to the indole ring even though the protonation only occur in the amine moiety.

Table 1. Fragment ions found in positive ionisation mode for detected metabolites.

Compound	Retention time (min)	*m/z*	Mass error (ppm)	Elemental composition	Fragment ion	Formula	Mass error (ppm)
5-MeO-MiPT*	3.24	247.1810	0.0	$C_{15}H_{23}N_2O^+$	174.0916	$C_{11}H_{12}NO^+$	-1.7
					159.0683	$C_{10}H_9NO^{+\cdot}$	-0.6
					143.0733	$C_{10}H_9N^{+\cdot}$	-1.4
					131.0732	$C_9H_9N^{+\cdot}$	-2.3
					86.0972	$C_5H_{12}N^+$	2.3
Metabolite 1	2.94	233.1649	-0.4	$C_{14}H_{21}N_2O^+$	160.0751	$C_{10}H_{10}NO^+$	-3.7
					142.0648	$C_{10}H_8N^+$	-2.5
					132.0799	$C_9H_{10}N^+$	-6.7
					117.0573	$C_8H_7N^{+\cdot}$	-0.1
					115.0536	$C_9H_7^{+\cdot}$	-5.1
					86.0961	$C_5H_{12}N^+$	-4.3
Metabolite 2	4.30	263.1753	-0.4	$C_{15}H_{23}N_2O_2^+$	174.0913	$C_{11}H_{12}NO^+$	-0.3
					159.0685	$C_{10}H_9NO^{+\cdot}$	4.2
					143.0731	$C_{10}H_9N^{+\cdot}$	1.2
					131.0723	$C_9H_9N^{+\cdot}$	-4.8
Metabolite 3	1.30	409.1965	-1.0	$C_{20}H_{29}N_2O_7^+$	336.1061	$C_{16}H_{18}NO_7^+$	-4.9
					233.1637	$C_{14}H_{21}N_2O^+$	-4.8
					160.0750	$C_{10}H_{10}NO^+$	-4.1
					86.0963	$C_5H_{12}N^+$	-1.5
Metabolite 4	1.72	439.2054	-4.9	$C_{21}H_{31}N_2O_8^+$	366.1159	$C_{17}H_{20}NO_8^+$	-6.6
					190.0855	$C_{11}H_{12}NO_2^+$	-4.1
					175.0634	$C_{10}H_9NO_2^{+\cdot}$	-4.1
					158.0592	$C_{10}H_8NO^+$	-5.6
					86.0961	$C_5H_{12}N^+$	-4.4

* The fragmentation of 5-MeO-MiPT corresponds to the observed in the analysis of *Estrella* sample.

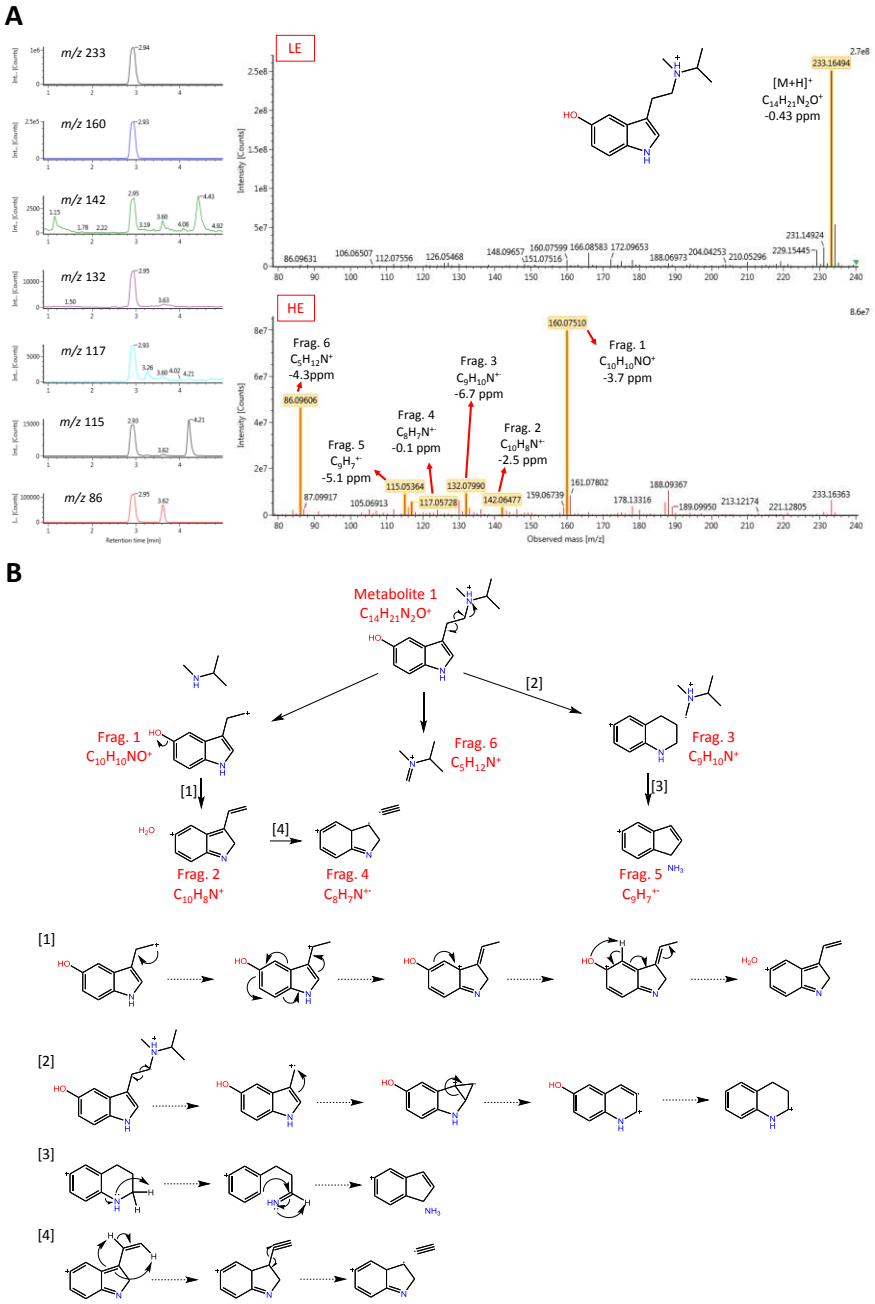


Figure 4. (**A**) LE and HE spectra of Metabolite 1 (right) and XICs with a ±20 mDa mass window (left). (**B**) Proposed fragmentation pathway for Metabolite 1 based on observed fragmentation.

N-oxide metabolite (Metabolite 2)

Metabolite 2 presented its $[M+H]^+$ at *m/z* 263.17531 ($C_{15}H_{23}N_2O_2^+$, rt 4.30 min), corresponding to a hydroxylation. This compound presented four fragment ions at *m/z* 174.0913 ($C_{11}H_{12}NO^+$), *m/z* 159.0685 ($C_{10}H_9NO^+$), *m/z* 143.0731 ($C_{10}H_9N^+$) and *m/z* 131.0723 ($C_9H_9N^+$) (**Table 1**), all of them shared with the 5-MeO-MiPT. These fragments would suggest the hydroxylation to occur in the amine group. This is in accordance with the neutral loss of 89.0841 Da observed (263→174), which corresponds to an elemental composition of $C_4H_{11}NO$. According to literature, the hydroxylation in an alkylic chain would produce the loss of a water molecule during CID fragmentation [38]. However, this fragment was not observed, indicating that the hydroxylation point would be the nitrogen atom. The formation of *N*-oxides in tryptamine analogues has been described in literature [28,39]. On this way, the putative structure of Metabolite 2 would be the *N*-oxide of 5-MeO-MiPT. MSE fragmentation spectrum of Metabolite 2 and the proposed fragmentation pathway for its structure can be found in **Figure S2**.

3.5. 5-MeO-MiPT Phase II metabolites

Glucuronide conjugation of Metabolite 1 (Metabolite 3)

Once identified the two phase I metabolites, phase II metabolites were investigated. A drug-unique peak in raw urine and serum samples was detected, fitting with the glucuronide conjugate of Metabolite 1 (*m/z* 409.1965, $C_{20}H_{29}N_2O_7^+$, rt 1.30 min). The position of the glucuronide conjugation was determined based on the observed fragmentation (**Table 1**). The "key" fragment was observed at *m/z* 336.1061 ($C_{16}H_{18}NO_7^+$), corresponding to the neutral loss of *N*-isopropylmethylamine (73.0892 Da), also present for 5-MeO-MiPT and Metabolite 1. This fragment indicates that the glucuronide conjugation should have occurred in the hydroxyl group of the indole ring, discarding the formation of an *N*-glucuronide. Therefore, this compound would correspond to the glucuronide of Metabolite 1. The fragment present at *m/z* 233.1637 involved the loss of the glucuronide, releasing the non-conjugated part ($C_{14}H_{21}N_2O^+$).

The other two observed fragments at *m/z* 160.0750 ($C_{10}H_{10}NO^+$) and *m/z* 86.0963 ($C_5H_{12}N^+$) were shared with Metabolite 1, as expected. For further information about the MS^E fragmentation spectrum and the proposed fragmentation pathway, consult **Figure S3**.

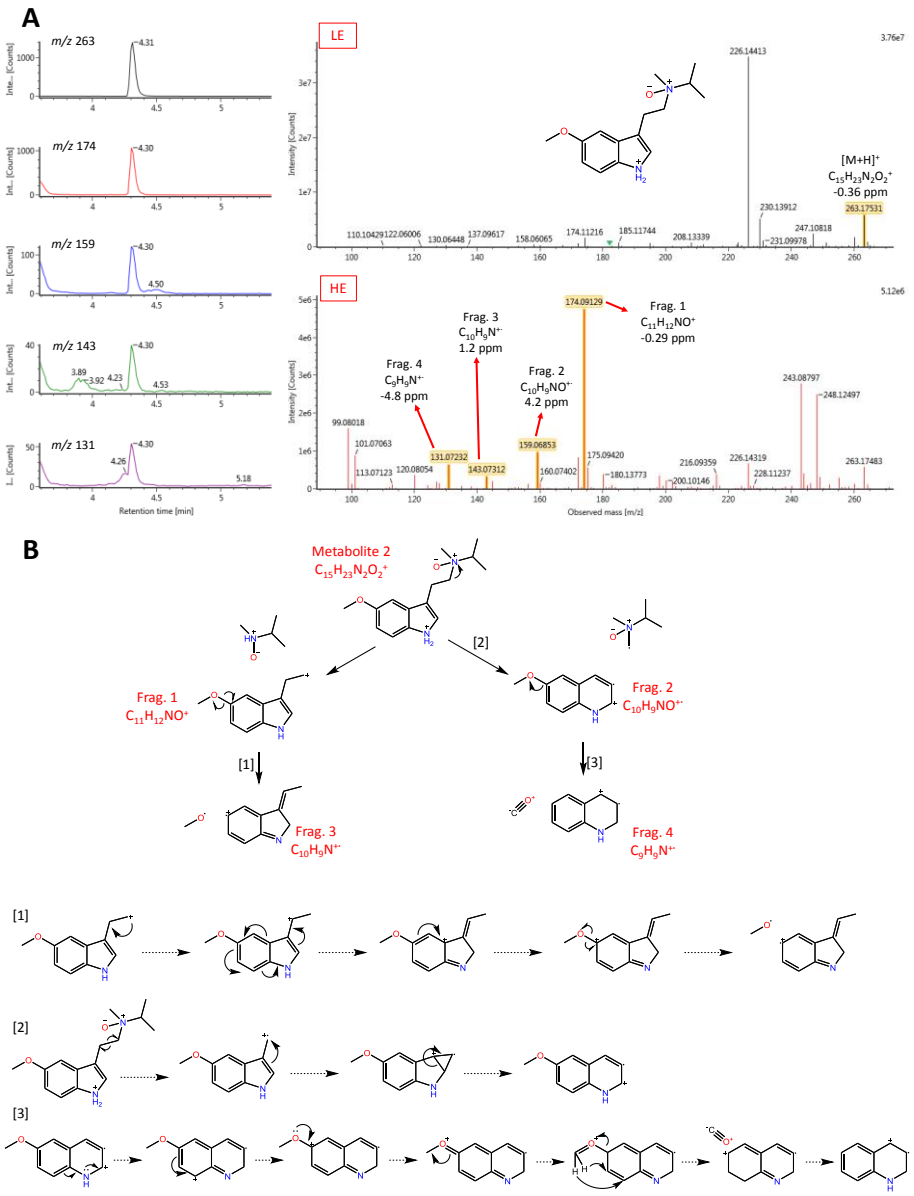


Figure S2. (**A**) LE and HE spectra of Metabolite 2 (right) and XICs with a ±20 mDa mass window (left). (**B**) proposed fragmentation pathway for Metabolite 2.

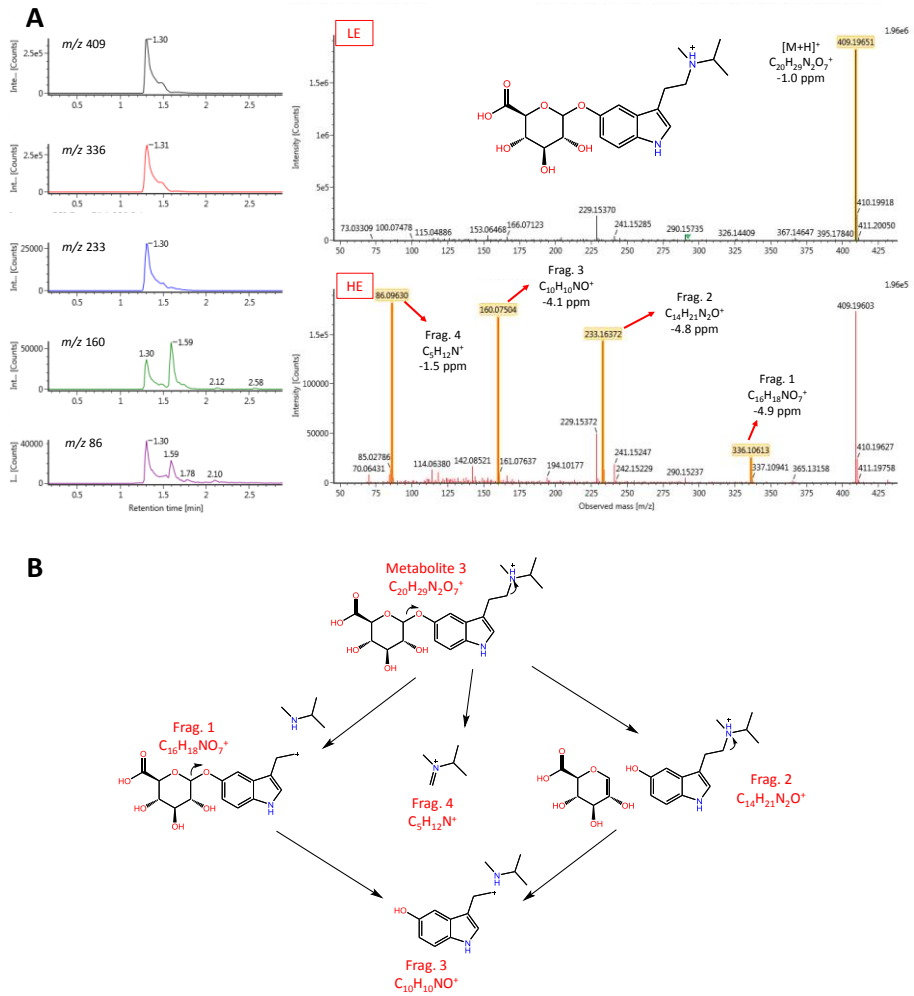


Figure S3. (**A)** LE and HE spectra of Metabolite 3 (right) and XICs with a ±20 mDa mass window (left). (**B**) proposed fragmentation pathway for Metabolite 3.

Glucuronide conjugation of hydroxyindole metabolite (Metabolite 4)

The second phase II metabolite would correspond to a glucuronide conjugation of a non-detected phase I metabolite, an indole-hydroxylated metabolite (*m/z* 439.20534, $C_{21}H_{31}N_2O_8^+$, rt 1.72 min). The exact position of the hydroxyl group was not determined, but it was enclosed in the indole ring according to its fragmentation. Fragment at *m/z* 366.1159 ($C_{17}H_{20}NO_8^+$) corresponded to the well-known *N*-isopropylmethylamine loss (73.0892 Da), indicating that the

glucuronide, and thus the hydroxylation, was present in the indole ring. Another familiar fragment was at *m/z* 86.0961 ($C_5H_{12}N^+$), corresponding to the functionalised amine. Fragment at *m/z* 190.0855 ($C_{11}H_{12}NO_2^+$) corresponded to the loss of the glucuronide conjugation of fragment at *m/z* 366. The other two fragments at *m/z* 175.0634 ($C_{10}H_9NO_2^+$) and *m/z* 158.0592 ($C_{10}H_8NO^+$) were also justified based on the proposed metabolite structure (**Table 1**). **Figure S4**, the MS/MS fragmentation spectra of this metabolite and the proposed fragmentation pathway can be consulted. In this case, MS^E fragmentation spectrum was not clean enough for observing the product ions of Metabolite 4, thus MS/MS experiments were carried out to better match the fragmentation observed to the metabolite structure.

In order to enhance the confidence on the fragmentation observed in MS^E, MS/MS spectra were also acquired for Metabolite 1, Metabolite 2 and Metabolite 3 (**Figure S5** to **S8**, available at the end of this research article).

After the identification of the four metabolites previously described, a plausible metabolic pathway of the 5-MeO-MiPT was proposed (**Figure 5**).

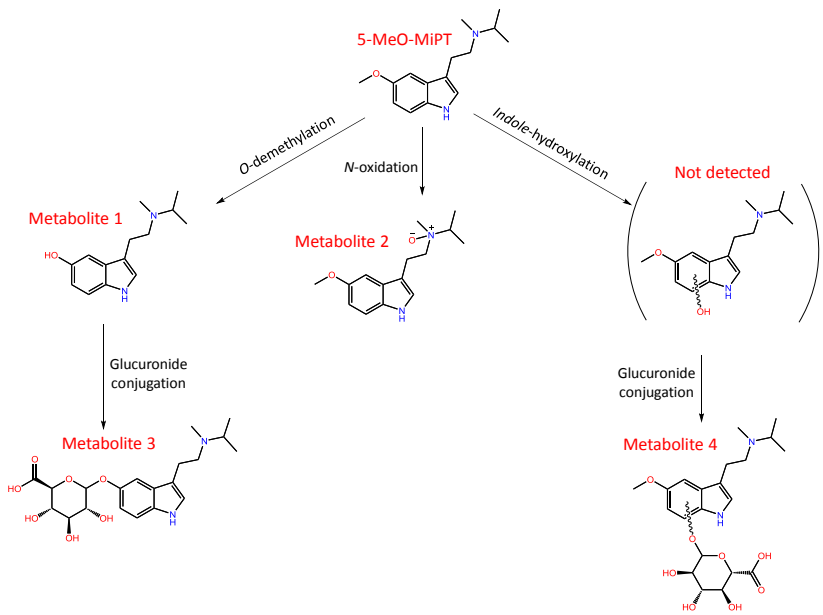


Figure 5. Proposed metabolic pathway for 5-MeO-MiPT.

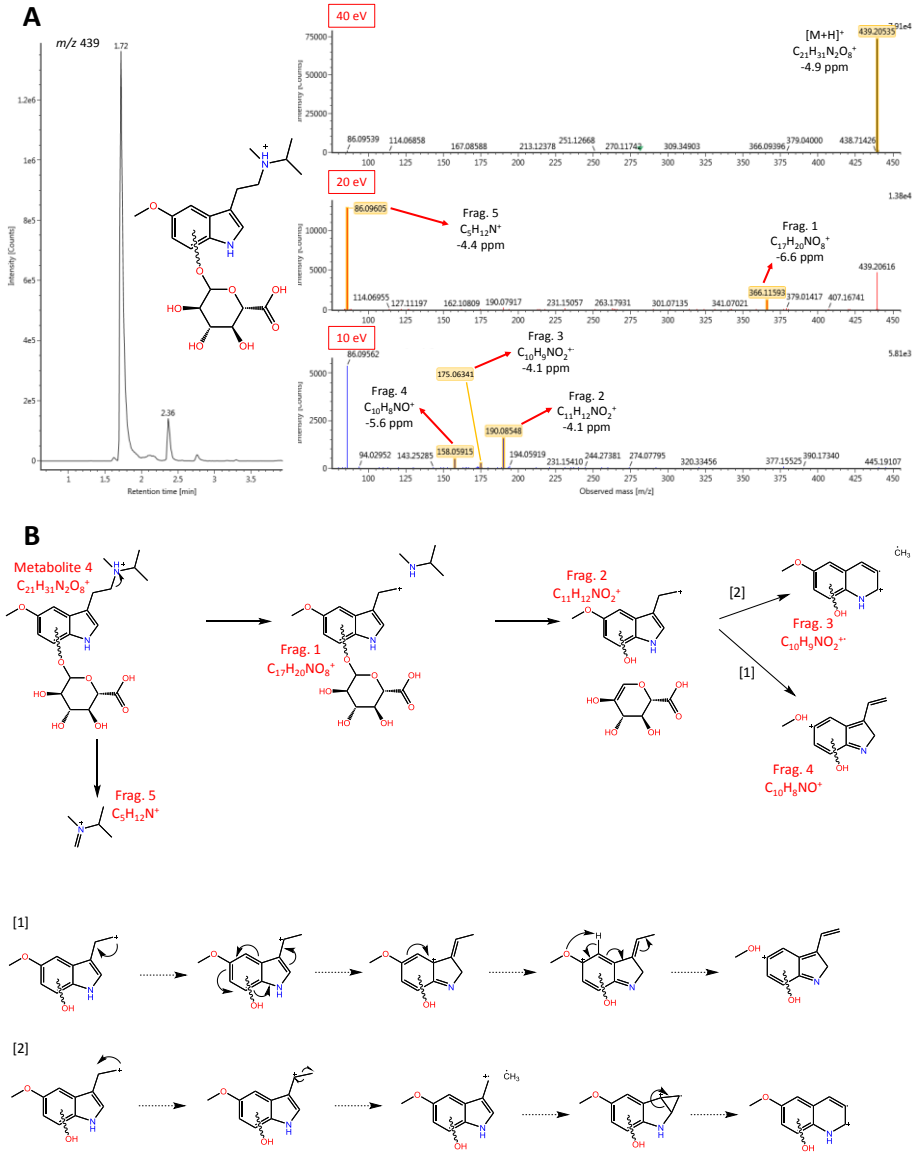


Figure S4. (**A**) MS/MS spectra acquired at 10, 20 and 40 eV for Metabolite 4 (right) and XIC of the [M+H]+ in the LE function with a ±20 mDa mass window. (**B**) proposed fragmentation pathway for Metabolite 4.

3.6. Implications of the obtained results

A total of four 5-MeO-MiPT metabolites were elucidated in serum and urine: two phase I and two phase II metabolites. The two phase I metabolites tentatively identified corresponded to the *O*-demethylated (Metabolite 1) and the *N*-oxide

(Metabolite 2). Our study reveals that 5-MeO-MiPT is mostly metabolized as demethylation of the methoxy group in the indole ring. On the one hand, this tendency is similar to the one reported for the analogue 5-MeO-DiPT, where the demethylated metabolite was the most significant transformation product detected [30,32,33]. On the other hand, the *N*-deisopropylated metabolite described for 5-MeO-DiPT has not been detected for 5-MeO-MiPT, surely due to the change in the amine functionalisation. Moreover, the Metabolite 2 detected in this work, corresponding to the oxidation in the amine moiety as *N*-oxide, has not been reported for 5-MeO-DiPT. Nevertheless, this biotransformation has been recently found for the tryptamine *N*,*N*-diallyltryptamine (DALT) [28,39]. The metabolism study of DALT derivatives revealed a vast number of biotransformations. However, in the case of 5-MeO-MiPT, only the formation of the amine oxidation as *N*-oxide has been found.

Regarding phase II metabolites, the glucuronide of the demethylated phase I metabolite (Metabolite 3), and the glucuronide of the hydroxyindole metabolite (Metabolite 4), were detected. The no-detection of the hydroxyindole phase I metabolite in the hydrolysed urine could indicate that the response of this metabolite was too low to be detected or that the corresponding glucuronide is not hydrolysed under the conditions used, as it has been observed for two biomarkers of testosterone consumption [40].

In this study, a 5-MeO-MiPT dose of 0.27 mg/kg was injected to mice in order to elucidate metabolites and to study their pharmacokinetics in serum and urine. This dose, as explained previously, was based on a rough quantification of the 5-MeO-MiPT detected in the two pill samples, and a typical human consumer weighing 75 kg. However, metabolism studies of 5-MeO-DiPT [32] and DALT derivatives [28,39] in male Wistar rats referred much higher doses (10 mg/kg and 20 mg/kg, respectively) that used in this work. Surely, the use of higher doses facilitates the detection of higher number of metabolites, but it is possible that several of the identified metabolites at high-dose metabolism studies in rats cannot be found in human urine after consumption of a typical dose. In front of

this dilemma, we preferred to use realistic doses, based on legal highs analyses or experiences described by consumers in order to obtain putative biological consumption markers, despite that some metabolites observed at higher dose can be ignored. On the basis of our results, Metabolite 1 and its glucuronide (Metabolite 3), in addition to the parent compound, might be candidates as consumption markers, an issue that will be discussed in the next subsection.

3.7. Prevalence and detectability of 5-MeO-MiPT metabolites in serum and urine

The pharmacokinetic study of 5-MeO-MiPT revealed that most of the parent compound is demethylated and conjugated as glucuronide in the first 20 min, as shown in **Figure 6A**. It should be remarked that experiences were performed in mice, which metabolism is faster than human. To have representative curves between percentage of each compound and excretion time, the response of each individual compound was related with the total response of all detected compounds by assuming that each compound gave the same response in the instrument. This approach was used due to the non-availability of standards for the metabolites detected. This figure shows that in bloodstream, the parent 5-MeO-MiPT has a drop of 75% in the first 30 min, while Metabolite 3 presents its maximum response at 20 min. For Metabolite 1 and Metabolite 4, the response in bloodstream did not exceed 6% respect the initial 5-MeO-MiPT. At 60 min, most of 5-MeO-MiPT was removed from bloodstream by urine excretion or metabolic pathways, while there was still around 30% of Metabolite 3.

Regarding hydrolysed urine, Metabolite 1 presented the highest response, while 5-MeO-MiPT only represented 38% respect to Metabolite 1 (**Figure 6B**). Metabolite 2 was minor (around 2%) and could not be detected at 3 hours. An important decrease between 3-4 hours was observed in the concentration of 5-MeO-MiPT and Metabolite 1. Nevertheless, after 6 hours, Metabolite 1 was still detected, at a concentration of 30 % respect to its concentration at 1 hour.

As expected, Metabolite 3 and Metabolite 4 were not observed due to the enzymatic hydrolysis of glucuronides. In this way, Metabolite 1 would be the most suitable biological consumption marker of 5-MeO-MiPT.

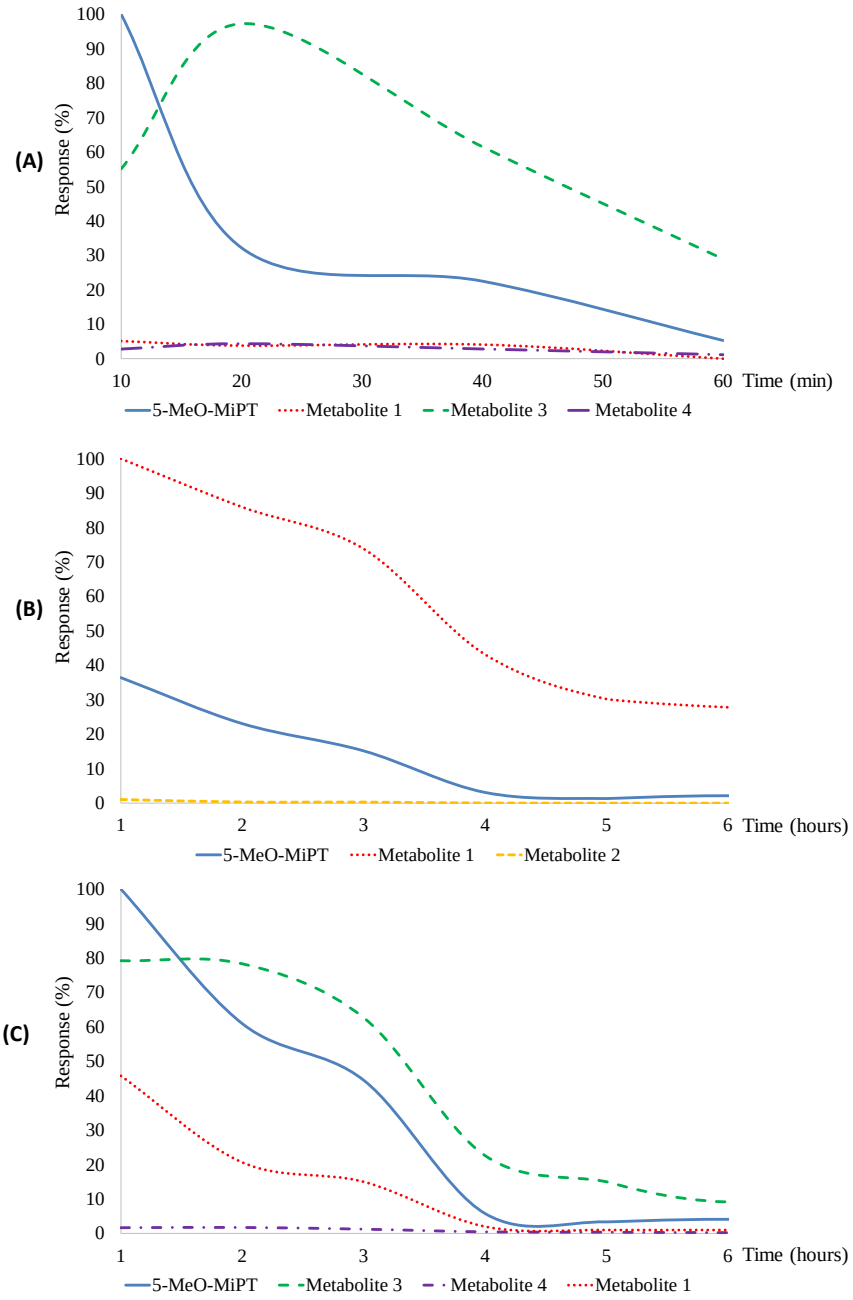


Figure 6. Pharmacokinetic of 5-MeO-MiPT and its metabolites in: (**A**) blood serum, (**B**) hydrolysed urine, and (**C**) diluted raw urine.

In relation to the diluted raw urine (**Figure 6C**), the major compound found at 1 hour, in terms of response, was the parent 5-MeO-MiPT. Metabolite 3 represented around 80%, whereas Metabolite 1 was 50%. However, Metabolite 4 did not exceed the 2% of the response, being not detected at 4 hours. The elimination of 5-MeO-MiPT and its metabolites revealed that Metabolite 3 concentration remains constant for the first 2 hours, while the 5-MeO-MiPT and Metabolite 1 show a decrease. Similarly to hydrolysed urine, the most important concentration fall was observed between 3 and 4 hours. At 6 hours, the concentration of 5-MeO-MiPT represent around the 5% of its initial concentration, and Metabolite 3 the 10%.

Once evaluated the pharmacokinetics of 5-MeO-MiPT, plausible consumption biomarkers can be proposed. The consumption of this tryptamine can be monitored in hydrolysed urine by the parent compound and the O-demethylated metabolite (Metabolite 1). The detection of these biomarkers should be performed in hydrolysed urine, as the glucuronide cleavage of Metabolite 3 would increase the concentration of Metabolite 1. Both compounds were still detected after 24 hours of drug administration, as it can be seen in **Figure 7**, demonstrating that the proposed biomarkers reveal the consumption of 5-MeO-MiPT. Despite Metabolite 1 is the most abundant one in hydrolysed urine, it should not be used as 5-MeO-MiPT consumption marker individually. The detection of Metabolite 1 without the simultaneous detection of 5-MeO-MiPT would generate a reasonable doubt about which is the tryptamine that have been consumed: 5-MeO-MiPT or the synthetic analogue of psilocin, 4-OH-MiPT. Thus, the presence of both 5-MeO-MiPT and Metabolite 1 after 24 hours in urine would reveal the consumption of this tryptamine.

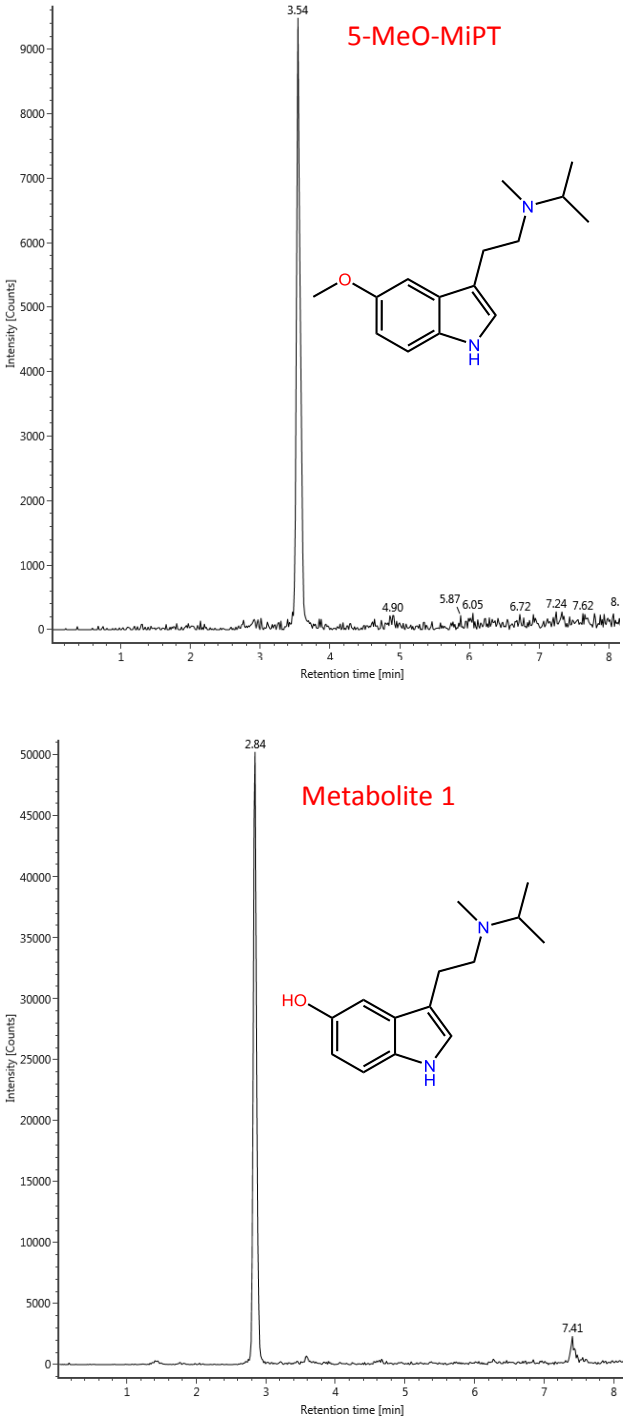


Figure 7. XICs corresponding to the protonated molecule of 5-MeO-MiPT (top) and Metabolite 1 (bottom) in hydrolysed urine, collected 24 hours after administration.

4. Conclusions

Due to the difficulties associated to the metabolism studies in humans, in this work the *in vivo* approach has been applied using adult male mice of the inbred strain C57BLJ/6 as model. This has allowed the establishment of potential consumption markers of the synthetic tryptamine 5-MeO-MiPT. By the use of UHPLC-HRMS, two phase I and two phase II metabolites have been elucidated. The accurate-mass data acquired under MS^E mode allowed the tentative identification of three metabolites, but elucidation of the fourth one required MS/MS experiments in order to obtain cleaner spectra. Nevertheless, all the metabolites were additionally confirmed by MS/MS experiments. After evaluating the results, the most important metabolite found was the O-demethylated (Metabolite 1) and its glucuronide (Metabolite 3).

The pharmacokinetic study revealed that 5-MeO-MiPT was rapidly metabolized, being almost completely removed in bloodstream after 60 min. In the case of urine samples, an important decrease of 5-MeO-MiPT and the two major metabolites was observed between 3 and 4 hours, although these three compounds were still detected after 6 hours. The parent tryptamine 5-MeO-MiPT and the O-demethylated metabolite were both detected in hydrolysed urine collected 24 hours after administration. Both compounds are proposed as biological markers for monitoring 5-MeO-MiPT consumption in hydrolysed consumer's urine, discarding the O-demethylated glucuronide because it would be transformed in Metabolite 1 after the hydrolysis step. The detection of the parent compound is crucial for differentiating the consumption of two chemically-related tryptamines, 5-MeO-MiPT and the psilocin analogue 4-OH-MiPT. Data reported in this work will be useful for developing analytical methodologies for 5-MeO-MiPT consumption detection in hospitals and research centres.

Acknowledgements

David Fabregat-Safont, Juan Vicente Sancho, Félix Hernández and María Ibáñez acknowledge financial support from Generalitat Valenciana (Group of Excellence Prometeo II 2014/023, from the Ministerio de Economía y Competitividad in Spain (Project: CTQ2015-65603-P) and from NPS-Euronet (HOME/2014/JDRUG/AG/DRUG/7086), co-funded by the European Commission. This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein. David Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (Grant FPU15/02033). Manuela Barneo-Muñoz and Ferran Martinez-Garcia acknowledge financial support from Generalitat Valenciana (Group of Excellence PROMETEO2016/076) and from University Jaume I (UJI-B2016-45). Authors also acknowledge Spanish Society of Chromatographic and Related Techniques (SECyTA) for the First Prize in the XII Edition of the Prizes José Antonio García Dominguez to the Best Poster Communication awarded at the XVI Scientific Meeting of the Spanish Society of Chromatographic and Related Techniques celebrated in Seville, Spain (SECyTA2016).

References

[1] S. Gibbons, "Legal Highs" – novel and emerging psychoactive drugs: a chemical overview for the toxicologist, Clin. Toxicol. 50 (2012) 15–24. doi:10.3109/15563650.2011.645952.

[2] European Monitoring Centre for Drugs and Drug Addiction, European Drug Report 2016, EMCDDA–Europol Jt. Publ. (2016) 80. doi:10.2810/88175.

[3] D. Favretto, J.P. Pascali, F. Tagliaro, New challenges and innovation in forensic toxicology: Focus on the "New Psychoactive Substances," J. Chromatogr. A. 1287 (2013) 84–95. doi:10.1016/j.chroma.2012.12.049.

[4] J.H. Halpern, Hallucinogens and dissociative agents naturally growing in the United States, Pharmacol. Ther. 102 (2004) 131–138. doi:10.1016/j.pharmthera.2004.03.003.

[5] M.S. Riga, G. Soria, R. Tudela, F. Artigas, P. Celada, The natural hallucinogen 5-MeO-DMT, component of Ayahuasca, disrupts cortical function in rats: reversal by antipsychotic drugs, Int. J. Neuropsychopharmacol. 17 (2014) 1269–1282. doi:10.1017/S1461145714000261.

[6] S.C. Smolinske, R. Rastogi, S. Schenkel, Foxy methoxy: a new drug of abuse., J. Med. Toxicol. 1 (2005) 22–5. http://www.ncbi.nlm.nih.gov/pubmed/18072099.

[7] C. Sogawa, N. Sogawa, J. Tagawa, A. Fujino, K. Ohyama, M. Asanuma, M. Funada, S. Kitayama, 5-Methoxy-N,N-diisopropyltryptamine (Foxy), a selective and high affinity inhibitor of serotonin transporter, Toxicol. Lett. 170 (2007) 75–82. doi:10.1016/j.toxlet.2007.02.007.

[8] R. Meatherall, P. Sharma, Foxy, a designer tryptamine hallucinogen., J. Anal. Toxicol. 27 (2003) 313–7. doi:NO_DOI.

[9] A.A. Muller, New Drugs of Abuse Update: Foxy Methoxy, J. Emerg. Nurs. 30 (2004) 507–508. doi:10.1016/j.jen.2004.07.037.

[10] D.M. Copton, M.C. Selinger, E.K. Testa, K.D. Larkins, An examination of the effects of 5-methoxy-N,N-di(iso)propyltryptamine hydrochloride (Foxy) on cognitive development in rats, Psychol. Rep. 98 (2006) 651–661. doi:10.2466/pr0.98.3.651-661.

[11] K. Noworyta-Sokołowska, K. Kamińska, G. Kreiner, Z. Rogóż, K. Gołembiowska, Neurotoxic Effects of 5-MeO-DIPT: A Psychoactive Tryptamine Derivative in Rats, Neurotox. Res. 30 (2016) 606–619. doi:10.1007/s12640-016-9654-0.

[12] Drug Enforcement Administration (DEA), Controlled Subsances - Alphabetical Order, Drug Enforcemen Administration, USA, 2017.

[13] Danish Medicines Agency, Executive order no. 557 of 31 May 2011 on euphoriant substances, Denmark, 2011.

[14] A. Shulgin, A. Shulgin, TIHKAL: The Continuation, Transform Press, 1997.

[15] E. Shimizu, H. Watanabe, T. Kojima, H. Hagiwara, M. Fujisaki, R. Miyatake, K. Hashimoto, M. Iyo, Combined intoxication with methylone and 5-MeO-MIPT, Prog. Neuro-Psychopharmacology Biol. Psychiatry. 31 (2007) 288–291. doi:10.1016/j.pnpbp.2006.06.012.

[16] E. Tanaka, T. Kamata, M. Katagi, H. Tsuchihashi, K. Honda, A fatal poisoning with 5-methoxy-N,N-diisopropyltryptamine, Foxy, Forensic Sci. Int. 163 (2006) 152–154. doi:10.1016/j.forsciint.2005.11.026.

[17] Observatorio español de la droga y las toxicomanías. Ministerio de Sanidad Servicios Sociales e Igualdad, Informe 2016. Alcohol, tabaco y drogas ilegales en España, (2016).

[18] M. Ibañez, J. V. Sancho, L. Bijlsma, A.L.N. Van Nuijs, A. Covaci, F. Hernandez, Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances, TrAC - Trends Anal. Chem. 57 (2014) 107–117. doi:10.1016/j.trac.2014.02.009.

[19] D. Fabregat-Safont, I. Fornís, M. Ventura, C. Gil, N. Calzada, J.V. Sancho, F. Hernández, M. Ibáñez, Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain., Drug Test. Anal. (2017). doi:10.1002/dta.2182.

[20] M. Concheiro, M. Castaneto, R. Kronstrand, M.A. Huestis, Simultaneous determination of 40 novel psychoactive stimulants in urine by liquid chromatography–high resolution mass spectrometry and library matching, J. Chromatogr. A. 1397 (2015) 32–42. doi:10.1016/j.chroma.2015.04.002.

[21] M. Pettersson Bergstrand, A. Helander, O. Beck, Development and application of a multi-component LC–MS/MS method for determination of designer benzodiazepines in urine, J. Chromatogr. B. 1035 (2016) 104–110. doi:10.1016/j.jchromb.2016.08.047.

[22] C. Bell, C. George, A.T. Kicman, A. Traynor, Development of a rapid LC-MS/MS method for direct urinalysis of designer drugs, Drug Test. Anal. 3 (2011) 496–504. doi:10.1002/dta.306.

[23] A.A. Azaryan, A.Z. Temerdashev, E. V. Svetlichnaya, A.G. Kal'nitskii, I.A. Balabaev, Determination of some cathinones, tropane alkaloids, and "pharmaceutical narcotics" in urine, J. Anal. Chem. 71 (2016) 955–964. doi:10.1134/S1061934816090021.

[24] M. Ibáñez, Ó.J. Pozo, J. V. Sancho, T. Orengo, G. Haro, F. Hernández, Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 151–164. doi:10.1007/s00216-015-9088-1.

[25] O.J. Pozo, M. Ibáñez, J. V. Sancho, J. Lahoz-Beneytez, M. Farre, E. Papaseit, R. de la Torre, F. Hernandez, Mass Spectrometric Evaluation of Mephedrone In Vivo Human Metabolism: Identification of Phase I and Phase II Metabolites, Including a Novel Succinyl Conjugate, Drug Metab. Dispos. 43 (2014) 248–257. doi:10.1124/dmd.114.061416.

[26] J. Welter, M.R. Meyer, P. Kavanagh, H.H. Maurer, Studies on the metabolism and the detectability of 4-methyl-amphetamine and its isomers 2-methyl-amphetamine and 3-methyl-amphetamine in rat urine using GC-MS and LC-(high-resolution)-MS n, Anal. Bioanal. Chem. 406 (2014) 1957–1974. doi:10.1007/s00216-013-7595-5.

[27] M.R. Meyer, C. Vollmar, A.E. Schwaninger, E.U. Wolf, H.H. Maurer, New cathinone-derived designer drugs 3-bromomethcathinone and 3-fluoromethcathinone: studies on their metabolism in rat urine and human liver microsomes using GC-MS and LC-high-resolution MS and their detectability in urine, J. Mass Spectrom. 47 (2012) 253–262. doi:10.1002/jms.2960.

[28] J.A. Michely, S.D. Brandt, M.R. Meyer, H.H. Maurer, Biotransformation and detectability of the new psychoactive substances N,N-diallyltryptamine (DALT) derivatives 5-fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT in urine using GC-MS, LC-MSn, and LC-HR-MS/MS, Anal. Bioanal. Chem. 409 (2017) 1681–1695. doi:10.1007/s00216-016-0117-5.

[29] S. Anizan, K. Ellefsen, M. Concheiro, M. Suzuki, K.C. Rice, M.H. Baumann, M.A. Huestis, 3,4-Methylenedioxypyrovalerone (MDPV) and metabolites quantification in human and rat plasma by liquid chromatography–high resolution mass spectrometry, Anal. Chim. Acta. 827 (2014) 54–63. doi:10.1016/j.aca.2014.04.015.

[30] S. Narimatsu, R. Yonemoto, K. Masuda, T. Katsu, M. Asanuma, T. Kamata, M. Katagi, H. Tsuchihashi, T. Kumamoto, T. Ishikawa, S. Naito, S. Yamano, N. Hanioka, Oxidation of 5-methoxy-N,N-diisopropyltryptamine in rat liver microsomes and recombinant cytochrome P450 enzymes, Biochem. Pharmacol. 75 (2008) 752–760. doi:10.1016/j.bcp.2007.09.019.

[31] T. Kanamori, K. Kuwayama, K. Tsujikawa, H. Miyaguchi, Y. Togawa-Iwata, H. Inoue, A model system for prediction of the in vivo metabolism of designer drugs using three-dimensional culture of rat and human hepatocytes, Forensic Toxicol. 29 (2011) 142–151. doi:10.1007/s11419-011-0116-3.

[32] T. Kanamori, K. Kuwayama, K. Tsujikawa, H. Miyaguchi, Y. Iwata, H. Inoue, T. Kishi, In Vivo Metabolism of 5-Methoxy-N,N-diisopropyltryptamine in Rat, J. Heal. Sci. 52 (2006) 425–430. doi:10.1248/jhs.52.425.

[33] T. Kamata, M. Katagi, H.T. Kamata, A. Miki, N. Shima, K. Zaitsu, M. Nishikawa, E. Tanaka, K. Honda, H. Tsuchihashi, Metabolism of the psychotomimetic tryptamine derivative 5-methoxy-N,N-diisopropyltryptamine in humans: identification and quantification of its urinary metabolites, Drug Metab. Dispos. 34 (2005) 281–287. doi:10.1124/dmd.105.005835.

[34] M.J. Jin, C. Jin, J.Y. Kim, M.K. In, O.S. Kwon, H.H. Yoo, A Quantitative Method for Simultaneous Determination of 5-Methoxy-N,N-Diisopropyltryptamine and its Metabolites in Urine Using Liquid Chromatography-Electrospray lonization-Tandem Mass Spectrometry, J. Forensic Sci. 56 (2011) 1044–1048. doi:10.1111/j.1556-4029.2011.01753.x.

[35] X. Matabosch, O.J. Pozo, N. Monfort, C. Pérez-Mañá, M. Farré, J. Segura, R. Ventura, Detection and characterization of betamethasone metabolites in human urine by LC-MS/MS, Drug Test. Anal. 7 (2015) 663–672. doi:10.1002/dta.1770.

[36] X. Matabosch, O.J. Pozo, E. Papaseit, M. Farré, J. Marcos, J. Segura, R. Ventura, Detection and characterization of triamcinolone acetonide metabolites in human urine by liquid chromatography/tandem mass spectrometry after intramuscular administration, Rapid Commun. Mass Spectrom. 28 (2014) 1829–1839. doi:10.1002/rcm.6965.

[37] X. Matabosch, O.J. Pozo, C. Pérez-Mañá, E. Papaseit, J. Segura, R. Ventura, Detection and characterization of prednisolone metabolites in human urine by LC-MS/MS, J. Mass Spectrom. 50 (2015) 633–642. doi:10.1002/jms.3571.

[38] R. Ramanathan, A. Su, N. Alvarez, N. Blumenkrantz, S.K. Chowdhury, K. Alton, J. Patrick, Liquid Chromatography/Mass Spectrometry Methods for Distinguishing N -Oxides from Hydroxylated Compounds, Anal. Chem. 72 (2000) 1352–1359. doi:10.1021/ac9911692.

[39] J.A. Michely, A.G. Helfer, S.D. Brandt, M.R. Meyer, H.H. Maurer, Metabolism of the new psychoactive substances N,N-diallyltryptamine (DALT) and 5-methoxy-DALT and their detectability in urine by GC–MS, LC–MS n , and LC–HR–MS–MS, Anal. Bioanal. Chem. 407 (2015) 7831–7842. doi:10.1007/s00216-015-8955-0.

[40] A. Kotronoulas, A. Gomez-Gomez, J. Segura, R. Ventura, J. Joglar, O.J. Pozo, Evaluation of two glucuronides resistant to enzymatic hydrolysis as markers of testosterone oral administration, J. Steroid Biochem. Mol. Biol. 165 (2017) 212–218. doi:10.1016/j.jsbmb.2016.06.006.

MS/MS spectra

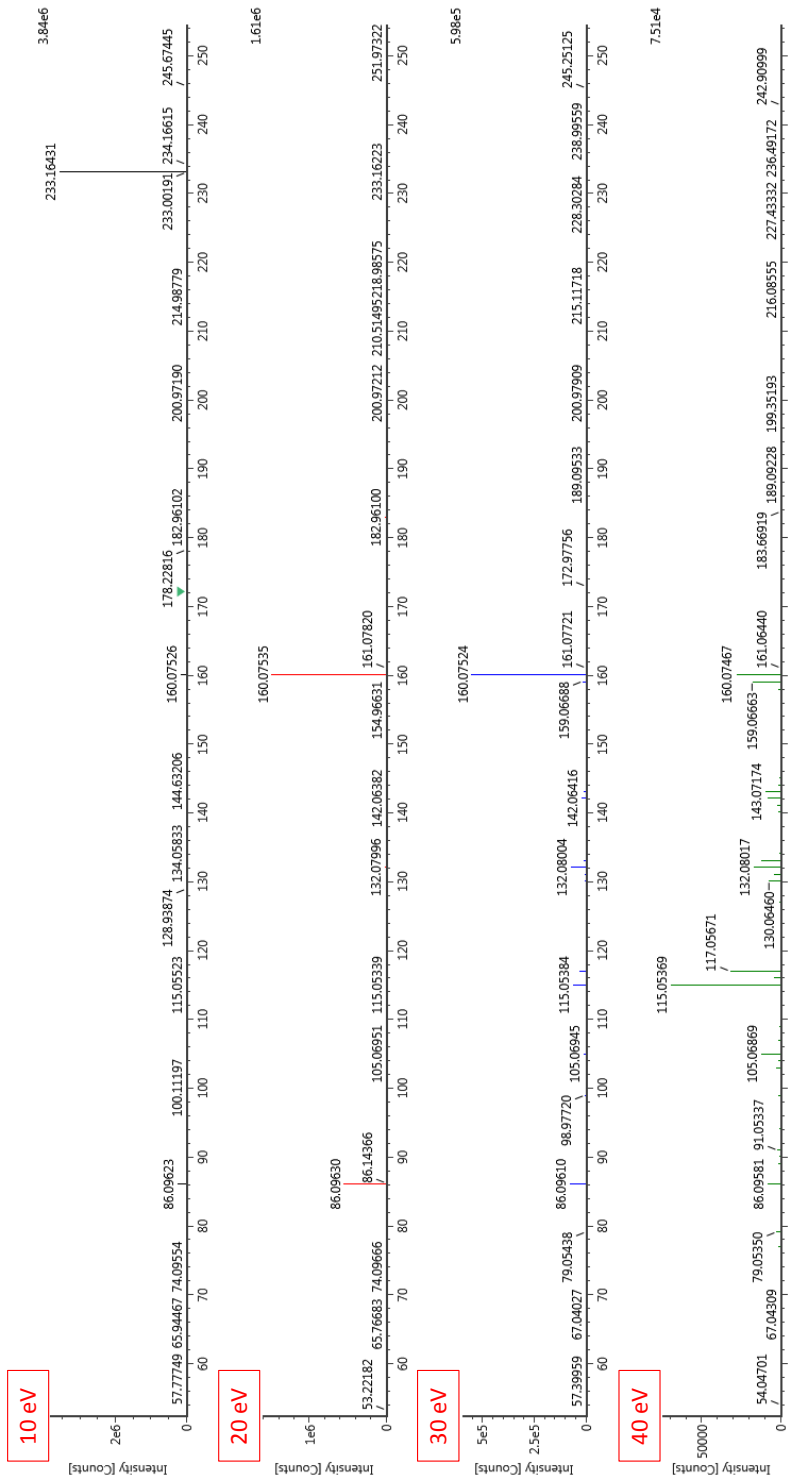


Figure S5. MS/MS spectra collected at 10, 20, 30 and 40 eV for Metabolite 1.

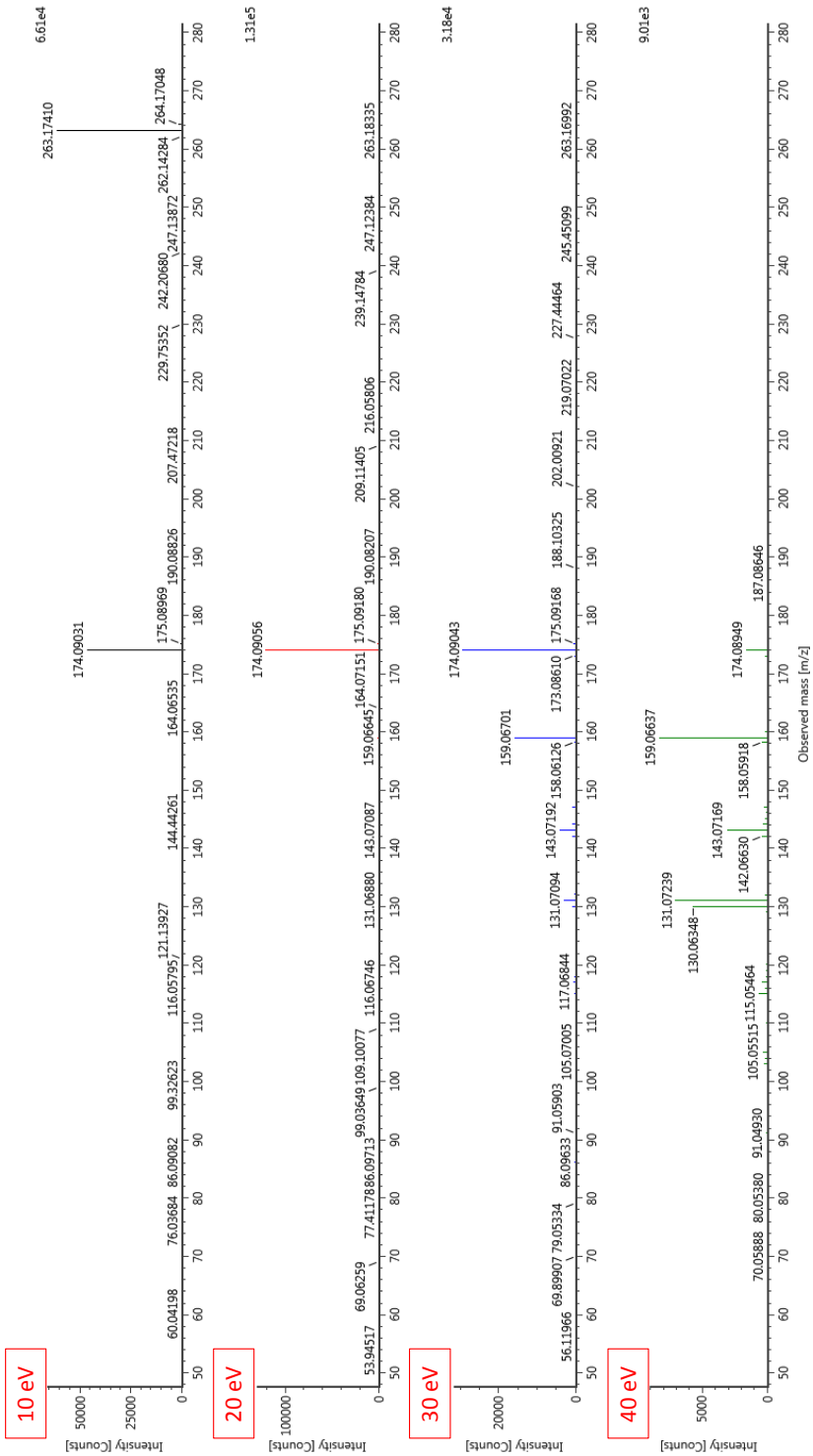


Figure S6. MS/MS spectra collected at 10, 20, 30 and 40 eV for Metabolite 2.

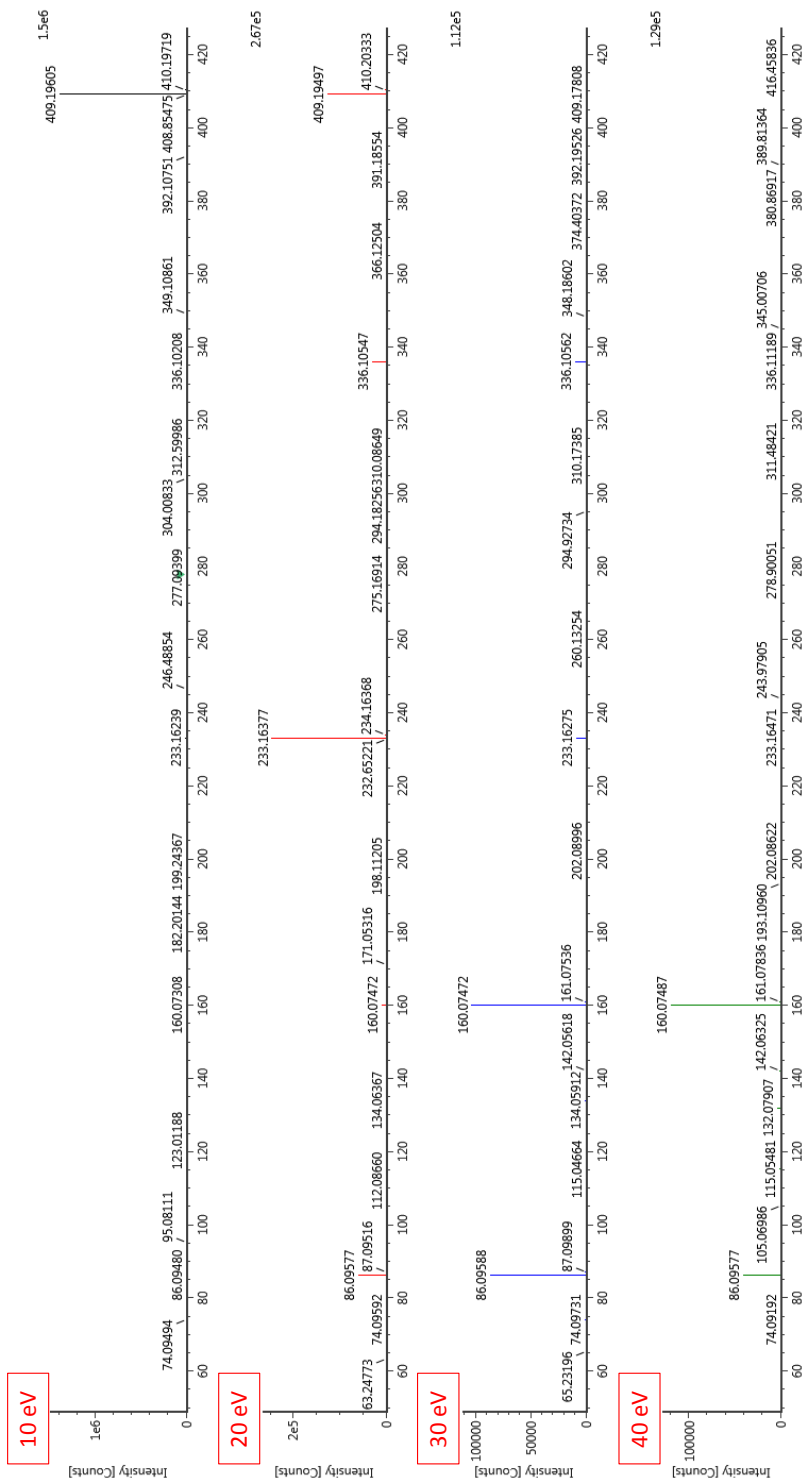


Figure S7. MS/MS spectra collected at 10, 20, 30 and 40 eV for Metabolite 3.

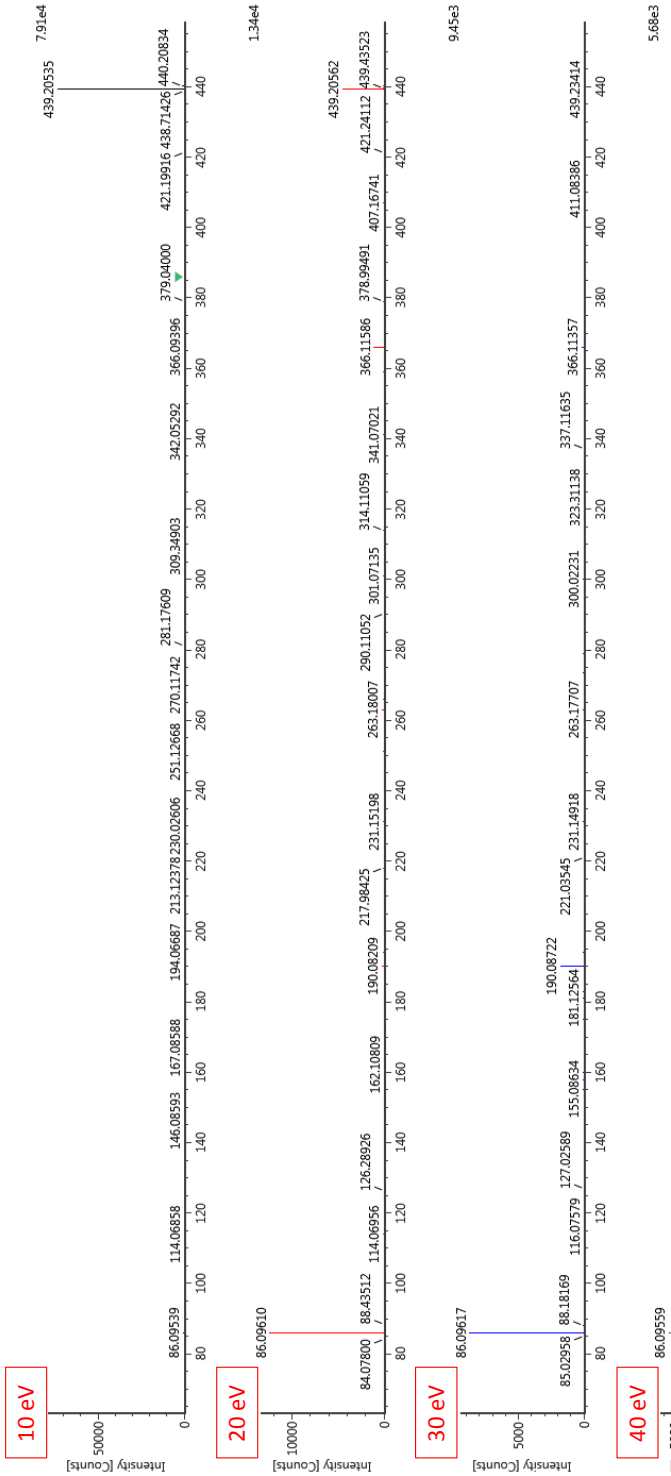


Figure S8. MS/MS spectra collected at 10, 20, 30 and 40 eV for Metabolite 4.

5.3. Research article X

Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

Understanding the pharmacokinetics of synthetic cathinones evaluation of the blood-brain barrier permeability of 13 related compounds in rats

Journal:	*Addiction Biology*
Manuscript ID	AB-02-2020-0032.R2
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	30-Sep-2020
Complete List of Authors:	IBANEZ, MARIA; University Jaume I, RESEARCH INSTITUTE FOR PESTICIDES AND WATER Fabregat-Safont, David; University Jaume I, RESEARCH INSTITUTE FOR PESTICIDES AND WATER HERNÁNDEZ, FÉLIX; University Jaume I, RESEARCH INSTITUTE FOR PESTICIDES AND WATER SANCHO, JUAN; University Jaume I, RESEARCH INSTITUTE FOR PESTICIDES AND WATER CARBÓN, XOÁN; ENERGY CONTROL VENTURA, MIREIA; ENERGY CONTROL STOVE, CHRISTOPHE; Ghent University, Faculty of Pharmaceutical Sciences MARTINEZ-GARCIA, FERRAN; University Jaume I, Predepartmental Unit of Medicine BARNEO-MUÑOZ, MANUELA; University Jaume I, Predepartmental Unit of Medicine
Keywords:	Blood-brain barrier, synthetic cathinones, pharmacology

Accepted in Addiction Biology

Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats

David Fabregat-Safont [1], Manuela Barneo-Muñoz [2], Xoán Carbón [3], Félix Hernández [1], Ferran Martinez-Garcia [2], Mireia Ventura [3], Christophe P. Stove [4], Juan V. Sancho [1], María Ibáñez [1*]

[1] Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain.

[2] Predepartmental Unit of Medicine, University Jaume I. Unitat Mixta de Neuroanatomia Funcional NeuroFun-UVEG-UJI. Avda. Sos Baynat s/n, 12071, Castellón, Spain.

[3] Energy Control (Asociación Bienestar y Desarrollo), c/ Independencia 384, 08041, Barcelona, Spain.

[4] Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium.

Abstract

Synthetic cathinones are the second most commonly seized new psychoactive substance family in Europe. These compounds have been related to several intoxication cases, including fatalities. Although the pharmacological effects, metabolism and pharmacokinetics of cathinones have been studied, there is little information about the permeability of these compounds through the blood-brain barrier (BBB). This is an important parameter to understand the behaviour and potency of cathinones. In this work, 13 selected cathinones have been analysed in telencephalon tissue from Sprague-Dawley rats intraperitoneally dosed at 3 mg/kg. Our results revealed a direct relationship between compound polarity and BBB permeability, with higher permeability for the more polar cathinones. The chemical moieties present in the cathinone had an important impact on the BBB permeability, with lengthening of the α-alkyl chain or functionalization of the aromatic ring with alkyl moieties resulting in lower concentration in telencephalon tissue. Our data suggest that transport of cathinones is a carrier-mediated process, similar to cocaine transport across the BBB.

Keywords Blood-brain barrier, new psychoactive substances, synthetic cathinones, pharmacokinetics, pharmacology, toxicological analysis.

Introduction

The consumption of synthetic cathinones represents an important public health problem, according to the most recent report from the United Nations Office on Drugs and Crime [1], which illustrates that this new psychoactive substance (NPS) family is one of the most commonly seized worldwide, together with synthetic cannabinoids [1]. Most of the cathinone seizures are powder, together with pills and similar products. These compounds have also been found as adulterants in "classical" illegal drugs such as cocaine, illustrating that their prevalence of consumption could be underestimated [2,3]. In addition to the data obtained by seizure analysis, the public health problem related to cathinones is also illustrated by numerous intoxication cases related to these substances, and even some fatalities [4–6]. The synthetic cathinones prevalence can also be illustrated by analytical data obtained from wastewater analysis, illustrating that these compounds are being consumed worldwide [7,8].

It is almost impossible to ban all the cathinone derivatives existing nowadays due to the continuous change in structure of new compounds appearing on the market. Besides, new compounds that could replace banned ones surface in mere weeks, in a similar way to what occurs with synthetic cannabinoids [9]. To face this public health problem, the scientific community must be able to provide information about novel compounds, their chemical, pharmacological and toxicological properties. Thus, a notable number of papers have been published, as illustrated by the reviews available in literature about the metabolism of these substances [10–12], the associated pharmacological behaviour [13,14], toxicology [5,15], and even their neurotoxicity [16].

An important pharmacological issue to highlight is how cathinones affect endogenous compounds, producing a psychoactive effect. Several studies have demonstrated that cathinones act as non-selective monoamine uptake inhibitors, increasing the levels of dopamine and serotonin [17,18], producing effects similar to cocaine [19,20]. Thus, the potency of cathinones and other NPS may be studied using

in vitro approaches [21–23], in a similar way to synthetic cannabinoids [24]. Although *in vitro* studies provide valuable information about the intrinsic potency of a compound, the [in vivo] effect must be determined by the extent to which a compound reaches its site of action. One of the key barriers in this context is the blood-brain barrier (BBB), modulating the exchange of compounds between the brain and the blood [25]. The BBB is a complex system that presents different "entry routes" that can be used by drugs or hormones [25], such as passive diffusion (usually used by non-polar compounds such as steroids) and carrier-mediated influx [25] (used by some psychoactive substances such as cocaine [26]), whereby a specific transporter helps the compound to cross the BBB and reach the brain. To complement *the in vitro* data and better understand the pharmacokinetics (and *in vivo* potency) of cathinones, it is therefore essential to generate accurate data on the BBB permeability of these compounds [26].

This work is the first to quantify an extensive series of cathinones in brain samples from rats intraperitoneally injected with these compounds, with the objective of relating the permeability through the BBB with their structure. To this aim, we have developed and validated [27–30] advanced analytical methodology based on ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for the determination of 13 cathinones (**Figure 1**) in Sprague-Dawley rats' telencephalon tissue. UHPLC coupled to mass spectrometry (MS) plays an essential role in cathinone analysis, using both high-resolution MS (HRMS) and low-resolution MS/MS. Thus, most studies about the identification of novel cathinones [31–33] and the elucidation of their metabolites [34–36] have utilized UHPLC-HRMS, taking profit of the full-spectrum acquisition and high mass accuracy provided by this technique. UHPLC-MS/MS is the preferred technique for the accurate and sensitive determination of a predetermined list of compounds, and has often been used for studying the pharmacokinetics and pharmacodynamics of cathinones [20,37–40].

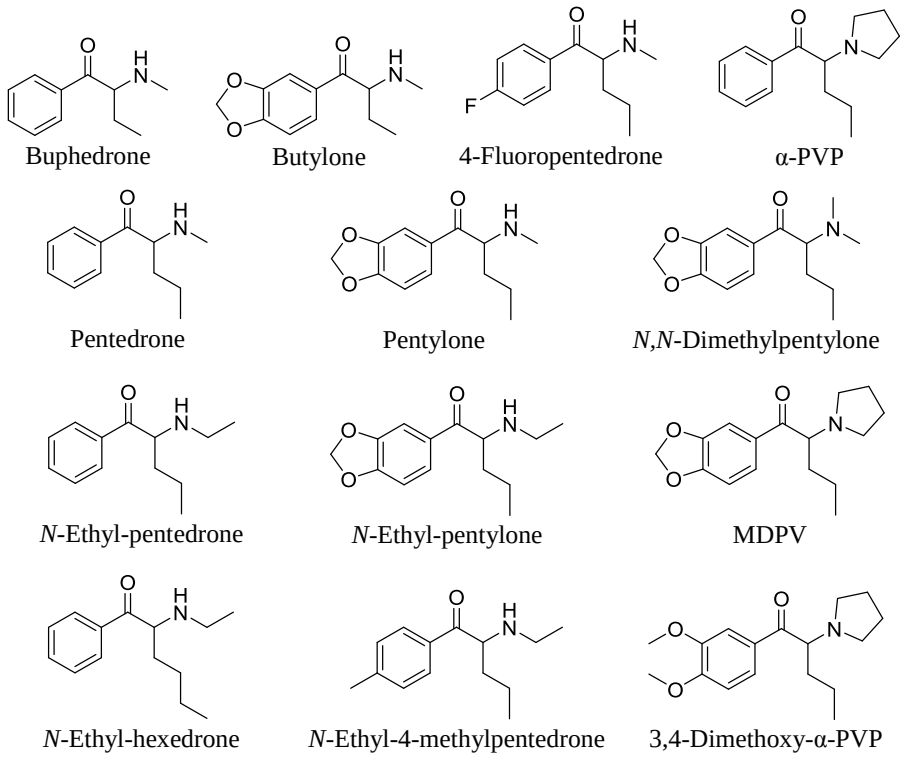


Figure 1. Structures of the 13 synthetic cathinones selected for the study.

As shown in **Figure 1**, taking pentedrone as a template, the cathinones investigated in this study differ in the amine functionalization, aromatic ring substitutions and/or length of the alkyl chain of the alpha carbon atom. The studied compounds were thoroughly selected in order to cover most of the possible combinations of moiety changes usually appearing in cathinones. Using a human *in vitro* BBB permeability model, Simmler et al. readily assessed the transendothelial transport of a series of cathinones [17], whereas the permeability of three cathinones with different alkyl chain length (methylone, butylone and pentylone) was evaluated by Grecco et al. using Sprague-Dawley rats [41]. Our work further elaborates on this by testing *in vivo* an extensive set of well-chosen cathinones with different changes in the moieties of the molecule.

Materials and methods

Reagents and chemicals

Research chemicals containing cathinones were provided by Energy Control (ABD Foundation, Barcelona, Spain). All the compounds were characterized and purity-tested by UHPLC-HRMS and nuclear magnetic resonance, following the same procedures already reported in literature [42,43]. Cathinone stock solutions were prepared at approximately 1 mg/mL in methanol (0.01 mg accuracy). HPLC-grade water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC-grade methanol, HPLC-grade acetonitrile, HPLC-grade ethanol, formic acid and acetone were purchased from Scharlau (Scharlab, Barcelona, Spain). Physiological saline solution was purchased from Laboratorios ERN (Barcelona, Spain).

Animal testing

For animal experiments, dose and brain dissection time were selected based on the information available in literature. In this work, a realistic 3 mg/kg dose 40 was preferred over the very large doses (around 20 mg/kg) used in other pharmacokinetic studies [39,41]. This is relevant because extremely high doses could affect the metabolome, metabolic routes or active transport - among other parameters - that could be involved in the pharmacology and pharmacokinetics of these compounds. It should be noted that, depending on the compound, the doses reported by users are vastly different. For example, in the case of pentedrone, the compound used as reference in this work, 30 to 60 mg has been described as a typical dose when using intravenous route (roughly 0.5 to 1.0 mg/kg) [44]. However, for the aim of this work, it seemed reasonable to use similar doses for all the tested compounds, in order to facilitate the interpretation of the results and avoid the influence of different cathinone concentrations on the data obtained.

Peters and colleagues [40] quantified three cathinones in rat brains by LC-MS/MS, dosing the rats at 3 mg/kg for methylone and mephedrone, and at 1 mg/kg for MDPV. Pharmacokinetic data obtained revealed that the highest concentration was achieved 20 min after administration. Also another study [45], studying the variation of monoamine transporters in brain tissue from rats injected at 1, 3 and 10 mg/kg, reported the highest concentration in brain tissue 20 min after injection. Based on these studies, 3 mg/kg was selected as the dosage to be administered to the rats for all the compounds, and the brain was dissected 20 min post-injection.

Thus, thirty female Sprague-Dawley rats (8 weeks of age), weighing between 280 and 320 g, were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Animals were housed in groups of 3 animals in polypropylene plastic cages under controlled temperature (24 ±2 ºC) and lighting conditions (12h:12h; lights ON at 8 am), with ad libitum access to food and water. Before drug administration, animals were handled and habituated to the experimental room for one week. Experiments were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/60-EEC) and approved by the Valencian Region government ethical committee (Generalitat Valenciana, Direcció General d'Agricultura, Ganaderia i Pesca, ref. 2019/VSC/PEA/0048).

Two rats were dosed per compound and the brains were pooled in order to avoid possible animal differences. In total, twenty-six rats received intraperitoneal injections of the 13 different cathinones at a dose of 3 mg/kg in 300 µL of physiological saline solution containing 5% ethanol – the latter for increasing the solubility of the synthetic cathinones. Four additional animals were injected with the same volume of the vehicle and used to obtain blank brain tissue samples, to be used to prepare quality control samples (QCs) and matrix-matched calibration curves. After 20 min, rats were anesthetized with CO_2 and decapitated immediately. The brain was dissected (avoiding blood that could contaminate it), and the telencephalon (both cerebral hemispheres) was isolated, quickly frozen in liquid nitrogen and stored at -23 ºC until analysis.

Sample treatment

Brain tissue samples were homogenized and crushed with dry ice (Praxair, Valencia, Spain) using an electric grinder, followed by a -23 ºC overnight storage for CO_2 evaporation. After that, approximately 250 mg were accurately weighted (±0.1 mg) in 1.5 mL polypropylene tubes, and 750 µL of acetonitrile containing 1% of formic acid were added. Samples were extracted for 30 min under agitation using a vortex (Velp Scientifica, Usmate Velate, Italy) at 1200 rpm. After keeping extracts for 30 min at -23 ºC, samples were centrifuged at 12000 rpm for 10 min. Finally, the supernatant was diluted with ultrapure water for UHPLC-MS/MS analysis: in the case of samples used for method validation, the supernatant was 10-fold diluted, whereas for the brain samples obtained after administration of cathinones, supernatant was 1000-fold diluted.

The sample treatment procedure was adapted from literature [19], with the only difference being the homogenization procedure. In the present study, homogenization was performed using an electric grinder and dry ice, followed by extraction with acetonitrile and 1% formic acid, a freezing step as clean-up and dilution of the supernatant with HPLC-grade water. Sample weight, extraction volumes and dilutions were designed according to information available in literature [40], with some modifications to improve method sensitivity.

Instrumentation

Samples were analysed using an Acquity UPLC™ H-Class liquid chromatography system (Waters Corp, Mildford, MA, USA) coupled to a Xevo TQ-S mass spectrometer (Waters Corp, Manchester, UK) equipped with a triple quadrupole mass analyser, using a Z-Spray electrospray interface (ESI).

Chromatographic separation was performed in reverse phase, using a Cortecs UPLC™ T3 2.1x100 mm, 1.6 µm analytical column (Waters Corp, Wexford, Ireland), maintained at 40 ºC. Mobile phases were water (solvent A) and acetonitrile (solvent B), both with 0.1% formic acid, at a flow rate of 0.4 mL/min and changing as follows:

0 min 10% B, 0.5 min 10% B, 5.5 min 40% B, 5.6 min 99% B, 8.0 min 99% B, 8.1 min 10% B (total run time 10 min). Injection volume was 20 µL.

ESI was operated in positive ionization mode using a capillary voltage of 1.0 kV. Nitrogen was used as desolvation and cone gas, at 1200 L/h and 250 L/h, respectively. Source temperature was 150 ºC, and desolvation temperature 650 ºC. Cone voltage and collision energies were optimized for each compound. Argon (99.995 %, Praxair) was used as collision gas. 3 selected reaction monitoring (SRM) transitions were acquired per compound. Dwell times were automatically selected in order to acquire 12 points/peak, being at least 0.08 s/transition.

UHPLC-MS/MS data were acquired and processed using MassLynx 4.1 software (Waters Corp, Manchester, UK) and TargetLynx application (Waters Corp, Manchester, UK).

Results

UHPLC-MS/MS optimization

MS/MS optimization was performed by direct infusion into the MS system of individual solutions of the cathinones at 1 µg/L. An ESI source was selected due to the presence of an easily-protonatable nitrogen in all the synthetic cathinones. The capillary voltage was optimized using pentedrone, MDPV and naphyrone (the latter not being included in the in vivo study) as model compounds. Cone voltage and precursor ion selection was performed using individual solutions, testing different cone voltages from 10 to 50 V. As expected, the precursor ion selected was, in all the cases, the protonated molecule ($[M+H]^+$). Once the precursor ion as selected, different collision energies (from 5 to 50 eV, in steps of 5 eV) were tested in order to evaluate the fragmentation of the compounds and thus, select the most specific and sensitive product ions.

Up to 3 SRM transitions (*Q* quantification transition; q_1 and q_2 first and second confirmation transitions, respectively) were selected for each cathinone in order to increase the confidence of compound identification based on the calculation of two ion ratios (q_1/Q and q_2/Q). The optimized MS/MS conditions for the 14 synthetic cathinones (the 13 used for treating the animals plus naphyrone) are shown in **Table S1**.

Chromatographic separation was accurately optimized in order to separate two pairs of isomeric cathinones (*N*,*N*-dimethylpentylone vs *N*-ethyl-pentylone, and *N*-ethyl-hexedrone vs *N*-ethyl-4-methylpentedrone) that present interferences in their SRM transitions. In order to enhance chromatographic resolution, a Cortecs UPLC™ T3 2.1 x 100 mm, 1.6 µm analytical column was selected. Initially, chromatographic performance was assessed by comparing peak shape and sensitivity using H_2O:methanol and H_2O:acetonitrile with a generic elution gradient (0.3 mL/min, 0% organic solvent at 0 min linearly increased to 99% at 10 min). Peak shape and sensitivity were higher for all the compounds using acetonitrile. After that, acidity of the solvents was tested using formic acid. In this case, a concentration of 0.1% of formic acid produced the highest sensitivity and also the narrowest peaks. Finally, the addition of NH_4Ac was also assessed, but no improvements were observed and thus, the use of this modifier was discarded.

Based on the retention times observed for the cathinones, different elution gradients were evaluated. The best chromatographic separation for the two pairs of isomeric cathinones was achieved using the gradient described in the Instrumentation section: maintained from 0 to 0.5 min at 10% of acetonitrile, and linearly increased until 40% at 5.5 min. Finally, flow rate was slightly optimized in order to obtain narrower peaks and enhance the separation of the isomeric cathinones. A flow rate of 0.4 mL/min was selected as the optimal flow, as it provided narrower peaks and did not produce the co-elution of these isomers. This chromatographic method allowed the chromatographic separation of these isomers at 5% of baseline, as can be observed in **Figure S1**.

Table S1. Selected SRM transitions for the validated compounds. Retention time (RT), cone voltage (CV) and collision energy (CE) are included.

Compound	Elemental Composition	RT (min)	Precursor ion	CV (V)	*Q* transition		*q*$_1$ transition		*q*$_2$ transition	
					Product ion	CE (eV)	Product ion (*q*$_1$/*Q*)	CE (eV)	Product ion (*q*$_2$/*Q*)	CE (eV)
Buphedrone	$C_{11}H_{15}NO$	2.3	178.1	30	132.1	15	91.0 (0.98)	20	77.0 (0.12)	30
Butylone	$C_{12}H_{15}NO_3$	2.6	222.1	20	174.1	20	146.1 (0.42)	20	131.1 (0.32)	30
Pentedrone	$C_{12}H_{17}NO$	3.4	192.1	20	132.1	15	91.0 (0.36)	15	144.1 (0.22)	25
Pentylone	$C_{13}H_{17}NO_3$	3.7	236.1	30	188.1	20	175.1 (0.42)	25	86.0 (0.30)	20
N-Ethyl-pentedrone	$C_{13}H_{19}NO$	3.7	206.2	30	146.1	15	91.0 (0.96)	15	118.1 (0.83)	20
4-Fluoropentedrone	$C_{13}H_{16}FNO$	3.8	210.1	20	150.1	15	109.0 (0.11)	15	135.0 (0.26)	25
N,*N*-Dimethylpentylone	$C_{14}H_{19}NO_3$	3.9	250.1	30	100	20	135.1 (0.72)	20	149.0 (0.56)	20
α-PVP	$C_{15}H_{21}NO$	4	232.2	30	91	20	126.1 (0.23)	20	105.0 (0.27)	25
N-Ethyl-pentylone	$C_{14}H_{19}NO_3$	4	250.1	20	202.1	15	174.0 (0.17)	30	135.1 (0.11)	20
MDPV	$C_{16}H_{21}NO_3$	4.2	276.2	20	126.1	25	135.0 (0.73)	25	175.0 (0.61)	20
3,4-dimethoxy-α-PVP	$C_{17}H_{25}NO_3$	4.2	292.2	20	151	30	126.1 (0.73)	30	221.1 (1.55)	15
N-Ethyl-hexedrone	$C_{14}H_{21}NO$	4.7	220.2	20	146.1	15	91.0 (0.35)	15	130.1 (0.25)	25
N-Ethyl-4-methylpentedrone	$C_{14}H_{21}NO$	4.8	220.2	20	144	30	105.1 (0.88)	20	175.1 (1.12)	10
Naphyrone	$C_{19}H_{23}NO$	6.1	282.2	20	141	30	211.1 (0.76)	15	126.1 (0.34)	30

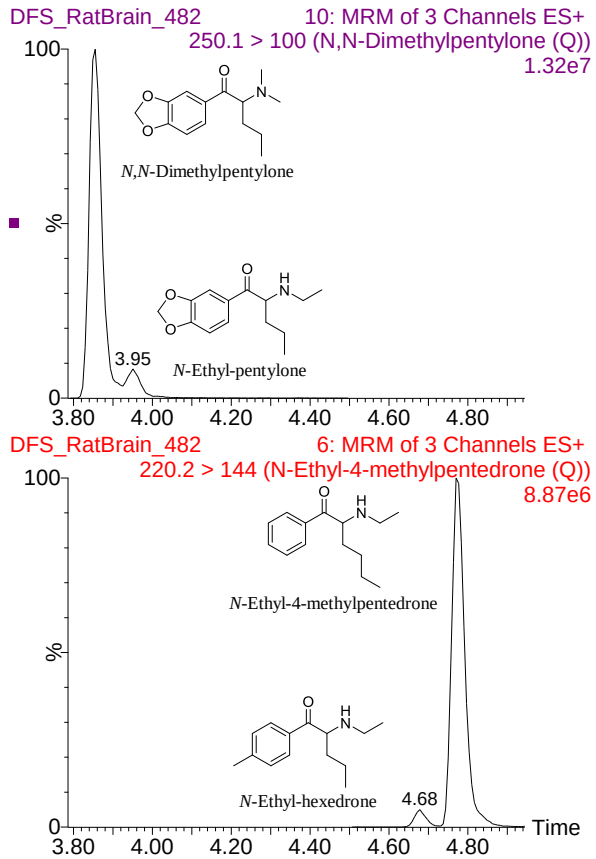


Figure S1. Chromatographic separation of isomeric cathinones at 5% of baseline. **Top** SRM for the quantification trace of *N,N*-dimethylpentylone (3.8 min). A chromatographic peak can be observed at 3.95 min, corresponding to *N*-ethyl-pentylone. **Bottom** SRM for the quantification trace of *N*-ethyl-4-methylpentedrone (4.78 min). A chromatographic peak can be observed at 4.68 min, corresponding to *N*-ethyl-hexedrone.

Method validation

The analytical methodology was validated in terms of specificity, linearity, matrix effect, accuracy, precision, lower limits of quantification (LLOQs) and limits of detection (LODs).

Specificity was assessed by the analysis of blank telencephalon samples. No chromatographic peaks at the expected retention time for all the compounds were observed for the selected SRM transitions.

Linearity was evaluated by analysing matrix-matched calibration curves at 10 concentration levels, from 1 to 1000 ng/L. A linear model was accepted if, upon back-calculation, >75% of the standards from the true concentrations was within the 15% of the nominal value [27,28].

By comparing the absolute areas of peaks and slopes of the standard lines between solvent and matrix-matched calibration, the absence or presence of "absolute" *matrix effect* was assessed. A value of 100% indicates that no absolute matrix effect was observed. A value of >100% indicates an ionization enhancement and a value of <100% an ionization suppression, following the procedure available in the literature [29,30].

Accuracy of the analytical procedure was evaluated by means of recovery experiments using blank brain samples spiked at 1 and 10 ng/g, in quintuplicate for each spiked level. Recoveries between 85 and 115% (80-120% at LLOQ) were considered satisfactory.

Precision was evaluated at 2 concentration levels (1 and 10 ng/g) as the repeatability in terms of relative standard deviations (RSD), considering RSDs lower than 15% (20% at LLOQ) as satisfactory.

LLOQs were established as the lowest level validated with acceptable accuracy and precision (%bias and %RSD within 20%), while LODs were established for a signal-to-noise ratio (S/N) of 3 from the chromatographic peak of the sample spiked at the LLOQ.

For the fortification of a blank brain sample, a mix of the 14 synthetic cathinones was prepared in acetone, in order to promote the miscibility of the compounds in the crushed telencephalon tissue. After 60 min equilibration, fortified samples were extracted using the described procedure.

Table S2 shows the results obtained during the validation of the method, including recoveries and RSD at the two levels studied, LODs, LLOQs and correlation coefficients.

For all the compounds, correlation coefficients higher than 0.99 were obtained in the range of 1 to 1000 ng/L using matrix-matched calibration.

Table S2. UHPLC-MS/MS method validation results for the selected synthetic cathinones in telencephalon tissue samples (n=5).

Compound	Recovery (RSD) (%)		LOD (pg/g)	LOQ (ng/g)	Correlation coefficient (r)
	1 ng/g	10 ng/g			
Buphedrone	103 (6)	85 (8)	22.7	1.0	0.99985
Butylone	101(8)	89 (9)	1.3	1.0	0.99995
Pentedrone	97 (9)	96 (8)	8.2	1.0	0.99983
Pentylone	105 (8)	89 (8)	4.2	1.0	0.99982
N-Ethyl-pentedrone	98 (9)	98 (7)	14.4	1.0	0.99960
4-Fluoropentedrone	112 (7)	86 (8)	8.6	1.0	0.99897
N,N-Dimethylpentylone	100 (8)	92 (8)	2.5	1.0	0.99993
α-PVP	107 (8)	100 (8)	1.9	1.0	0.99995
N-Ethyl-pentylone	104 (8)	95 (8)	4.0	1.0	0.99998
MDPV	111 (7)	96 (8)	1.9	1.0	0.99990
3,4-dimethoxy-α-PVP	82 (6)	96 (9)	3.5	1.0	0.99716
N-Ethyl-hexedrone	106 (8)	102 (8)	4.0	1.0	0.99983
N-Ethyl-4-methylpentedrone	113 (7)	94 (8)	1.8	1.0	0.99958
Naphyrone	111 (8)	108 (9)	0.2	1.0	0.99965

Table S3 shows the matrix-effect for *N*-ethyl-pentylone, including the information on the signal suppression at each concentration level for both solvent and matrix-matched calibration lines, and also for their slopes. Additionally, **Table S4** shows a summary of the matrix effect study for all the cathinones validated. It is important to highlight that the matrix effects were overall limited (between 91% and 106%, except for buphedrone (121.6%) and pentedrone (125.1%)), with CV's below 15%. Recoveries ranged from 82 to 113% for the 1 ng/g level (LLOQ), and from 85 to 108% for the 10 ng/g level (**Table S2**).

Table S3. Matrix effect observed for *N*-ethyl-pentylone at each concentration level, and, for the slope, for solvent and matrix-matched calibration curves.

St. Conc. (ppt)	Peak area		Matrix effect (%)[b]
	Solvent	Matrix-matched	
1	1289.6	1260.6	97.7
2.5	2165.6	2114.4	97.6
5	4110.0	3975.6	96.7
10	6999.1	6843.5	97.8
25	16881.3	16633.7	98.5
50	33606.2	33342.0	99.2
100	66933.6	66551.9	99.4
250	170041.9	162359.1	95.5
500	334794.2	326551.1	97.5
1000	638222.2	621185.5	97.3
Mean			97.7
S.D. (±)			1.2
C.V. (%)			1.2
Slope[a]	642.61	625.22	97.3
R^2	0.9994	0.9994	

S.D. = standard deviation.
C.V. = coefficient of variation.
[a] Calculated from the equation y=mx + b; each standard line was constructed using ten different concentrations.
[b] Matrix effect calculated as ME (%) = M/S x 100, where M is matrix-matched area and S solvent area. A value of >100% indicates ionization enhancement, and a value of <100% signal suppression.

Importantly, in both cases, RSDs were lower than 10% (**Table S2**), illustrating the high precision of the developed methodology. **Figure S2** shows the SRM transitions for the 14 validated compounds at the LLOQ level. The LLOQ for all the synthetic cathinones was set at 1 ng/g, while the LODs, calculated theoretically based on the S/N ratio obtained for the LLOQ level, were between 0.2 and 23 pg/g (**Table S2**), further indicating the high sensitivity of synthetic cathinones analysed by ESI-MS/MS.

Table S4. Matrix effect observed for the validated cathinones.

Compound	Parameter[a]	Solvent	Matrix-matched	Matrix effect (%)[a]
Buphedrone	Mean			121.6
	S.D. (±)			33.3
	C.V. (%)			27.4
	Slope	248.71	235.45	94.7
Pentedrone	Mean			125.1
	S.D. (±)			43.8
	C.V. (%)			35.0
	Slope	593.02	572.63	96.6
N-ethyl-pentedrone	Mean			92.8
	S.D. (±)			12.8
	C.V. (%)			13.8
	Slope	523.24	508.69	97.2
4-fluoropentedrone	Mean			93.1
	S.D. (±)			8.9
	C.V. (%)			9.6
	Slope	1036.04	997.32	96.3
N-ethyl-hexedrone	Mean			92.7
	S.D. (±)			3.0
	C.V. (%)			3.2
	Slope	540.81	519.20	96.0
N-ethyl-4-methylpentedrone	Mean			101.0
	S.D. (±)			15.8
	C.V. (%)			15.7
	Slope	837.42	791.15	94.5
Butylone	Mean			95.1
	S.D. (±)			2.9
	C.V. (%)			3.1
	Slope	867.81	848.64	97.8

S.D. = standard deviation.
C.V. = coefficient of variation.
[a] Calculated as in **Table S3.**

Table S4. Matrix effect observed for the validated cathinones (continuation).

Compound	Parameter[a]	Solvent	Matrix-matched	Matrix effect (%)[a]
α-PVP	Mean			92.0
	S.D. (±)			4.4
	C.V. (%)			4.8
	Slope	1978.08	1890.81	95.6
Pentylone	Mean			106.0
	S.D. (±)			9.9
	C.V. (%)			9.4
	Slope	480.33	475.77	99.1
N,*N*-dimethylpentylone	Mean			91.5
	S.D. (±)			7.3
	C.V. (%)			8.0
	Slope	1049.18	1048.83	100.0
N-ethyl-pentylone	Mean			97.7
	S.D. (±)			1.2
	C.V. (%)			1.2
	Slope	642.61	625.22	97.3
MDPV	Mean			97.7
	S.D. (±)			3.6
	C.V. (%)			3.7
	Slope	1252.12	1229.22	98.2
3,4-dimethoxy-α-PVP	Mean			92.8
	S.D. (±)			5.3
	C.V. (%)			5.7
	Slope	1010.13	978.44	96.9
Naphyrone	Mean			91.5
	S.D. (±)			3.4
	C.V. (%)			3.7
	Slope	6671.27	6290.21	94.3

S.D. = standard deviation.
C.V. = coefficient of variation.
[a] Calculated as in **Table S3.**

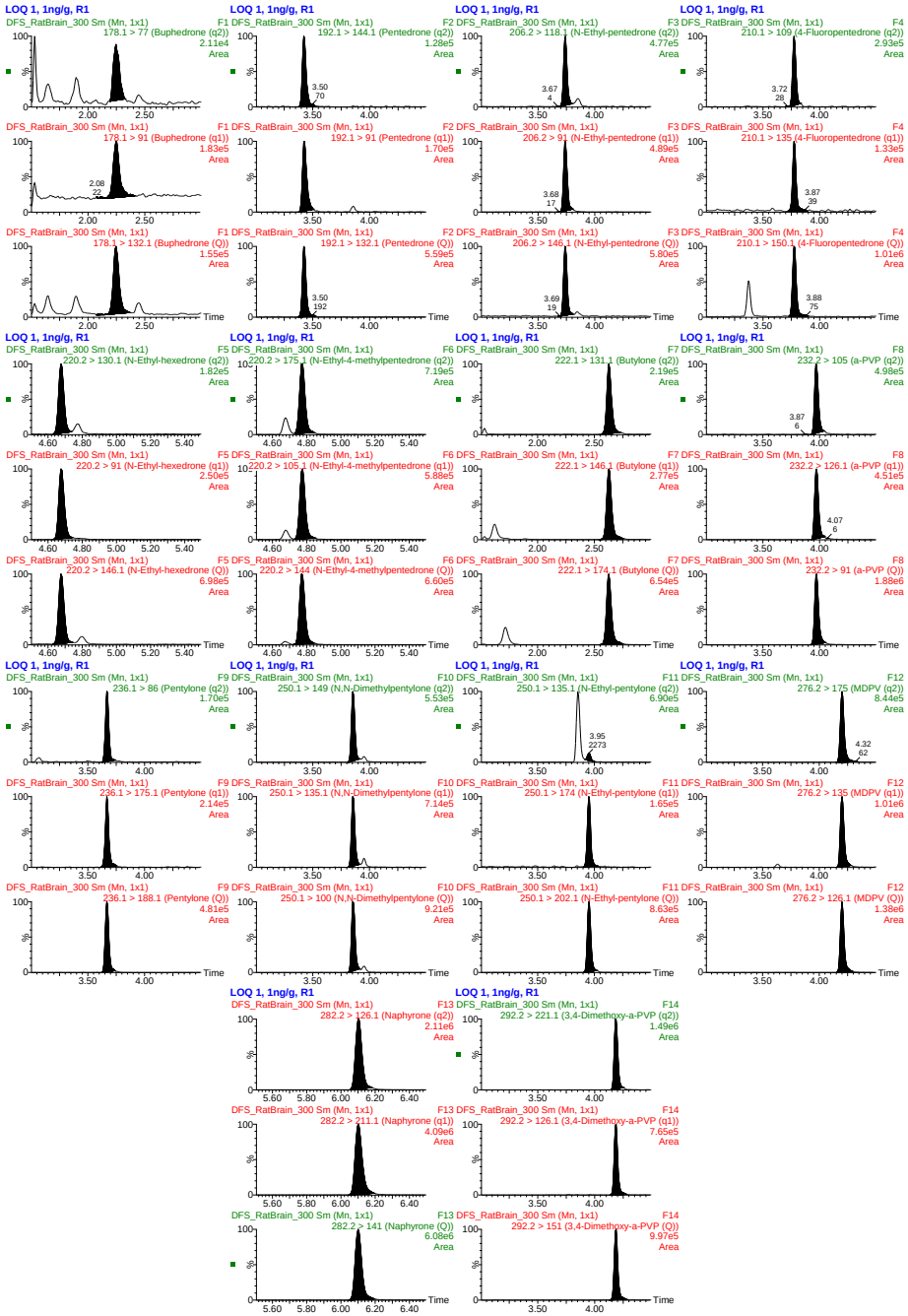


Figure S2. LC-MS/MS (SRM) chromatograms obtained for the 14 synthetic cathinones at the LLOQ level (1 ng/g).

Cathinone concentration found in telencephalon tissue

Cathinone concentrations found in brain displayed wide differences, from 762 ng/g brain tissue for *N*,*N*-dimethylpentylone to 10596 ng/g for *N*-ethyl-pentylone, the concentrations for most of the remaining compounds ranging between 1000 and 4000 ng/g. In all cases, the concentrations were well above the analytical performance, in terms of sensitivity and limits of quantification of our methodology. In addition, reliability of the analytical methodology was supported by analysis of quality control (QC) samples in duplicate, spiked at 1 and 10 ng/g, included in the sample batch. Recoveries between 70 and 120% were obtained, confirming the correct quantification of the cathinones in telencephalon tissue.

The concentrations found in telencephalon samples for all the compounds are shown in **Tables 1-3**. The differences observed in the cathinone levels suggest that the BBB permeability of these compounds is structure-dependent, being associated with their polarity, as discussed further.

Discussion

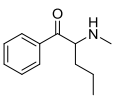
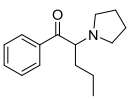
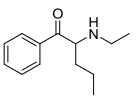
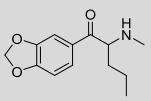
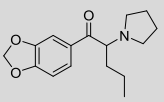
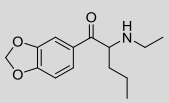
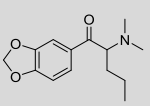
Cathinone penetration through the blood-brain barrier

The main objective of our study was to evaluate the relationship between the structure of cathinones and their BBB permeability, in order to get better acquainted with the pharmacological behaviour of these substances.

Pronounced concentration differences were observed in the telencephalon for different cathinones, ranging from 762 ng/g (*N*,*N*-dimethylpentylone) to 10596 ng/g (*N*-ethyl-pentylone). This difference is surprising given the very high structural similarity of these two compounds, which only differ in the amine functionalization (dimethyl vs. ethyl).

Table 1 shows the concentrations found in telencephalon tissue for cathinones that differ by the functionalization of the amine moiety (*N*). In the two groups (those without aromatic ring substitution and those with a 3,4-methylenedioxy substituent), cathinones with an *N*-methyl (pentedrone and pentylone) moiety were found at a higher concentration than those with a pyrrolidine ring (α-PVP and MDPV). Regarding compounds with an *N*-ethyl group, *N*-ethyl-pentedrone had lower permeability than the *N*-methyl analogue whereas *N*-ethyl-pentylone had a higher permeability than the *N*-methyl analogue. It is also remarkable that the cathinone with a *N*,*N*-dimethyl group (*N*,*N*-dimethylpentylone) seemed to have the lowest permeability of the BBB.

Table 1. Concentration levels found in rat telencephalon tissue for cathinones differing on the amine (*N*) functionalization (rats dosed at 3 mg/kg).

Change in the *N* functionalization			
Compound	**Brain tissue conc. (ng/g)**	**Ratio found/dosed (μg/mg)**	**Structure**
Pentedrone	3,718	1.03	
α-PVP	3,054	0.84	
N-Ethyl-pentedrone	1,432	0.40	
Pentylone	3,113	0.86	
MDPV	863	0.24	
N-Ethyl-pentylone	10,596	2.94	
N,*N*-Dimethylpentylone	762	0.21	

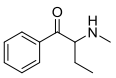
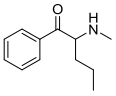
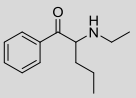
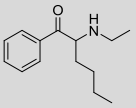
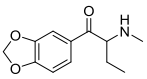
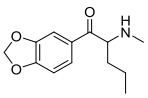
N-ethyl-pentylone was, by far, the cathinone with the highest concentration in telencephalon tissue. Also known as ephylone or bk-EBDP, this substance is a recently reported cathinone that has been involved in numerous recent intoxication cases [46,47] including 151 deaths between 2014 and 2018 [48], which raises high concerns regarding the toxicity of this compound. The high *N*-ethyl-pentylone concentration found in telencephalon tissue is in line with a recent study about the pharmacokinetic behaviour of this cathinone, which also suggested a high BBB permeability [39], which could explain its elevated toxicity.

Another common modification seen in cathinone analogues is altering the length of the alkyl chain. In this study, we evaluated three pairs of cathinones that only differed from each other in the length of the alkyl chain. (**Table 2**): buphedrone and pentedrone, *N*-ethyl-pentedrone and *N*-ethyl-hexedrone, and butylone and pentylone. In all three cases, lengthening the alkyl chain led to a reduction of the BBB permeability, as shown in **Table 2**. These results are in concordance with data reported in a similar study [41], where the permeability of methylone, butylone and pentylone through the BBB was evaluated. The reported concentrations in cerebrospinal fluid were around 13 mg/L for butylone and 7 mg/L for pentylone after dosing Sprague-Dawley rats at 20 mg/kg. These results are coherent with those of the present study, where around 6,000 and 3,700 ng/g butylone and pentylone, respectively, were found in telencephalon for rats dosed at 3 mg/kg.

Based on these data, the increment of the non-polarity of the cathinones due to the increase of the alkyl chains produces a reduction of the BBB permeability. Strangely, the most potent cathinone analogues in terms of dose reported by consumers are those that have a three-carbon alkyl chain: MDPV, pentylone, α-PVP, pentedrone, etc., with the dose being higher if the length is shortened or increased further in most cases [49]. This could indicate that the mechanisms of toxicity of these compounds are not directly linked to their BBB permeability.

As can be seen in the case studies for bk-EBDP intoxications, users frequently report a long duration of action for this compound, which is not so common for other cathinones. Perhaps the duration of effects indicates that bk-EBDP lingers in the body for an unusually long amount of time, and some of the toxicity may stem from this phenomenon.

Table 2. Concentration levels found in rat telencephalon tissue for cathinones differing on the alkyl chain length (rats dosed at 3 mg/kg).

Alkyl chain length			
Compound	**Brain tissue conc. (ng/g)**	**Ratio found/dosed (µg/mg)**	**Structure**
Buphedrone	6,067	1.69	
Pentedrone	3,718	1.03	
N-Ethyl-pentedrone	1,432	0.40	
N-Ethyl-hexedrone	1,160	0.32	
Butylone	4,659	1.29	
Pentylone	3,113	0.86	

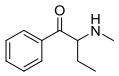
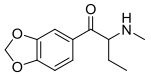
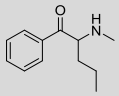
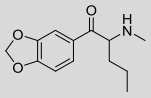
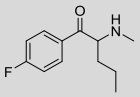
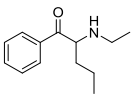
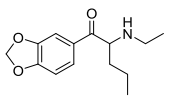
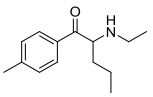
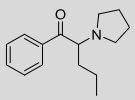
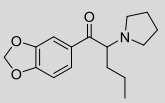
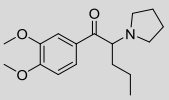
The last typical change in the cathinone structure is functionalization of the aromatic ring. As can be observed in **Table 3**, the functionalizations studied were the addition of a 3,4-methylenedioxy moiety, a methyl group, a 3,4-dimethoxy group, and the addition of an halogen atom (in this case, a fluorine). Three of the four cathinone couples with/without a 3,4-methylenedioxy moiety (buphedrone and butylone, pentedrone and pentylone, and α-PVP and MDPV) presented a

reduction of the permeability through the BBB when this moiety was added to the molecule (**Table 3**), and it could also be related to the increment of the non-polarity of the compound by this modification.

The remarkably low brain tissue concentration (860 ng/g) for MDPV is in line with previously reported concentrations for MDPV in rat brain, quantified around 260 ng/g at 30 min when dosing a rat at 1 mg/kg 38. Only for the couple *N*-ethyl-pentedrone and *N*-ethyl-pentylone, the cathinone with the 3,4-methylenedioxy moiety presented a higher concentration in telencephalon tissue. Similar to the results obtained when analysing the *N*-functionalization (**Table 1**), *N*-ethyl-pentylone produced an unexpected result when compared to the other cathinones. The higher telencephalon concentration of 4-fluoropentedrone, when compared with pentedrone, suggests that the presence of a halogen atom may increase BBB permeability, potentially due to an increment of the compound's polarity. The reason for the increment of the permeability observed when adding a methyl group (*N*-ethyl-pentedrone vs *N*-ethyl-4-methylpentedrone) or a 3,4-dimethoxy group (α-PVP vs 3,4-dimethoxy-α-PVP) is unclear. It is possible that the presence of these terminal methyl groups allows for easier passing through the BBB. In order to confirm this, more cathinones with these aromatic ring changes should be evaluated.

The concentration differences discussed above, when changing the *N*-functionalization, alkyl chain length and aromatic ring substitution, point at a positive correlation between polarity and BBB permeability, as also suggested by others [41]. However, based on an *in vitro* model using TY09 conditionally immortalized human brain capillary endothelial cells, Simmler and colleagues suggested the opposite: these authors reported that a decrease in polarity of cathinones produces an increment of the permeability of the BBB, with non-polar cathinones presenting a particularly high transendothelial permeability [17].

Table 3. Concentration levels found in rat telencephalon tissue for cathinones differing on the aromatic ring substitutions (rats dosed at 3 mg/kg).

Aromatic ring substitution			
Compound	**Brain tissue conc. (ng/g)**	**Ratio found/dosed (µg/mg)**	**Structure**
Buphedrone	6,067	1.69	
Butylone	4,659	1.29	
Pentedrone	3,718	1.03	
Pentylone	3,113	0.86	
4-Fluoropentedrone	4,806	1.34	
N-Ethyl-pentedrone	1,432	0.40	
N-Ethyl-pentylone	10,596	2.94	
N-Ethyl-4-methylpentedrone	2,541	0.71	
α-PVP	3,054	0.85	
MDPV	863	0.24	
3,4-dimethoxy-α-PVP	2,083	0.58	

Although these in vitro data apparently contradict our findings and those of the previous literature [38,41], the use of live animals instead of a cell culture is a closer representation of the real pharmacokinetic behaviour of these compounds in a process as complex as BBB permeability.

In fact, the BBB is composed not just of endothelial cells, but also includes associated cell elements such as astrocyte endfeet, pericytes and microglia. There are several important routes of transport across the BBB [25], passive diffusion and the carrier-mediated influx being the most common ones related to psychoactive substances. The coexistence of both influx processes for cocaine through the BBB has been reported in literature using an *in vivo* model with Swiss mice; here, the carrier mediated influx rate was 3.4 times greater than its passive diffusion rate [26]. The same publication indicates that MDPV is also a substrate for the cocaine transporter. Based on this evidence and in line with the data presented in this work as well as in literature [41], it can be deduced that the penetration of cathinones through the BBB is a carrier-mediated process. An exhaustive study about the solute carrier transporters involved in cathinone transport should be performed in order to confirm this hypothesis.

In addition to compound polarity and moieties present in the distinct structures, different physicochemical properties of the compounds such as logP and logD, topological polar surface area, number of rotatable bonds, fraction of sp_3 carbons, heavy atom count, among other parameters, can be also be related to BBB permeability. For the compounds studied in this work, topological polar surface area values (obtained from PubChem) were evaluated for 11 compounds. No relationship between this parameter and BBB permeability was found. No additional parameters were found in compound databases, consequently those should be determined experimentally or theoretically to evaluate their contribution to BBB permeability.

In summary, the relationship between cathinone structure and their ability to cross the blood-brain barrier was studied in this work. To this aim, telencephalon tissues from Sprague-Dawley rats dosed at 3 mg/kg with 13 different cathinones were analyzed by a validated UHPLC-MS/MS procedure. The results obtained showed that permeability of cathinones is related to their polarity, with better crossing of the BBB with increasing polarity. These findings are in accordance with previously published data using rats [41] but differ from those obtained using *in vitro* experiments [17], demonstrating the importance of not solely relying on *in vitro* data. Less polar *N* functionalizations, such as the presence of a pyrrolidine ring, reduced the cathinone transport through the BBB. In a similar way, cathinones with longer alkyl chain were less able to cross the BBB. Non-polar aromatic ring substitutions such as 3,4-methylenedioxy reduced BBB permeability, while the presence of a fluorine atom increased BBB transport. All this data, together with information available in literature from similar studies [41] and the BBB transport [26] suggest that cathinones cross the BBB through a carrier-mediated process. Additionally, this study shows that studying the pharmacology and pharmacokinetics of cathinones, and NPS as a whole, is crucial for a better understanding of the *in vivo* potency of these compounds, complementing other studies such as dopamine and serotonin uptake inhibition. Our future work will be focused on the study of additional cathinones that will appear on the continuously evolving NPS market, in order to support the role of carrier-mediated processes in the BBB passage of cathinones.

Acknowledgements

D. Fabregat-Safont, J.V. Sancho, F. Hernández and M. Ibáñez acknowledge financial support from the University Jaume I (UJI-B2018-19). D. Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (FPU15/02033). F. Martinez-Garcia and M. Barneo-Muñoz acknowledge financial support from Ministerio de Economía y Competitividad-

FEDER (BFU2016-77691-C2-1-P), the Generalitat Valenciana (PROMETEO/2016/076) and the Universitat Jaume I de Castelló (UJI-B2016-45). X. Carbón and M. Ventura acknowledge the grants from Subdirecció General de Drogodependències, Departament de Salut, Generalitat de Catalunya and Plan Nacional sobre Drogas. Authors also acknowledge Esther Fuentes-Ferragud for helping during method development.

Competing Interests

The authors declare that they have no competing interests.

Author contribution

D.F-S., J.V.S and M.I. conceived the work. M.B-M. and F.M-G. performed animal experiment and brain dissection. D.F-S. and M.I. performed sample treatment, instrumental analysis and data process. D.F-S., J.V.S, M.B-M., F.M-G, X.C, M.V. and M.I. interpreted and discussed the results. F.H. and F.M-G contributed with new reagents and analytical tools. D.F-S and M.I. wrote the first draft of the manuscript. J.V.S, M.B-M., F.M-G, X.C, M.V., C.S. and F.H. provided useful comments and feedback for the manuscript.

References

1. United Nations Office on Drugs and Crime. World Drug Report 2020.; 2020.

2. Oliver CF, Palamar JJ, Salomone A, et al. Synthetic cathinone adulteration of illegal drugs. Psychopharmacology (Berl). 2019;236(3):869-879. doi:10.1007/s00213-018-5066-6

3. Giné CV, Espinosa IF, Vilamala MV. New psychoactive substances as adulterants of controlled drugs. A worrying phenomenon? Drug Test Anal. 2014;6(7-8):819-824. doi:10.1002/dta.1610

4. Zaami S, Giorgetti R, Pichini S, Pantano F, Marinelli E, Busardò FP. Synthetic cathinones related fatalities: An update. Eur Rev Med Pharmacol Sci. 2018;22(1):268-274. doi:10.26355/eurrev-201801-14129

5. Majchrzak M, Celiński R, Kuś P, Kowalska T, Sajewicz M. The newest cathinone derivatives as designer drugs: an analytical and toxicological review. Forensic Toxicol. September 2017. doi:10.1007/s11419-017-0385-6

6. Kraemer M, Boehmer A, Madea B, Maas A. Death cases involving certain new psychoactive substances: A review of the literature. Forensic Sci Int. 2019;298:186-267. doi:10.1016/j.forsciint.2019.02.021

7. Bijlsma L, Celma A, López FJ, Hernández F. Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations. Curr Opin Environ Sci Heal. 2019;9:1-12. doi:10.1016/j.coesh.2019.03.002

8. Celma A, Sancho J V., Salgueiro-González N, et al. Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology. J Chromatogr A. 2019;1602:300-309. doi:10.1016/j.chroma.2019.05.051

9. Bijlsma L, Ibáñez M, Miserez B, et al. Mass spectrometric identification and structural analysis of the third-generation synthetic cannabinoids on the UK market since the 2013 legislative ban. Forensic Toxicol. 2017;35(2):376-388. doi:10.1007/s11419-017-0368-7

10. Zawilska JB, Wojcieszak J. Designer cathinones—An emerging class of novel recreational drugs. Forensic Sci Int. 2013;231(1-3):42-53. doi:10.1016/j.forsciint.2013.04.015

11. Coppola M, Mondola R. 3,4-Methylenedioxypyrovalerone (MDPV): Chemistry, pharmacology and toxicology of a new designer drug of abuse marketed online. Toxicol Lett. 2012;208(1):12-15. doi:10.1016/j.toxlet.2011.10.002

12. Zaitsu K, Katagi M, Tsuchihashi H, Ishii A. Recently abused synthetic cathinones, α-pyrrolidinophenone derivatives: a review of their pharmacology, acute toxicity, and metabolism. Forensic Toxicol. 2014;32(1):1-8. doi:10.1007/s11419-013-0218-1

13. Gatch MB, Dolan SB, Forster MJ. Comparative Behavioral Pharmacology of Three Pyrrolidine-Containing Synthetic Cathinone Derivatives. J Pharmacol Exp Ther. 2015;354(2):103-110. doi:10.1124/jpet.115.223586

14. Coppola M, Mondola R. Synthetic cathinones: Chemistry, pharmacology and toxicology of a new class of designer drugs of abuse marketed as "bath salts" or "plant food." Toxicol Lett. 2012;211(2):144-149. doi:10.1016/j.toxlet.2012.03.009

15. Kelly JP. Cathinone derivatives: A review of their chemistry, pharmacology and toxicology. Drug Test Anal. 2011;3(7-8):439-453. doi:10.1002/dta.313

16. Angoa-Pérez M, Anneken JH, Kuhn DM. Neurotoxicology of Synthetic Cathinone Analogs. In: Brain Imaging in Behavioral Neuroscience. ; 2016:209-230. doi:10.1007/7854_2016_21

17. Simmler L, Buser T, Donzelli M, et al. Pharmacological characterization of designer cathinones in vitro. Br J Pharmacol. 2013;168(2):458-470. doi:10.1111/j.1476-5381.2012.02145.x

18. Baumann MH, Ayestas MA, Partilla JS, et al. The Designer Methcathinone Analogs, Mephedrone and Methylone, are Substrates for Monoamine Transporters in Brain Tissue. Neuropsychopharmacology. 2012;37(5):1192-1203. doi:10.1038/npp.2011.304

19. Olesti E, Rodríguez-Morató J, Gomez-Gomez A, Ramaekers JG, de la Torre R, Pozo OJ. Quantification of endogenous neurotransmitters and related compounds by liquid chromatography coupled to tandem mass spectrometry. Talanta. 2019;192(July 2018):93-102. doi:10.1016/j.talanta.2018.09.034

20. Olesti E, De Toma I, Ramaekers JG, et al. Metabolomics predicts the pharmacological profile of new psychoactive substances. J Psychopharmacol. 2019;33(3):347-354. doi:10.1177/0269881118812103

21. Simmler LD, Liechti ME. Interactions of Cathinone NPS with Human Transporters and Receptors in Transfected Cells. In: ; 2016:49-72. doi:10.1007/7854_2016_20

22. Simmler LD, Buchy D, Chaboz S, Hoener MC, Liechti ME. In Vitro Characterization of Psychoactive Substances at Rat, Mouse, and Human Trace Amine-Associated Receptor 1. J Pharmacol Exp Ther. 2016;357(1):134-144. doi:10.1124/jpet.115.229765

23. Rickli A, Hoener MC, Liechti ME. Monoamine transporter and receptor interaction profiles of novel psychoactive substances: Para-halogenated amphetamines and pyrovalerone cathinones. Eur Neuropsychopharmacol. 2015;25(3):365-376. doi:10.1016/j.euroneuro.2014.12.012

24. Noble C, Cannaert A, Linnet K, Stove CP. Application of an activity-based receptor bioassay to investigate the in vitro activity of selected indole- and indazole-3-carboxamide-based synthetic cannabinoids at CB1 and CB2 receptors. Drug Test Anal. 2018;(August):1-11. doi:10.1002/dta.2517

25. Abbott NJ, Patabendige AAK, Dolman DEM, Yusof SR, Begley DJ. Structure and function of the blood–brain barrier. Neurobiol Dis. 2010;37(1):13-25. doi:10.1016/j.nbd.2009.07.030

26. Chapy H, Smirnova M, Andre P, et al. Carrier-Mediated Cocaine Transport at the Blood-Brain Barrier as a Putative Mechanism in Addiction Liability. Int J Neuropsychopharmacol. 2015;18(1):pyu001-pyu001. doi:10.1093/ijnp/pyu001

27. European Medicines Agency. Guideline on bioanalytical method validation, EMEA/CHMP/EWP/192217/2009. 2011.

28. Food and Drug Administration. Bioanalytical Method Validation, FDA-2013-D-1020. Presented at the: 2018.

29. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS. Anal Chem. 2003;75(13):3019-3030. doi:10.1021/ac020361s

30. Constanzer ML, Chavez-Eng CM, Fu I, Woolf EJ, Matuszewski BK. Determination of dextromethorphan and its metabolite dextrorphan in human urine using high performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry: a study of selectivity of a tandem mass spectrometric assa. J Chromatogr B. 2005;816(1-2):297-308. doi:10.1016/j.jchromb.2004.11.049

31. Fabregat-Safont D, Carbón X, Gil C, et al. Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. 2018;36(2):447-457. doi:10.1007/s11419-018-0422-0

32. Doi T, Akiko Asada B, Akihiro Takeda B, et al. Identification and characterization of a-PVT, a-PBT, and their bromothienyl analogs found in illicit drug products. Forensic Toxicol. 2016;34(1):76-93. doi:10.1007/s11419-015-0288-3

33. Qian Z, Jia W, Li T, Hua Z, Liu C. Identification and analytical characterization of four synthetic cannabinoids ADB-BICA, NNL-1, NNL-2, and PPA(N)-2201. Drug Test Anal. 2017;9(1):51-60. doi:10.1002/dta.1990

34. Ibáñez M, Pozo ÓJ, Sancho J V., Orengo T, Haro G, Hernández F. Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry. Anal Bioanal Chem. 2016;408(1):151-164. doi:10.1007/s00216-015-9088-1

35. Swortwood MJ, Ellefsen KN, Wohlfarth A, et al. First metabolic profile of PV8, a novel synthetic cathinone, in human hepatocytes and urine by high-resolution mass spectrometry. Anal Bioanal Chem. 2016;408(18):4845-4856. doi:10.1007/s00216-016-9599-4

36. Helfer AG, Turcant A, Boels D, et al. Elucidation of the metabolites of the novel psychoactive substance 4-methyl- N -ethyl-cathinone (4-MEC) in human urine and pooled liver microsomes by GC-MS and LC-HR-MS/MS techniques and of its detectability by GC-MS or LC-MS n standard screening approach. Drug Test Anal. 2015;7(5):368-375. doi:10.1002/dta.1682

37. López-Arnau R, Martínez-Clemente J, Carbó M, Pubill D, Escubedo E, Camarasa J. 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. 2013;45:64-72. doi:10.1016/j.pnpbp.2013.04.007

38. Horsley RR, Lhotkova E, Hajkova K, et al. Behavioural, Pharmacokinetic, Metabolic, and Hyperthermic Profile of 3,4-Methylenedioxypyrovalerone (MDPV) in the Wistar Rat. Front Psychiatry. 2018;9(APR):1-13. doi:10.3389/fpsyt.2018.00144

39. Lin Z, Chen Y, Li J, et al. Pharmacokinetics of N- ethylpentylone and its effect on increasing levels of dopamine and serotonin in the nucleus accumbens of conscious rats. Addict Biol. 2019;(November 2018):e12755. doi:10.1111/adb.12755

40. Peters JR, Keasling R, Brown SD, Pond BB. Quantification of Synthetic Cathinones in Rat Brain Using HILIC–ESI-MS/MS. J Anal Toxicol. 2016;(July):718-725. doi:10.1093/jat/bkw074

41. Grecco GG, Kisor DF, Magura JS, Sprague JE. Impact of common clandestine structural modifications on synthetic cathinone "bath salt" pharmacokinetics. Toxicol Appl Pharmacol. 2017;328:18-24. doi:10.1016/j.taap.2017.05.010

42. Fabregat-Safont D, Carbón X, Ventura M, et al. Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci Rep. 2017;7(1). doi:10.1038/s41598-017-06778-9

43. Fabregat-Safont D, Carbón X, Ventura M, Fornís I, Hernández F, Ibáñez M. Characterization of a recently detected halogenated aminorex derivative: para-fluoro-4-methylaminorex (4′F-4-MAR). Sci Rep. 2019;9(1):8314. doi:10.1038/s41598-019-44830-y

44. Expert Committee on Drug Dependence. Pentedrone Critical Review Report.; 2016. https://www.who.int/medicines/access/controlled-substances/4.6_Pentedrone_CritReview.pdf?ua=1.

45. Baumann MH, Ayestas MA, Partilla JS, et al. The Designer Methcathinone Analogs, Mephedrone and Methylone, are Substrates for Monoamine Transporters in Brain Tissue. Neuropsychopharmacology. 2012;37(5):1192-1203. doi:10.1038/npp.2011.304

46. Krotulski AJ, Papsun DM, De Martinis BS, Mohr ALA, Logan BK. N-Ethyl Pentylone (Ephylone) Intoxications: Quantitative Confirmation and Metabolite Identification in Authentic Human Biological Specimens. J Anal Toxicol. 2018;42(7):467-475. doi:10.1093/jat/bky025

47. Thirakul P, S. Hair L, L. Bergen K, M. Pearson J. Clinical Presentation, Autopsy Results and Toxicology Findings in an Acute N-Ethylpentylone Fatality. J Anal Toxicol. 2017;41(4):342-346. doi:10.1093/jat/bkx004

48. Drug Enforcement Administration (DEA). Schedules of Controlled Substances: Temporary Placement of N-Ethylpentylone in Schedule I. Vol 83.; 2018. https://www.federalregister.gov/documents/2018/06/13/2018-12669/schedules-of-controlled-substances-temporary-placement-of-n-ethylpentylone-in-schedule-i.

49. Debruyne D, Loilier M, Cesbron A, Le Boisselier R, Bourgine J. Emerging drugs of abuse: current perspectives on substituted cathinones. Subst Abuse Rehabil. May 2014:37. doi:10.2147/SAR.S37257

5.4. Discussion of the results obtained

In vivo models provide different approaches for an accurate assessment of the pharmacological behaviour of NPS, including metabolic profiling, pharmacokinetics, tissue absorption (and distribution), and excretion. The research presented in **Chapter 5** illustrates that *in vivo* models are versatile enough for understanding physiological processes involved with NPS, such as the permeability of the BBB for a certain type of drugs.

Scientific article IX presents the elucidation of the main metabolites of the synthetic tryptamine 5-MeO-MiPT by the use of C57BL/J6 adult male mice, as well as the pharmacokinetic of circulatory metabolites in blood, and excretion rate in urine. The results obtained from urine analysis revealed the most adequate consumption biomarkers for this compound, as well as the determination of long-term metabolites that could be detected 24 h after drug administration. Furthermore, **scientific article X** includes, on the one hand, the validation of an analytical methodology for the quantification of 13 synthetic cathinones in rat cerebrum tissue by UHPLC-MS/MS; and, on the other hand, an accurate evaluation of the permeability of these synthetic cathinones through the BBB, and their relationship with the different moieties present in compound structure. In this case, Sprague-Dawley adult female rats were used.

The results obtained in **research article IX** illustrate that 5-MeO-MiPT is mainly metabolised through phase I *O*-demethylation, followed by phase II *O*-glucuronidation, being the last one the most important in blood and urine. Additional minor metabolites were obtained after an *N*-oxidation in the tertiary amine, and indole hydroxylation plus glucuronidation. Few days after the publication of our article, the metabolic pathway of 5-MeO-MiPT using pHLM, and also blood and urine samples from a consumer, was published by Katharina Elisabeth Grafinger and colleagues [29]. This publication reported different phase I metabolites after *in vitro* incubation of 100 µM of and 5-MeO-MiPT during 120 min, and analysis by UHPLC-HRMS (QTOF, 5600 TripleTof from Sciex),

including the *O*-demethylated, *N*-oxide, and indole hydroxylated metabolites reported in our work [29]. It is important to point out that the indole hydroxylated metabolite was not directly detected in our work, but it was detected as the glucuronide conjugated phase II metabolite. In total, 4 and 7 metabolites were detected in blood and urine, respectively [29]. The results from these real samples reported by Grafinger showed that parent compound and the *O*-demethylated metabolite (29% of parent response) were the consumption markers with highest responses found in blood sample, while parent compound, *N*-demethylated (34%) and *N*-oxide (19%) were the main compounds detected in urine [29]. It has to be noted that the consumption biomarker proposed in our work (this is, the *O*-demethylated metabolite) represented only the 8% of parent response in the urine sample [29].

Grafinger and co-authors stated that the differences between their results and those presented in **research article IX**, especially in the phase II metabolites, could be produced by the interspecies differences- clearance of a free drug between smaller animals and large species [29]. Additionally, Grafinger and colleagues used an *in silico* prediction tool for metabolite prediction which, as stated by the own authors, was not able to predict phase II metabolites, including glucuronides [29]. Finally, the sample treatment used by Grafinger was based in protein precipitation for blood samples, and in a dilute-and-shoot approach for urine samples [29]. So, if only phase I metabolites were searched for during UHPLC-HRMS analysis, and no enzymatic hydrolysis was performed for glucuronide cleavage, it is logical that the results obtained by Grafinger and our study differ at first sight. As illustrated in **research article IX** (**Figure 6**), the main circulatory metabolite, as well as the main excreted through urine, was the glucuronide conjugated of the *O*-demethyl. So, it is possible that this phase II metabolite, determined as the major compound after *in vivo* evaluation in mice, was also present in the blood and urine samples from the patient, but it was not found due to the inability of the *in silico* prediction tool for proposing phase II metabolites. It is also possible that the results of the prevalence of phase I

metabolites would be different if enzymatic hydrolysis had been performed by Grafinger. In this sense, enzymatic hydrolysis is mandatory for the cleavage of the conjugated moieties that could mask phase I metabolites.

So, the results presented by Grafinger and those included in **research article IX** should be considered as complementary: on the one hand, although Grafinger's work is performed with human samples, there are some important aspects which have not been adequately treated; on the other hand, our results (obtained with an *in vivo* approach) could not completely reflect the human metabolic behaviour of 5-MeO-MiPT.

Regarding **scientific article X**, an accurate discussion of the obtained results has been presented in the article text. In this case, up to 13 synthetic cathinones were quantified in Sprague-Dawley female rat cerebrum tissue, dosed with 3 mg/kg of each compound individually. Summarising, a direct relationship between the structure of the synthetic cathinones and the permeability through the BBB was found, suggesting that the permeability is decreased when non-polar moieties are present in cathinone structure. These results are not in agreement with the data obtained by Linda Simmler and co-authors after *in vitro* evaluation [30], who suggested that the permeability through the BBB was increased with the non-polarity of the cathinone. Nevertheless, a similar study from Gregory Grecco and colleagues, who quantified 3 related synthetic cathinones in rat brain, indicated that the length of the α alkyl chain of these compounds was related to the drug levels found in brain, decreasing the cathinone levels as the α alkyl chain was longer [27]. Similar to the discussion of **research article VIII** in **Chapter 4**, and the previously discussed **research article IX**, some differences can be found in *in vitro* and *in vivo* models, which in some cases can be justified (as commented in **research article VIII** and **IX**), and in other cases can be contradictory, as in this case. The results from Simmler suggested that cathinones present a passive diffusion through the BBB, but the results from Grecco and our study indicated that cathinones could present an active transport. The key of the study was found in an article from Hélène Chapy, who evaluated the carrier-mediated cocaine

transport at the BBB, but also found that the synthetic cathinone MDPV was a substrate of the cocaine transporter [31]. So, with the results from Chapy [31], together with the data from Grecco [27] and our study, it was suggested that synthetic cathinones present a carrier-mediated process in the BBB, which is increased with the polarity of the moieties present in the compound.

The information presented in **Chapter 5** illustrates the versatility of *in vivo* models for performing NPS ADME experiments, such as metabolite profiling and pharmacokinetics, or quantification in tissues and evaluation of the permeability of different compounds in a certain organ.

5.5. Literature

1. Korfmacher, W. A. Using Mass Spectrometry for Drug Metabolism Studies. (CRC Press, 2004).

2. Pozo, O. 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. Drug Metab. Dispos. 43, 248–257 (2014).

3. Olesti, E. et al. Pharmacokinetics of Mephedrone and Its Metabolites in Human by LC-MS/MS. AAPS J. 19, 1767–1778 (2017).

4. Franz, F., Haschimi, B., King, L. A. & Auwärter, V. Extraordinary long detection window of a synthetic cannabinoid metabolite in human urine – Potential impact on therapeutic decisions. Drug Test. Anal. 12, 391–396 (2020).

5. Ibáñez, M. et al. Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry. Anal. Bioanal. Chem. 408, 151–164 (2016).

6. Jang, M. et al. Determination of urinary metabolites of XLR-11 by liquid chromatography–quadrupole time-of-flight mass spectrometry. Anal. Bioanal. Chem. 408, 503–516 (2016).

7. Krotulski, A. J., Papsun, D. M., De Martinis, B. S., Mohr, A. L. A. & Logan, B. K. N-Ethyl Pentylone (Ephylone) Intoxications: Quantitative Confirmation and Metabolite Identification in Authentic Human Biological Specimens. J. Anal. Toxicol. 42, 467–475 (2018).

8. Jones, M. J., Hernandez, B. S., Janis, G. C. & Stellpflug, S. J. A case of U-47700 overdose with laboratory confirmation and metabolite identification. Clin. Toxicol. 3650, 1–5 (2016).

9. Higashikawa, Y. & Suzuki, S. Studies on 1-(2-phenethyl)-4-(N-propionylanilino)piperidine (fentanyl) and its related compounds. VI. Structure-analgesic activity relationship for fentanyl, methyl-substituted fentanyls and other analogues. Forensic Toxicol. 26, 1–5 (2008).

10. Banister, S. D. et al. The chemistry and pharmacology of putative synthetic cannabinoid receptor agonist (SCRA) new psychoactive substances (NPS) 5F- PY- PICA, 5F- PY- PINACA, and their analogs. Drug Test. Anal. 11, 976–989 (2019).

11. Nordmeier, F., Richter, L. H. J., Schmidt, P. H., Schaefer, N. & Meyer, M. R. Studies on the in vitro and in vivo metabolism of the synthetic opioids U-51754, U-47931E, and methoxyacetylfentanyl using hyphenated high-resolution mass spectrometry. Sci. Rep. 9, 13774 (2019).

12. Kanamori, T. et al. In Vivo Metabolism of 5-Methoxy-N,N-diisopropyltryptamine in Rat. J. Heal. Sci. 52, 425–430 (2006).

13. Xu, D. et al. Analysis of AMB-FUBINACA Biotransformation Pathways in Human Liver Microsome and Zebrafish Systems by Liquid Chromatography-High Resolution Mass Spectrometry. Front. Chem. 7, 1–9 (2019).

14. Richter, L. H. J. et al. Tools for studying the metabolism of new psychoactive substances for toxicological screening purposes – A comparative study using pooled human liver S9, HepaRG cells, and zebrafish larvae. Toxicol. Lett. 305, 73–80 (2019).

15. Schaefer, N. et al. Metabolic patterns of JWH-210, RCS-4, and THC in pig urine elucidated using LC-HR-MS/MS: Do they reflect patterns in humans? Drug Test. Anal. 9, 613–625 (2017).

16. Grafinger, K. E., Wilke, A., König, S. & Weinmann, W. Investigating the ability of the microbial model Cunninghamella elegans for the metabolism of synthetic tryptamines. Drug Test. Anal. 11, 721–729 (2019).

17. Maurer, H. H. & Brandt, S. D. New Psychoactive Substances. Pharmacology, Clinical, Forensic and Analytical Toxicology. Handbook of Experimental Pharmacology 252, (Springer International Publishing, 2018).

18. Calinski, D. M., Kisor, D. F. & Sprague, J. E. A review of the influence of functional group modifications to the core scaffold of synthetic cathinones on drug pharmacokinetics. Psychopharmacology (Berl). 236, 881–890 (2019).

19. Watanabe, S., Vikingsson, S., Åstrand, A., Gréen, H. & Kronstrand, R. Biotransformation of the New Synthetic Cannabinoid with an Alkene, MDMB-4en-PINACA, by Human Hepatocytes, Human Liver Microsomes, and Human Urine and Blood. AAPS J. 22, 13 (2020).

20. Richter, L. H. J., Maurer, H. H. & Meyer, M. R. Metabolic fate of the new synthetic cannabinoid 7'N-5F-ADB in rat, human, and pooled human S9 studied by means of hyphenated high-resolution mass spectrometry. Drug Test. Anal. 11, 305–317 (2019).

21. 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).

22. Horsley, R. R. et al. Behavioural, Pharmacokinetic, Metabolic, and Hyperthermic Profile of 3,4-Methylenedioxypyrovalerone (MDPV) in the Wistar Rat. Front. Psychiatry 9, 1–13 (2018).

23. Concheiro, M. et al. Nonlinear Pharmacokinetics of (±)3,4-Methylenedioxymethamphetamine (MDMA) and Its Pharmacodynamic Consequences in the Rat. Drug Metab. Dispos. 42, 119–125 (2013).

24. Peters, J. R., Keasling, R., Brown, S. D. & Pond, B. B. Quantification of Synthetic Cathinones in Rat Brain Using HILIC–ESI-MS/MS. J. Anal. Toxicol. 40, 718–725 (2016).

25. Olesti, E. et al. Quantification of endogenous neurotransmitters and related compounds by liquid chromatography coupled to tandem mass spectrometry. Talanta 192, 93–102 (2019).

26. Olesti, E. et al. Metabolomics predicts the pharmacological profile of new psychoactive substances. J. Psychopharmacol. 33, 347–354 (2019).

27. Grecco, G. G., Kisor, D. F., Magura, J. S. & Sprague, J. E. Impact of common clandestine structural modifications on synthetic cathinone "bath salt" pharmacokinetics. Toxicol. Appl. Pharmacol. 328, 18–24 (2017).

28. Grecco, G. G., Kisor, D. F. & Sprague, J. E. Pharmacokinetic data of synthetic cathinones in female Sprague-Dawley rats. Data Br. 21, 1045–1050 (2018).

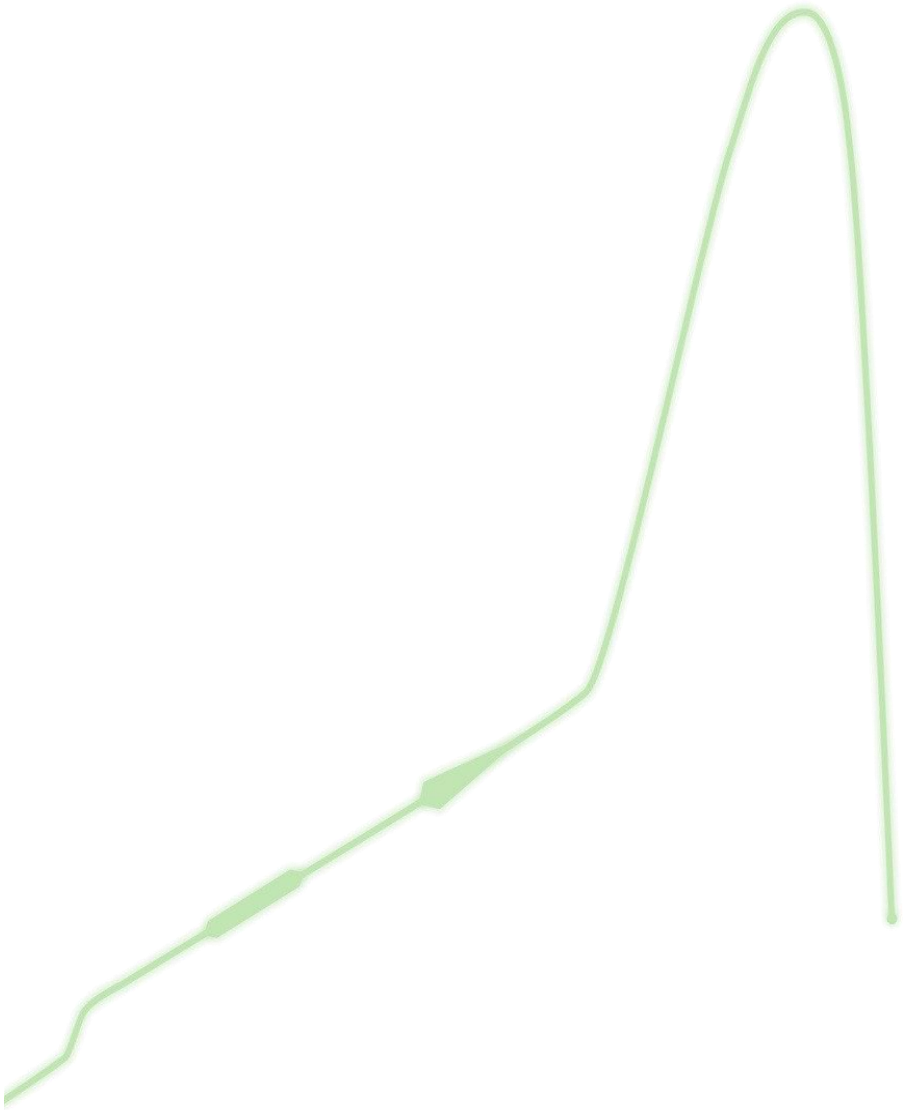
29. Grafinger, K. E., Hädener, M., König, S. & Weinmann, W. Study of the in vitro and in vivo metabolism of the tryptamine 5-MeO-MiPT using human liver microsomes and real case samples. Drug Test. Anal. 10, 562–574 (2018).

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

31. Chapy, H. et al. Carrier-Mediated Cocaine Transport at the Blood-Brain Barrier as a Putative Mechanism in Addiction Liability. Int. J. Neuropsychopharmacol. 18, pyu001–pyu001 (2015).

CHAPTER 6

SUSPECT SCREENING OF SYNTHETIC CANNABINOIDS IN URINE



CHAPTER 6
SUSPECT SCREENING OF SYNTHETIC CANNABINOIDS IN URINE

6.1. Introduction

6.2. Research article XI

> "Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples".
> *Journal of Pharmaceutical and Biomedical Analysis, 2020; 186:113298.*

6.3. Research article XII

> "Variation in the pattern of synthetic cannabinoid use by a female patient during 2018".
> *Adicciones, 2020; 32:228.*

6.4. Discussion of the results obtained

6.5. Literature

6.1. Introduction

LC-MS is one of the most powerful analytical tools for toxicological analysis [1], including the quantification of drugs in human samples [2,3], determination of toxins [4,5], doping control [6,7], and endocrine disrupting compounds [8,9]. An especially interesting application of LC-MS in toxicology is the analysis of biological samples to obtain information about the prevalence of certain substances in a certain population. This technique has been widely applied for the study of illicit drugs consumption in festivals, through urine and saliva analysis [10], as well as for the determination of NPS consumption prevalence, through the analysis of overdose case samples [11] or wastewater for covering a wider population [12,13]. Indeed, the use of LC-MS for the analysis of urine samples to determine NPS consumption is a hot topic in the last years [14,15]. Thus, different European projects, such as NPS-Euronet, have been financed dealing with the analysis of urine and wastewater samples for stablishing NPS consumption patterns [16].

Nevertheless, as presented and discussed in **Chapters 4** and **5**, the analysis of urine samples (and wastewater) for determining NPS consumption is hampered by the metabolic transformations that suffer these substances after consumption [17]. In the case of synthetic cathinones, they are not highly metabolised and parent compound is typically the main consumption biomarker in urine [18,19]. In other cases, for example synthetic opioids, the parent compound is also present in urine, but there are some metabolites that could present higher concentration levels and thus, they should be used as consumption markers [20,21]. However, SCRA are completely metabolised, as discussed in **Chapter 4** (**research article VII**), so their determination in urine must be performed by the screening of their major metabolites [22,23]. In consequence, it is necessary to stablish the metabolic profiling of these compounds for proposing the most suitable biomarkers. As an illustrative example, the SCRA XLR-11 was detected in 2011 in Russia [24], the *in vitro* metabolite profiling was reported in 2013 using pHH [25], and finally the urinary metabolites, and thus consumption biomarkers of this compound, were reported

in 2016 [26]. It can be noticed that it could take a long time to perform metabolism experiments for all the reported SCRA, in order to obtain a metabolite list that could be used for suspect screening in urine samples. At this point, collaborative compound databases present an interesting tool for obtaining suspect compound lists to be used during LC-MS analysis [27].

In some specific cases, the number of compounds that should be searched in urine samples can be limited depending on different criteria. Traditionally, the studies dealing with illicit drug consumption have made use of self-reported data through questionnaires and/or interviews; however, the information provided by the users could not reflect the real consumption behaviour [10], as in some cases, users do not know what substance are they consuming. Nevertheless, in this chapter, it is presented a different criterion for the selection of SCRA consumption markers that should be searched for in urine analysis. If the geographical origin of the products consumed is accurately determined, for example those collected in specific medical centres, the compounds to be determined can be selected based on the SCRA availability in this area.

The studies included in the present chapter have been performed in collaboration with different medical services of the Valencian region, and more specifically with the Hospital Provincial de Castelló (**research article XI**) and with the medical staff of two Addictive Behaviour Unit (ABU) in Valencia (**research article XII**), in order to determine the SCRA being consumed by teenagers in different situations.

Research article XI presents the investigation of the SCRA consumption prevalence among teenagers in 5 juvenile offenders' centres from the Valencian region during 2016. In this study, the medical services of the centres suspicioned that teenagers consumed synthetic cannabinoids during their permits with stay at home for avoiding drug tests performed when they return to the centre. Some of these teenagers recognised having bought herbal blends in a local smartshop of Valencia (Spain) and to local dealers. The results obtained in the analysis of the

legal highs available in that smartshop have already been presented in **Chapter 2**. So, the major metabolites of the SCRA detected in these herbal blends were searched for in literature, and the suspect compound list was prepared for the UHPLC-HRMS suspect screening. The XLR-11 and UR-144 metabolites were found in urine of different teenagers.

A specific case is presented in **research article XII**, in which the SCRA consumption patterns of a female teenager who was under treatment in an ABU in Valencia along 2018 was studied. In the first half-year, the patient recognised the consumption of the same herbal blend consumed by teenagers in the juvenile offenders' centres. This fact was confirmed by detecting the main metabolites of XLR-11 and UR-144 in her urine. Nevertheless, in the second half-year, a novel SCRA (5F-ADB) was detected in some herbs seized in two ABUs in Valencia, as well as in a new herbal blend available in the local smartshop. The main metabolites of 5F-ADB were detected in the urine samples from the patient collected after summer, illustrating that her consumption patterns had changed. This change was related to the ban in Spain of XLR-11 in July 2018, showing that SCRA availability (and thus, consumption patterns) are highly affected by the current legislation.

6.2. Research article XI

Journal of Pharmaceutical and Biomedical Analysis 186 (2020) 113298

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples

David Fabregat-Safont[a], María Ibáñez[a,*], Abel Baquero[b], Juan Vicente Sancho[a], Félix Hernández[a], Gonzalo Haro[b,c,**]

[a] Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, Castelló, Spain
[b] Department of Medicine, University Cardenal Herrera-CEU, CEU Universities, Castelló, Spain
[c] Department of Psychiatry, Consorcio Hospitalario Provincial de Castelló, Spain

ARTICLE INFO

Article history:
Received 3 February 2020
Received in revised form 30 March 2020
Accepted 2 April 2020
Available online 13 April 2020

Keywords:
Juvenile offenders' centres
Synthetic cannabinoids
Drug analysis
XLR-11
UR-144

ABSTRACT

The use of synthetic cannabinoids (SCs), which escape conventional detection systems, may be a good alternative to elude routine drug analysis for cannabis. The detection of these drugs in urine is unusual due to their complete and fast metabolism, therefore requiring alternative strategies. In this work, an investigation has been made on SCs consumption by minors (less than 18 years old) in juvenile offenders' centres. 667 urine samples (from 127 minors) were collected after their permits with stay at home. We also studied the SCs from 7 herbal blends available at the smartshop frequented by the minors. Both, urine and herbal blends, were analysed by liquid chromatography coupled to high resolution mass spectrometry. The analysis of urine confirmed the absence of more than 200 SCs investigated. Thus, the focus was made on metabolites reported for those SCs identified in the herbal blends collected from the smart-shop. The major metabolites of XLR-11 and UR-144 (N-pentanoic acid and N-(5-hydroxypentyl)) were found in several urine samples. Apart from the main metabolites included in the initial searching, a thorough investigation of more metabolites for these SCs was additionally performed, including MS/MS experiments for the tentative identification of compounds detected in the urine samples. The 16 samples positive to the XLR-11 metabolites were assigned to 6 minors, only 2 of which had recognized consumption. On the basis of the results obtained, preventive and therapeutic interventions must be implemented to reduce the consumption of psychoactive substances and to improve the risk-perception of these substances by minors.

1. Introduction

The consumption of cannabis has gradually increased in Spain, as in most other countries, over the last decade. In Europe, cannabis is consumed by up to 30.4 % of the population in Spain and accounts for 29.9 % of related admissions for treatment [1]. The legal consequences of consumption vary depending on the judicial system: cannabis use is legalised in some areas with no repercussions while, in others, its use can have civil or even criminal repercussions. At this point, synthetic cannabinoids (SCs) can be used as a replacement of cannabis. In addition to the low perception of risk [2–4] and high availability and low cost of these substances, the detection of SCs and their metabolites in humans is difficult, due to the high number of existing compounds and the variability in their chemical structures. All these aspects surely contribute to the increased use of SCs.

According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), more than 160 SCs have been identified in herbal blends or spices since 2008. These herb mixtures, sold as 'legal' cannabis substitutes, are readily available in smartshops and via the internet. As stated by this organism, 80,000 products containing new psychoactive substances (NPS) were seized in Europe in 2015 and SCs accounted for 29 % of these. Moreover, many novel SCs are identified in herbal mixtures every year, illustrating the ongoing appearance of new substances [5–7]. High-resolution mass spectrometry (HRMS) is much useful for identifying SCs in herbal blends [8,9] but standardized analytical strategies are required for identification of SCs in forensic samples, especially in urine, to advance in the knowledge of this topic. This would

* Corresponding author at: Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain.
** Corresponding author at: Research group TXP, Medicine Department, Universidad Cardenal Herrera CEU, Calle Grecia, 31, 12006, Castellón, Spain.
E-mail addresses: ibanezm@uji.es (M. Ibáñez), gonzalo.haro@uchceu.es (G. Haro).

https://doi.org/10.1016/j.jpba.2020.113298
0731-7085/

Journal of Pharmaceutical and Biomedical Analysis, 2020; 186:113298

Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples

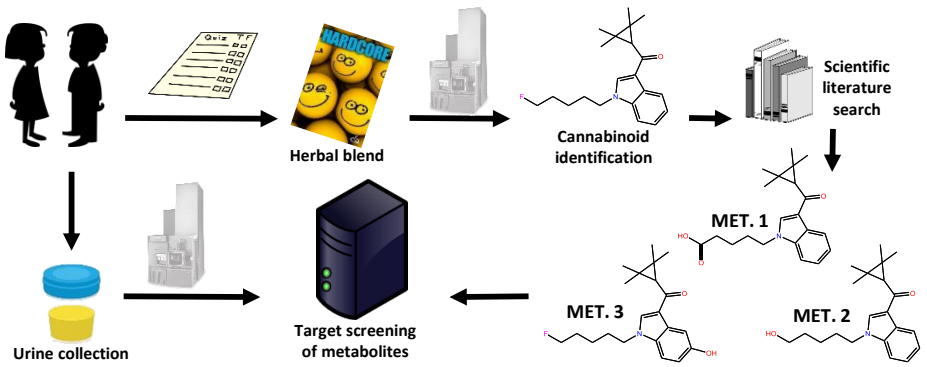
David Fabregat-Safont [a], María Ibáñez [a,*], Abel Baquero [b], Juan V. Sancho [a], Félix Hernández [a], Gonzalo Haro [b,c,*]

[a] Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castelló, Spain.

[b] Department of Medicine, University Cardenal Herrera-CEU, CEU Universities, Castelló, Spain.

[c] Department of Psychiatry. Consorcio Hospitalario Provincial de Castelló, Spain.

Graphical abstract



Abstract

The use of synthetic cannabinoids (SCs), which escape conventional detection systems, may be a good alternative to elude routine drug analysis for cannabis. The detection of these drugs in urine is unusual due to their complete and fast metabolism, therefore requiring alternative strategies. In this work, an investigation has been made on SCs consumption by minors (less than 18 years old) in juvenile offenders' centres. 667 urine samples (from 127 minors) were collected after their permits with stay at home. We also studied the SCs from 7 herbal blends available at the smartshop frequented by the minors. Both, urine and herbal blends, were analysed by liquid chromatography coupled to high resolution mass spectrometry. The analysis of urine confirmed the absence of more than 200 SCs investigated. Thus, the focus was made on metabolites reported for those SCs identified in the herbal blends collected from the smart-shop. The major metabolites of XLR-11 and UR-144 (*N*-pentanoic acid and *N*-(5-hydroxypentyl)) were found in several urine samples. Apart from the main metabolites included in the initial searching, a thorough investigation of more metabolites for these SCs was additionally performed, including MS/MS experiments for the tentative identification of compounds detected in the urine samples. The 16 samples positive to the XLR-11 metabolites were assigned to 6 minors, only 2 of which had recognized consumption. On the basis of the results obtained, preventive and therapeutic interventions must be implemented to reduce the consumption of psychoactive substances and to improve the risk-perception of these substances by minors.

Keywords Juvenile offenders' centres; Synthetic cannabinoids; Drug analysis; XLR-11; UR-144.

1. Introduction

The consumption of cannabis has gradually increased in Spain, as in most other countries, over the last decade. In Europe, cannabis is consumed by up to 30.4% of the population in Spain and accounts for 29.9% of related admissions for treatment [1]. The legal consequences of consumption vary depending on the judicial system: cannabis use is legalised in some areas with no repercussions while, in others, its use can have civil or even criminal repercussions. At this point, synthetic cannabinoids (SCs) can be used as a replacement of cannabis. In addition to the low perception of risk [2–4] and high availability and low cost of these substances, the detection of SCs and their metabolites in humans is difficult, due to the high number of existing compounds and the variability in their chemical structures. All these aspects surely contribute to the increased use of SCs.

According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), more than 160 SCs have been identified in herbal blends or spices since 2008. These herb mixtures, sold as "legal" cannabis substitutes, are readily available in smartshops and via the Internet. As stated by this organism, 80,000 products containing new psychoactive substances (NPS) were seized in Europe in 2015 and SCs accounted for 29% of these. Moreover, many novel SCs are identified in herbal mixtures every year, illustrating the ongoing appearance of new substances [5–7]. High-resolution mass spectrometry (HRMS) is much useful for identifying SCs in herbal blends [8,9] but standardized analytical strategies are required for identification of SCs in forensic samples, especially in urine, to advance in the knowledge of this topic. This would first require metabolism studies to establish appropriate urinary consumption-markers for SCs. Several studies have shown that it is near impossible to detect unaltered SCs in urine samples [10–13]. Thus, urine analysis of potential SC consumers should focus on the major metabolites [14], using powerful techniques, such as ultra-high performance liquid chromatography (UHPLC) coupled to HRMS [12,15,16] or tandem mass spectrometry (MS/MS) [17–19].

The prevalence of SC use in juvenile detention centres is commonly unknown, and data about its consumption is obtained only via self-reports [20], a standard procedure in the early stages of substance-use detection [21]. Given the suspect use of SCs in juvenile detention centres and the difficulties associated to their detection, we present a comprehensive strategy to reveal the consumption of SC by minors through the analysis of herbal blends presumably consumed. In parallel, a searching of the main metabolites of the SCs identified was performed in urine samples. Considering the high number of compounds investigated, HRMS with hybrid quadrupole-time of flight (QTOF) mass analyser was applied for analysis given the useful information (accurate-mass full-spectrum data) provided by this technique.

2. Materials and methods

2.1. Reagents and chemicals

HPLC-grade water was obtained by purifying demineralised water using a Milli-Q system from Millipore (Bedford, MA, USA). LC-MS grade methanol, acetone, and hydrochloric acid (HCl 37%) were purchased from Scharlau (Scharlab, Barcelona, Spain). Diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) was purchased from Merck (Darmstadt, Germany). β-glucuronidase from *E. Coli K12* (80 U/mL at 25 ºC) was purchased from Roche (Indianapolis, IN, USA).

1 M $H_2PO_4^-/HPO_4^{2-}$ buffer was prepared by dissolving the corresponding amount of (NH4)2HPO4 in HPLC-grade water and adjusting the pH to 7 with HCl.

2.2. Study design, herbal blends purchase and urine samples collection

127 teenagers, aged less than 18 years and linked to one of the five participant juvenile offenders' centres in the Valencian region (Spain) were included in this study. The inclusion criterion was to have therapeutic permits with stay at home, which entailed providing a urine sample for toxicological evaluation upon their return to the centre.

The subjects participated in a group interview, where they were asked about their use of NPS; if they mentioned their own consumption, we asked them about the commercial name of the NPS and how they acquired it.

Up to seven herbal blends suspicious to contain synthetic cannabinoids were acquired in a smartshop located near the juvenile offenders' centres participating in this study: *Oro Fantastico*, *Mazazo*, *Sonrisa Absoluta*, *Placaje*, *Sonrisa*, *Hardcore*, and *Tio Tieso* (**Figure 1** shows the packaging of these products). These herbal blends were purchased by the time of collecting urine samples from the teenagers (this is, in 2016).

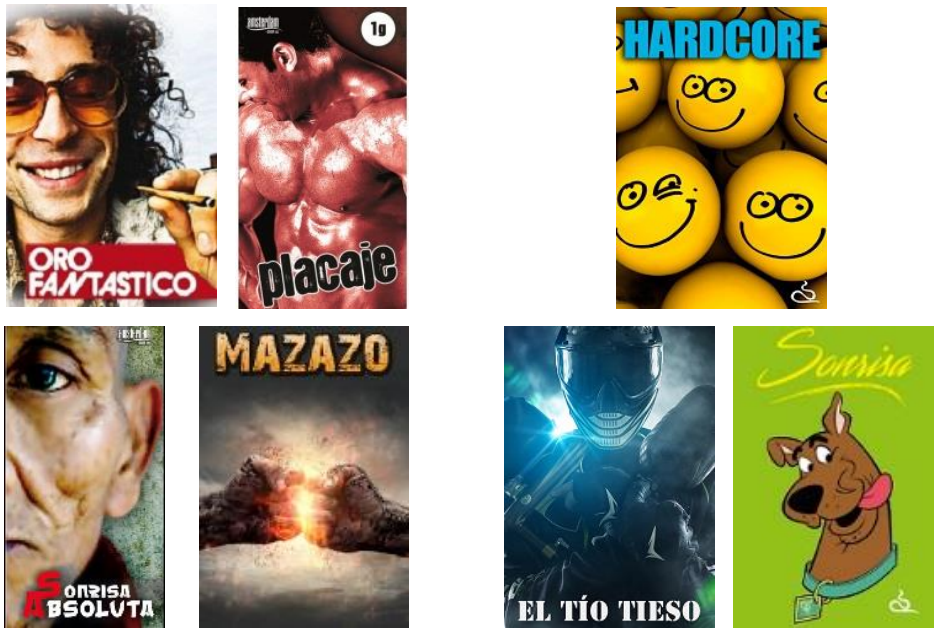


Figure 1. Front of the different herbal blend products purchased on the smartshop. **A** Products previously analysed and reported in literature. **B** Products recently analysed.

A total of 667 urine samples were collected between May and October 2016 from all the participants in the study. Individual urine samples were kept at -23 ºC after collection.

2.3. Sample treatment

Synthetic cannabinoids in the herbal blends were identified by HRMS, as described in literature [9]. Briefly, 0.1 g of herbal blend was extracted with 2 mL of acetone in a 2 mL propylene tube and introduced in an ultrasonic bath for 15 min. After centrifugation at 12000 rpm for 10 min, the supernatant was 1000-fold diluted with ultrapure water. Finally, the extract was transferred to a glass vial and 20 µL were injected into the UHPLC-HRMS system.

Urine samples were processed using an enzymatic hydrolysis procedure adapted from the literature [22,23], thus releasing the unconjugated compound. This process has been shown to be effective for cleaving NPS-derived glucuronides found in mice urine samples [24]. Briefly, 1 mL of urine was buffered with 0.4 mL of phosphate buffer; 16 µL of β-glucuronidase from *E. coli* strain *K12* was added and the sample was incubated for 1 h at 55 ±2 ºC. Samples were then frozen at -18 ºC for at least 3 h, thawed, and centrifuged at 12000 rpm for 15 min to remove any lipids and proteins. Finally, 20 µL were injected into the UHPLC-HRMS system.

2.4. Instrumentation

The herbal blends and urine samples were analysed using an ACQUITY UPLC ultra-high performance liquid chromatography (UHPLC) system (Waters Corp, Mildford, MA, USA) coupled to a XEVO G2 QTOF hybrid quadrupole time-of-flight (QTOF) mass spectrometer (Waters Corp, Manchester, UK) with an orthogonal Z-spray electrospray ionization (ESI) interface operating in positive ionization mode.

Chromatographic separation was performed using a Cortecs C18 100 x 2.1 mm 2.7 µm particle size analytical column (Waters Corp, Wexford, Ireland) at a flow rate of 0.3 mL/min. Mobile phases were H_2O (A) and methanol (B), both with 0.01% formic acid. The mobile phase gradient was performed as follows: 10% of B at 0 min, 90% of B at 14 min linearly increased, 90% of B at 16 min, and finally 10% B at 18 min in order to return to initial conditions.

The injection volume was 20 µL. The column temperature was set to 40ºC. The TOF resolution was ~20000 at FWHM at *m/z* 556 in positive ionisation mode. The range acquired by the MS system was from *m/z* 50 to 1000. A capillary voltage of 0.7 kV and a cone voltage of 20 V were used during all the chromatographic run. Nitrogen (Praxair, Valencia, Spain) was used as desolvation and nebulizing gas. The desolvation gas flow was set at 1000 L/h, while cone gas was set to 80 L/h. Argon 99.995% (Praxair) was used as a collision gas. The interface temperature was set to 600 ºC and the source temperature to 120 ºC. For MS^E experiments, two acquisition functions with different collision energies were created. Further details about the instrumental conditions can be found in literature [24], as well as the selection of the UHPLC-HRMS parameters for detecting NPS metabolites in urine samples from NPS consumers [25].

MS data were acquired in continuum mode using MassLynx software version 4.1 (Waters Corp, Manchester, UK), and processed with UNIFI scientific information system version 1.8 (Waters Corp, Manchester, UK).

2.5. Ethical consideration

This study was approved by the Research and Ethics Committee at the Consorcio Hospitalario Provincial (Castellon) on 26 September, 2014 (ref. 20141113), by the Conselleria de Benestar Social at the Generalitat Valenciana on 1 June, 2015 (registration 3 June, 2015, ref. 32833), and the Office of the Children's Justice Prosecutor, following the principles and requirements established in the Declaration of Helsinki and the European Council Convention for research on humans. The confidentiality of the participants and their data was guaranteed according to Organic Law 15/1999 on the Protection of Personal Data, and the subjects and their legal guardians signed their informed consent to their participation in the study.

3. Results

Given the extensive and fast metabolism of SCs in humans, a realistic approach to reveal the consumption of SCs is to monitor their major metabolites in urine [14]. In the present work, we included more than 200 SCs in our database, but we did not consider all their metabolic pathways due to the lack of information in some cases, particularly for the newest SCs. Therefore, we devised an alternative strategy of searching parent SCs in the herbal blends referred to by the minors participating in this study. After identifying the SCs present in those herbal blends, we investigated their main reported metabolites in urine samples.

3.1. UHPLC-HRMS screening strategy and herbal blends analysis

During the group interviews, 59 participants recognised to have ever consumed a SC marketed as Hardcore during therapy periods to avoid being caught by conventional urine analyses. They described acquiring this product in a smartshop close to the centres involved. On this way, we purchased all seven herbal blends available in the smartshop via its webpage (**Figure 1**).

Of the seven herbal blends acquired for this study, we had already analysed *Oro fantastico*, *Mazazo*, *Placaje*, and *Sonrisa absoluta* (**Figure 1A**), and had identified four SCs in them: JWH-081, JWH-250, JWH-203, and JWH-019 [8]; our repeated analysis in this study found no differences in their composition. We also analysed the new herbal products, *Hardcore*, *Sonrisa*, and *Tio tieso* (**Figure 1B**) by UHPLC-HRMS and cross-referenced the suspect peaks against our SC database [9]. Based on the observed accurate-mass data on fragmentation and information in the literature, we tentatively identified four additional SCs: XLR-11, UR-144, an UR-144 *N*-(5-chloropentyl) analogue, and 5F-AKB48 (5F-APINACA), as shown in **Table 1**. The identity of the SCs could not be unequivocally confirmed by comparison with the analytical reference standards as they were not available in our laboratory. Nevertheless, the fragmentation observed, its compatibility with the chemical structures of the NPS, and the agreement with HRMS data reported in literature, provided a high degree of

reliability in the tentative identification of the synthetic cannabinoids found in herbal blends.

Table 1. SCs identified in the herbal blend samples analysed in this work.

Herbal blend sample	Synthetic cannabinoids found
Oro Fantastico	JWH-081[a]
Sonrisa Absoluta	JWH-081[a]
	JWH-250[a]
Placaje	JWH-081[a]
	JWH-250[a]
	JWH-019[a]
	JWH-203[a]
Mazazo	JWH-081[a]
	JWH-250[a]
	JWH-019[a]
	JWH-203[a]
Hardcore	XLR-11[b]
	UR-144[b]
	UR-144 *N*-(5-chloropentyl) analogue [b]
Sonrisa	5F-AKB48[b]
	XLR-11[b]
	UR-144[b]
Tio Tieso	5F-AKB48[b]
	XLR-11[b]
	UR-144[b]

[a] Compound identified with reference standard.
[b] Compound tentatively identified based on the accurate-mass fragmentation observed and information available on literature.

As an illustrative example, the analysis of the herb mixture *Hardcore* was as follows: three chromatographic peaks were observed in the base peak intensity chromatogram (BPI) of the low-energy function (**Figure 2B**), at 13.40 min ($[M+H]^+$ *m/z* 330.2222), 13.99 min ($[M+H]^+$ *m/z* 346.1931), and 14.54 min ($[M+H]^+$ *m/z* 312.2327). These were tentatively identified as XLR-11, an UR-144 *N*-(5-chloropentyl) analogue, and UR-144, based on the accurate-mass collision-induced dissociation (CID) fragments observed in the high-energy function (**Figure 2A**). The fragments observed for XLR-11 and UR-144 also coincided with fragmentation profiles reported in the literature [26,27], while

fragmentation of the UR-144 *N*-(5-chloropentyl) analogue was justified based on the XLR-11 and UR-144 fragments. After CID ion evaluation, a plausible fragmentation pathway was proposed for the three SCs found in the Hardcore herbal blend **(Figure S1**). At the time of performing this study and collecting herbal blend samples from the smartshops (i.e. in 2016), there was a notable spread of XLR-11 and UR-144 in Spain.

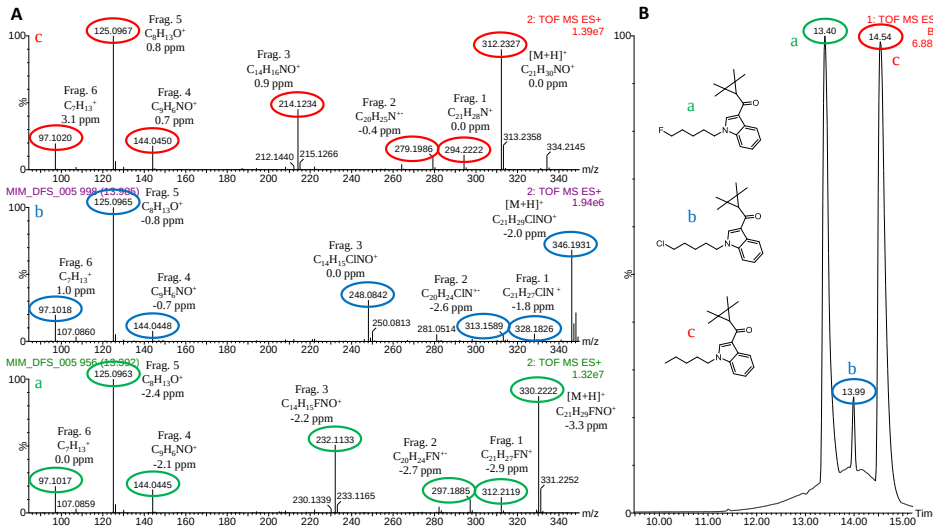


Figure 2. Tentative identification of three SCs (a: XLR-11, b: UR-144 N-(5-chloropentyl) analog, c: UR-144) in *Hardcore* herbal blend by UHPLC-HRMS. **A** Accurate-mass fragmentation observed for the three SCs. **B** BPI chromatogram of the herbal blend extract.

3.2. Detection of SC metabolites in urine samples

Once identified several SCs in the herbal blends, a suspect list containing the major metabolites (from two to four) for each compound was built [12,18]. No metabolites were found for the UR-144 *N*-(5-chloropentyl) analogue, a fact that was considered not much relevant because this substance was only found in *Hardcore* at very low abundance in comparison to the other two SCs (XLR-11 and UR-144) present in this product. **Table 2** shows the 19 metabolites to be investigated in urine samples based on the literature search. It can be seen that

UR-144 and XLR-11, the two main components of *Hardcore*, share two metabolites, and only XLR-11 presented an additional specific one.

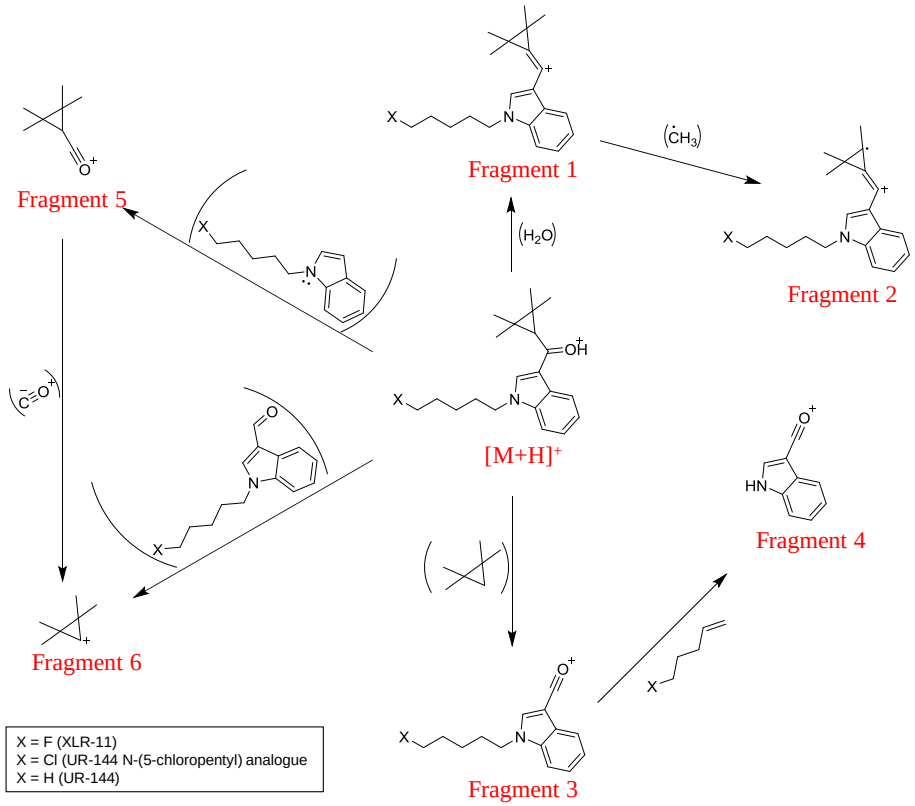


Figure S1. Proposed fragmentation pathway for the three SCs found in Hardcore herbal blend.

Two SC metabolites were found in 16 urine samples: *N*-pentanoic acid and *N*-(5-hydroxypentyl). **Figure 3** shows the tentative identification of both metabolites (**A**, *N*-pentanoic acid; **B**, *N*-(5-hydroxypentyl)) based on the accurate-mass fragmentation observed. The presence of these metabolites suggested the consumption of XLR-11 and/or UR-144, being not possible to stablish which of these was consumed as both share the same metabolites. The extracted-ion chromatogram at the exact mass of *N*-(6-hydroxyindole), another metabolite of XLR-11, showed a chromatographic peak, but the fragmentation pattern did not fit with that reported in previous works, with the hydroxylation point appearing

to be on the tetramethylcyclopropane ring (**Figure S2**). However, the potential consumption of this substance was supported by the tentative identification of the remaining two metabolites. This is in agreement with previous findings, where the main metabolites reported in urine samples from XLR-11 consumers were *N*-pentanoic acid and N-(5-hydroxypentyl) [28].

Table 2. Synthetic cannabinoid metabolites selected for the suspect screening of urine samples.

Synthetic cannabinoid	Target metabolites
JWH-081	JWH-081 *N*-(5-hydroxypentyl) metabolite
	JWH-081 *N*-pentanoic acid metabolite
	JWH-081 5-hydroxyindole metabolite
	JWH-081 4-hydroxynaphthyl metabolite
JWH-250	JWH-250 *N*-(5-hydroxypentyl) metabolite
	JWH-250 *N*-pentanoic acid metabolite
	JWH-250 5-hydroxyindole metabolite
JWH-203	JWH-203 *N*-(5-hydroxypentyl) metabolite
	JWH-203 *N*-pentanoic acid metabolite
	JWH-203 5-hydroxyindole metabolite
JWH-019	JWH-019 *N*-(6-hydroxyhexyl) metabolite
	JWH-019 *N*-pentanoic acid metabolite
	JWH-019 5-hydroxyindole metabolite
XLR-11	XLR-11/UR-144 *N*-(5-hydroxypentyl) metabolite*
	XLR-11 6-hydroxyindole metabolite
	XLR-11/UR-144 *N*-pentanoic acid metabolite*
UR-144	XLR-11/UR-144 *N*-(5-hydroxypentyl) metabolite*
	XLR-11/UR-144 *N*-pentanoic acid metabolite*
5F-AKB48	AKB48 *N*-(5-hydroxypentyl) metabolite
	AKB48 *N*-pentanoic acid metabolite
	5F-AKB48 *N*-(4-hydroxypentyl) metabolite

*Common metabolites for XLR-11 and UR-144.

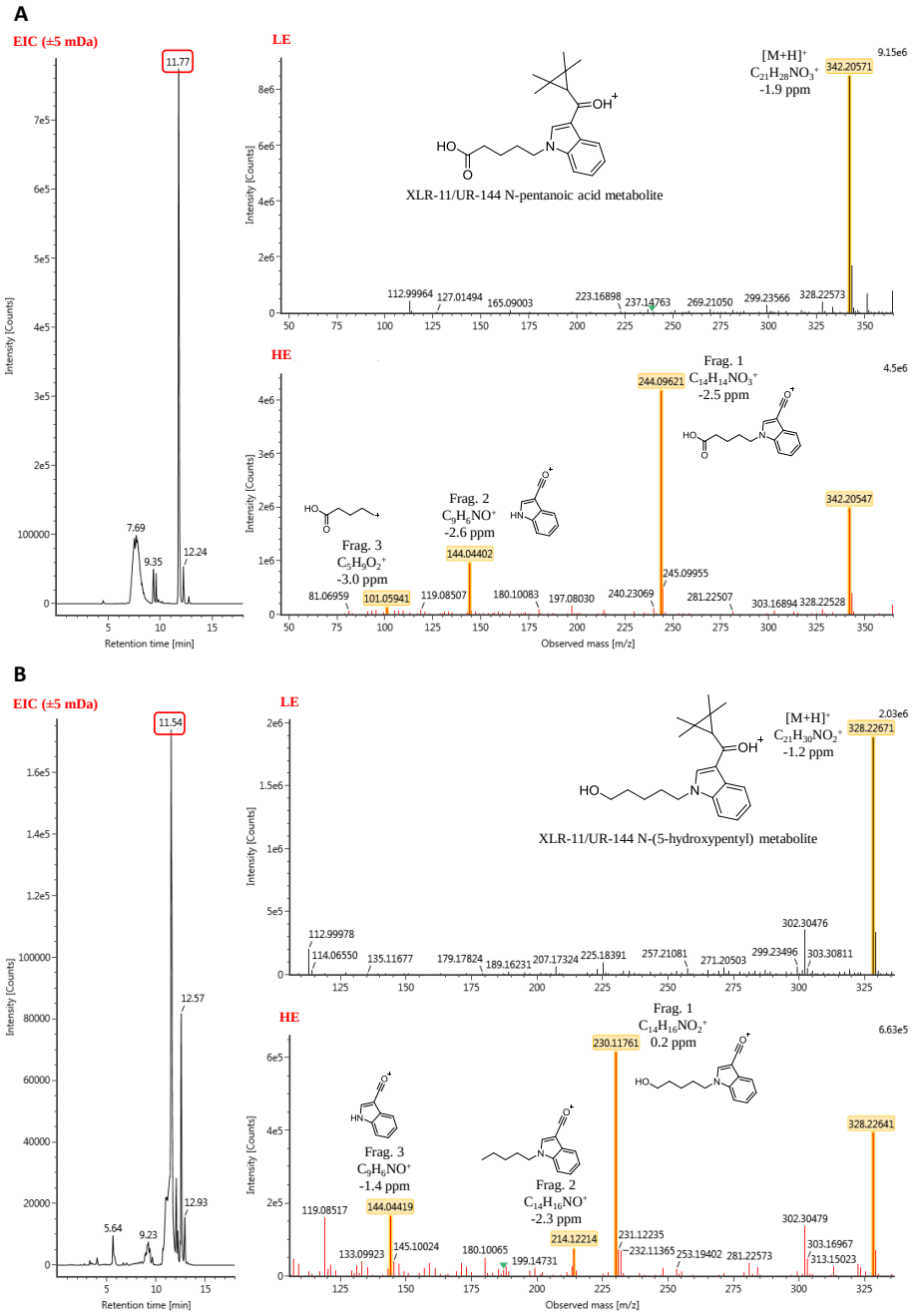


Figure 3. Tentative identification of the two major XLR-11 human metabolites in a urine sample. EIC (left) and accurate-mass fragmentation (right). **A** *N*-pentanoic acid metabolite. **B** *N*-(5-hydroxypentyl) metabolite.

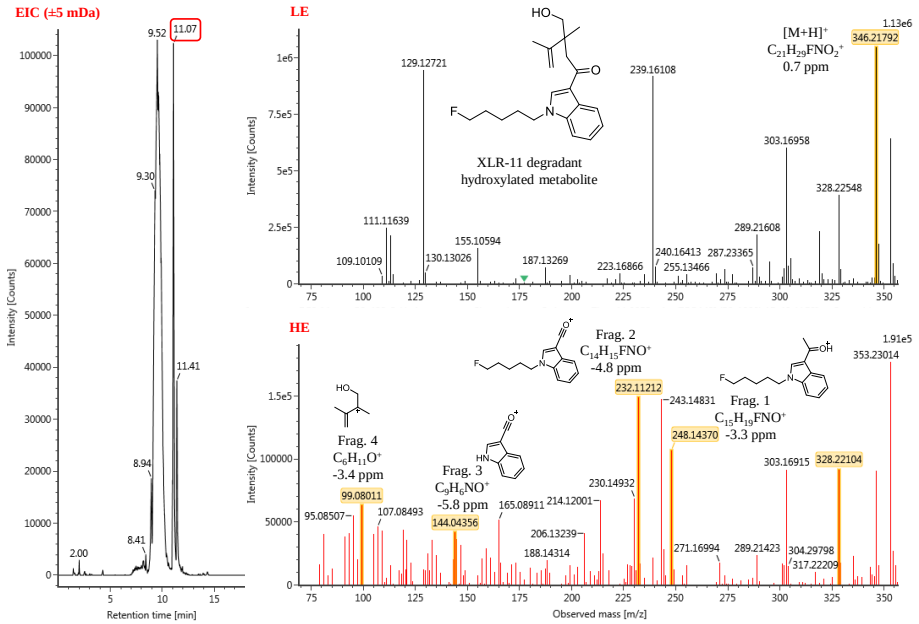
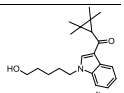
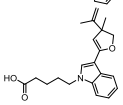
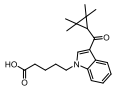
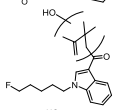
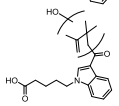
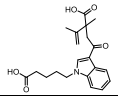


Figure S2. Tentative identification of the third XLR-11 found in a urine sample. EIC (left) and accurate-mass fragmentation (right).

3.3. Confirmation of the consumption of XLR-11 or UR-144

The 16 urine samples positive to the major metabolites of XLR-11/UR-144 were re-processed, searching for additional minor metabolites described in urine from XLR-11 consumers [28]. This step was performed in order to confirm whether the cannabinoid consumed was XLR-11 or UR-144. In total, 12 phase I metabolites were searched in positive urine samples. In some cases, the same biotransformations occurred on different moieties of the molecule, and therefore two (or more) metabolites had the same elemental composition. For example, four metabolites corresponded to hydroxylations on different carbon atoms of the tetramethylcyclopropane ring. **Table 3** shows the XLR-11 metabolites selected for the screening of individual urine samples.

Table 3. XLR-11 Phase I metabolites selected for the suspect screening of individual urine samples (based on [28]).

Metabolite	Biotransformation	Elemental composition	Proposed Structure
M1[1]	Defluorination to hydroxylation	$C_{21}H_{29}NO_2$	
M2	Defluorination to carboxylic acid + hydroxylation + dehydration (2 metabolites described: M2-1, M2-2)	$C_{21}H_{25}NO_3$	
M3[2]	Defluorination to carboxylic acid (2 metabolites described: M3-1, M3-2)	$C_{21}H_{27}NO_3$	
M4[3]	Hydroxylation	$C_{21}H_{28}FNO_2$	
M5	Defluorination to carboxylic acid + hydroxylation (4 metabolites described: M5-1, M5-2, M5-3, M5-4)	$C_{21}H_{27}NO_4$	
M6	Defluorination to carboxylic acid + carboxylation (2 metabolites described: M6-1, M6-2)	$C_{21}H_{25}NO_5$	

[1] XLR-11/UR-144 *N*-(5-hydroxypentyl) metabolite
[2] XLR-11/UR-144 *N*-pentanoic acid metabolite
[3] XLR-11 degradant hydroxylated metabolite

Up to six XLR-11 metabolites were found in the 16 urine samples (**Table 3**). To obtain cleaner spectra and enhance reliability in the metabolite tentative identifications, additional MS/MS experiments were performed to obtain the accurate-mass product ion spectra, comparing the fragmentation observed with that described in the literature [28]. In the particular case of M5, two chromatographic peaks were observed corresponding to two hydroxylation metabolites. According to literature [28], the structures of these metabolites would correspond to hydroxylation in different points of the degraded tetramethylcyclopropane ring. However, with only the HRMS data available, it is not possible to determine either the exact position of the hydroxyl group, or if the hydroxylation occurred in the degraded tetramethyl cyclopropane ring or in the intact ring. To obtain such information, both compounds should be synthetized and analysed by UHPLC-HRMS in order to unequivocally identify the exact structure of the metabolite. The MS/MS spectra of the detected metabolites at 10, 20, 30, and 40 eV collision energies, and the fragment-structure

justifications are detailed in at the end of this research article (**Figures S3-S8**). For fragment structure justification, the biotransformation is placed in the structure to facilitate the fragmentation interpretation.

With all information obtained after a comprehensive analysis by HRMS, the 16 urine samples positives to XLR-11 metabolites could be assigned to 6 minors based on the anonymous urine sample codification. Only two adolescents had recognized consumption in the administered questionnaires, while the remaining 4 did not recognize any SCs consumption.

4. Discussion

The consumption of SCs seems not very common in the juvenile offenders' centres from the Valencian region, but a few cases have been found in this work. In the present study, 29 out of 127 participants admitted having ever used SCs, although our survey did not record when the consumption had occurred. Therefore, indication of SC consumption did not necessarily imply that the urine sample collected would produce a positive result, or maybe the opposite case: some consumers did not recognise consumption in the survey, thus should be cross-referenced with toxicological analysis. Analysis of the herbal blends reported to have been consumed by the participants allowed the detection of several SCs. Subsequent urine analysis demonstrated the presence of major metabolites of XLR-11 and UR-144, supporting the consumption of the suspect products by some participants. In our study, SC consumption was only detected in 6 of the 29 self-referred cases. Considering the high number of SCs reported until now and the much higher number of potential metabolites, some of them being still unknown, it seems wise to focus the investigation on major metabolites of the active compounds identified in the products (e.g. herbal blends) within the "distribution area" of the minors. To increase the detection rate, the analysis could be focused on the appropriate target compounds.

Both, toxicological and consumption information, can then be obtained from users in a synchronised way [29]. The application of advanced analytical techniques, such as UHPLC-HRMS, allows performing wide-scope screening of large number of suspect compounds, without the need of reference standards available. The use of appropriate databases containing as many metabolites reported as possible is a good strategy to increase the number of compounds under investigation. This makes feasible to detect more SC consumers via their urines analysis from among those who do not recognise their consumption via surveys or interviews. In our study, we detected 4 cases of minors who, in the group interviews and questionnaires, denied the use of synthetic cannabinoids. The final, unequivocal identification of the detected metabolites in urine would have required the acquisition of the corresponding analytical reference standards. The main limitation when investigating NPS metabolites is the non-commercial availability for many of them. However, in the present work the careful interpretation of mass spectra, their compatibility with the chemical structure, and the agreement with previous data reported gave a high degree of confidence to their identification.

5. Conclusions

In this work, SCs consumption among teenagers confined in juvenile offenders' centres has been investigated through the analysis of 667 urine samples collected from 127 participants, and the analysis of the herbal blends potentially consumed by the minors. Based on the metabolic behaviour of SC, the screening strategy applied by UHPLC-HRMS to urine samples was focused on the major metabolites reported for the SCs that were identified in the herbal blends potentially consumed by the teenagers. In this way, the main metabolites of XLR-11 and UR-14 were identified in 16 urines, corresponding to 6 teenagers.

These two substances, XLR-11 and UR-144, were banned in Europe a few years ago (e.g. in Germany the maximum spread was reached between 2012 and 2015), and other synthetic cannabinoids, such as 5F-ADB, AB-FUBINACA and MDMB-CHMICA, replaced them. However, at the time of performing this work, both compounds were still in use in Spain, and in fact they were identified in the herbal blends sold in the local smartshop nearby the juvenile offenders' centre. In September 2018, the herbal blends containing these two SCs were removed from the market, being replaced by a new one containing 5F-ADB (see [30]).

The results from our study demonstrate that SCs are occasionally consumed in juvenile offenders' centres in the Valencian region. The different approaches to reveal SCs consumption must be efficiently synchronised, so that information obtained from interviews and questionnaires must be matched to the composition of the products from the smartshops where the consumers acquire these substances, as well as to the urine analysis results within few hours of their last consumption.

Funding

D. Fabregat-Safont, J.V. Sancho, F. Hernández and M. Ibáñez acknowledge financial support from Generalitat Valenciana [PROMETEO/2019/040], Ministerio de Economía y Competitividad in Spain [Project: CTQ2015-65603-P] and University Jaume I [UJI-B2018-19]. D. Fabregat-Safont, F. Hernández and M. Ibáñez also acknowledge NPS-Euronet [HOME/2014/JDRUG/AG/DRUG/7086], co-funded by the European Commission. This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein. D. Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant [Grant FPU15/02033]. G. Haro acknowledges financial support from Fundación C.V. de investigación del Hospital Provincial de Castellón (CAF 17-071).

Declaration of interest

No potential conflict of interest was reported by the authors.

CRediT authorship contribution statement

David Fabregat-Safont Methodology, Investigation, Writing - original draft. **María Ibáñez** Conceptualization, Methodology, Investigation, Writing - original draft, Supervision. **Abel Baquero** Conceptualization, Methodology, Investigation. **Juan Vicente Sancho** Methodology, Resources, Funding acquisition. **Félix Hernández** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition. **Gonzalo Haro** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

Acknowledgements

All authors acknowledge institutional and legal support and authorizations from Conselleria de Benestar Social, Generalitat Valenciana and altruistic collaboration of directors and staff of the juvenile offenders' centres.

References

[1] European Monitoring Centre for Drugs and Drug Addiction, European Drug Report 2017, EMCDDA–Europol Jt. Publ. (2017) 88. doi:10.2810/610791.

[2] H.B. Clayton, R. Lowry, C. Ashley, A. Wolkin, A.M. Grant, Health Risk Behaviors With Synthetic Cannabinoids Versus Marijuana, Pediatrics. 139 (2017) e20162675. doi:10.1542/peds.2016-2675.

[3] G. Haro, C. Ripoll, M. Ibáñez, T. Orengo, V.M. Liaño, E. Meneu, F. Hernández, F. Traver, Could Spice Drugs Induce Psychosis With Abnormal Movements Similar to Catatonia?, Psychiatry Interpers. Biol. Process. 77 (2014) 206–208. doi:10.1521/psyc.2014.77.2.206.

[4] K.G. Shanks, D. Winston, J. Heidingsfelder, G. Behonick, Case reports of synthetic cannabinoid XLR-11 associated fatalities, Forensic Sci. Int. 252 (2015) e6–e9. doi:10.1016/j.forsciint.2015.04.021.

[5] W. Jia, X. Meng, Z. Qian, Z. Hua, T. Li, C. Liu, Identification of three cannabimimetic indazole and pyrazole derivatives, APINACA 2 H -indazole analogue, AMPPPCA, and 5F-AMPPPCA, Drug Test. Anal. 9 (2017) 248–255. doi:10.1002/dta.1967.

[6] C. Liu, W. Jia, Z. Hua, Z. Qian, Identification and analytical characterization of six synthetic cannabinoids NNL-3, 5F-NPB-22-7 N , 5F-AKB-48-7 N , 5F-EDMB-PINACA, EMB-FUBINACA, and EG-018, Drug Test. Anal. (2017). doi:10.1002/dta.2160.

[7] K.N. Moore, D. Garvin, B.F. Thomas, M. Grabenauer, Identification of Eight Synthetic Cannabinoids, Including 5F-AKB48 in Seized Herbal Products Using DART-TOF-MS and LC-QTOF-MS as Nontargeted Screening Methods, J. Forensic Sci. (2017) 1–8. doi:10.1111/1556-4029.13367.

[8] M. Ibáñez, L. Bijlsma, A.L.N. van Nuijs, J. V. Sancho, G. Haro, A. Covaci, F. Hernández, Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends, J. Mass Spectrom. 48 (2013) 685–694. doi:10.1002/jms.3217.

[9] M. Ibañez, J. V. Sancho, L. Bijlsma, A.L.N. Van Nuijs, A. Covaci, F. Hernandez, Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances, TrAC - Trends Anal. Chem. 57 (2014) 107–117. doi:10.1016/j.trac.2014.02.009.

[10] X. Diao, J. Carlier, M. Zhu, S. Pang, R. Kronstrand, K.B. Scheidweiler, M.A. Huestis, In vitro and in vivo human metabolism of a new synthetic cannabinoid NM-2201 (CBL-2201), Forensic Toxicol. 35 (2017) 20–32. doi:10.1007/s11419-016-0326-9.

[11] N.B. Holm, A.J. Pedersen, P.W. Dalsgaard, K. Linnet, Metabolites of 5F-AKB-48, a synthetic cannabinoid receptor agonist, identified in human urine and liver microsomal preparations using liquid chromatography high-resolution mass spectrometry, Drug Test. Anal. 7 (2015) 199–206. doi:10.1002/dta.1663.

[12] K.B. Scheidweiler, M.J.Y. Jarvis, M.A. Huestis, Nontargeted SWATH acquisition for identifying 47 synthetic cannabinoid metabolites in human urine by liquid chromatography-high-resolution tandem mass spectrometry, Anal. Bioanal. Chem. 407 (2015) 883–897. doi:10.1007/s00216-014-8118-8.

[13] A. Wohlfarth, M.S. Castaneto, M. Zhu, S. Pang, K.B. Scheidweiler, R. Kronstrand, M.A. Huestis, Pentylindole/Pentylindazole Synthetic Cannabinoids and Their 5-Fluoro Analogs Produce Different Primary Metabolites: Metabolite Profiling for AB-PINACA and 5F-AB-PINACA, AAPS J. 17 (2015) 660–677. doi:10.1208/s12248-015-9721-0.

[14] X. Diao, M. Huestis, Approaches, Challenges, and Advances in Metabolism of New Synthetic Cannabinoids and Identification of Optimal Urinary Marker Metabolites, Clin. Pharmacol. Ther. 101 (2017) 239–253. doi:10.1002/cpt.534.

[15] A. V. Labutin, A.Z. Temerdashev, Nontarget screening of the markers of synthetic cannabinoids in urine using HPLC–MS/MS, J. Anal. Chem. 70 (2015) 1620–1628. doi:10.1134/S1061934815140087.

[16] K. Zaitsu, H. Nakayama, M. Yamanaka, K. Hisatsune, K. Taki, T. Asano, T. Kamata, M. Katagai, Y. Hayashi, M. Kusano, H. Tsuchihashi, A. Ishii, High-resolution mass spectrometric determination of the synthetic cannabinoids MAM-2201, AM-2201, AM-2232, and their metabolites in postmortem plasma and urine by LC/Q-TOFMS, Int. J. Legal Med. 129 (2015) 1233–1245. doi:10.1007/s00414-015-1257-4.

[17] T. Berg, L. Kaur, A. Risnes, S.M. Havig, R. Karinen, Determination of a selection of synthetic cannabinoids and metabolites in urine by UHPSFC-MS/MS and by UHPLC-MS/MS, Drug Test. Anal. 8 (2016) 708–722. doi:10.1002/dta.1844.

[18] M. Jang, I. Shin, J. Kim, W. Yang, Simultaneous quantification of 37 synthetic cannabinoid metabolites in human urine by liquid chromatography-tandem mass spectrometry, Forensic Toxicol. 33 (2015) 221–234. doi:10.1007/s11419-015-0265-x.

[19] K. Minakata, I. Yamagishi, H. Nozawa, K. Hasegawa, M. Suzuki, K. Gonmori, O. Suzuki, K. Watanabe, Sensitive identification and quantitation of parent forms of six synthetic cannabinoids in urine samples of human cadavers by liquid chromatography–tandem mass spectrometry, Forensic Toxicol. 35 (2017) 275–283. doi:10.1007/s11419-017-0354-0.

[20] D.W. Young, R. Dembo, C.E. Henderson, A national survey of substance abuse treatment for juvenile offenders, J. Subst. Abuse Treat. 32 (2007) 255–266. doi:10.1016/j.jsat.2006.12.018.

[21] A. Baquero Escribano, M.T. Beltrán Negre, G. Calvo Orenga, S. Carratalá Monfort, F. Arnau Peiró, S. Meca Zapatero, G. Haro Cortés, Consumo de krokodil por vía oral en España: a propósito de un caso, Adicciones. 28 (2016) 242. doi:10.20882/adicciones.828.

[22] X. Matabosch, O.J. Pozo, N. Monfort, C. Pérez-Mañá, M. Farré, J. Segura, R. Ventura, Detection and characterization of betamethasone metabolites in human urine by LC-MS/MS, Drug Test. Anal. 7 (2015) 663–672. doi:10.1002/dta.1770.

[23] X. Matabosch, O.J. Pozo, C. Pérez-Mañá, E. Papaseit, J. Segura, R. Ventura, Detection and characterization of prednisolone metabolites in human urine by LC-MS/MS, J. Mass Spectrom. 50 (2015) 633–642. doi:10.1002/jms.3571.

[24] D. Fabregat-Safont, M. Barneo-Muñoz, F. Martinez-Garcia, J.V. Sancho, F. Hernández, M. Ibáñez, Proposal of 5-methoxy- N -methyl- N -isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice, J. Chromatogr. A. 1508 (2017) 95–105. doi:10.1016/j.chroma.2017.06.010.

[25] M. Ibáñez, Ó.J. Pozo, J. V. Sancho, T. Orengo, G. Haro, F. Hernández, Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 151–164. doi:10.1007/s00216-015-9088-1.

[26] T. Sobolevsky, I. Prasolov, G. Rodchenkov, Detection of urinary metabolites of AM-2201 and UR-144, two novel synthetic cannabinoids, Drug Test. Anal. 4 (2012) 745–753. doi:10.1002/dta.1418.

[27] A. Wohlfarth, S. Pang, M. Zhu, A.S. Gandhi, K.B. Scheidweiler, H.F. Liu, M.A. Huestis, First metabolic profile of XLR-11, a novel synthetic cannabinoid, obtained by using human hepatocytes and high-resolution mass spectrometry, Clin. Chem. 59 (2013) 1638–1648. doi:10.1373/clinchem.2013.209965.

[28] M. Jang, I.S. Kim, Y.N. Park, J. Kim, I. Han, S. Baeck, W. Yang, H.H. Yoo, Determination of urinary metabolites of XLR-11 by liquid chromatography–quadrupole time-of-flight mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 503–516. doi:10.1007/s00216-015-9116-1.

[29] N.P. Lemos, Driving Under the Influence of Synthetic Cannabinoid Receptor Agonist XLR-11, J. Forensic Sci. 59 (2014) 1679–1683. doi:10.1111/1556-4029.12550.

[30] D. Fabregat-Safont, C. Ripoll, T. Orengo, J.V. Sancho, F. Hernández, M. Ibáñez, Variación en el patrón de consumo de cannabinoides sintéticos de una paciente a lo largo de 2018, Adicciones. (2020). doi:10.20882/adicciones.1379.

MS/MS spectra

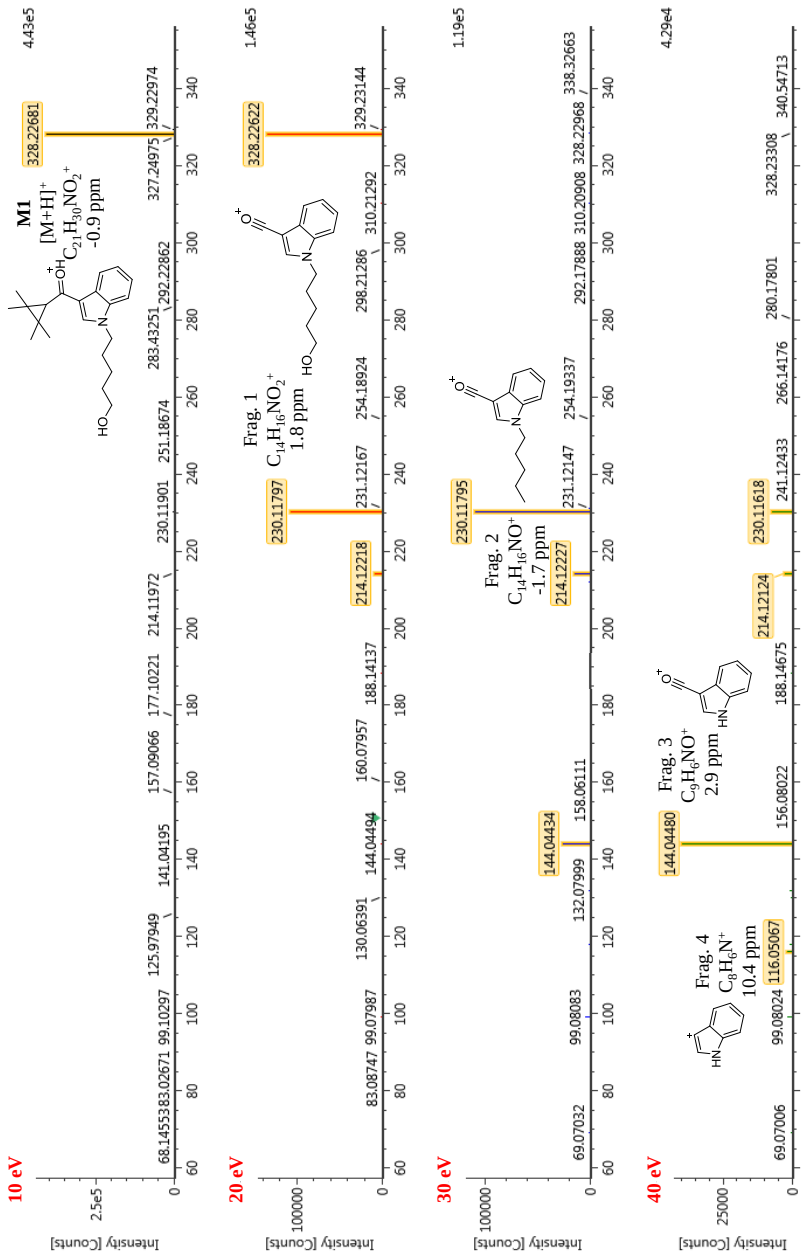


Figure S3. MS/MS spectra at different collision energies for the metabolite M1 of the XLR-11.

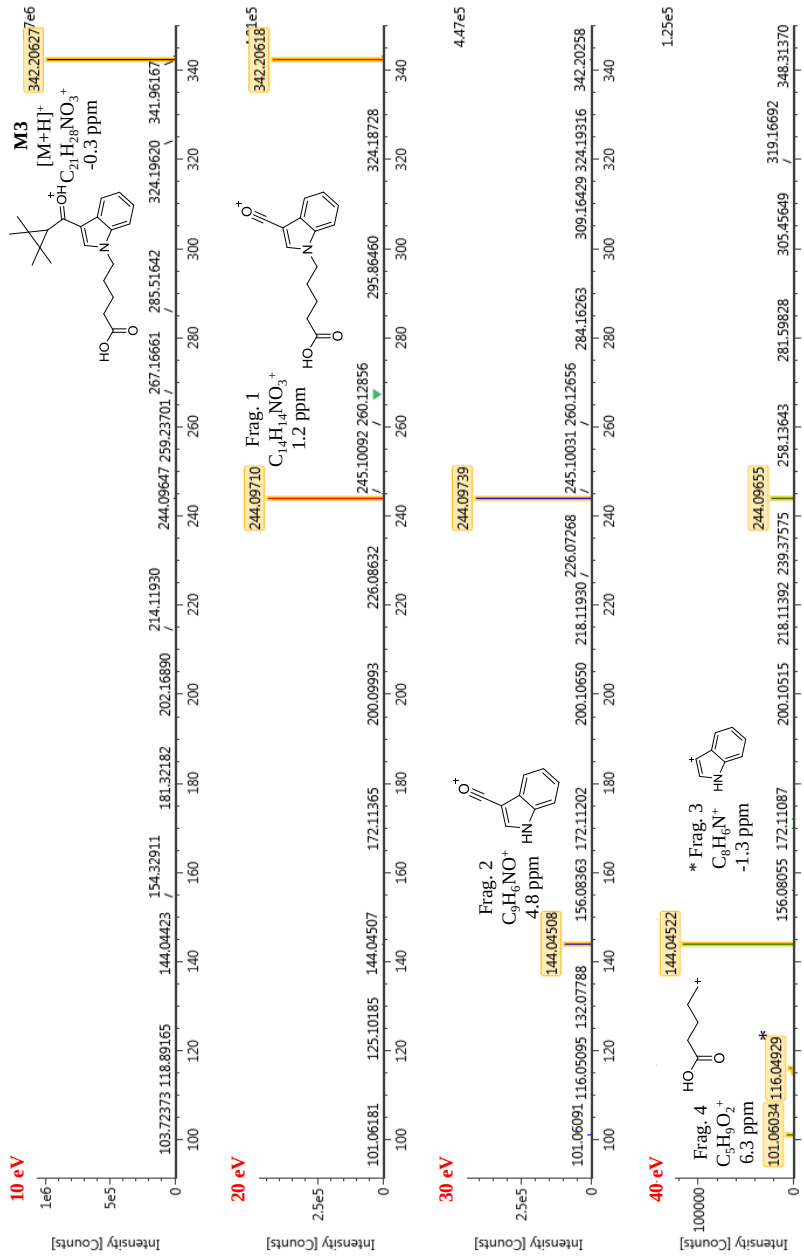


Figure S4. MS/MS spectra at different collision energies for the metabolite M3 of the XLR-11.

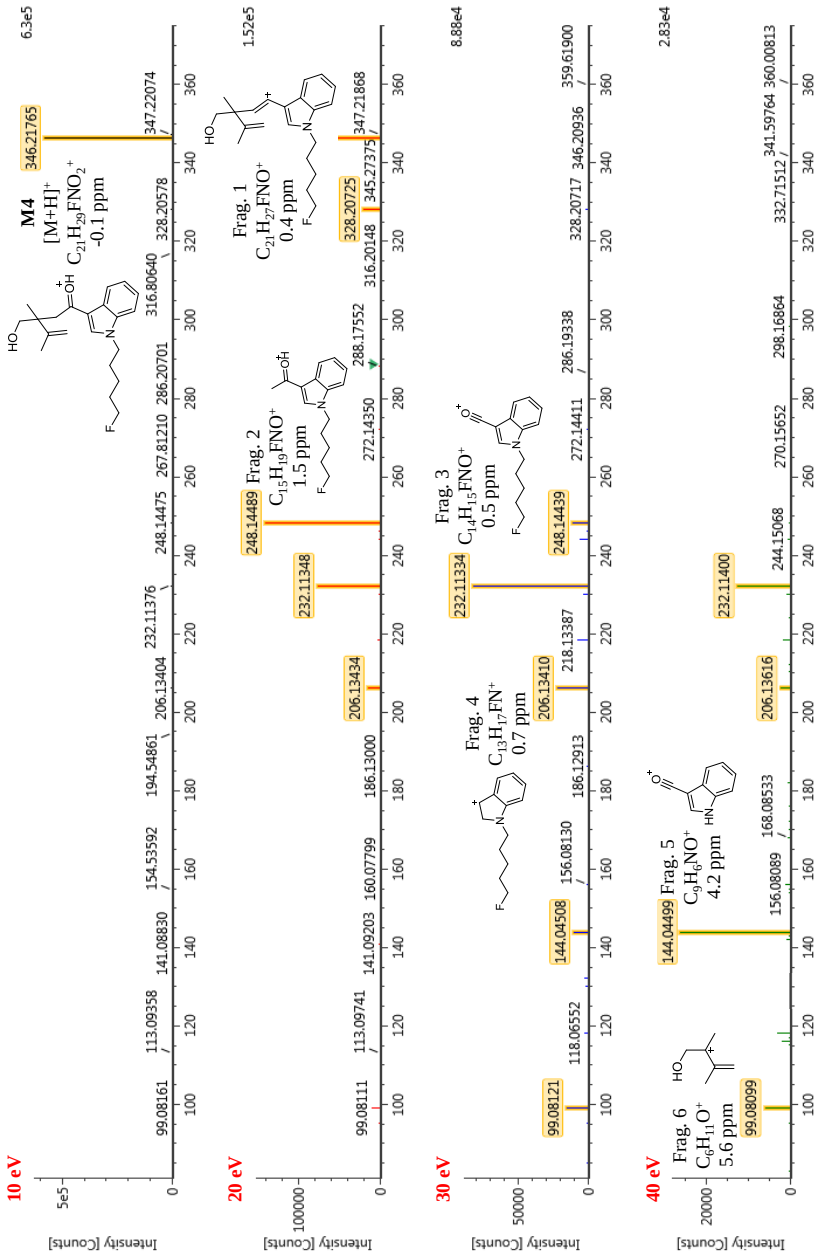


Figure S5. MS/MS spectra at different collision energies for the metabolite M4 of the XLR-11.

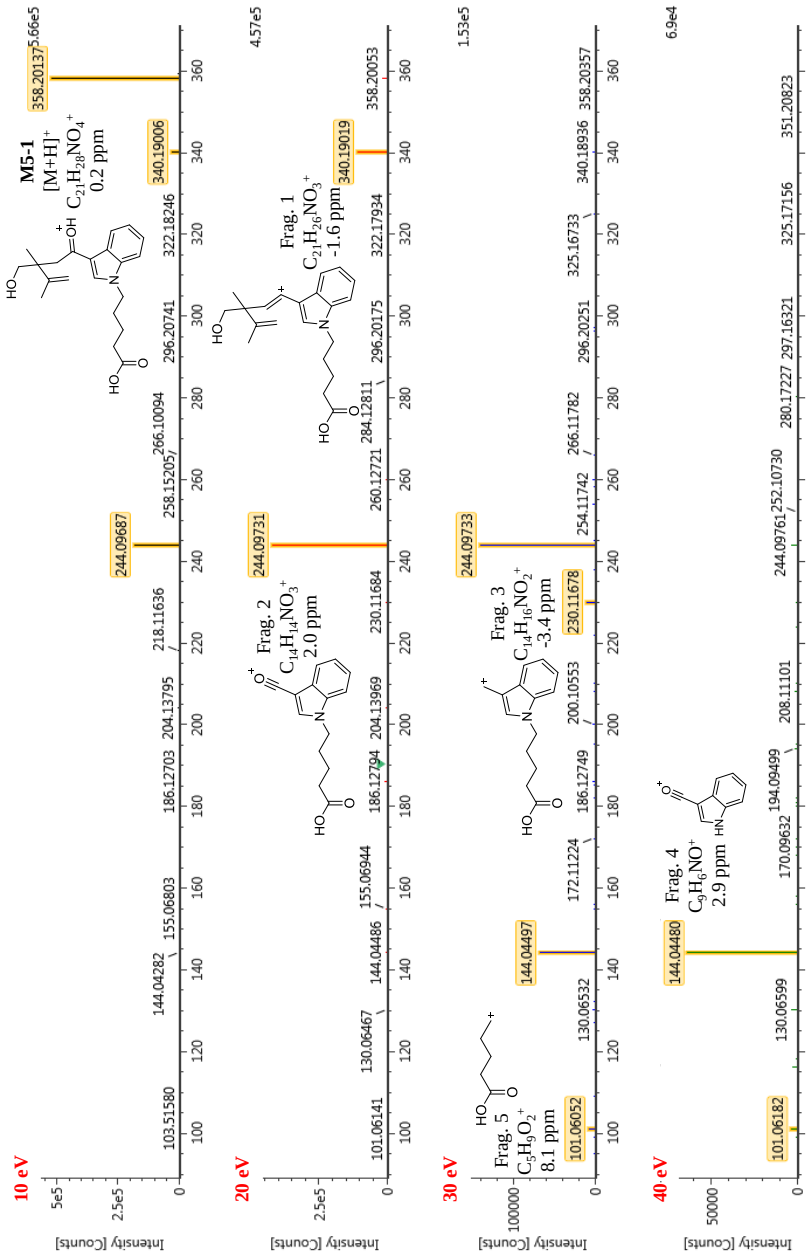


Figure S6. MS/MS spectra at different collision energies for the metabolite M5-1 of the XLR-11.

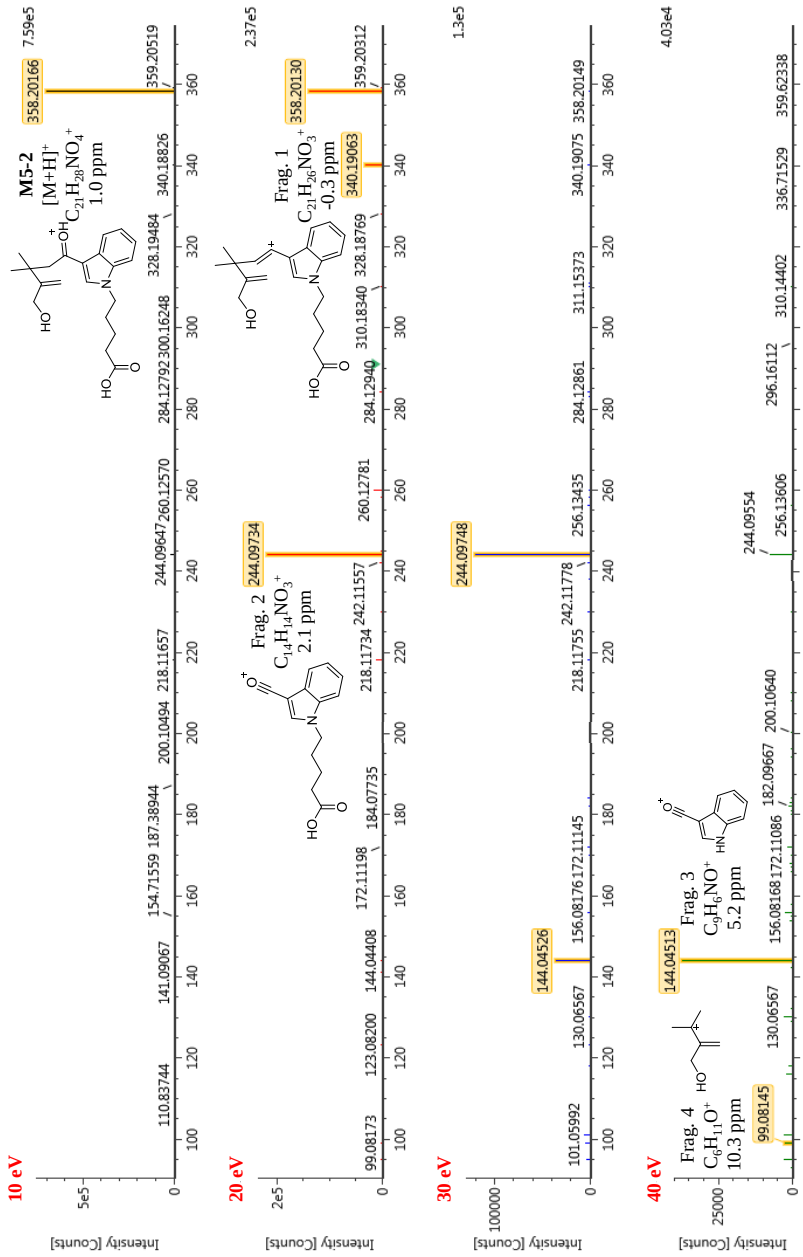


Figure S7. MS/MS spectra at different collision energies for the metabolite M5-2 of the XLR-11.

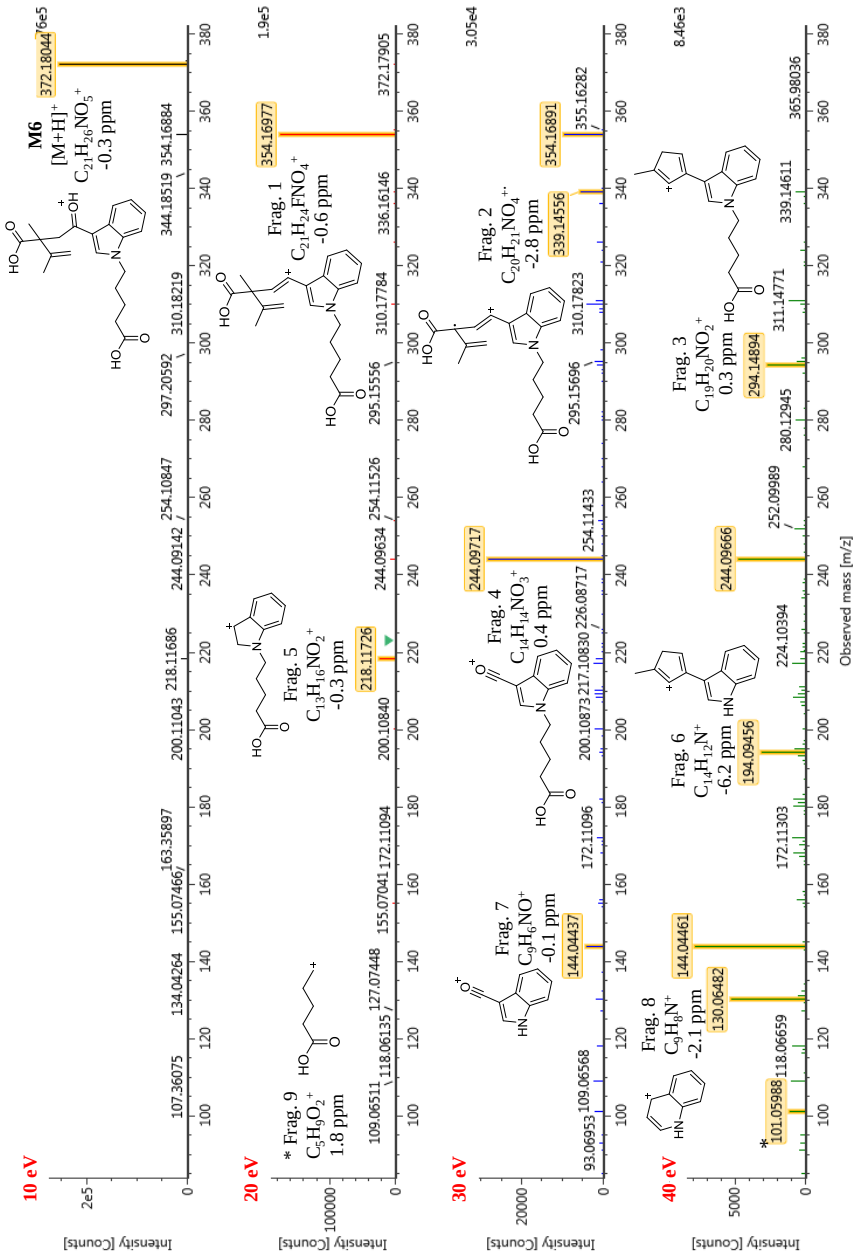


Figure S8. MS/MS spectra at different collision energies for the metabolite M6 of the XLR-11.

6.3. Research article XII

letter to the editor adicciones vol. 32 no. 3 · 2020

Variation in the pattern of synthetic cannabinoid use by a female patient during 2018

Variación en el patrón de consumo de cannabinoides sintéticos de una paciente a lo largo de 2018

David Fabregat-Safont*, Carmen Ripoll**, Teresa Orengo***, Juan V. Sancho*, Félix Hernández*, María Ibáñez*.

* Research Institute for Pesticides and Water. University Jaume I, Castellón. Spain.
** Addictions Treatment Unit Campanar, Valencia. Spain.
*** Addictions Treatment Unit Grao-Clínico, Valencia. Spain.

The use of synthetic cannabinoids (SCs) is, nowadays, an important social concern. A quick search on the literature shows how these compounds have been related to numerous intoxication cases (Adams et al., 2017; Kusano et al., 2018). The Spanish Early Warning System (SEAT) has notified the detection of seven new SCs since 2014 (Observatorio Español de las Drogas y las Adicciones, 2017). In addition, several SC have been reported in herbal blends acquired in Spanish shops (Ibáñez et al., 2013). However, detecting SC use through urine analysis cannot be carried out directly since these substances are rapidly and completely metabolized, making it necessary to detect their main metabolites (Scheidweiler, Jarvis & Huestis, 2015).

This study is a follow-up of an SC user attending an Addictive Behaviour Unit (ABU) in Valencia (Spain). We analysed the patient's urine samples, as well as the herbs and cigarettes which we took from her. Herbs sold in the smart shop in which the patient was thought to have been buying were also analysed. The change observed in the pattern of use was linked to the prohibition of certain cannabinoids in Spain (Ministerio de Sanidad Consumo y Bienestar Social, 2018).

Urine and herbal samples were collected in the ABUs participating in the study, following the protocol approved by the local ethics committee (Ref: DGNRI6 14-24-10). The urine and herbs seized from the patient were collected at the ABU which was treating her, while the second ABU impounded herbs and cigarettes from other patients. Additionally, herbs were bought at a well-known smart shop in the town where the patient lives. The analysis of herbs and urine was carried out following the methodology described in the literature (Fabregat-Safont et al., 2017; Ibáñez et al., 2013).

The analyses were performed by high performance liquid chromatography-high resolution mass spectrometry. An Acquity I-Class liquid chromatograph (Waters, Mildford, MA, USA) was coupled to a Vion IMS Qtof system (Waters, Manchester, UK) using electrospray ionization.

When the patient went to the ABU, she was a 17-year-old adolescent who had been adopted aged 6 from an East European country. Parental pressure compelled her to visit the ABU, where she sought counselling regarding cannabis use because she lacked any awareness of the problem. She has been receiving continuous child psychiatry treatment since the age of 11 for behavioural disorders and mild mental retardation. She does not have a good relationship with her parents, who, moreover, are not very effective in handling the patient. She has displayed self-harm behaviours and has been in trouble with the Child Prosecution Office, without further consequences. She has dropped out of school.

In interviews conducted before the summer, the patient admitted to consuming a certain herb called *Hardcore* (Figure 1A). The analysis of this product allowed the identification of three SCs, belonging to the same family: XLR-11, UR-144 and, at lower concentration level, the chlorinated N-pentyl analogue of UR-144. Once the product's compo-

Received: July 2019; Accepted: November 2019.

Send correspondence to:
María Ibáñez, Instituto Universitario de Plaguicidas y Aguas, Universitat Jaume I de Castelló.
E-mail: ibanezm@uji.es

ADICCIONES, 2020 · VOL. 32 NO. 3 · PAGES 228-230

Adicciones, 2020; 32:228

Variation in the pattern of synthetic cannabinoid use by a female patient during 2018

David Fabregat-Safont*, Carmen Ripoll**, Teresa Orengo***,
Juan V. Sancho*, Félix Hernández*, María Ibáñez*

* Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071, Castellón, Spain.
** Addictions Treatment Unit Campanar, Valencia, Spain.
*** Addictions Treatment Unit Grao-Clínico, Valencia, Spain.

The use of synthetic cannabinoids (SCs) is, nowadays, an important social concern. A quick search on the literature shows how these compounds have been related to numerous intoxication cases (Adams et al., 2017; Kusano et al., 2018). The Spanish Early Warning System (SEAT) has notified the detection of seven new SCs since 2014 (Observatorio Español de las Drogas y las Adicciones, 2017). In addition, several SC have been reported in herbal blends acquired in Spanish shops (Ibáñez et al., 2013). However, detecting SC use through urine analysis cannot be carried out directly since these substances are rapidly and completely metabolized, making it necessary to detect their main metabolites (Scheidweiler, Jarvis & Huestis, 2015).

This study is a follow-up of an SC user attending an Addictive Behaviour Unit (ABU) in Valencia (Spain). We analysed the patient's urine samples, as well as the herbs and cigarettes which we took from her. Herbs sold in the smart shop in which the patient was thought to have been buying were also analysed. The change observed in the pattern of use was linked to the prohibition of certain cannabinoids in Spain (Ministerio de Sanidad Consumo y Bienestar Social, 2018).

Urine and herbal samples were collected in the ABUs participating in the study, following the protocol approved by the local ethics committee (Ref: DGNRI6 14-24-10). The urine and herbs seized from the patient were collected at the ABU which was treating her, while the second ABU impounded herbs and cigarettes from other patients. Additionally, herbs were bought at a well-known smart shop in the town where the patient lives. The analysis of herbs and urine was carried out following the methodology described in the literature (Fabregat-Safont et al., 2017; Ibáñez et al., 2013).

The analyses were performed by high performance liquid chromatography-high resolution mass spectrometry. An Acquity I-Class liquid chromatograph (Waters, Mildford, MA, USA) was coupled to a Vion IMS Qtof system (Waters, Manchester, UK) using electrospray ionization.

When the patient went to the ABU, she was a 17-yearold adolescent who had been adopted aged 6 from an East European country. Parental pressure compelled her to visit the ABU, where she sought counselling regarding cannabis use because she lacked any awareness of the problem. She has been receiving continuous child psychiatry treatment since the age of 11 for behavioural disorders and mild mental retardation. She does not have a good relationship with her parents, who, moreover, are not very effective in handling the patient. She has displayed selfharm behaviours and has been in trouble with the Child Prosecution Office, without further consequences. She has dropped out of school.

In interviews conducted before the summer, the patient admitted to consuming a certain herb called *Hardcore* (**Figure 1A**). The analysis of this product allowed the identification of three SCs, belonging to the same family: XLR-11, UR-144 and, at lower concentration level, the chlorinated *N*-pentyl analogue of UR-144. Once the product's composition was known, the patient's urine was analysed, looking for the metabolites described for XLR-11 and UR-144 (Jang et al., 2016). The main metabolite of XRL-11 (N-pentanoic acid) was detected in two of the three urine samples collected from the patient during the first half of 2018.

Figure 1. Samples of use analysed. **A** *Hardcore* herb containing XLR-11 and UR-144. **B, C** Herbs seized in the ABUs containing 5F-ADB. **D** Cigarette seized at the ABU containing 5F-ADB. **E** *Matador* herb containing 5F-ADB.

However, in the urine collected after the summer, no metabolite of XLR-11 was found. Thus, either the subject had stopped using SCs, or her pattern of use had changed. A quick search of Spanish legislation revealed that XLR11 had been banned, as published in the Boletín Oficial del Estado (Official State Gazette) on July 12, 2018 (Ministerio de Sanidad Consumo y Bienestar Social, 2018). It seemed logical to think that the subject had changed her consumption habits, although it was unknown which substance she might be smoking at that time. Weeks later, various herb samples were seized (**Figure 1B**), and the analyses revealed a dangerous new SC known as 5F-ADB or 5F-MDMB-PINACA. A high level of potency is attributed to this compound (Banister et al., 2016) and it has been linked to various deaths (Hasegawa et al., 2015; Kusano et al., 2018).

Once this new SC was identified, urine samples were reprocessed searching for the three metabolites described for 5F-ADB (Kusano et al., 2018). In two of the six samples collected after the summer, the three main metabolites of 5F-ADB were detected. The results obtained suggest that the prohibition of XLR-11 prompted manufacturers and/or sellers of new psychoactive substances to replace this product for one that was not legislated.

A few weeks later, a third herb (**Figure 1C**) and a cigarette (**Figure 1D**) were taken from two subjects treated in the second participating ABU. In addition, a new herbal blend available at the local smart shop was purchased (*Matador*, **Figure 1E**). We found 5F-ADB in all these samples, suggesting that this substance started being used frequently in Valencia after the summer.

Our work illustrates how the prohibition of SCs can alter their patterns of use. These results suggest that trends in the use of SCs depend largely on current legislation, so when a cannabinoid is banned, it disappears from the market and is replaced by a new compound, which quickly reaches the streets.

Acknowledgements

The authors acknowledge the funding received from the Spanish Ministry of Economy and Competitiveness (Project CTQ2015-65603-P) and from the Universitat Jaume I (Proyecto UJI-B2018-19). David Fabregat-Safont acknowledges the Spanish Ministry of Education, Culture and Sport for his pre-doctoral grant (Grant FPU15/02033).

Conflicts of interest

The authors declare no conflicts of interest.

References

Adams, A. J., Banister, S. D., Irizarry, L., Trecki, J., Schwartz, M. & Gerona, R. (2017). "Zombie" Outbreak Caused by the Synthetic Cannabinoid AMB-FUBINACA in New York. New England Journal of Medicine, 376, 235–242. doi:10.1056/NEJMoa1610300.

Banister, S. D., Longworth, M., Kevin, R., Sachdev, S., Santiago, M., Stuart, J.,… Kassiou, M. (2016). Pharmacology of Valinate and tert -Leucinate Synthetic Cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and Their Analogues. ACS Chemical Neuroscience, 7, 1241–1254. doi: 10.1021/acschemneuro.6b00137.

Fabregat-Safont, D., Barneo-Muñoz, M., Martinez-Garcia, F., Sancho, J. V., Hernández, F. & Ibáñez, M. (2017). Proposal of 5-methoxy-N-methyl-N-isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. Journal of Chromatography A, 1508. doi:10.1016/j.chroma.2017.06.010.

Hasegawa, K., Wurita, A., Minakata, K., Gonmori, K., Yamagishi, I., Nozawa, H.,… Suzuki, O. (2015). Identification and quantitation of 5-fluoro-ADB, one of the most dangerous synthetic cannabinoids, in the stomach contents and solid tissues of a human cadaver and in some herbal products. Forensic Toxicology, 33, 112–121. doi:10.1007/s11419-014-0259-0.

Ibáñez, M., Bijlsma, L., van Nuijs, A. L. N., Sancho, J. V., Haro, G., Covaci, A. & Hernández, F. (2013). Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends. Journal of Mass Spectrometry, 48, 685–694. doi:10.1002/jms.3217.

Jang, M., Kim, I. S., Park, Y. N., Kim, J., Han, I., Baeck, S.,… Yoo, H. H. (2016). Determination of urinary metabolites of XLR-11 by liquid chromatography–quadrupole time-of-flight mass spectrometry. Analytical and Bioanalytical Chemistry, 408, 503–516. doi:10.1007/s00216-015- 9116-1.

Kusano, M., Zaitsu, K., Taki, K., Hisatsune, K., Nakajima, J., Moriyasu, T.,… Ishii, A. (2018). Fatal intoxication by 5F–ADB and diphenidine: Detection, quantification, and investigation of their main metabolic pathways in humans by LC/MS/MS and LC/Q-TOFMS. Drug Testing and Analysis, 10, 284–293. doi:10.1002/dta.2215.

Ministerio de Sanidad Consumo y Bienestar Social (2018). BOE-A-2018-10185. Boletín Oficial Del Estado, 175, 73005-73006.

Observatorio Español de las Drogas y las Adicciones (2017). INFORME 2017. Alcohol, tabaco y drogas ilegales en España. Observatorio Español de La Droga y Las Toxicomanías. Ministerio de Sanidad y Servicios Sociales.

Scheidweiler, K. B., Jarvis, M. J. Y. & Huestis, M. A. (2015). Nontargeted SWATH acquisition for identifying 47 synthetic cannabinoid metabolites in human urine by liquid chromatography-high-resolution tandem mass spectrometry. Analytical and Bioanalytical Chemistry, 407, 883–897. doi:10.1007/s00216-014-8118-8.

6.4. Discussion of the results obtained

The monitoring of NPS consumption through the UHPLC-HRMS analysis of urine samples from a specific population, following a suspect screening strategy based on the detection of specific biomarkers (metabolites), can be improved with the information about legal highs potentially consumed. The studies included in **Chapter 6**, focused on monitoring the SCRA consumption among teenagers from the Valencian region, illustrate that a previous analysis of the herbal blends suspected to be consumed for SCRA identification allows an accurate literature search for selecting the most adequate metabolites. Obviously, the best option would be to include in this list the metabolites of all known SCRA. Unfortunately, nowadays exist more than 200 compounds, for most of which no metabolism data are available in literature. In this case, a good approach is to cross the data of where the urine samples were collected and the SCRA present in the herbal blends sold in the smartshops surrounding that region, in order to focus the screening on the interesting compounds.

Research article XI presents how the teenagers in juvenile offenders' centres used herbal blends during theirs permits with stay at home for evading routine drug urine analysis. As recognised by the teenagers in previous interviews, the herbal blends were purchased in a local smartshop. The analysis of these herbal blends, together with the data of legal highs previously acquired in the same smartshop, allowed the identification of the SCRA potentially consumed by this population. As SCRA are completely metabolised in the liver, as showed in **research article VII**, the analysis of urine samples must be focused on the major metabolites of these compounds. So, after an accurate literature search, a suspect list was built and used for the HRMS suspect screening. The metabolites of XLR-11 and UR-144 were detected in the urine samples from 6 teenagers. After that, it was deduced that the herbal blend *Hardcore*, containing XLR-11 and UR-114, was the product consumed.

As showed in **Chapter 2**, additional herbal blends were available in this smartshop (**Figure 2.3** and **Table 2.2**). *Hardcore*, *Sonrisa* and *Tío* were sold until summer 2018, when they were replaced by *Matador* (containing 5F-ADB), *Bull* and *Storm* (both with the recently reported DMBA-CHMINACA [28]). As illustrated in **research article XII**, this change in the herbal blends was encouraged by the XLR-11 ban in Spain in July 2018. This study shows that the SCRA consumption patterns of teenagers are highly dependent on the availability of herbal blends in the smartshops of their region. It is important to remark the crucial collaboration of the medical services involved in this study, as they seized the herbs that revealed the presence of 5F-ADB in Valencia and thus, alerted us about the availability of new herbal blends in the local smartshop (*Matador*, *Bull* and *Storm*). Again, the 5F-ADB consumption was assessed by the detection of the major metabolites reported for this compound. **Research article XII** was published as a "Letter to the editor". Due to the format of this type of publication, some information about this study could not be included. So, the main analytical aspects of this work are described and discussed below.

6.4.1. Research article XII analytical aspects

Sample treatment and instrumental analysis

Sample analysis was performed following the procedure described in **research article XI** for herbal blends and urine samples. But in this case, extracts were analysed by UHPLC-IMS-HRMS, using a Vion IMS Qtof (Waters, Manchester, UK) equipped with an ion mobility separator (IMS), and presenting an IMS-Q-HRMS design. The chromatographic separation was the same than the one used in **research article XI**. The Vion Qtof was operated as follows. The ESI was operated in positive ionisation mode, using a capillary voltage of 0.8 kV, cone voltage at 20 V, source temperature at 120 ºC, desolvation gas at 600 ºC, cone gas flow at 250 L/h and desolvation gas at 1000 L/h, using nitrogen (Praxair, Valencia, Spain) in both cases. Nitrogen was also used as a gas for the IMS cell and for the collision cell. The resolution of the TOF analyser was ~36000 for the

m/z 556 ion. The $HDMS^E$ acquisition mode was used (this is, an MS^E with an ion mobility separation), obtaining information about the protonated molecule in the low energy function (Function 2, energy of collision of 6 eV) and its fragments in the high energy function (Function 3, collision energy ramp of 21 to 56 eV). Calibration was performed bimonthly using the manufacturer's recommended mix Major Mix IMS/Qtof Calibration Kit (Waters). The mass accuracy was corrected throughout the analysis time using a solution of leucine enkephalin at 100 ng/mL, perfused and measured continuously by the Vion IMS Qtof system (Function 1). The data were acquired and processed using the UNIFI software (version 1.8.2, Waters).

Detecting XLR-11 metabolites in urine samples

As described in **research article XII**, the major metabolite of XLR-11 (XLR-11 *N*-pentanoic acid) was detected in different urine samples from the user collected before summer 2018. **Figure 6.1** shows the $HDMS^E$ spectrum, as well as the nw-EIC from $[M+H]^+$ of this metabolite.

It is important to point out the absence of fragment ions coming from the matrix in the HE spectrum acquired with the IMS-HRMS system (**Figure 6.1B**). As the fragment ions are filtered based on the drift time observed for precursor ion (in this case, the $[M+H]^+$, *m/z* 342.2067, $C_{21}H_{28}NO_3^+$, 1.1 ppm), most of the ions coming from co-eluting compounds are "removed", and a cleaner spectra is obtained. For XLR-11, two fragment ions at *m/z* 244.0974 ($C_{14}H_{14}NO_3^+$, 2.3 ppm) and 144.0446 ($C_9H_6NO^+$, 1.8 ppm) were observed.

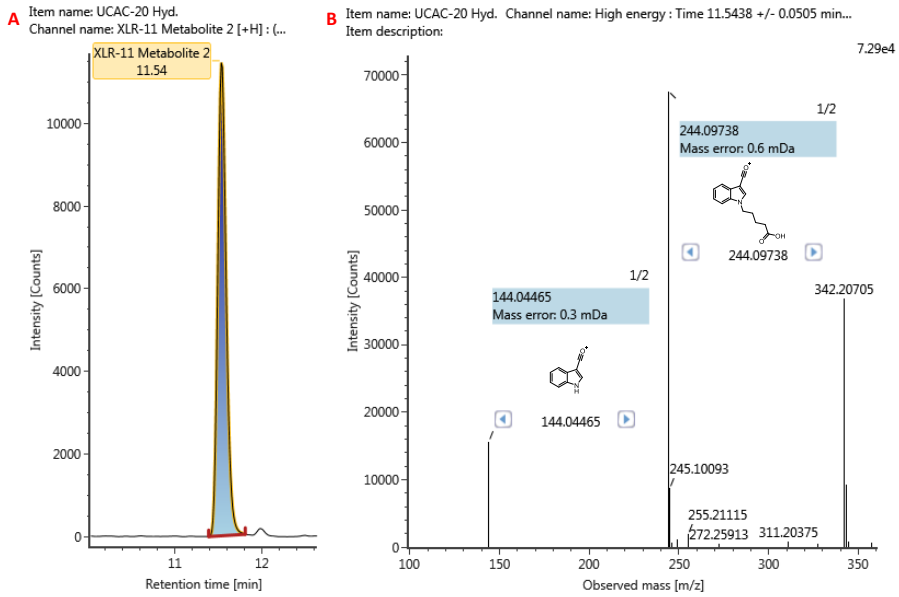


Figure 6.1. Detection of the XLR-11 *N*-pentanoic acid metabolite in a urine sample collected before summer 2018. **A** nw-EIC from protonated molecule, extracted with a ±5 mDa window. **B** HE spectrum from XLR-11 *N*-pentanoic acid metabolite.

5F-ADB and its metabolites in herbal blends and urine samples, respectively

As commented in **research article XII**, 5F-ADB was detected in different seized samples, as well as in the *Matador* herbal blend, after summer 2018. When this SCRA was found, urine samples previously collected were reprocessed, searching for the major metabolites described for this compound [29]. In this case, three major phase I metabolites had been reported: *O*-demethylated, oxidative defluorinated, and *N*-pentanoic acid metabolites [29]. The three metabolites were tentatively identified in urine samples from the teenager, as showed in **Figure 6.2**.

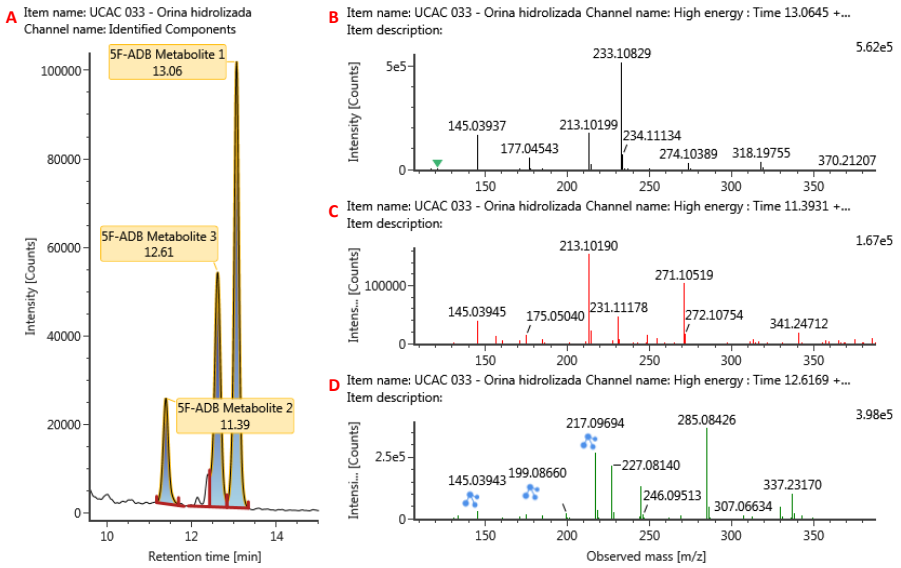


Figure 6.2. Tentative identification of the major metabolites of 5F-ADB in the teenager urine sample, collected after summer 2018. **A** nw-EIC from $[M+H]^+$ *O*-demethylated (Metabolite 1), oxidative defluorinated (Metabolite 2), and *N*-pentanoic acid (Metabolite 3). HE spectra from Metabolite 1 (**B**), 2 (**C**) and 3 (**D**).

The HRMS fragmentation data used for the tentative identification of the 5F-ADB in the seized samples, as well as for its major metabolites in urine samples, are listed in **Table 6.1**. The fragment ions observed for 5F-ADB, as well as for the detected metabolites, were justified based on the compound structure. Moreover, all the fragment ions observed for metabolites were in accordance with the fragmentation observed for the 5F-ADB parent compound after considering mass shifts produced by the biotransformations.

The 5F-ADB fragmentation is similar to that observed for 5F-APP-PICA and AMB-FUBINACA in **research article VII**, suggesting that most of the SCRA follow similar fragmentation pathways.

Table 6.1. HRMS data for 5F-ADB and its metabolites.

Compound	$[M+H]^+$ (*m/z*)	Elemental composition	Mass error (ppm)	Fragment ion (*m/z*)	Elemental composition	Mass error (ppm)
5F-ADB	378.2193	$C_{20}H_{29}FN_3O_3^+$	1.6	318.1979	$C_{18}H_{25}FN_3O^+$	0.7
				233.1086	$C_{13}H_{14}FN_2O^+$	0.4
				213.1023	$C_{13}H_{13}N_2O^+$	0.1
				177.0458	$C_9H_5FN_2O^+$	-0.3
				145.0396	$C_8H_5N_2O^+$	-0.6
Metabolite 1	364.2031	$C_{19}H_{27}FN_3O_3^+$	0.0	318.1976	$C_{18}H_{25}FN_3O^+$	-0.2
				233.1083	$C_{13}H_{14}FN_2O^+$	-0.8
				213.1020	$C_{13}H_{13}N_2O^+$	-1.2
				177.0454	$C_9H_5FN_2O^+$	-2.5
				145.0394	$C_8H_5N_2O^+$	-1.9
Metabolite 2	362.2078	$C_{19}H_{28}N_3O_4^+$	1.0	316.2022	$C_{18}H_{26}N_3O_2^+$	0.7
				231.1118	$C_{13}H_{15}N_2O_2^+$	-4.4
				213.1019	$C_{13}H_{13}N_2O^+$	-1.6
				145.0395	$C_8H_5N_2O^+$	-1.3
Metabolite 3	376.1869	$C_{19}H_{26}N_3O_5^+$	0.5	330.1811	$C_{18}H_{24}N_3O_3^+$	-0.4
				245.0920	$C_{13}H_{13}N_2O_3^+$	-0.4
				217.0969	$C_{12}H_{13}N_2O_2^+$	-1.0
				199.0866	$C_{12}H_{11}N_2O^+$	0.1
				145.0394	$C_8H_5N_2O^+$	-1.4

One of the benefits of using IMS-HRMS for toxicological analysis is the acquisition of "pseudo" MS/MS spectra, as the HRMS data are also filtered by the drift time observed in IMS cell. Additionally, if an IMS calibration solution is used, collision cross section (CCS) values can be automatically calculated from drift time, obtaining an orthogonal parameter that can be used for compound identification.

The information presented in **Chapter 6** illustrates one of the applications of LC-MS in toxicology: the suspect screening of SCRA metabolites in urine samples from herbal blends users. As SCRA are completely metabolised, a compound suspect list containing information on their main metabolites must be generated for UHPLC-HRMS analysis.

6.5. Literature

1. Polettini, A. Applications of LC-MS in Toxicology. (Pharmaceutical Press, 2006).

2. Valen, A., Leere Øiestad, Å. M., Strand, D. H., Skari, R. & Berg, T. Determination of 21 drugs in oral fluid using fully automated supported liquid extraction and UHPLC-MS/MS. Drug Test. Anal. 9, 808–823 (2017).

3. Bidny, S., Gago, K., Chung, P., Albertyn, D. & Pasin, D. Simultaneous Screening and Quantification of Basic, Neutral and Acidic Drugs in Blood Using UPLC-QTOF-MS. J. Anal. Toxicol. 41, 181–195 (2017).

4. Beltrán, E. et al. Development of sensitive and rapid analytical methodology for food analysis of 18 mycotoxins included in a total diet study. Anal. Chim. Acta 783, 39–48 (2013).

5. Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F. & Krska, R. Optimization and validation of a quantitative liquid chromatography–tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices. J. Chromatogr. A 1362, 145–156 (2014).

6. Pitarch-Motellón, J., Sancho, J. V., Ibáñez, M., Pozo, O. & Roig-Navarro, A. F. Determination of selected endogenous anabolic androgenic steroids and ratios in urine by ultra high performance liquid chromatography tandem mass spectrometry and isotope pattern deconvolution. J. Chromatogr. A 1515, 172–178 (2017).

7. Marcos, J. & Pozo, O. J. Current LC–MS methods and procedures applied to the identification of new steroid metabolites. J. Steroid Biochem. Mol. Biol. 162, 41–56 (2016).

8. Fabregat-Cabello, N., Pitarch-Motellón, J., Sancho, J. V., Ibáñez, M. & Roig-Navarro, A. F. Method development and validation for the determination of selected endocrine disrupting compounds by liquid chromatography mass spectrometry and isotope pattern deconvolution in water samples. Comparison of two extraction techniques. Anal. Methods 8, 2895–2903 (2016).

9. Silveira, R. S., Rocha, B. A., Rodrigues, J. L. & Barbosa, F. Rapid, sensitive and simultaneous determination of 16 endocrine-disrupting chemicals (parabens, benzophenones, bisphenols, and triclocarban) in human urine based on microextraction by packed sorbent combined with liquid chromatography tandem mass spectrom. Chemosphere 240, 124951 (2020).

10. Gjerde, H. et al. Drug Use by Music Festival Attendees: A Novel Triangulation Approach Using Self-Reported Data and Test Results of Oral Fluid and Pooled Urine Samples. Subst. Use Misuse 54, 2317–2327 (2019).

11. Helander, A. & Bäckberg, M. Epidemiology of NPS Based Confirmed Overdose Cases: The STRIDA Project. in 461–473 (2018). doi:10.1007/164_2018_134

12. Bijlsma, L., Celma, A., López, F. J. & Hernández, F. Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations. Curr. Opin. Environ. Sci. Heal. 9, 1–12 (2019).

13. González- Mariño, I. et al. Spatio- temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring. Addiction 115, 109–120 (2020).

14. Kimble, A. N. & DeCaprio, A. P. Systematic analysis of novel psychoactive substances. II. Development of a screening/confirmatory LC-QqQ-MS/MS method for 800+ compounds and metabolites in urine. Forensic Chem. 16, 100189 (2019).

15. Cheng, W.-C. & Dao, K.-L. Prevalence of drugs of abuse found in testing of illicit drug seizures and urinalysis of selected population in Hong Kong. Forensic Sci. Int. 299, 6–16 (2019).

16. Istituto di Ricerche Farmacologiche Mario Negri IRCCS. NPS Euronet. Available at: http://www.npseuronet.eu/. (Accessed: 13th April 2020)

17. Maurer, H. H. & Brandt, S. D. New Psychoactive Substances. Pharmacology, Clinical, Forensic and Analytical Toxicology. Handbook of Experimental Pharmacology 252, (Springer International Publishing, 2018).

18. Pozo, O. 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. Drug Metab. Dispos. 43, 248–257 (2014).

19. Ibáñez, M. et al. Analytical strategy to investigate 3,4-methylenedioxypyrovalerone (MDPV) metabolites in consumers' urine by high-resolution mass spectrometry. Anal. Bioanal. Chem. 408, 151–164 (2016).

20. Richeval, C., Gaulier, J.-M., Romeuf, L., Allorge, D. & Gaillard, Y. Case report: relevance of metabolite identification to detect new synthetic opioid intoxications illustrated by U-47700. Int. J. Legal Med. 133, 133–142 (2019).

21. Vikingsson, S. et al. LC-QTOF-MS Identification of Major Urinary Cyclopropylfentanyl Metabolites Using Synthesized Standards. J. Anal. Toxicol. 43, 607–614 (2019).

22. Gundersen, P. O. M., Spigset, O. & Josefsson, M. Screening, quantification, and confirmation of synthetic cannabinoid metabolites in urine by UHPLC-QTOF-MS. Drug Test. Anal. 11, 51–67 (2019).

23. Staeheli, S. N. et al. Liquid chromatography–tandem mass spectrometry screening method using information- dependent acquisition of enhanced product ion mass spectra for synthetic cannabinoids including metabolites in urine. Drug Test. Anal. 11, 1369–1376 (2019).

24. Shevyrin, V. et al. Identification and analytical properties of new synthetic cannabimimetics bearing 2,2,3,3-tetramethylcyclopropanecarbonyl moiety. Forensic Sci. Int. 226, 62–73 (2013).

25. Wohlfarth, A. et al. First Metabolic Profile of XLR-11, a Novel Synthetic Cannabinoid, Obtained by Using Human Hepatocytes and High-Resolution Mass Spectrometry. Clin. Chem. 59, 1638–1648 (2013).

26. Jang, M. et al. Determination of urinary metabolites of XLR-11 by liquid chromatography–quadrupole time-of-flight mass spectrometry. Anal. Bioanal. Chem. 408, 503–516 (2016).

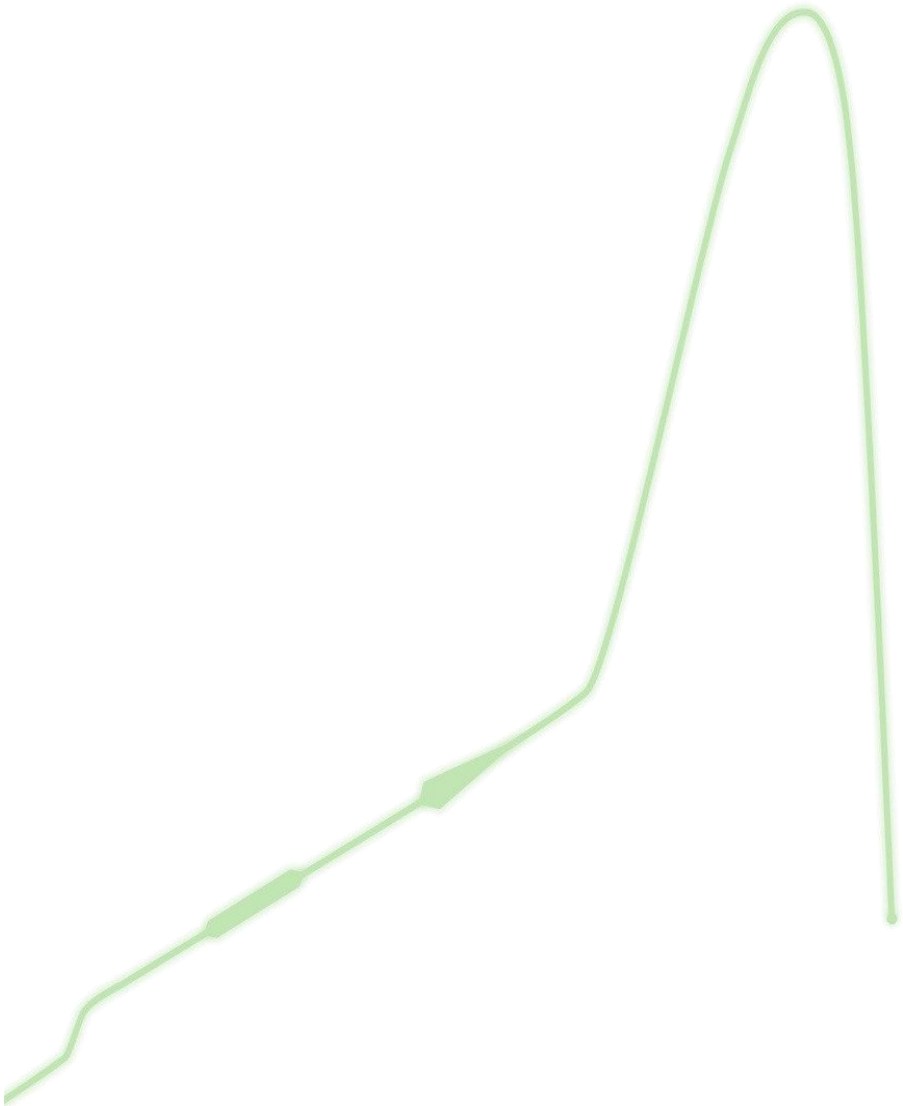
27. Mardal, M. et al. HighResNPS.com: An Online Crowd-Sourced HR-MS Database for Suspect and Non-targeted Screening of New Psychoactive Substances. J. Anal. Toxicol. 43, 520–527 (2019).

28. Lee, J. H. et al. Identification and characterization of an indazole-3-carboxamide class synthetic cannabinoid: 2-[1-(cyclohexylmethyl)-1H-indazole-3-carboxamido]-3,3-dimethylbutanoic acid (DMBA-CHMINACA). Forensic Sci. Int. 291, 167–174 (2018).

29. Kusano, M. et al. Fatal intoxication by 5F–ADB and diphenidine: Detection, quantification, and investigation of their main metabolic pathways in humans by LC/MS/MS and LC/Q-TOFMS. Drug Test. Anal. 10, 284–293 (2018).

CHAPTER 7

CONCLUSIONS AND FUTURE WORK



7.1. Conclusions

The **main conclusion** of this Doctoral Thesis is the undeniable need for high specialization in analytical chemistry for toxicological and forensic sciences. In this sense, MS-based techniques, and especially HRMS, plays an important role. The data generated need an in-depth and accurate processing for obtaining all the valuable information, required for an adequate interpretation of the results.

The forensic toxicology field includes the study of different aspects of the NPS but, similarly to how toxicological analysis needs analytical chemistry, analytical chemistry requires the collaboration of a wide range of disciplines to achieve this adequate understanding of the results obtained during NPS research, such as the pharmaceutical, medical, legal, and social sciences.

From the studies presented in this Doctoral Thesis, the following **specific conclusions** can be highlighted:

1) The need of continuous analysis for monitoring the NPS present in seizures, legal highs, research chemicals, and similar products, using HRMS with API sources, that facilitates compound identification thanks to the accurate-mass information obtained.
2) The combination of HRMS and NMR for the unequivocal compound identification when no analytical reference standard is available. For unreported substances, single-crystal X-ray diffraction can be used for a final identification.
3) The free-available publication of complete analytical data obtained by GC-MS, FTIR, HRMS and NMR for unreported compounds is highly recommended, to allow toxicological laboratories the tentative identification of these compounds.
4) Triple quadrupole mass analysers are a feasible alternative for performing NPS suspect screening based on the fragmentation of the compounds, especially for the analysis of consumption products when no HRMS instrumentation is available.

5) The *in vitro* and *in vivo* metabolism profiling results obtained for NPS should be complemented with additional models to obtain proven data for an accurate selection of the potential consumption biomarkers.
6) pHH, despite of their high cost and low throughput compared with pHLM or pS9, are the most adequate *in vitro* model for the identification of liver metabolites.
7) SCRA should be a priority for metabolite profiling studies, as these compounds are completely metabolised and thus, their determination in human urine samples must be performed through the determination of their major metabolites.
8) The use of *in vitro* assays based on the determination of the affinity of these compounds for specific receptors, have demonstrated the applicability for determining their potency.
9) Nevertheless, the pharmacological properties of the studied compounds, especially those related to the distribution and absorption in different tissues, must be considered for an accurate potency study, as slight modifications in the structure of a certain type of NPS can lead to high differences in their permeability through organ barriers.
10) When performing urine analysis in a certain population for determining NPS consumption, the geographical availability of NPS should also be considered, trying to select the most adequate biomarkers for the compounds potentially consumed.
11) The use of SCRA among teenagers has been demonstrated; so it would be necessary to apply risk prevention strategies for this group.

Most of the studies included in this thesis have been developed in collaboration with specialists in complementary disciplines. So, as final note for this thesis, as well as for any scientific work, the high-quality research cannot be performed individually: collaboration is always mandatory for obtaining valuable results that can be useful for scientific community, and maybe be the seed for future studies in the leading edge of knowledge.

7.1. Conclusions

La **principal conclusió** d'aquesta Tesi Doctoral és la innegable necessitat d'una alta especialització en química analítica per a les ciències toxicològiques i forenses. En aquest sentit, les tècniques basades en MS, i especialment en HRMS, juguen un paper important. Les dades generades necessiten un processament profund i precís per a obtindre tota la informació valuosa, a fi d'obtindre una adequada interpretació dels resultats.

El camp de la toxicologia forense inclou l'estudi de diferents aspectes de les NPS però, de manera similar a com l'anàlisi toxicològica necessita de la química analítica, la química analítica requereix de la col·laboració d'una àmplia gamma de disciplines per a aconseguir aquesta adequada comprensió dels resultats obtinguts durant la investigació amb NPS, com ara les ciències farmacèutiques, mèdiques, legals i socials.

Dels estudis presentats en aquesta Tesi Doctoral es poden destacar les següents **conclusions específiques**:

1) La necessitat d'una anàlisi contínua per a monitorar les NPS presents en les confiscacions, *legal highs*, *research chemicals* i productes similars, utilitzant HRMS amb fonts API, que facilita la identificació dels compostos gràcies a la informació de massa exacta obtinguda.
2) La combinació de HRMS i RMN per a la identificació inequívoca de compostos quan no es disposa de patrons de referència analítica. En el cas de les substàncies no reportades, pot utilitzar-se la difracció de raigs X de monocristall per a una identificació final.
3) Es recomana encaridament la publicació en accés obert de dades analítiques completes obtinguts per CG-MS, FTIR, HRMS i RMN per als compostos no reportats, a fi de permetre als laboratoris toxicològics la identificació provisional d'aquests compostos.

4) Els analitzadors de tipus triple quadrupol són una alternativa viable per a dur a terme anàlisi de tipus *suspect screening*, basats en la fragmentació de les NPS, especialment per a l'anàlisi de productes de consum i quan no es disposa d'instrumentació de HRMS.
5) Els resultats de les rutes metabòliques obtingudes mitjançant models *in vitro* i *in vivo* obtinguts per a les NPS han de complementar-se amb models addicionals per a obtindre dades contrastades que permeten una selecció precisa dels possibles biomarcadors de consum.
6) La incubació amb pHH, malgrat el seu alt cost i baix rendiment en comparació amb els pHLM o pS9, són el model *in vitro* més adequat per a la identificació de metabòlits produïts al fetge.
7) Els SCRA han de ser una prioritat per als estudis de metabolisme, ja que aquests compostos es metabolitzen completament i, per tant, la seua determinació en mostres d'orina humana ha de realitzar-se mitjançant la determinació dels seus principals metabòlits.
8) L'ús d'assajos *in vitro* basats en la determinació de l'afinitat d'aquests compostos per receptors específics, han demostrat l'aplicabilitat per a estimar la seua potència.
9) No obstant això, les propietats farmacològiques dels compostos estudiats, especialment les relacionades amb la distribució i l'absorció en diferents teixits, han de tindre's en compte per a un estudi precís de la potència, ja que lleugeres modificacions en l'estructura d'un determinat tipus de NPS poden donar lloc a grans diferències en la seua permeabilitat a través de les barreres dels òrgans.
10) Quan es realitza l'anàlisi d'orina en una determinada població per a determinar el consum de NPS, s'ha de considerar també la disponibilitat geogràfica de NPS, tractant de seleccionar els biomarcadors més adequats per als compostos potencialment consumits.
11) S'ha demostrat l'ús dels SCRA entre els adolescents, per la qual cosa seria necessari aplicar estratègies de prevenció de riscos per a aquest grup de població.

La majoria dels estudis inclosos en aquesta tesi s'han desenvolupat en col·laboració amb especialistes en altres disciplines científiques. Per tant, com a nota final de la tesi, així com de qualsevol treball científic, la investigació d'alta qualitat no pot realitzar-se de manera individual: la col·laboració és sempre obligatòria per a obtindre resultats valuosos que puguen ser útils per a la comunitat científica, i tal vegada siga la llavor de futurs estudis a la frontera del coneixement.

7.1. Conclusiones

La **principal conclusión** de esta Tesis Doctoral es la innegable necesidad de una alta especialización en química analítica para las ciencias toxicológicas y forenses. En este sentido, las técnicas basadas en MS, y especialmente en HRMS, juegan un papel importante. Los datos generados necesitan un procesamiento profundo y preciso para obtener toda la información valiosa, a fin de obtener una adecuada interpretación de los resultados.

El campo de la toxicología forense incluye el estudio de diferentes aspectos de las NPS pero, de forma similar a como el análisis toxicológico necesita de la química analítica, la química analítica requiere de la colaboración de una amplia gama de disciplinas para lograr esta adecuada comprensión de los resultados obtenidos durante la investigación con NPS, tales como las ciencias farmacéuticas, médicas, legales y sociales.

De los estudios presentados en esta Tesis Doctoral se pueden destacar las siguientes **conclusiones específicas**:

1) La necesidad de un análisis continuo para monitorizar las NPS presentes en las incautaciones, *legal highs*, *research chemicals* y productos similares, utilizando HRMS con fuentes API, que facilita la identificación de los compuestos gracias a la información de masa exacta obtenida.
2) La combinación de HRMS y RMN para la identificación inequívoca de compuestos cuando no se dispone de patrones de referencia analítica. En el caso de las sustancias no reportadas, puede utilizarse la difracción de rayos X de monocristal para una identificación final.
3) Se recomienda encarecidamente la publicación en acceso abierto de datos analíticos completos obtenidos por CG-MS, FTIR, HRMS y RMN para los compuestos no reportados, a fin de permitir a los laboratorios toxicológicos la identificación provisional de estos compuestos.

4) Los analizadores de tipo triple cuadrupolo son una alternativa viable para llevar a cabo análisis de tipo *suspect screening*, basados en la fragmentación de las NPS, especialmente para el análisis de productos de consumo y cuando no se dispone de instrumentación de HRMS.
5) Los resultados de las rutas metabólicas obtenidas mediante modelos *in vitro* e *in vivo* obtenidos para las NPS deben complementarse con modelos adicionales para obtener datos contrastados que permitan una selección precisa de los posibles biomarcadores de consumo.
6) La incubación con pHH, a pesar de su alto costo y bajo rendimiento en comparación con los pHLM o pS9, son el modelo *in vitro* más adecuado para la identificación de metabolitos producidos en el hígado.
7) Los SCRA deben ser una prioridad para los estudios de metabolismo, ya que estos compuestos se metabolizan completamente y, por lo tanto, su determinación en muestras de orina humana debe realizarse mediante la determinación de sus principales metabolitos.
8) El uso de ensayos *in vitro* basados en la determinación de la afinidad de estos compuestos por receptores específicos, han demostrado la aplicabilidad para estimar su potencia.
9) No obstante, las propiedades farmacológicas de los compuestos estudiados, especialmente las relacionadas con la distribución y la absorción en diferentes tejidos, deben tenerse en cuenta para un estudio preciso de la potencia, ya que ligeras modificaciones en la estructura de un determinado tipo de NPS pueden dar lugar a grandes diferencias en su permeabilidad a través de las barreras de los órganos.
10) Al realizar análisis de orina en una determinada población para determinar el consumo de NPS, se debe considerar también la disponibilidad geográfica de NPS, tratando de seleccionar los biomarcadores más adecuados para los compuestos potencialmente consumidos.

11) Se ha demostrado el uso de los SCRA entre los adolescentes, por lo que sería necesario aplicar estrategias de prevención de riesgos para este grupo de población.

La mayoría de los estudios incluidos en esta tesis se han desarrollado en colaboración con especialistas en otras disciplinas científicas. Por lo tanto, como nota final de ls tesis, así como de cualquier trabajo científico, la investigación de alta calidad no puede realizarse de forma individual: la colaboración es siempre obligatoria para obtener resultados valiosos que puedan ser útiles para la comunidad científica, y tal vez sea la semilla de futuros estudios en la frontera del conocimiento.

7.2. Suggestions for future work

Most of the studies presented in this Doctoral Thesis have been developed at the Research Institute for Pesticides and Water of Universitat Jaume I (Castelló de la Plana, Spain), and two studies were performed at the Forensic Medicine Department (Forensic Chemistry Section) of University of Copenhagen (Copenhagen, Denmark). This thesis includes all the work performed during 4 years of collaboration with different institutions. Continuing with these collaborations, additional research can be proposed and developed in the next years.

It is important to continue the monitoring of the NPS that are being consumed in Spain in collaboration with Energy Control for a better understanding of the consumption patterns and drug trends. Additionally, it is also interesting the identification and full-analytical characterisation of novel NPS not previously reported, deepening into their origin, as presented in **Chapter 2**.

The development of novel analytical strategies for compound identification is also an important research line. Similar to the approach performed in **research article V** for cathinone identification, this methodology could be explored for SCRA or synthetic opioids identification in seizures, as these compounds also present similar fragmentation pathways. Moreover, the application of common fragment and neutral loss searches during HRMS analysis will help for the direct elucidation of unknown compounds, or molecules not considered, during suspect screening analyses.

The information presented in **Chapter 4**, about the *in vitro* model application for metabolism studies, could be complemented with the determination of the *in vivo* metabolites using mice or rats, in order to increase the confidence on the adequation of the proposed consumption biomarkers. Additionally, sandwich-cultured hepatocytes *in vitro* experiments could also be performed for determining the biliary efflux of the elucidated metabolites.

An interesting research line encompasses the ADME experiments, offering a wide range of possibilities to be explored in the field of NPS research. **Chapter 5** presents two applications of *in vivo* models: metabolite profiling and pharmacokinetics. Further studies using mice and rats could be developed, such as the determination of metabolites and their distribution over time in different tissues such as brain, liver, kidney, blood, and finally urine excretion. These studies will provide additional information for understanding NPS metabolism, toxicity, potency, and excretion.

As showed in **Chapter 6**, the SCRA consumption among teenagers under treatment and/or control is a serious problem. So, additional studies for NPS consumption monitoring in this population should be performed in collaboration with medical and social services.

Research article XII present one of the benefits of IMS-HRMS instruments for suspect screening strategy: obtaining cleaner fragmentation spectra that could help for compound identification. The use of IMS-HRMS instruments has increased in the last years thanks to the development of different IMS technologies that can be coupled to HRMS, such as the Drift tube (Agilent Technologies), the Travelling-Wave (Waters Corporation), and the Trapped-IMS (Bruker Daltonics). So, it is likely that the use of IMS-HRMS systems for toxicological and forensic analysis will be a hot topic soon.

CCS values from the ionised molecules can be directly determined based on drift time, and they are commonly used as an additional identification parameter. Nevertheless, it would be interesting the application of these values for finding potential metabolites. Similar to the mass defect filter approach for metabolite detection, it is possible that CCS values of related compound (for example, of metabolites) will present a small variation and thus, they can be used for compound filtering. So, this is an interesting application of the IMS-HRMS data that can be explored for metabolite profiling experiments.

There is still much work to be done in the NPS research field, and many papers to be written.

7.2. Suggeriments per a treballs futurs

La majoria dels estudis presentats en aquesta Tesi Doctoral s'han desenvolupat en l'Institut Universitari de Plaguicides i Aigües de la Universitat Jaume I (Castelló de la Plana, Espanya), i dos estudis es van realitzar en el Departament de Medicina Forense (Secció de Química Forense) de la Universitat de Copenhaguen (Copenhaguen, Dinamarca). Aquesta tesi inclou tot el treball realitzat durant 4 anys de col·laboració amb diferents institucions. Continuant amb aquestes col·laboracions, es poden proposar i desenvolupar investigacions addicionals en els pròxims anys.

És important continuar el monitoratge de les NPS que es consumeixen a Espanya en col·laboració amb Energy Control per a entendre els patrons i tendències de consum. A més, també és interessant la identificació i caracterització analítica completa de les noves NPS no reportades, aprofundint a l'origen, tal com es presenta en el **Capítol 2**.

El desenvolupament de noves estratègies analítiques per a la identificació de compostos és també una important línia d'investigació. De manera similar a l'enfocament realitzat en l'**article d'investigació V** per a la identificació de catinones, aquesta metodologia podria explorar-se per a la identificació de SCRA o opiacis sintètics en les confiscacions, ja que aquests compostos també presenten vies de fragmentació similars. A més, l'aplicació d'estratègies de tipus *common fragment ion* i *common neutral loss* durant l'anàlisi mitjançant HRMS ajudarà a l'elucidació directa de compostos desconeguts, o molècules no considerades, durant anàlisis de tipus *suspect screening*.

La informació presentada en el **Capítol 4**, sobre l'aplicació de models *in vitro* per a estudis de metabolisme, podria complementar-se amb la determinació dels metabòlits *in vivo* utilitzant ratolins o rates, a fi d'augmentar la confiança en la idoneïtat dels biomarcadors de consum proposats. A més, podrien realitzar-se també experiments in vitro d'hepatòcits cultivats tipus sandvitx per a determinar l'efluent biliar dels metabòlits elucidats.

Una línia d'investigació interessant són els experiments de tipus ADME, oferint una àmplia gamma de possibilitats a explorar en el camp de la investigació amb NPS. En el **Capítol 5** es presenten dues aplicacions dels models *in vivo*: l'elucidació de metabòlits i la seua farmacocinètica. Podrien desenvolupar-se més estudis amb ratolins i rates, com la determinació dels metabòlits i la seua distribució al llarg del temps en diferents teixits com el cervell, el fetge, el ronyó, la sang i, finalment, l'excreció d'orina. Aquests estudis proporcionaran informació addicional per a comprendre el metabolisme, la toxicitat, la potència i l'excreció de les NPS.

Com es mostra en el **Capítol 6**, el consum de SCRA entre els adolescents sota tractament i/o control és un problema greu. Per tant, s'han de realitzar estudis addicionals per a la vigilància del consum de NPS en aquesta població, en col·laboració amb els serveis mèdics i socials.

En l'**article d'investigació XII** es presenta un dels beneficis dels instruments IMS-HRMS per a anàlisi de tipus *suspect screening*: obtindre espectres de fragmentació més nets que podrien ajudar a la identificació dels compostos. L'ús d'instruments IMS-HRMS ha augmentat en els últims anys gràcies al desenvolupament de diferents tecnologies IMS que poden acoblar-se a HRMS, com el *Drift tube* (Agilent Technologies), *Travelling-Wave* (Waters Corporation) i *Trapped-IMS* (Bruker Daltonics). Per tant, és probable que l'ús dels sistemes IMS-HRMS per a l'anàlisi toxicològica i forense siga prompte un tema candent.

Els valors de CCS de les molècules ionitzades poden determinar-se directament a partir del *drift time*, i s'utilitzen comunament com un paràmetre d'identificació addicional. No obstant això, seria interessant l'aplicació d'aqueixos valors per a trobar possibles metabòlits. De manera similar a l'estratègia *mass defect filtering* per a la detecció de metabòlits, és possible que els valors de CCS d'un compost relacionat (per exemple, de metabòlits) presenten una xicoteta variació i, per tant, puguen utilitzar-se per al filtrat de compostos. Així doncs, es tracta d'una

interessant aplicació de les dades de l'IMS-HRMS que pot explorar-se per als experiments d'elucidació de metabòlits.

Encara queda molta faena per fer en el camp de la investigació de les NPS, i molts treballs per escriure.

7.2. Sugerencias para Trabajos futuros

La mayoría de los estudios presentados en esta Tesis Doctoral se han desarrollado en el Instituto Universitario de Plaguicidas y Aguas de la Universitat Jaume I (Castelló de la Plana, España), y dos estudios se realizaron en el Departamento de Medicina Forense (Sección de Química Forense) de la Universidad de Copenhague (Copenhague, Dinamarca). Esta tesis incluye todo el trabajo realizado durante 4 años de colaboración con diferentes instituciones. Continuando con estas colaboraciones, se pueden proponer y desarrollar investigaciones adicionales en los próximos años.

Es importante continuar la monitorización de las NPS que se consumen en España en colaboración con Energy Control para entender los patrones y tendencias de consumo. Además, también es interesante la identificación y caracterización analítica completa de las nuevas NPS no reportadas, profundizando en su origen, tal y como se presenta en el **Capítulo 2**.

El desarrollo de nuevas estrategias analíticas para la identificación de compuestos es también una importante línea de investigación. De forma similar al enfoque realizado en el **artículo de investigación V** para la identificación de catinonas, esta metodología podría explorarse para la identificación de SCRA u opiáceos sintéticos en las incautaciones, ya que estos compuestos también presentan vías de fragmentación similares. Además, la aplicación de estrategias de tipo *common fragment ion* y *common neutral loss* durante el análisis mediante HRMS ayudará a la elucidación directa de compuestos desconocidos, o moléculas no consideradas, durante análisis de tipo *suspect screening*.

La información presentada en el **Capítulo 4**, sobre la aplicación de modelos *in vitro* para estudios de metabolismo, podría complementarse con la determinación de los metabolitos *in vivo* utilizando ratones o ratas, a fin de aumentar la confianza en la idoneidad de los biomarcadores de consumo propuestos. Además, podrían realizarse también experimentos *in vitro* de hepatocitos cultivados tipo sándwich para determinar el efluente biliar de los metabolitos elucidados.

Una línea de investigación interesante son los experimentos de tipo ADME, ofreciendo una amplia gama de posibilidades a explorar en el campo de la investigación con NPS. En el **Capítulo 5** se presentan dos aplicaciones de los modelos *in vivo*: la elucidación de metabolitos y su farmacocinética. Podrían desarrollarse más estudios con ratones y ratas, como la determinación de los metabolitos y su distribución a lo largo del tiempo en diferentes tejidos como el cerebro, el hígado, el riñón, la sangre y, por último, la excreción de orina. Estos estudios proporcionarán información adicional para comprender el metabolismo, la toxicidad, la potencia y la excreción de las NPS.

Como se muestra en el **Capítulo 6**, el consumo de SCRA entre los adolescentes bajo tratamiento y/o control es un problema grave. Por lo tanto, se deben realizar estudios adicionales para la vigilancia del consumo de NPS en esta población, en colaboración con los servicios médicos y sociales.

En el **artículo de investigación XII** se presenta uno de los beneficios de los instrumentos IMS-HRMS para análisis de tipo *suspect screening*: obtener espectros de fragmentación más limpios que podrían ayudar a la identificación de los compuestos. El uso de instrumentos IMS-HRMS ha aumentado en los últimos años gracias al desarrollo de diferentes tecnologías IMS que pueden acoplarse a HRMS, como el *Drift tube* (Agilent Technologies), *Travelling-Wave* (Waters Corporation) y *Trapped-IMS* (Bruker Daltonics). Por lo tanto, es probable que el uso de los sistemas IMS-HRMS para el análisis toxicológico y forense sea pronto un tema candente.

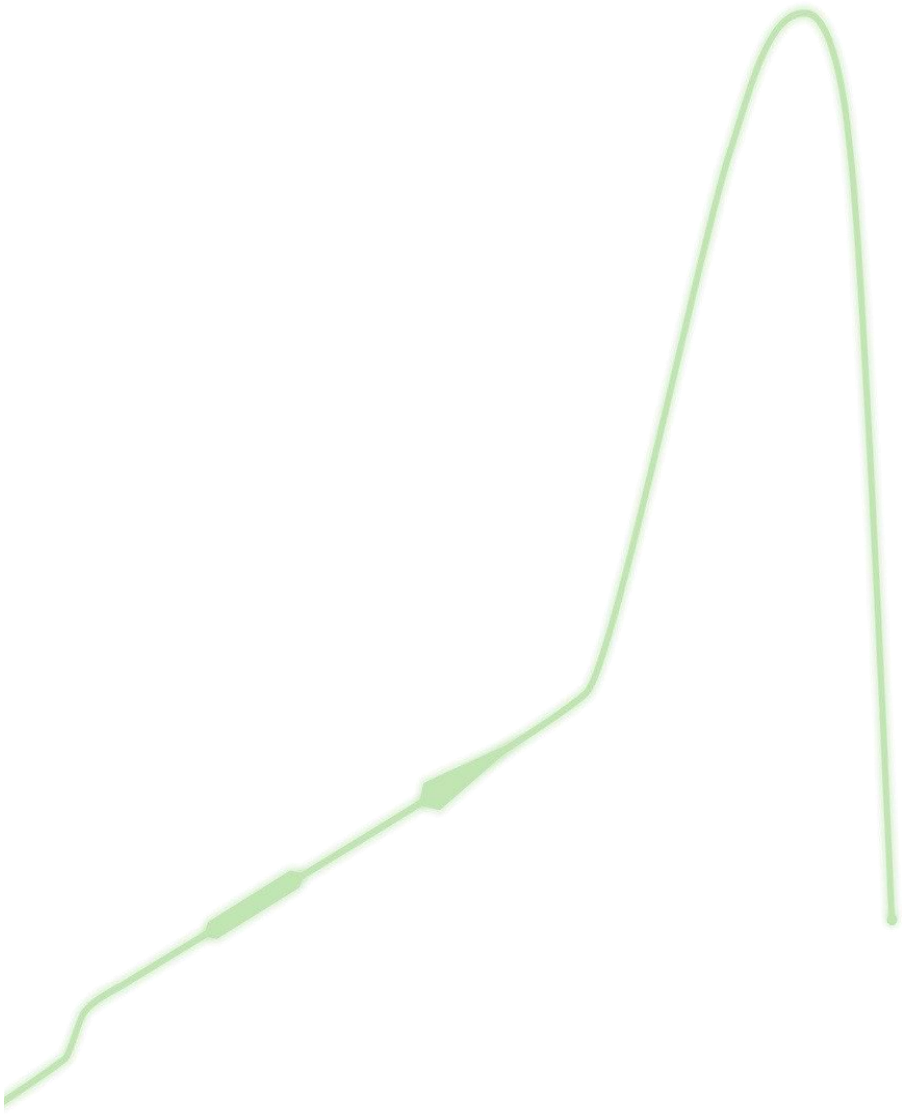
Los valores de CCS de las moléculas ionizadas pueden determinarse directamente en base al *drift time*, y se utilizan comúnmente como un parámetro de identificación adicional. No obstante, sería interesante la aplicación de esos valores para encontrar posibles metabolitos. De manera similar a la estrategia *mass defect filtering* para la detección de metabolitos, es posible que los valores de CCS de un compuesto relacionado (por ejemplo, de metabolitos) presenten una pequeña variación y, por lo tanto, puedan utilizarse para el filtrado de

compuestos. Así pues, se trata de una interesante aplicación de los datos del IMS-HRMS que puede explorarse para los experimentos de elucidación de metabolitos.

Todavía queda mucho trabajo por hacer en el campo de la investigación de las NPS, y muchos trabajos por escribir.

ANNEX

CO-AUTHOR AGREEMENT



UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana,
14th September 2020

I, **María Ibáñez Martínez**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Fornís, I., Ventura, M., Gil, C., Calzada, N., Sancho, J.V., Hernández, F., Ibáñez, M.: Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test. Anal. 9, 1073–1080 (2017). https://doi.org/10.1002/dta.2182

2. Fabregat-Safont, D., Barneo-Muñoz, M., Martinez-Garcia, F., Sancho, J.V., Hernández, F., Ibáñez, M.: Proposal of 5-methoxy- N -methyl- N -isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. J. Chromatogr. A. 1508, 95–105 (2017). https://doi.org/10.1016/j.chroma.2017.06.010

3. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Guillamón, E., Sancho, J. V., Hernández, F., Ibáñez, M.: Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017). https://doi.org/10.1038/s41598-017-06778-9

4. Fabregat-Safont, D., Carbón, X., Gil, C., Ventura, M., Sancho, J. V., Hernández, F., Ibáñez, M.: Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. 36, 447–457 (2018). https://doi.org/10.1007/s11419-018-0422-0

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

5. Fabregat-Safont, D., Mardal, M., Noble, C., Cannaert, A., Stove, C.P., Sancho, J.V., Linnet, K., Hernández, F., Ibáñez, M.: Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Test. Anal. 11, 1358–1368 (2019). https://doi.org/10.1002/dta.2659

6. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Hernández, F., Ibáñez, M.: Characterization of a recently detected halogenated aminorex derivative: para-fluoro-4-methylaminorex (4'F-4-MAR). Sci. Rep. 9, 8314 (2019). https://doi.org/10.1038/s41598-019-44830-y

7. Fabregat-Safont, D., Sancho, J. V., Hernández, F., Ibáñez, M.: Rapid tentative identification of synthetic cathinones in seized products taking advantage of the full capabilities of triple quadrupole analyzer. Forensic Toxicol. 37, 34–44 (2019). https://doi.org/10.1007/s11419-018-0432-y

8. Fabregat-Safont, D., Mardal, M., Sancho, J. V., Hernández, F., Linnet, K., Ibáñez, M.: Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation. J. Pharm. Anal. 10, 147–156 (2020). https://doi.org/10.1016/j.jpha.2019.12.006

9. Fabregat-Safont, D., Ripoll, C., Orengo, T., Sancho, J.V., Hernández, F., Ibáñez, M.: Variación en el patrón de consumo de cannabinoides sintéticos de una paciente a lo largo de 2018. Adicciones. 32, 228 (2020). https://doi.org/10.20882/adicciones.1379

10. Fabregat-Safont, D., Ibáñez, M., Baquero, A., Sancho, J.V., Hernández, F., Haro, G.: Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples. J. Pharm. Biomed. Anal. 186, 113298 (2020). https://doi.org/10.1016/j.jpba.2020.113298

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

11. Fabregat-Safont, D., Felis-Brittes, D., Mata-Pesquera, M., Sancho, J.V., Hernández, F., Ibáñez, M.: Direct and Fast Screening of New Psychoactive Substances Using Medical Swabs and Atmospheric Solids Analysis Probe Triple Quadrupole with Data-Dependent Acquisition. J. Am. Soc. Mass Spectrom. 31, 1610–1614 (2020). https://doi.org/10.1021/jasms.0c00112

12. Fabregat-Safont, D., Barneo-Muñoz, M., Carbón, X., Hernández, F., Martinez-Garcia, F., Ventura, M., Stove, CP., Sancho, J.V., Ibáñez, M.: Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. Under revision in Addiction Biology.

Signed, **María Ibáñez Martínez**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana,
14th September 2020

I, **Juan Vicente Sancho Llopis**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Fornís, I., Ventura, M., Gil, C., Calzada, N., Sancho, J.V., Hernández, F., Ibáñez, M.: Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test. Anal. 9, 1073–1080 (2017). https://doi.org/10.1002/dta.2182

2. Fabregat-Safont, D., Barneo-Muñoz, M., Martinez-Garcia, F., Sancho, J.V., Hernández, F., Ibáñez, M.: Proposal of 5-methoxy- N -methyl- N -isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. J. Chromatogr. A. 1508, 95–105 (2017). https://doi.org/10.1016/j.chroma.2017.06.010

3. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Guillamón, E., Sancho, J. V., Hernández, F., Ibáñez, M.: Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017). https://doi.org/10.1038/s41598-017-06778-9

4. Fabregat-Safont, D., Carbón, X., Gil, C., Ventura, M., Sancho, J. V., Hernández, F., Ibáñez, M.: Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. 36, 447–457 (2018). https://doi.org/10.1007/s11419-018-0422-0

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

5. Fabregat-Safont, D., Mardal, M., Noble, C., Cannaert, A., Stove, C.P., Sancho, J.V., Linnet, K., Hernández, F., Ibáñez, M.: Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Test. Anal. 11, 1358–1368 (2019). https://doi.org/10.1002/dta.2659

6. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Hernández, F., Ibáñez, M.: Characterization of a recently detected halogenated aminorex derivative: para-fluoro-4-methylaminorex (4'F-4-MAR). Sci. Rep. 9, 8314 (2019). https://doi.org/10.1038/s41598-019-44830-y

7. Fabregat-Safont, D., Sancho, J. V., Hernández, F., Ibáñez, M.: Rapid tentative identification of synthetic cathinones in seized products taking advantage of the full capabilities of triple quadrupole analyzer. Forensic Toxicol. 37, 34–44 (2019). https://doi.org/10.1007/s11419-018-0432-y

8. Fabregat-Safont, D., Mardal, M., Sancho, J. V., Hernández, F., Linnet, K., Ibáñez, M.: Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation. J. Pharm. Anal. 10, 147–156 (2020). https://doi.org/10.1016/j.jpha.2019.12.006

9. Fabregat-Safont, D., Ripoll, C., Orengo, T., Sancho, J.V., Hernández, F., Ibáñez, M.: Variación en el patrón de consumo de cannabinoides sintéticos de una paciente a lo largo de 2018. Adicciones. 32, 228 (2020). https://doi.org/10.20882/adicciones.1379

10. Fabregat-Safont, D., Ibáñez, M., Baquero, A., Sancho, J.V., Hernández, F., Haro, G.: Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples. J. Pharm. Biomed. Anal. 186, 113298 (2020). https://doi.org/10.1016/j.jpba.2020.113298

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

11. Fabregat-Safont, D., Felis-Brittes, D., Mata-Pesquera, M., Sancho, J.V., Hernández, F., Ibáñez, M.: Direct and Fast Screening of New Psychoactive Substances Using Medical Swabs and Atmospheric Solids Analysis Probe Triple Quadrupole with Data-Dependent Acquisition. J. Am. Soc. Mass Spectrom. 31, 1610–1614 (2020). https://doi.org/10.1021/jasms.0c00112

12. Fabregat-Safont, D., Barneo-Muñoz, M., Carbón, X., Hernández, F., Martinez-Garcia, F., Ventura, M., Stove, CP., Sancho, J.V., Ibáñez, M.: Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. Under revision in Addiction Biology.

Signed, **Juan Vicente Sancho Llopis**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana,
14th September 2020

I, **Félix Javier Hernández Hernández**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Fornís, I., Ventura, M., Gil, C., Calzada, N., Sancho, J.V., Hernández, F., Ibáñez, M.: Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test. Anal. 9, 1073–1080 (2017). https://doi.org/10.1002/dta.2182

2. Fabregat-Safont, D., Barneo-Muñoz, M., Martinez-Garcia, F., Sancho, J.V., Hernández, F., Ibáñez, M.: Proposal of 5-methoxy- N -methyl- N -isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. J. Chromatogr. A. 1508, 95–105 (2017). https://doi.org/10.1016/j.chroma.2017.06.010

3. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Guillamón, E., Sancho, J.V., Hernández, F., Ibáñez, M.: Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017). https://doi.org/10.1038/s41598-017-06778-9

4. Fabregat-Safont, D., Carbón, X., Gil, C., Ventura, M., Sancho, J. V., Hernández, F., Ibáñez, M.: Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. 36, 447–457 (2018). https://doi.org/10.1007/s11419-018-0422-0

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

5. Fabregat-Safont, D., Mardal, M., Noble, C., Cannaert, A., Stove, C.P., Sancho, J.V., Linnet, K., Hernández, F., Ibáñez, M.: Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Test. Anal. 11, 1358–1368 (2019). https://doi.org/10.1002/dta.2659

6. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Hernández, F., Ibáñez, M.: Characterization of a recently detected halogenated aminorex derivative: para-fluoro-4-methylaminorex (4'F-4-MAR). Sci. Rep. 9, 8314 (2019). https://doi.org/10.1038/s41598-019-44830-y

7. Fabregat-Safont, D., Sancho, J. V., Hernández, F., Ibáñez, M.: Rapid tentative identification of synthetic cathinones in seized products taking advantage of the full capabilities of triple quadrupole analyzer. Forensic Toxicol. 37, 34–44 (2019). https://doi.org/10.1007/s11419-018-0432-y

8. Fabregat-Safont, D., Mardal, M., Sancho, J. V., Hernández, F., Linnet, K., Ibáñez, M.: Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation. J. Pharm. Anal. 10, 147–156 (2020). https://doi.org/10.1016/j.jpha.2019.12.006

9. Fabregat-Safont, D., Ripoll, C., Orengo, T., Sancho, J.V., Hernández, F., Ibáñez, M.: Variación en el patrón de consumo de cannabinoides sintéticos de una paciente a lo largo de 2018. Adicciones. 32, 228 (2020). https://doi.org/10.20882/adicciones.1379

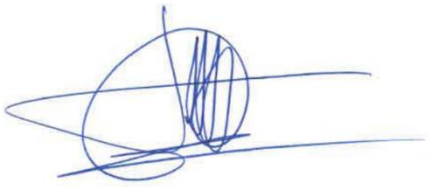
10. Fabregat-Safont, D., Ibáñez, M., Baquero, A., Sancho, J.V., Hernández, F., Haro, G.: Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples. J. Pharm. Biomed. Anal. 186, 113298 (2020). https://doi.org/10.1016/j.jpba.2020.113298

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

11. Fabregat-Safont, D., Felis-Brittes, D., Mata-Pesquera, M., Sancho, J.V., Hernández, F., Ibáñez, M.: Direct and Fast Screening of New Psychoactive Substances Using Medical Swabs and Atmospheric Solids Analysis Probe Triple Quadrupole with Data-Dependent Acquisition. J. Am. Soc. Mass Spectrom. 31, 1610–1614 (2020). https://doi.org/10.1021/jasms.0c00112

12. Fabregat-Safont, D., Barneo-Muñoz, M., Carbón, X., Hernández, F., Martinez-Garcia, F., Ventura, M., Stove, CP., Sancho, J.V., Ibáñez, M.: Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. Under revision in Addiction Biology.



Signed, **Félix Javier Hernández Hernández**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castellón, 28,
july, 2020

I, **Daniela Felis Brittes Poco**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Felis-Brittes, D., Mata-Pesquera, M., Sancho, J. V., Hernández, F., Ibáñez, M.: Direct and Fast Screening of New Psychoactive Substances Using Medical Swabs and Atmospheric Solids Analysis Probe Triple Quadrupole with Data-Dependent Acquisition. J. Am. Soc. Mass Spectrom. 31, 1610–1614 (2020). https://doi.org/10.1021/jasms.0c00112

Signed, **Daniela Felis Brittes Poco**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana, 28th July 2020

I, **María Mata Pesquera**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Felis-Brittes, D., Mata-Pesquera, M., Sancho, J. V., Hernández, F., Ibáñez, M.: Direct and Fast Screening of New Psychoactive Substances Using Medical Swabs and Atmospheric Solids Analysis Probe Triple Quadrupole with Data-Dependent Acquisition. J. Am. Soc. Mass Spectrom. 31, 1610–1614 (2020). https://doi.org/10.1021/jasms.0c00112

Signed, **María Mata Pesquera**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castellón de la Plana, 27th July 2020

I, **Eva María Guillamón Torres**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Guillamón, E., Sancho, J. V., Hernández, F., Ibáñez, M.: Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017). https://doi.org/10.1038/s41598-017-06778-9

Signed, **Eva María Guillamón Torres**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana,
September 15, 2020

I, **Fernando Martínez García**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Barneo-Muñoz, M., Martinez-Garcia, F., Sancho, J.V., Hernández, F., Ibáñez, M.: Proposal of 5-methoxy- N -methyl- N -isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. J. Chromatogr. A. 1508, 95–105 (2017). https://doi.org/10.1016/j.chroma.2017.06.010

2. Fabregat-Safont, D., Barneo-Muñoz, M., Carbón, X., Hernández, F., Martinez-Garcia, F., Ventura, M., Stove, CP., Sancho, J.V., Ibáñez, M.: Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. Under revision in Addiction Biology.

Firmado Fernando Martinez Garcia
el día 15/09/2020 con un certificado
emitido por AVVC

Signed, **Fernando Martínez García**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castellón de la Plana, 15, September, 2020

I, **Manuela Barneo Muñoz**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Barneo-Muñoz, M., Martinez-Garcia, F., Sancho, J.V., Hernández, F., Ibáñez, M.: Proposal of 5-methoxy- N -methyl- N -isopropyltryptamine consumption biomarkers through identification of in vivo metabolites from mice. J. Chromatogr. A. 1508, 95–105 (2017). https://doi.org/10.1016/j.chroma.2017.06.010

2. Fabregat-Safont, D., Barneo-Muñoz, M., Carbón, X., Hernández, F., Martinez-Garcia, F., Ventura, M., Stove, CP., Sancho, J.V., Ibáñez, M.: Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. Under revision in Addiction Biology.

Signed, **Manuela Barneo Muñoz**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Barcelona, 17th September 2020

I, **Mireia Ventura Vilamala**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Fornís, I., Ventura, M., Gil, C., Calzada, N., Sancho, J.V., Hernández, F., Ibáñez, M.: Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test. Anal. 9, 1073–1080 (2017). https://doi.org/10.1002/dta.2182

2. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Guillamón, E., Sancho, J. V., Hernández, F., Ibáñez, M.: Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017). https://doi.org/10.1038/s41598-017-06778-9

3. Fabregat-Safont, D., Carbón, X., Gil, C., Ventura, M., Sancho, J. V., Hernández, F., Ibáñez, M.: Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. 36, 447–457 (2018). https://doi.org/10.1007/s11419-018-0422-0

4. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Hernández, F., Ibáñez, M.: Characterization of a recently detected halogenated aminorex derivative: para-fluoro-4-methylaminorex (4'F-4-MAR). Sci. Rep. 9, 8314 (2019). https://doi.org/10.1038/s41598-019-44830-y

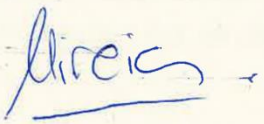
5. Fabregat-Safont, D., Barneo-Muñoz, M., Carbón, X., Hernández, F., Martinez-Garcia, F.,

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Ventura, M., Stove, CP., Sancho, J.V., Ibáñez, M.: Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. Under revision in Addiction Biology.



Signed, **Mireia Ventura Vilamala**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Barcelona,
25/9/2020

I, **Xoán Carbón Mallol**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Guillamón, E., Sancho, J. V., Hernández, F., Ibáñez, M.: Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017). https://doi.org/10.1038/s41598-017-06778-9

2. Fabregat-Safont, D., Carbón, X., Gil, C., Ventura, M., Sancho, J. V., Hernández, F., Ibáñez, M.: Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. 36, 447–457 (2018). https://doi.org/10.1007/s11419-018-0422-0

3. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Hernández, F., Ibáñez, M.: Characterization of a recently detected halogenated aminorex derivative: para-fluoro-4-methylaminorex (4′F-4-MAR). Sci. Rep. 9, 8314 (2019). https://doi.org/10.1038/s41598-019-44830-y

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

4. Fabregat-Safont, D., Barneo-Muñoz, M., Carbón, X., Hernández, F., Martinez-Garcia, F., Ventura, M., Stove, CP., Sancho, J.V., Ibáñez, M.: Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. Under revision in Addiction Biology.

Signed, **Xoán Carbón Mallol**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

BARCELONA Place, day,
22th september 2020 month, year

I, **Iván Fornís Espinosa**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Fornís, I., Ventura, M., Gil, C., Calzada, N., Sancho, J.V., Hernández, F., Ibáñez, M.: Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test. Anal. 9, 1073–1080 (2017). https://doi.org/10.1002/dta.2182

2. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Guillamón, E., Sancho, J. V., Hernández, F., Ibáñez, M.: Updating the list of known opioids through identification and characterization of the new opioid derivative 3,4-dichloro-N-(2-(diethylamino)cyclohexyl)-N-methylbenzamide (U-49900). Sci. Rep. 7, 6338 (2017). https://doi.org/10.1038/s41598-017-06778-9

3. Fabregat-Safont, D., Carbón, X., Ventura, M., Fornís, I., Hernández, F., Ibáñez, M.: Characterization of a recently detected halogenated aminorex derivative: para-fluoro-4-methylaminorex (4′F-4-MAR). Sci. Rep. 9, 8314 (2019). https://doi.org/10.1038/s41598-019-44830-y

Signed, **Iván Fornís Espinosa**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Barcelona 22th September 2020 Place, day, month, year

I, **Cristina Gil Lladanosa**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Fornís, I., Ventura, M., Gil, C., Calzada, N., Sancho, J.V., Hernández, F., Ibáñez, M.: Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test. Anal. 9, 1073–1080 (2017). https://doi.org/10.1002/dta.2182

2. Fabregat-Safont, D., Carbón, X., Gil, C., Ventura, M., Sancho, J. V., Hernández, F., Ibáñez, M.: Reporting the novel synthetic cathinone 5-PPDI through its analytical characterization by mass spectrometry and nuclear magnetic resonance. Forensic Toxicol. 36, 447–457 (2018). https://doi.org/10.1007/s11419-018-0422-0

Signed, **Cristina Gil Lladanosa**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Pinós (Lleida), 23th September, 2020

I, **Núria Calzada Álvarez**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Fornís, I., Ventura, M., Gil, C., Calzada, N., Sancho, J.V., Hernández, F., Ibáñez, M.: Identification and characterization of a putative new psychoactive substance, 2-(2-(4-chlorophenyl)acetamido)-3-methylbutanamide, in Spain. Drug Test. Anal. 9, 1073–1080 (2017). https://doi.org/10.1002/dta.2182

Signed, **Núria Calzada Álvarez**

in accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Copenhagen,
28th July, 2020

I, **Marie Mardal**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Mardal, M., Noble, C., Cannaert, A., Stove, C.P., Sancho, J. V., Linnet, K., Hernández, F., Ibáñez, M.: Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Test. Anal. 11, 1358–1368 (2019). https://doi.org/10.1002/dta.2659

2. Fabregat-Safont, D., Mardal, M., Sancho, J. V., Hernández, F., Linnet, K., Ibáñez, M.: Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation. J. Pharm. Anal. 10, 147–156 (2020). https://doi.org/10.1016/j.jpha.2019.12.006

Signed, **Marie Mardal**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Pennsylvania -USA,

27th, July, 2020

I, **Carolina Noble**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Mardal, M., Noble, C., Cannaert, A., Stove, C.P., Sancho, J. V., Linnet, K., Hernández, F., Ibáñez, M.: Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Test. Anal. 11, 1358–1368 (2019). https://doi.org/10.1002/dta.2659

Carolina Noble, Ph.D.
Forensic Toxicologist

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Copenhagen,
28th July, 2020

I, **Kristian Linnet**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Mardal, M., Noble, C., Cannaert, A., Stove, C.P., Sancho, J. V., Linnet, K., Hernández, F., Ibáñez, M.: Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Test. Anal. 11, 1358–1368 (2019). https://doi.org/10.1002/dta.2659

2. Fabregat-Safont, D., Mardal, M., Sancho, J. V., Hernández, F., Linnet, K., Ibáñez, M.: Metabolic profiling of four synthetic stimulants, including the novel indanyl-cathinone 5-PPDi, after human hepatocyte incubation. J. Pharm. Anal. 10, 147–156 (2020). https://doi.org/10.1016/j.jpha.2019.12.006

Signed, **Kristian Linnet**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

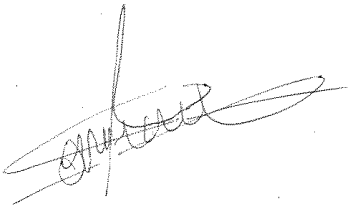
UNIVERSITAT JAUME I
Escola de Doctorat · ED

Ghent, 18 September 2020

I, **Annelies Cannaert**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Mardal, M., Noble, C., Cannaert, A., Stove, C.P., Sancho, J. V., Linnet, K., Hernández, F., Ibáñez, M.: Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Test. Anal. 11, 1358–1368 (2019). https://doi.org/10.1002/dta.2659



Signed, **Annelies Cannaert**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Ghent,
Belgium,
18/09/2020

I, **Christophe P. Stove**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Mardal, M., Noble, C., Cannaert, A., Stove, C.P., Sancho, J. V., Linnet, K., Hernández, F., Ibáñez, M.: Comprehensive investigation on synthetic cannabinoids: Metabolic behavior and potency testing, using 5F-APP-PICA and AMB-FUBINACA as model compounds. Drug Test. Anal. 11, 1358–1368 (2019). https://doi.org/10.1002/dta.2659.

2. Fabregat-Safont, D., Barneo-Muñoz, M., Carbón, X., Hernández, F., Martinez-Garcia, F., Ventura, M., Stove, CP., Sancho, JV., Ibáñez, M.: Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats. Under revision in Addiction Biology.

LABORATORIUM VOOR TOXICOLOGIE
UNIVERSITEIT GENT
PROF. DR. C. STOVE
Ottergemsesteenweg 460
B-9000 GENT

Signed, **Christophe P. Stove**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castellón, 15 September, 2020

I, **Abel Baquero Escribano**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Ibáñez, M., Baquero, A., Sancho, J.V., Hernández, F., Haro, G.: Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples. J. Pharm. Biomed. Anal. 186, 113298 (2020). https://doi.org/10.1016/j.jpba.2020.113298

Signed, **Abel Baquero Escribano**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

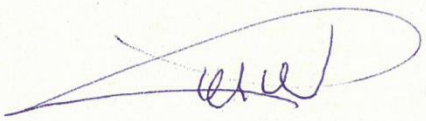
UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló, 15,
September,
2020

I, **Gonzalo Rafael Haro Cortés**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Ibáñez, M., Baquero, A., Sancho, J.V., Hernández, F., Haro, G.: Investigation on the consumption of synthetic cannabinoids among teenagers by the analysis of herbal blends and urine samples. J. Pharm. Biomed. Anal. 186, 113298 (2020). https://doi.org/10.1016/j.jpba.2020.113298



Signed, **Gonzalo Rafael Haro Cortés**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Place, day,
month, year

I, **Carmen Ripoll Alandés**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Ripoll, C., Orengo, T., Sancho, J.V., Hernández, F., Ibáñez, M.: Variación en el patrón de consumo de cannabinoides sintéticos de una paciente a lo largo de 2018. Adicciones. 32, 228 (2020). https://doi.org/10.20882/adicciones.1379

Signed, **Carmen Ripoll Alandés**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

Annex. Co-author agreement

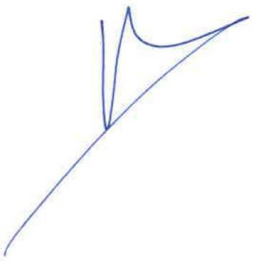
UNIVERSITAT JAUME I
Escola de Doctorat · ED

Valencia, 30th
July 2020

I, **Teresa Orengo Caus**, hereby authorise **David Fabregat Safont** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Fabregat-Safont, D., Ripoll, C., Orengo, T., Sancho, J.V., Hernández, F., Ibáñez, M.: Variación en el patrón de consumo de cannabinoides sintéticos de una paciente a lo largo de 2018. Adicciones. 32, 228 (2020). https://doi.org/10.20882/adicciones.1379



Signed, **Teresa Orengo Caus**

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...)
"Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"