

Ana Sofia da Costa Almeida

Semi-preparative enantioresolution, racemization and enantioselectivity studies with MDPV and binding affinity studies with synthetic cathinones

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Toxicologia Analítica
Clínica e Forense

Trabalho realizado sob orientação da Professora Doutora Carla Fernandes e do Professor
Doutor Fernando Remião

October 2022

Supervisors

Supervisor: Prof. Dr. Carla Fernandes

Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas,
Faculdade de Farmácia, Universidade do Porto

Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto,
Terminal de Cruzeiros do Porto de Leixões

Cosupervisor: Prof. Dr. Fernando Remião

UCIBIO – Applied Molecular Biosciences Unit, REQUIMTE, laboratory of Toxicology,
Department of Biological Sciences, Faculty of Pharmacy, University of Porto

Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy,
University of Porto

DE ACORDO COM A LEGISLAÇÃO EM VIGOR, NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTA DISSERTAÇÃO.

This work was carried out in Laboratory of Organic and Pharmaceutical Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto and Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto. This research was supported by national funds by FCT (UIDB/04423/2020, UIDB/MULTI/04378/2020, UIDP/04423/2020 LA/P/0140/2020, PTDC/CTA-AMB/6686/2020 projects) and BYT-Plus 2021/22 scholarship from CIIMAR and Amadeu Dias Foundation.



Some of the results of this work were/will be presented as poster/oral communications:

Poster communications:

A.S. Almeida, F. Remião, C. Fernandes, Enantioseparation and racemization of MDPV by liquid chromatography, Congress 2022 APCF-TOXRUN, 7th-8th April 2022 (**Annex 1**)

A.S. Almeida, F. Remião, C. Fernandes, Semi-preparative enantioresolution of MDPV enantiomers and racemization study by liquid chromatography, 14th Edition of IJUP, 4th-6th May 2022 (**Annex 2**)

A.S. Almeida, F. Remião, C. Fernandes, Semi-preparative enantioresolution, racemization and cytotoxicity studies with MDPV enantiomers, Blue Think Conference 2022 CIIMAR, 27th September 2022 (**Annex 3**)

A.S. Almeida, B. Silva, S. Cravo, F. Remião, C. Fernandes, Development and validation of an UHPLC-UV method for MDPV quantification to evaluate enantioselectivity on absorption using the Caco-2 cell line, 12th National Chromatography Meeting, 6th-8th December 2022 (**Annex 4**)

Oral communications:

A.S. Almeida, B. Silva, S. Cravo, F. Remião, C. Fernandes, Semi-preparative separation and racemization study of MDPV enantiomers by liquid chromatography and absorption study on Caco-2 model, XXVI Encontro Galego-Portugués de Química, 16th-18th November 2022 (**Annex 5**)

Abstracts and presented posters available in Annexes.

Articles in International Peer-Reviewed Journals:

T. Cardoso*, A.S. Almeida*, F. Remião, C. Fernandes, Enantioresolution and binding affinity studies on human serum albumin: Recent applications and trends, Chemosensors 2021, 9, 304. <https://doi.org/10.3390/chemosensors9110304>

* These authors contributed equally to this work.

A.S. Almeida, B. Silva, P.G. Pinho, F. Remião, C. Fernandes, Synthetic cathinones: recent developments, enantioselectivity studies and enantioseparation methods, Molecules 2022, 27(7), 2057. <https://doi.org/10.3390/molecules27072057>

ACKNOWLEDGMENTS

Depois deste longo ano de trabalho, não podia deixar de agradecer a todas as pessoas que tornaram a realização desta dissertação possível. Gostaria de agradecer:

À minha orientadora, a professora Carla Fernandes, pelo acompanhamento, motivação, ensinamentos que me transmitiu e confiança que sempre depositou em mim. Por todas as oportunidades que me deu durante o meu, ainda pequeno, percurso na investigação. Por toda a simpatia e carinho que sempre demonstrou.

Ao professor Fernando Remião, o meu coorientador, pela oportunidade de trabalhar com ele e por todo o apoio e disponibilidade ao longo deste ano.

À Dra. Sara Cravo por todo o apoio no HPLC, por estar sempre disponível para me ajudar, responder às minhas dúvidas e arranjar soluções para os problemas que foram surgindo.

Ao Doutor João Pedro Silva pela ajuda no início da minha aventura na cultura celular e pela disponibilidade para responder sempre às minhas dúvidas.

À Doutora Bárbara Silva pelo grande apoio na parte experimental do meu trabalho, por toda a simpatia, disponibilidade para me ajudar e conhecimentos que me transmitiu.

A todos os professores que fizeram parte do meu percurso académico.

A todos os meus colegas de laboratório por me terem recebido muito bem e me ajudarem sempre que precisava. Um especial agradecimento à Cláudia, Joana Cardoso e Liliana pela companhia e amizade.

A todos os meus amigos de longa data (Leonor, Diva, Francisco, Ana Moutinho, Helena, Emanuel, Joana Gomes, Ângela, Joana Teixeira, Filipa Carvalhosa...) por terem estado do meu lado durante estes anos todos, por ouvirem sempre os meus desabafos e por serem uma boa “distração” durante alturas mais stressantes.

Por fim e, sem dúvida, não menos importante, à minha família, em especial à minha mãe e à minha irmã, por me tornarem na pessoa que sou hoje, por todo o amor e por estarem sempre comigo durante todos os bons e maus momentos.

ABSTRACT

Synthetic cathinones, such as 3,4-methylenedioxypyrovalerone (MDPV), are widely abused due to their psychostimulant effects. Being chiral compounds, studying the influence of stereochemistry in their biological/toxicological properties (enantioselectivity) is of great importance as well as the racemization phenomena since enantiomers can undergo racemization in certain temperatures and acidic/basic environments. Moreover, enantioresolution methods that allow the separation of the enantiomers of synthetic cathinones have been reported, being liquid chromatography (LC) using chiral stationary phases (CSPs) the technique of choice. Additionally, LC has also been widely used in binding affinity studies with human serum albumin (HSA).

In this dissertation, the semi-preparative enantioresolution of MDPV and a racemization study were performed by LC using amylose-derived CSPs. The enantiomers were successfully separated with good enantioselectivity and resolution and collected with high enantiomeric ratios (e.r.) and recovery rates. The racemization was only affected by higher temperatures. For 48 h at room temperature and 24 h at 37 °C the enantiomers remained stable.

The potential enantioselectivity of MDPV in cytotoxicity and expression of neuroplasticity-involved proteins was evaluated *in vitro* in the SH-SY5Y cell line. Furthermore, the potential enantioselectivity of MDPV in the passage across the Caco-2 monolayer was also evaluated. To detect and quantify MDPV, an UHPLC-UV method was developed and validated. No statistically significant differences were found between the enantiomers (no enantioselectivity) in any of the assays except for the P_{app} values obtained in the permeability assay in the basolateral to apical direction.

Additionally, using selected synthetic cathinones, information on the HSA binding site of these compounds was obtained by displacement chromatography using an HSA chiral column and (S)-ibuprofen and warfarin as competitors. Competition was observed between the examined compounds and both competitors, except for racemic pentedrone with warfarin, consistent with an allosteric competition with a non-cooperative binding mechanism.

Keywords: Binding affinity studies; Enantiomeric resolution; Enantioselectivity; Liquid chromatography; MDPV; Racemization; Synthetic cathinones

RESUMO

Catinonas sintéticas, como a 3,4-metilenodioxipirovalerona (MDPV), são amplamente consumidas devido às suas propriedades psicostimulantes. Sendo compostos quirais, o estudo da influência da estereoquímica nas suas propriedades biológicas/toxicológicas (enantiosseletividade) é de grande importância, assim como o estudo do fenómeno da racemização uma vez que enantiómeros podem racemizar em determinadas temperaturas e em ambientes ácidos/básicos. Para além disso, têm sido descritos métodos que permitem a separação dos enantiómeros de catinonas sintéticas, sendo a cromatografia líquida (CL) com a utilização de fases estacionárias quirais (FEQs) a técnica mais escolhida. A CL tem sido também muito utilizada em estudos de afinidade com albumina sérica humana (ASH).

Neste trabalho, foi realizada a enantiorresolução semi-preparativa da MDPV e um estudo de racemização por CL utilizando FEQs derivadas de amilose. Os enantiómeros foram separados com sucesso com uma boa enantiosseletividade e resolução e recolhidos com altas razões enantioméricas (r.e.) e taxas de recuperação. A racemização foi apenas afetada por temperaturas mais elevadas. Durante 48 h à temperatura ambiente e 24 h a 37 °C, os enantiómeros mantiveram-se estáveis.

A potencial enantiosseletividade da MDPV na citotoxicidade e expressão de proteínas envolvidas na neuroplasticidade foi avaliada *in vitro* na linha celular SH-SY5Y. Além disso, a potencial enantiosseletividade da MDPV na passagem através da monocamada das Caco-2 foi também avaliada. Para detetar e quantificar a MDPV, um método de UHPLC-UV foi desenvolvido e validado. Não foram encontradas diferenças estatisticamente significativas entre os enantiómeros em nenhum dos ensaios (não houve enantiosseletividade), exceto nos valores de P_{app} obtidos no ensaio de permeabilidade na direção basolateral para apical.

Adicionalmente, utilizando catinonas sintéticas selecionadas, foi obtida informação sobre o local de ligação na ASH destes compostos através de um estudo cromatográfico de competição utilizando uma coluna quiral de ASH e como competidores o (S)-ibuprofeno e a varfarina. Foi observada competição entre os compostos estudados e ambos os competidores, exceto para a pentedrona racémica com a varfarina, consistente com competição alostérica com um mecanismo de ligação não cooperativo

Palavras-chave: Catinonas sintéticas; Cromatografia líquida; Enantiosseletividade; Estudos de afinidade; MDPV; Racemização; Resolução enantiomérica

INDEX

ACKNOWLEDGMENTS	v
ABSTRACT	vi
RESUMO	vii
FIGURE INDEX	xii
TABLE INDEX	xvi
ABBREVIATIONS AND SYMBOLS.....	xvii
INTRODUCTION	1
1. RELEVANCE OF CHIRALITY AND ENANTIOSELECTIVITY IN PHARMACO/TOXYCODYNAMIC AND PHARMACO/TOXYCOCYNETIC EVENTS	2
2. SYNTHETIC CATHINONES: RECENT DEVELOPMENTS, ENANTIOSELECTIVITY STUDIES AND ENANTIOSEPARATION METHODS	4
2.1. Introduction	5
2.2. Classification of Synthetic Cathinones.....	7
2.3. Chronological evolution and recent developments of cathinone derivatives	9
2.4. Toxicokinetic properties.....	15
2.5. Mechanism of action and effects	17
2.6. Enantioselectivity studies	19
2.7. Enantiomeric resolution.....	25
2.8. Conclusions	35
2.9. Updates on novel synthetic cathinones and enantioselectivity studies with synthetic cathinones between February to October 2022	35
3. BINDING AFFINITY STUDIES ON HUMAN SERUM ALBUMIN	38
3.1. Human Serum Albumin	38
3.2. High-Performance Affinity Liquid Chromatography	39
AIMS.....	42
MATERIAL AND METHODS	44
1. Semi-preparative enantioresolution of the enantiomers of MDPV.....	45

1.1.	Reagents and samples	45
1.2.	Sample preparation.....	45
1.3.	Instrumentation and chromatographic conditions	45
1.4.	Hydrochloride formation	45
1.5.	Chromatographic parameters determination	45
2.	Racemization study with the enantiomers of MDPV	46
2.1.	Reagents and samples	46
2.2.	Sample preparation.....	46
2.3.	Instrumentation and chromatographic conditions	46
3.	Enantioselectivity studies with the enantiomers of MDPV in the SH-SY5Y cell line	47
3.1.	Reagents	47
3.2.	Cell culture	47
3.3.	MTT reduction assay.....	48
3.4.	Neutral Red (NR) assay	48
3.5.	Preparation of total protein extracts.....	48
3.6.	Western Blotting analysis	49
3.7.	Statistical analysis.....	49
4.	Permeability assay with the enantiomers of MDPV using the Caco-2 cell line .	50
4.1.	Reagents	50
4.2.	Instrumental and chromatographic conditions	50
4.3.	Method validation	50
4.3.1.	Specificity	50
4.3.2.	Linearity.....	51
4.3.3.	Accuracy.....	51
4.3.4.	Precision.....	51
4.3.5.	LOD and LOQ	52

4.3.6. Stability.....	52
4.4. Caco-2 cell culture	52
4.4.1. NR assay.....	52
4.4.2. Permeability assay	53
4.5. Statistical analysis.....	54
5. Displacement experiment with synthetic cathinones on HSA	54
5.1. Reagents and samples	54
5.2. Sample preparation.....	54
5.3. Instrumentation and chromatographic conditions	54
5.4. Determination of the association equilibrium constant (K_i).....	55
RESULTS AND DISCUSSION.....	56
1. Semi-preparative enantioresolution of the enantiomers of MDPV.....	57
2. Racemization study with the enantiomers of MDPV	59
3. Enantioselectivity studies with the enantiomers of MDPV in the SH-SY5Y cell line	64
3.1. Effects of the enantiomers of MDPV in cytotoxicity.....	64
3.2. Effects of MDPV enantiomers in the expression of proteins involved in neuroplasticity	65
4. Permeability assay with the enantiomers of MDPV using the Caco-2 cell line .	69
4.1. Chromatographic method.....	70
4.2. Method validation	71
4.2.1. Specificity	71
4.2.2. Linearity.....	72
4.2.3. Accuracy.....	72
4.2.4. Precision.....	73
4.2.5. LOD and LOQ	73
4.2.6. Stability.....	73
4.3. Cell viability assay.....	74

4.4. Permeability assay	74
5. Displacement experiment with synthetic cathinones on HSA	78
CONCLUSIONS AND FUTURE WORK.....	82
REFERENCES	83
ANNEXS.....	102

FIGURE INDEX

Figure 1 – New psychoactive substances reported for the first time from 2005 to 2020 (October) divided by categories [20].	5
Figure 2 – Structures of cathinone (1) and amphetamine (2).	6
Figure 3 – Some bioactive components of khat: cathine (3); ascorbic acid (4); α -terpineol (5); myricetin (6); and celastrol (7).	6
Figure 4 – Core structure of cathinone derivatives.	7
Figure 5 – Examples of synthetic cathinones from each group based on the substitution pattern: 8-18 from the <i>N</i> -alkylated cathinones, 19-22 from the pyrrolidinophenone cathinones, 23-26 from the 3,4-methylenedioxy cathinones and 27 and 28 from the mixed cathinones.	9
Figure 6 - Timeline of events related to the history of synthetic cathinones.	9
Figure 7 - Structures of the most recent cathinone derivatives 29-48 .	12
Figure 8 - Structures of some of the most recent cathinone derivatives 49-69 .	13
Figure 9 - Structures of cathinone derivatives synthesized in controlled laboratories (70-80).	14
Figure 10 - General metabolic pathways of synthetic cathinones: a) main metabolic pathways of <i>N</i> -alkylated cathinones; b) reduction of the β -keto moiety to an alcohol; c) hydroxylation and further oxidation of the methyl substituent of the aromatic ring to a carboxylic acid; d) metabolism of the 3,4-methylenedioxy ring; e) metabolism of the pyrrolidinyl ring to a lactam.	16
Figure 11 - Modes of action of synthetic cathinones at the central nervous system. (DA: dopamine; NA: noradrenaline; 5-HT: 5-hydroxytryptamine (serotonin); DAT: dopamine transporters; NAT: noradrenaline transporters; SERT: serotonin transporters; VMAT ₂ : vesicular monoamine transporter-2).	18
Figure 12 - Effects of the enantiomers and racemate of mephedrone on monoamine release via DAT and SERT [73]. DAT: dopamine transporters; MPP ⁺ : 1-methyl-4-phenylpyridinium (substrate for DAT); SERT: serotonin transporters; 5HT: 5-hydroxytryptamine (serotonin).	20
Figure 13 - Effects of <i>S</i> -(-)-4-methylcathinone and <i>R</i> -(+)-4methylcathinone on monoamine release via DAT, SERT and NET ((a) and (b), respectively) and on ICSS ((c) and (d), respectively) [74]. DAT: dopamine transporters; NET: norepinephrine transporters; SERT: serotonin transporters; ICSS: intracranial self-stimulation. * indicates significant increases and # indicates significant decreases in ICSS rates relative to vehicle for at least one stimulation frequency as determined by analysis of frequency–rate curves in panels (a,b).	21

Figure 14 - Avoidance tests with racemic α -PVP (A), S- α -PVP (B) and R- α -PVP (C) [81]. *Significantly different from 1.5, 3, and 5	22
Figure 15 – Enantioselectivity studies on the absorption of methylone (24) and pentedrone (14) using Caco-2 cell line. *** $p < 0.001$, **** $p < 0.0001$	23
Figure 16 – Enantioselectivity studies on neurocytotoxicity of methylone (24) and pentedrone (14) towards dopaminergic SH-SY5Y cells and the role of the efflux transporter multidrug resistance associated protein 1 (MRP1) and P-glycoprotein (P-gp). ** $p < 0.01$, **** $p < 0.0001$. (Reprint with permission from [86], Copyright (2021) ELSEVIER).....	24
Figure 17 - Chromatograms of the semipreparative enantioseparation of pentedrone (A) and methylone (B). (Reprint with permission from [116], Copyright (2018) ELSEVIER)..	32
Figure 18 – Chiral separation of three different cathinone derivatives (ethcathinone, 3-MEC and 4-CEC) by HPLC-UV [122].	33
Figure 19 – Methods used for the enantiomeric resolution of cathinones. HPLC: high performance liquid chromatography; LC: liquid chromatography; GC: gas chromatography; CE: capillary electrophoresis; CEC: capillary electrochromatography; SFC: super critical fluid chromatography.	34
Figure 20 - Types of CSPs used for the enantiomeric resolution of cathinones.	34
Figure 21 – Structures of the most recent cathinone derivatives (2019-2022) 81-107	36
Figure 22 – HSA's structure (from Protein Data Bank:1AO6) with Sudlow's site I and Sudlow's site II, the main binding sites, evidenced.	39
Figure 23 – Chromatogram of semi-preparative separation of the enantiomers of MDPV; Tris-3,5-dimethylphenylcarbamate amylose home-made column; Mobile phase: Hex:EtOH:DEA (97:3:0.1); Flow rate: 1.5 mL/min; UV detection: 254 nm.	57
Figure 24 – Chromatograph of the first enantiomeric fraction (E1); Tris-3,5-dimethylphenylcarbamate amylose home-made column; Mobile phase: Hex:EtOH:DEA (97:3:0.1); Flow rate: 1.5 mL/min; UV detection: 254 nm.	58
Figure 25 – Chromatograph of the second enantiomeric fraction (E2); Tris-3,5-dimethylphenylcarbamate amylose home-made column; Mobile phase: Hex:EtOH:DEA (97:3:0.1); Flow rate: 1.5 mL/min; UV detection: 254 nm.	59
Figure 26 – Chromatogram of the analytical separation of the enantiomers of MDPV; Lux Amylose-I ® column; Mobile phase: Hex:EtOH:DEA (97:3:0.1); Flow rate: 1 mL/min; UV detection: 254 nm.	60

Figure 27 - Reduction of MTT (A) and NR uptake (B) in undifferentiated and dopaminergic SH-SY5Y cells exposed to the individual enantiomers of MDPV in three concentrations (0.773; 1.165 and 1.506 mM) for 24 h. Results were expressed as mean $\pm$ SD obtained from four independent experiments, run in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. respective control (undifferentiated or differentiated cells without treatment)	65
Figure 28 – Effects of MDPV enantiomers (E1 and E2) in BDNF expression in undifferentiated SH-SY5Y cells. The expression was normalized by β -actin. Results are expressed as mean $\pm$ SD for the fold change relatively to control for at least four independent experiments (n=4) performed in duplicate.	67
Figure 29 - Effects of MDPV in Cdk5 expression in undifferentiated SH-SY5Y cells. The expression was normalized by β -actin. Results are expressed as mean $\pm$ SD for the fold change relatively to control for at least four independent experiments (n=4) performed in duplicate.	69
Figure 30 - UV spectra of MDPV [100 μ M in HBSS (+/+)], mobile phase [25 mM ammonium acetate:ACN:FA (75:25:0.1)] and HBSS(+/+).	70
Figure 31 - Chromatogram of MDPV [100 μ M in HBSS(+/+)]. Chromatographic conditions: Kinetex® EVO C18 column, 25 mM ammonium acetate:ACN:FA (75:25:0.1) as the mobile phase, flow rate: 0.12 mL/min, UV detection 236 nm.	71
Figure 32 – Chromatogram of blank sample [HBSS (+/+)]. Chromatographic conditions: Kinetex® EVO C18 column, 25 mM ammonium acetate:ACN:FA (75:25:0.1) as the mobile phase, flow rate: 0.12 mL/min, UV detection 236 nm.	72
Figure 33 – Average calibration curve (plot of concentration of MDPV vs peak area).	72
Figure 34 – Cytotoxicity evaluation in Caco-2 cells exposed to racemic MDPV (0-1500 μ M) for 24 h performed by the NR assay. Results are expressed as mean $\pm$ SD from 4 independent experiments (performed in triplicate). * $p < 0.05$, **** $p < 0.0001$ vs. control (0 μ M).	74
Figure 35 – Monitoring of TEER values from day 4 to day 21 after seeding of Caco-2 cells. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments.	75
Figure 36 – Percentage of cumulative quantity of the enantiomers of MDPV transported through the Caco-2 monolayer in each time point. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments (performed in duplicate).	76

Figure 37 – Mass balance obtained for the enantiomers of MDPV after the permeability assay with Caco-2 cells. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments (performed in duplicate).	76
Figure 38 – P_{app} values obtained for the enantiomers of MDPV after the permeability assay with Caco-2 cells. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments (performed in duplicate). * $p < 0.05$, *** $p < 0.0001$	77
Figure 39 – Efflux ratios obtained for the enantiomers of MDPV after the permeability assay with Caco-2 cells. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments (performed in duplicate).	77
Figure 40 - Results from displacement chromatography experiments with synthetic cathinones in the absence and presence of increasing concentrations of the competitors expressed by the plot of $1/k$ of the analyte versus concentration of competitor.....	80

TABLE INDEX

Table 1 - Development of analytical methods for enantioresolution of synthetic cathinones from 2018 to 2021.	27
Table 2 - Chromatograms from the racemization study after exposing E1 to different temperatures (RT, 37 °C and 70 °C) for 48 h, and respective e.r. values.....	61
Table 3 – Chromatograms from the racemization study after exposing E2 to different temperatures (RT, 37 °C and 70 °C) for 48 h, and respective e.r. values.....	61
Table 4 – Chromatograms from the racemization study after exposing E1 to different temperatures (RT, 37 °C and 70 °C), in the presence of DIPEA for 48 h, and respective e.r. values.....	62
Table 5 - Chromatograms from the racemization study after exposing E2 to different temperatures (RT, 37 °C and 70 °C), in the presence of DIPEA for 48 h, and respective e.r. values.....	63
Table 6 – Linear regression data for evaluation of linearity.....	72
Table 7 – Accuracy data.....	73
Table 8 – Inter-day and intra-day precision data.....	73
Table 9 – Sample stability after 6 weeks of storage expressed as the %peak area variation relative to day 1.....	73
Table 10 – Data obtained for mass balance, P_{app} values and efflux ratios expressed as mean $\pm$ SD.	78
Table 11 – k values obtained for selected synthetic cathinones in presence of the competitors warfarin and (S)-ibuprofen (0-20 μ M).	79
Table 12 – K_I values obtained by displacement chromatography analysis. Values were determined by the slope and intercept of the plot of $1/k$ of the analyte vs increasing concentrations of the competitor.....	81

ABREVIATIONS AND SYMBOLS

%CV - Coefficient of variation

2,4-DMEC - 2,4-Dimethylethcathinone

2,4-DMMC - 2,4-Dimethylmethcathinone

2,4-DMPPP - 2,4-Dimethyl- α -pyrrolidinopropiophenone

2,4-iso-DMC - 2,4-Dimethylisocathinone

2-Me- α -PVP - 1-(2-Methylphenyl)-2-(pyrrolidin-1-yl) pentan-1-one

3,4-Dimethoxy- α -PVP - 3,4-Dimethoxy- α -pyrrolidinopentiophenone

3-FMC - 3-Fluoromethcathinone

3F-NEB - 2-(Ethylamino)-1-(3-fluorophenyl)butan-1-one

3F-N-ethylhexedrone - 2-(Ethylamino)-1-(3-fluorophenyl)hexan-1-one

3F- α -PHP - 1-(3-Fluorophenyl)-2-(pyrrolidin-1-yl)-hexan-1-one

3F- α -PiHP - 1-(3-Fluorophenyl)4-methyl-2-(pyrrolidin-1-yl)pentan-1-one

3-methyl-N-propylcathinone - 2-(Propylamino)-1-(3-methylphenyl)-1-propanone

4 F-PBP - 4'-Fluoro- α -pyrrolidinobutyrophenone

4-BEC - 4-Bromoethcathinone

4-Br-PPP - 4-Bromo- α -pyrrolidinopropiophenone

4-Br-PVP - 1-(4-Bromophenyl)-2-(pyrrolidin-1-yl)pentan-1-one

4-Cl-3MMC - 4-Chloro-3-methylmethcathinone

4-Cl-PPP - 1-(4-Chlorophenyl)-2-(pyrrolidin-1-yl)propan-1-one

4-Cl- α -EAPP - 1-(4-Chlorophenyl)-2-(ethylamino)pentan-1-one

4-Cl- α -PHP - 1-(4-Chlorophenyl)-2-(pyrrolidin-1-yl)hexan-1-one]

4-Ethyl- α -PVP - 1-(4-Ethylphenyl)-2-(pyrrodin-1-yl)pentan-1-one

4-FMC - 4-Fluoromethcathinone

4F-PHP - 1-(4-Fluorophenyl)-2-(pyrrolidin-1-yl)hexanone

4-F- α -PHPP - 4-Fluoro- α -pyrrolidinoheptanophenone

4-F- α -PVP - 4-Fluoro- α -pyrrolidinopentiophenone

4-MEC - 4-Methyl-N-ethylcathinone

4-Methoxy- α -PHPP - 4-methoxy- α -pyrrolidinoheptanophenone

4-Methoxy- α -POP - 4-Methoxy- α -pyrrolidinooctanophenone

4-Methoxy- α -PVP - 4-Methoxy- α -pyrrolidinovalerophenone)

4-Methylhexedrone - 2-(Methylamino)-1-(4-methylphenyl)-1-hexanone

4-MPD - 4-Methylpentedrone

5-BPDI - 1-(2,3-Dihydro-1H-inden-5-yl)-2-(pyrrolidin-1-yl)hexan-1-one
5-HT - 5-Hydroxytryptamine
5-PPDI - 1-(2,3-Dihydro-1*H*-inden-5-yl)-2-(pyrrolidin-1-yl)butan-1-one
ACN - Acetonitrile
AGP - α -Acid glycoprotein
ANOVA - Analysis of variance
AP - Apical
ATCC - American Tissue Culture Collection
BDNF - Brain-derived neurotrophic factor
BGE - Background electrolyte
bk-MDA - 2-Amino-1-(benzo[d][1,3]dioxol-5-yl)propan-1-one
bk-PDP - 1-(1,3-Benzodioxol-5-yl)-2-(ethylamino)-1-pentanone
BL - Basolateral
BMAPN - 2-(Methylamino)-1-(naphthalen-2-yl) propan-1-one
BSA - Bovine serum albumin
BTA - 2'-Bromotartranilic acid
ButOH - Butanol
CBH - Cellobiohydrolase I
CD - Cyclodextrin
Cdk5 - Cyclin-dependent kinase 5
CE - Capillary electrophoresis
CEC - Capillary electrochromatography
CIP - Cahn-Ingold-Prelog
CNS - Central nervous system
COMT - Catechol *O*-methyltransferase
CSP - Chiral stationary phase
DAD - Diode array detection
DAT - Dopamine transporters
DEA - Diethylamine
DEB - *N,N*-Diethylbuphedrone
DEP - *N,N*- Diethylpentedrone
DIPEA - Diisopropylethylamine
DMB - *N,N*-Dimethylbuphedrone
DMEM - Dulbecco's modified Eagle's medium

DMP - *N,N*-Dimethylpentedrone
DMSO - Dimethyl sulfoxide
DNFP-L-V - *N*α-(2,4-Dinitro-5-fluorophenyl)-L-valinamide
DTT - Dithiothreitol
e.r. - Enantiomeric ratio
ECACC - European Collection of Cell Culture
EKS - Electrokinetic supercharging
EMCDDA - European Monitoring Centre for Drugs and Drug Addiction
EPH - Ephedrone or Methcathinone
EtOH - Ethanol
FA - Formic acid
FBS - Fetal bovine serum
FDA - Food Drug Administration
GC - Gas chromatography
HBSS (-/-) - Hank's balanced salt solution without calcium and magnesium
HBSS (+/+) - Hank's balanced salt solution with calcium and magnesium
Hex - Hexane
Hexedrone - α-Methylaminohexanophenone
Hexylone - 1-(1,3-Benzodioxol-5-yl)-2-(methylamino)-hexan-1-one
HILIC - Hydrophilic interaction chromatography
HLCs - Human hepatocyte-like cells
hOCT1 - Human organic cation transporter
HP - Human plasma
HPALC - High-performance affinity liquid chromatography
HPLC - High performance liquid chromatography
HRMS - High resolution mass spectrometry
HRP - horseradish peroxidase
HSA - Human serum albumin
ICH - International Conference on Harmonization
ICSS - Intracranial self-stimulation
IPA - Propan-2-ol
Isohexedrone - 4-Methyl-2-(methylamino)-1-phenylpentan-1-one
Isohexylone - 1-(1,3-Benzodioxol-5-yl)-4-methyl-2-(methylamino)pentan-1-one
IUPAC - International Union of Applied Chemistry

LC - Liquid chromatography
LOD - Limit of detection
LOQ - Limit of quantification
L-TPC - Trifluoroacetyl-L-prolyl chloride
MCF - (1*R*)-(-)-Menthylchloroformate
MDMA - 3,4-Methylenedioxymethamphetamine
MDPiHP - 1-(1,3-Benzodioxol-5-yl)-4-methyl-2-pyrrolidin-1-yl-pentan-1-one
MDPV - 3,4-Methylenedioxypyrovalerone
MDPV8 - 1-(1,3-Benzodioxol-5-yl)-2-(pyrrolidin-1-yl)-heptan-1-one
MeOH - Methanol
MEPH - Mephedrone
MFPHP - 1-(4-Fluoro-3-methyl-phenyl)-2-pyrrolidin-1-yl-hexan-1-one
MFPVP - 1-(4-Fluoro-3-methylphenyl)-2-(pyrrolidin-1-yl)pentan-1-one
MPHP - 4-Methyl- α -pyrrolidinohexanophenone
MPP - 2-Methyl-1-phenyl-2-(pyrrolidin-1-yl)propan-1-one
MPP⁺ - 1-Methyl-4-phenylpyridinium
MRP1 - Multidrug resistance associated protein 1
MRPs - Multidrug resistance proteins
MS - Mass spectrometry
MTPA - α -Methoxy- α -trifluoromethylphenylacetic acid
MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
***N,N*-Diethylhexedrone** - 2-(Diethylamino)-1-phenylhexan-1-one
***N,N*-Diethylpentylone** - 1-(1,3-Benzodioxol-5-yl)-2-(diethylamino)pentan-1-one
NAT - Noradrenaline transporters
***N*-Butylpentylone** - 1-(1,3-Benzodioxol-5-yl)-2-(butylamino)pentan-1-one
NCI - Negative ion chemical ionization
***N*-cyclohexylmethylone** - 1-(Benzo-1,3-dioxol-5-yl)-2-(cyclohexylamino)propan-1-one
NEP - *N*-Ethyl-pentylone
NET - Norepinephrine transporter
***N*-Ethylheptedrone** - 2-(Ethylamino)-1-phenylheptan-1-one
***N*-Ethylheptylone** - 1-(1,3-Benzodioxol-5-yl)-2-(ethylamino)heptan-1-one
NPLC - Normal-phase liquid chromatography
N-PP - α -Propyloaminopentiophenone
NPS - New psychoactive substances

NR - Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride)

P-gp - P-glycoprotein

PMSF - Phenylmethanesulfonyl

POSC - Polar organic solvent chromatography

RA - Retinoic acid

RPLC - Reversed-phase liquid chromatography

RT - Room temperature

SD - Standard deviation

SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SERT - Serotonin transporter

SFC - Super critical fluid chromatography

SPE - Solid-phase extraction

TEA - Triethylamine

TEER - Transepithelial electrical resistance

TFA - Trifluoroacetic acid

TPA - Tetradecanoyl phorbol 13-acetate

TPBS - Tween phosphate buffered saline

UHPLC - Ultra-high-performance liquid chromatography

UV - Ultra-violet

VMAT₂ - Vesicular monoamine transporter-2

α -D2PV - 1,2-Diphenyl-2-(pyrrolidin-1-yl)ethan-1-one

α -EAPP - α -Ethylaminopentiophenone

α -PBT - α -Pyrrolidinobuthiophenone

α -PCYP - 2-Cyclohexyl-1-phenyl-2-pyrrolidin-1-yl-ethan-1-one

α -PHP - α -Pyrrolidinohexanophenone

α -PHPP - α -Pyrrolidinoheptanophenone

α -PiHP - 4-Methyl-1-phenyl-2-(pyrrolidin-1-yl)pentan-1-one

α -PipBP - α -Piperidinobuthiophenone

α -POP - α -Pyrrolidinooctanophenone

α -PVP - α -Pyrrolidinopentiophenone

α -PVT - α -Pyrrolidinopentiothiophenone

INTRODUCTION

1. RELEVANCE OF CHIRALITY AND ENANTIOSELECTIVITY IN PHARMACO/TOXYCODYNAMIC AND PHARMACO/TOXYCOCYNETIC EVENTS
2. SYNTHETIC CATHINONES: RECENT DEVELOPMENTS, ENANTIOSELECTIVITY STUDIES AND ENANTIOSEPARATION METHODS
3. BINDING AFFINITY STUDIES ON HUMAN SERUM ALBUMIN

1. RELEVANCE OF CHIRALITY AND ENANTIOSELECTIVITY IN PHARMACO/TOXYCODYNAMIC AND PHARMACO/TOXYCOCYNETIC EVENTS

Biological structures such as receptors, enzymes, transporters, and other binding molecules comprise intrinsic chirality and can recognize and discriminate between enantiomers by selective interactions leading to different biological responses. This phenomenon is called enantioselectivity [1]. Therefore, enantiomers can express differences in pharmacokinetic (absorption, distribution, metabolism and excretion), pharmacodynamic (drug-biotarget interactions) and toxicological events [2, 3].

For majority of drugs, the passage across cell membranes is a passive process through the phospholipid bilayer without the intermediation of proteins. As the enantioselectivity is only observed when the absorption process involves carrier molecules [4-6], the gastrointestinal absorption of drugs is expected to be similar between enantiomers unless there is involvement of a transport protein.

Drug distribution is controlled by two principal physicochemical properties: partition coefficient and protein binding. Enantioselectivity is not influenced by the partition coefficient, but enantiomers can possess differences in drug binding whether to plasma or tissue proteins [7]. This binding can be measured by the volume of distribution of the two enantiomers [5]. Moreover, human serum albumin (HSA) has been the most studied protein for enantioselective binding, despite some studies concerning other serum proteins, such as α -acid glycoprotein (AGP) [3, 8, 9]. Additionally, enantioselectivity in drug distribution by tissue binding differences was also described [6].

Enantioselective metabolism of drugs is the most common event responsible for enantioselectivity in pharmacokinetics meaning that enantiomers might be metabolized by different pathways or even the same pathways but to different extents [10]. First-pass metabolism is one of the reasons for enantioselective metabolism resulting in differences in oral bioavailability [11, 12]. Additionally, genetic polymorphisms in metabolic enzymes might also affect plasma concentrations in an enantioselective way [5, 12].

Active and passive processes might be involved in the elimination of drugs and their metabolites. For biliary excretion, passive diffusion relies on the gradient of concentrations between plasma and bile. Thus, this process is not enantioselective. However, active mechanisms can discriminate between enantiomers leading to different extents of elimination [7]. For renal excretion, glomerular filtration, active transport and renal metabolism can be involved in this process. Glomerular filtration is a passive nonenantioselective process,

therefore, enantioselectivity in renal elimination can result from active transport mechanisms or renal metabolism. Additionally, since plasma protein binding can be enantioselective, it can also result in apparent enantioselectivity in renal clearance of some drugs [7, 12]. Besides that, the interaction of enantiomers with the binding sites of P-glycoprotein, multidrug resistance proteins (MRPs), and organic anion and cation transporters, which are transport proteins involved in drug transport across canalicular cells and renal tubular cells, might be enantioselective [5].

Ideally, the therapeutic activity of a drug would remain in just one enantiomer and the adverse effects in the other. However, the different interactions of a pair of enantiomers with molecular targets usually lead to a range of different activity scenarios [2, 3]. For instance, enantiomers can present similar pharmacological/biological activity, but one can be more active than the other [2, 13]. Although there are only a few cases where this happens, some drugs can have equally bioactive enantiomers [2, 14]. Additionally, one of the enantiomers might have no activity at all [14]. Lastly, enantiomers can also have qualitatively different activities[15].

Furthermore, enantiomers can also present differences in toxicological properties. As well as pharmacological properties, toxicological properties can be identical between enantiomers, quantitatively or qualitatively different, or they can remain only in the pharmacological active enantiomer or the inactive one [2, 10].

2. SYNTHETIC CATHINONES: RECENT DEVELOPMENTS, ENANTIOSELECTIVITY STUDIES AND ENANTIOSEPARATION METHODS

Review article reprinted from Molecules 2022, 27(7), 2057
(<https://doi.org/10.3390/molecules27072057>)

Synthetic cathinones: recent developments, enantioselectivity studies and enantioseparation methods

A.S. Almeida^{1,2,3,4}, B. Silva^{2,4}, P.G. Pinho^{2,4}, F. Remião^{2,4}, C. Fernandes^{1,3}

¹Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal

²UCIBIO—Applied Molecular Biosciences Unit, REQUIMTE, Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal

³Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, s/n, 4450-208 Matosinhos, Portugal

⁴Associate Laboratory i4HB—Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal

Abstract:

New psychoactive substances represent a public health threat since they are not controlled by international conventions, are easily accessible online and are sold as a legal alternative to illicit drugs. Among them, synthetic cathinones are widely abused due to their stimulant and hallucinogenic effects. To circumvent the law, new derivatives are clandestinely synthesized and, therefore, synthetic cathinones keep emerging on the drug market, with their chemical and toxicological properties still unknown. In this review, a literature assessment about synthetic cathinones is presented focusing on the recent developments, which include more than 50 derivatives since 2014. A summary of their toxicokinetic and toxicodynamic properties are also presented. Furthermore, synthetic cathinones are chiral compounds, meaning that they can exist as two enantiomeric forms which may present different biological and toxicological activities. To analyze the enantiomers, the development of enantiomeric resolution methods for synthetic cathinones is crucial. Many methods have been reported over the years that include mostly chromatographic and electromigration techniques, with liquid chromatography using chiral stationary phases being the technique of choice. This review intended to present an overview of enantioselectivity studies and enantioseparation analysis regarding synthetic cathinones, highlighting the relevance of chirality and current trends.

2.1. Introduction

The use of new psychoactive substances (NPS) has been growing since 2000 [16]. These substances started to replace illicit drugs as legal alternatives being known as “legal highs”, “smart drugs” or “research chemicals” [17, 18]. They can be sold, as bath salts, plant fertilizers or air fresheners. Although these products are frequently labeled as “not for human consumption”, they are mostly purchased with that purpose. Therefore, NPS are defined as new narcotics or psychotropic substances, in pure form or in mixture preparations, that are not controlled by international conventions but can represent a public health concern [18, 19].

Between 1997 and 2020, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) was monitoring more than 820 NPS. Despite this number, it is possible to observe that since 2015 the number of NPS notified for the first time has been decreasing (**Figure 1**). In 2019, 53 NPS were reported for the first time and in 2020, until October, the number was 38 [20].

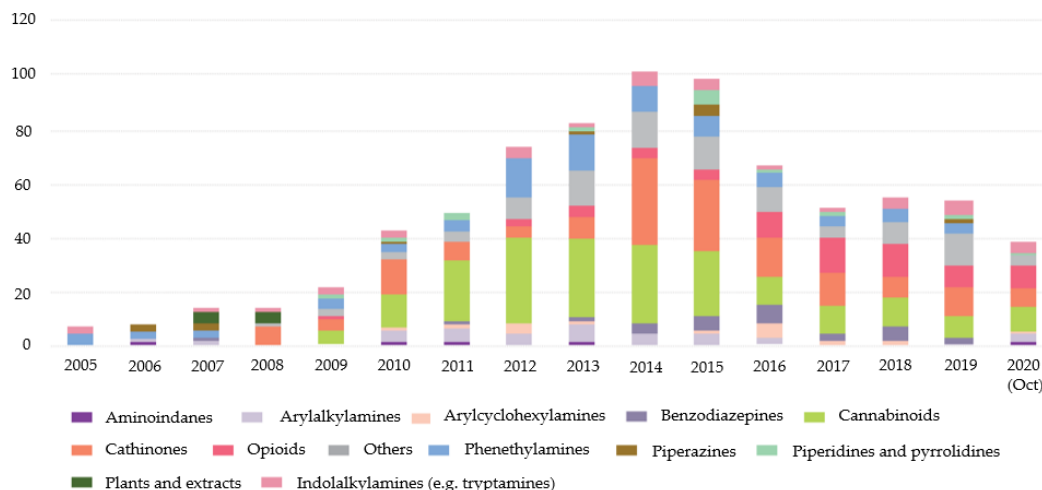


Figure 1 – New psychoactive substances reported for the first time from 2005 to 2020 (October) divided by categories [20].

The popularity of these drugs had a sharp increase due to their easy accessibility online. Human and animal studies involving these NPS are very limited and non-existent for some of them. Therefore, available information about the biological and toxicological properties of these substances is still very limited. The actual composition of NPS sold online can be very different from the package label and, therefore, consumers might purchase and use them mistakenly. All these factors explain why the world of new psychoactive substances represents a huge danger for public health [18, 19].

The two groups of NPS reported on a larger scale are synthetic cannabinoids and

synthetic cathinones, representing more than two-thirds of all available compounds since 2005 [20, 21]. The present paper will focus on synthetic cathinones, which comprise a vast group of compounds derived from cathinone (**1**), an alkaloid found in khat (*Catha edulis*) leaves, structurally identical and similar in action to amphetamine (**2**) (**Figure 2**) [22].

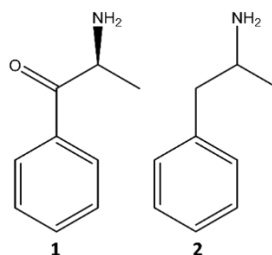


Figure 2 – Structures of cathinone (**1**) and amphetamine (**2**).

Moreover, chewing of fresh khat leaves has been a tradition for centuries in some cultures. The leaves contain more than 40 components such as alkaloids, flavonoids, amino acids, glycosides, sterols, vitamins and minerals (**Figure 3**) [23]. In 1930, cathine or (+)-norpseudoephedrine (**3**) was identified as the active principle of khat. However, the activity of cathine (**3**) was insufficient to be responsible for all the biological effects observed. Later on, in 1975, cathinone was isolated and found to be seven to ten times more bioactive than cathine (**3**) [21].

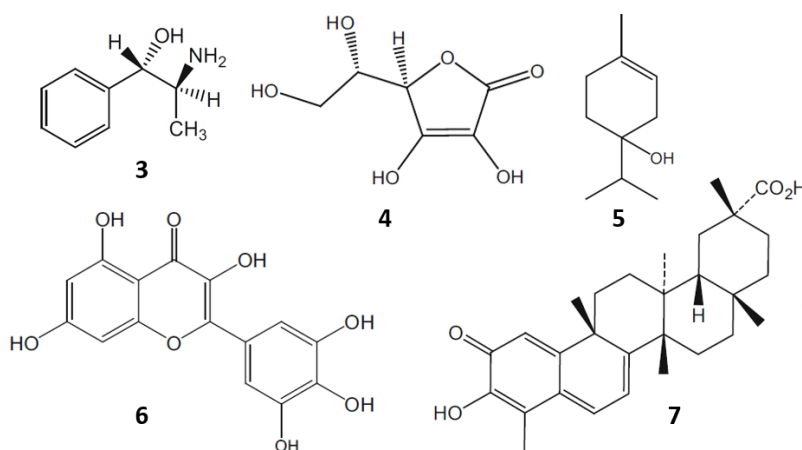


Figure 3 – Some bioactive components of khat: cathine (**3**); ascorbic acid (**4**); α -terpineol (**5**); myricetin (**6**); and celastrol (**7**).

Although both cathine and cathinone are internationally controlled, the World Health Organization considered that the evidence was insufficient to justify the international control of

khat. Nonetheless, they advised the development of campaigns to educate the public about the potential adverse effects of excessive use of khat [24]. Khat has been used as starting material to synthesize derivatives resulting in the first synthetic cathinones that, in the 2000's, emerged in the drug market [21]. Synthetic cathinones gradually became available in smartshops, internet and other drug paraphernalia stores [25]. They were commonly found as “bath salts” under names like Bloom, Ivory Wave, Vanilla Sky, Blue Silk, Purple Wave [22]. By 2013, approximately 600 “dark web” sites were identified in Europe that allowed the purchase of these compounds anonymously using untraceable digital currencies [26].

Synthetic cathinones are widely abused due to their stimulant and hallucinogenic effects, replacing 3,4-methylenedioxymethamphetamine (MDMA), cocaine and amphetamines, which are much more expensive. However, synthetic cathinones can be much more potent than the drugs that they are intended to mimic, increasing the risk of overdose and death [27].

In literature, some fundamental reviews about the development, pharmacokinetics, mechanisms of action, and biological/toxicological effects of synthetic cathinones can be found [21, 28-30]. One of the main objectives of this review is the reporting of new synthetic cathinones that have been clandestinely synthesized and emerged on the drug market in the last few years, to infer about current trends. Special focus is given to stereochemistry issues for this class of compounds which, in many studies, is still not considered. A compilation of enantioselectivity studies as well as enantioseparation analysis of synthetic cathinones is also presented, highlighting the relevance of chirality.

2.2. Classification of Synthetic Cathinones

Synthetic cathinones are β -keto phenethylamine derivatives presenting the same core structure. Moreover, they are structurally similar to amphetamine being the difference the presence of a keto group [29, 31]. Cathinone derivatives can be synthesized by the addition of several substituents at different sites of the cathinone scaffold as represented in **Figure 4** [32].

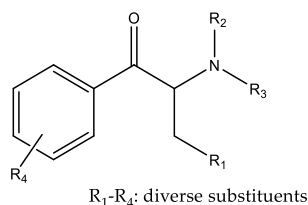


Figure 4 – Core structure of cathinone derivatives.

Consequently, synthetic cathinones can be divided into four groups based on the

substitution pattern (**Figure 5**). The first group, the *N*-alkylated cathinones (**8-18**), which includes methcathinone (**8**), mephedrone (**9**), dimethylcathinone (**10**) and ethcathinone (**11**), are *N*-substituted compounds with an unsubstituted or substituted phenyl ring. Most of the first synthetic cathinones are part of this group. Pyrrolidinophenone derivatives, the second group, contain in their structure a substituted or unsubstituted phenyl ring and a pyrrolidinyl ring in the side chain. Pyrovalerone (**19**), MPBP (**20**), naphyrone (**21**) and α -PVP (**22**) are examples. The third group are the 3,4-methylenedioxy cathinones to which belong pentylone (**23**), methylone (**24**), butylone (**25**) and ethylone (**26**). They are characterized by a 1,3-benzodioxol-5-yl ring and a straight side chain. Moreover, they present similar structure and pharmacological properties to MDMA. Lastly, the fourth group comprises mixed cathinones or 3,4-methylenedioxypyrrolidinophenones, for instance, MDPBP (**27**) and MDPV (**28**), which present in their structure a combination of the last two groups moieties: a methylenedioxy ring and a pyrrolidinyl side ring [29, 31].

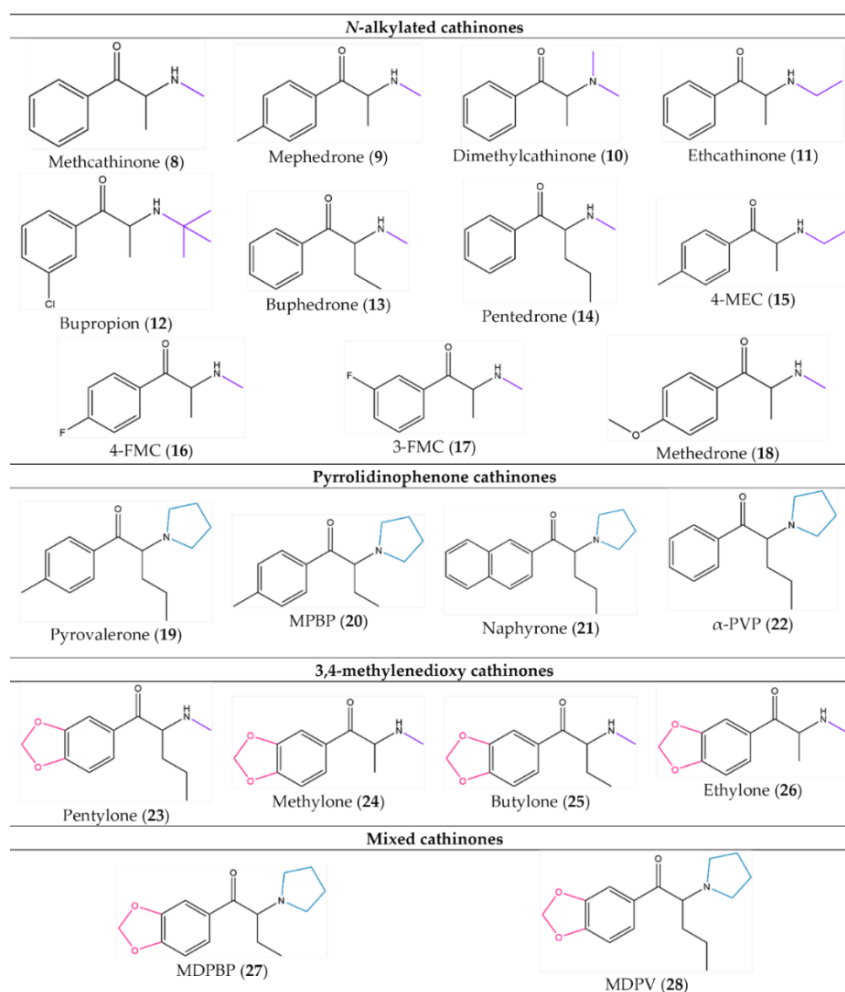


Figure 5 – Examples of synthetic cathinones from each group based on the substitution pattern: **8-18** from the *N*-alkylated cathinones, **19-22** from the pyrrolidinophenone cathinones, **23-26** from the 3,4-methylenedioxy cathinones and **27** and **28** from the mixed cathinones.

2.3. Chronological evolution and recent developments of cathinone derivatives

In **Figure 6** some of the most important landmarks of the history of synthetic cathinones are summarized.

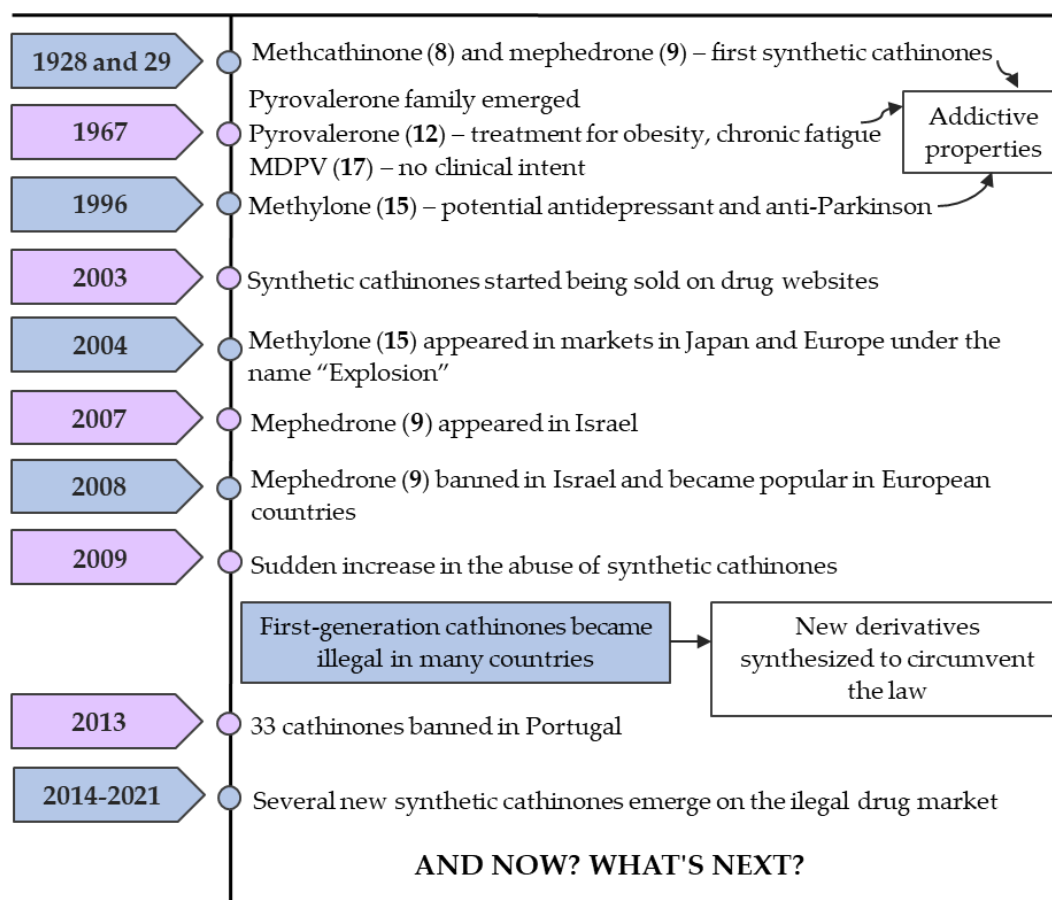


Figure 6 - Timeline of events related to the history of synthetic cathinones.

Methcathinone (α -methylamino-propiofenone or ephedrone (EPH), **(8)**) and mephedrone (4-methylmethcathinone or MEPH, **(9)**) (**Figure 5**) were the first synthetic cathinones arising in 1928 and 1929, respectively [17]. Methcathinone **(8)** was meant to reach the market as an antidepressant, but latter it was found to have powerful addictive properties [21, 33]. As a consequence, this synthetic derivative was responsible for several intoxications in the Soviet Union in the 70s and in the USA in the 90s being known in the streets as "Cat",

“Jeff” and “Mulka” [28].

The pyrrolidinophenone family comprises a range of compounds that began to be reported at the end of the 60s. Pyrovalerone (**19**, **Figure 5**) is a member of this family and was firstly synthesized as a treatment for obesity, chronic fatigue and lethargy but, due to its addictive potential, the clinical use was stopped after these reports of abuse [21]. However, other derivatives of this family, such as 3,4-methylenedioxypyrovalerone (MDPV (**28**), **Figure 5**) in 1967, were synthesized with no clinical intent [17, 21].

In 1996, methylone (3,4-methylenedioxy-*N*-methylcathinone or β k-MDMA, (**24**) appeared as a potential antidepressant and anti-Parkinson agent but this compound never reached the market due to their psychostimulant properties identical to MDMA [21, 34].

From the few cathinones synthesized with a medicinal intent, only bupropion (**12**) succeeded for that purpose, being currently used as an antidepressant and a support to smoking cessation [28, 35].

Synthetic cathinones had barely any attention until 2003 when they were first reported online on drug websites as a legal replacement to MDMA [22].

Around 2004, methylone (**24**) started to appear in markets in Japan and Europe under the name “Explosion” being also the first of these substances to be sold via smartshops and online [28]. In 2007, mephedrone (**9**) made its appearance on the market, first in Israel, being, however, banned in this country in 2008. After this, mephedrone (**9**), also known in the markets as “Meph”, “TopCat”, “Mcat”, “Meow Meow”, among other names, became more popular in European countries [21, 28]. Many drug users began to replace cocaine and ecstasy by mephedrone (**9**) due to a decrease in the purity and availability of the first two drugs. Additionally, mephedrone (**9**) was less expensive and more potent. This explains why, in 2009, there was a sudden increase in the abuse of synthetic cathinones, especially mephedrone (**9**) [28].

Later, this first-generation of cathinones became illegal in many countries. To overcome this, clandestine chemists started to modify their structures to obtain new derivatives that could circumvent the law. Thus, several new cathinones were synthesized such as buphedrone (**13**), butylone (**25**), ethylone (**26**), pentedrone (**14**) and its constitutional isomer 4-methyl-*N*-ethylcathinone (4-MEC, (**15**)). Additionally, flephedrone (4-fluoromethcathinone or 4-FMC (**16**)) and 3-fluoromethcathinone (3-FMC (**17**)), two derivatives of mephedrone (**9**) were also reported [29].

Naphyrone (naphthylpyrovalerone (**21**)), which appeared after mephedrone (**9**) was marketed in the UK under the name “Energy-1” (NRG-1) as a legal alternative [28].

Simultaneously with pentedrone (**14**), a derivative from the same group, α -pyrrolidinopentiophenone (α -PVP, (**22**)) also emerged [29]. The popularity of this compound increased greatly in Europe and USA, between 2011 and 2015, being found in the markets as “Flakka” or “Gravel”. After several fatal or almost fatal cases of abuse, α -PVP (**22**) began to be controlled internationally [28]. Moreover, in Portugal, in April of 2013, because of new legislative control measures, the commercialization and use of 33 cathinone derivatives were prohibited and all smartshops were closed [21, 36].

The chemical structures of the first cathinones are being constantly modified. Each year several new derivatives emerge on the illegal drug market. The chemical structures of synthetic cathinones (**29-69**) synthesized and reported since 2014 until today are represented in **Figures 7 and 8**.

In 2014, several new synthetic cathinones were found in the Japanese market being sold as “aroma liquids” and “fragrance powders”. They included 4-methoxy- α -PVP (**29**), α -EAPP (**30**), α -PHPP or PV8 (**31**), α -POP or PV9 (**32**), *N*-ethyl-4-methylpentedrone (**33**), MPHP (**34**), 3,4-dimethoxy- α -PVP (**35**), 4-F- α -PVP (**36**). Almost half a year later, α -PHP (**37**), 4-methoxy- α -PHPP (**38**), 4-methoxy- α -POP (**39**), and 4-F- α -PHPP (**40**) were also discovered [28, 29, 37-39].

Moreover, in Portugal, 4-F-PBP (**41**) was reported for the first time in 2015 [28, 40]. In the same year, the first thienyl cathinone derivatives, α -PVT (**42**), α -PBT (**43**) and bromothienyl analogs were identified [29, 41]. The thiothionone (**44**), another thienyl cathinone derivative was also discovered, at the same time [29, 42].

In 2016, propylone (**45**), *N*-ethylhexedrone (**46**), 4-chloro-pentedrone (**47**), α -PiHP (**48**), 4-Cl- α -EAPP (**49**) and 4-Cl- α -PHP (**50**) were identified for the first time [28, 43]. In the same year, brephedrone (**51**) was identified in street seized samples in Brazil. Although this synthetic cathinone was already reported in other countries before [44].

In 2017, an unknown compound found in seized drugs in the UK was identified and characterized as indapyrophenidone (**52**), a novel cathinone derivative [45]. Additionally, hexedrone (**53**), 4-BEC (**54**), 4-Cl-PPP (**55**) and 4-Br-PVP (**56**) were first reported in Poland [28, 46]. Furthermore, in the same year, three emerging cathinone derivatives: 4-MPD (**57**), 4F-PHP (**58**) and bk-EPDP (**59**) were detected, identified, and fully characterized [47].

In 2018, 5-PPDI (**61**), a novel synthetic cathinone, was identified and characterized in an unknown white powder [48].

One year later, seven other new synthetic cathinones were reported in Poland: 5-BPDI (**62**), *N*-propylcathinone (**63**), 2,4-DMEC (**64**), 2,4-DMMC (**65**), 2,4-DMPPP (**66**), 2,4-iso-DMC

(**67**) and 4-Br-PPP (**68**) [49]. Additionally, in the same year, *N*-butylhexedrone (**69**) was identified in seized material [50], which was, later on, characterized by spectroscopic and crystallographic analysis [51].

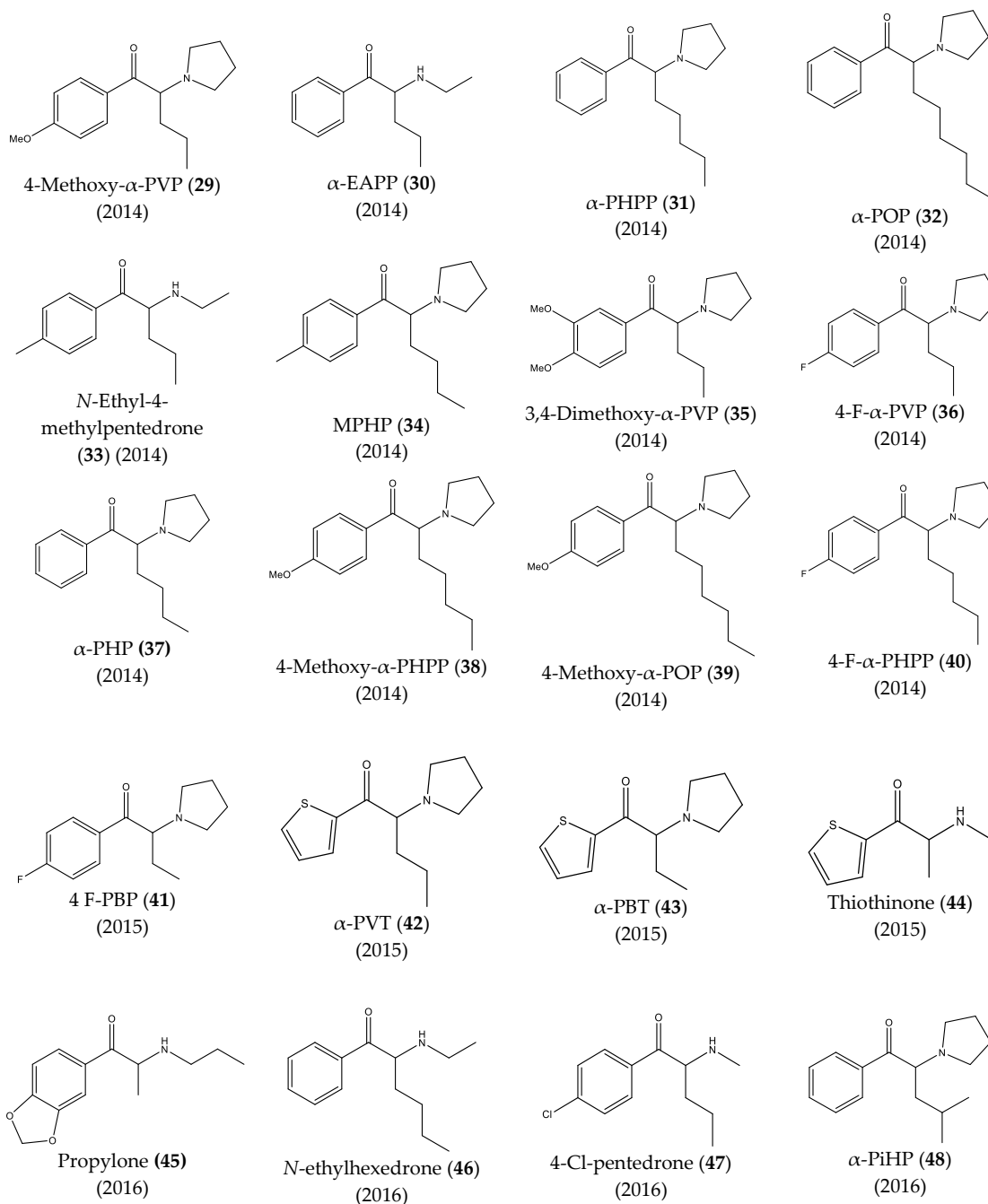


Figure 7 - Structures of the most recent cathinone derivatives **29-48**.

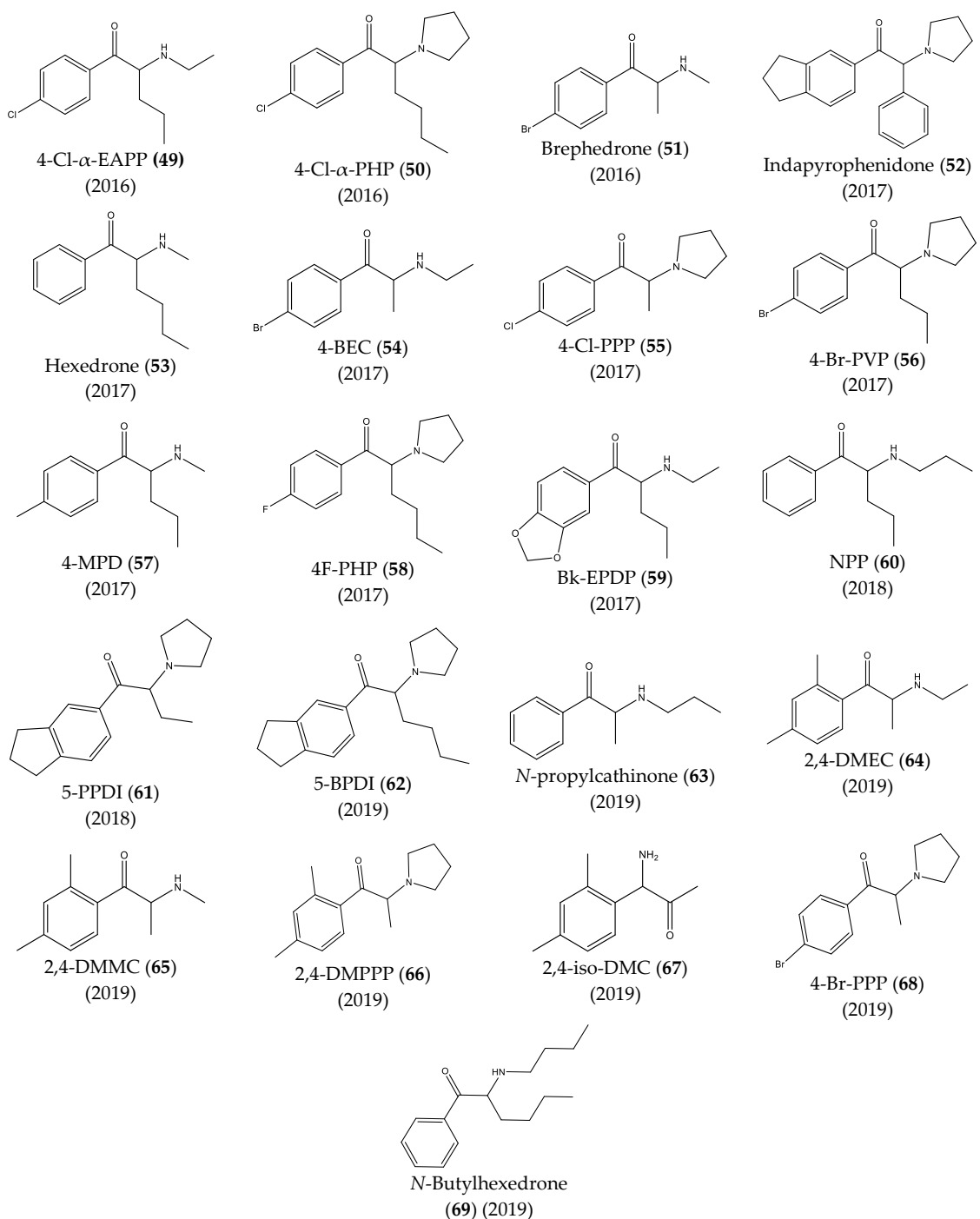


Figure 8 - Structures of some of the most recent cathinone derivatives **49-69**.

Besides that, some studies described novel synthetic cathinones (**70-80**) that were synthesized in controlled laboratories (instead of found in the drug market) with the purpose of studying potential effects and to develop analytical techniques for the identification and characterization of future cathinones (**Figure 9**).

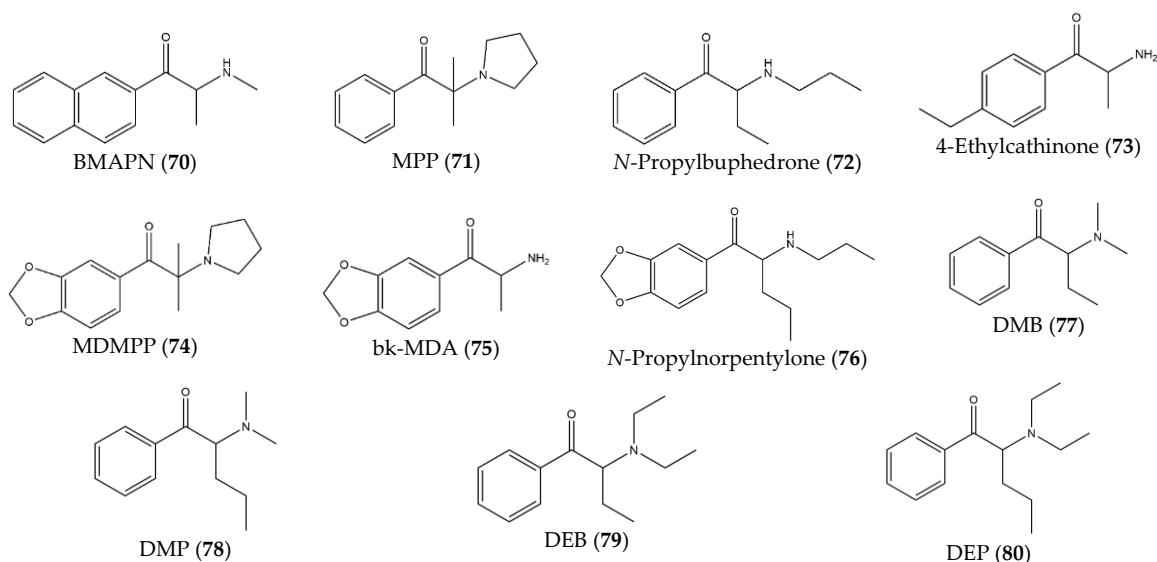


Figure 9 - Structures of cathinone derivatives synthesized in controlled laboratories (**70-80**).

For instance, Botanas *et al.* [52] synthesized a novel synthetic cathinone, BMAPN (**70**), with the purpose of studying its rewarding and reinforcing properties. Since this compound presents a naphthalene substituent on the aromatic ring, this study can be helpful to predict abuse potential of future cathinones with aromatic ring substitutions [52]. Moreover, Carlsson *et al.* [53] synthesized six novel synthetic cathinones: MPP, **71**), *N*-propylbuphedrone (**72**), 4-ethylcathinone (**73**), MDMPP (**74**), bk-MDA (**75**), *N*-propylnorpentylone (**76**). This study described the synthesis of these analogs and provided spectroscopic data [53]. With the same purpose, Gaspar *et al.* [54] synthesized four novel synthetic cathinones: DMB (**77**), DMP (**78**), DEB (**79**) and DEP (**80**).

Some of the new cathinone derivatives have been described in cases of abuse. For instance, Hasegawa *et al.* [55] reported a fatal poisoning case of a woman after oral ingestion of an “aroma liquid” type drug bought in a drug shop. This study identified and quantified PV9 (**32**) in the “aroma liquid” product as well as in *antemortem* and *postmortem* samples [55].

Majchrzak *et al.* [56] reported the first case of fatal poisoning with N-PP (**60**), a novel synthetic cathinone. This compound was identified in a white powder found in the scene and high concentrations were found in *postmortem* specimens collected from the autopsy [56]. Moreover, Pieprzyca *et al.* [57] reported two fatal poisoning cases in which PV8 (**31**) was detected and quantified in biological samples and found to be the cause of the deaths.

Adamowicz *et al.* [58] reported a fatal intoxication with α -PiHP (**48**). This substance was detected and quantified in all *postmortem* samples except in hair and was reported as the main

cause of death, although 4-CMC, *N*-ethylhexedrone, benzoylecgonine and MDMA were also detected in some analysed materials [58].

Currently, hundreds of synthetic cathinones have been identified and up to 250 new cathinone-related chemical entities are estimated to emerge every year [59]. The identification of these compounds and the implementation of a drug library with their structures and physicochemical and pharmacological properties are of great importance for chemists and toxicologists [29].

2.4. Toxicokinetic properties

Substituted cathinones are more frequently administered orally or by nasal insufflation (snorting). Other pathways, such as rectal administration, intravenous or intramuscular injection, smoking or inhalation, are less common but have also been reported [32]. Moreover, there have been occasionally described some cases of insertion of synthetic cathinones into the eye (eyeballing) [60].

In most cases, when compared to amphetamines, synthetic cathinones present a lower ability to cross the blood–brain barrier since the β -keto group increases their polarity. However, for the pyrrolidine derivatives, the presence of a pyrrolidine ring decreases their polarity increasing the blood–brain barrier permeability [18, 61].

Synthetic cathinones can be metabolized by several pathways from phase I and phase II reactions (**Figure 10**).

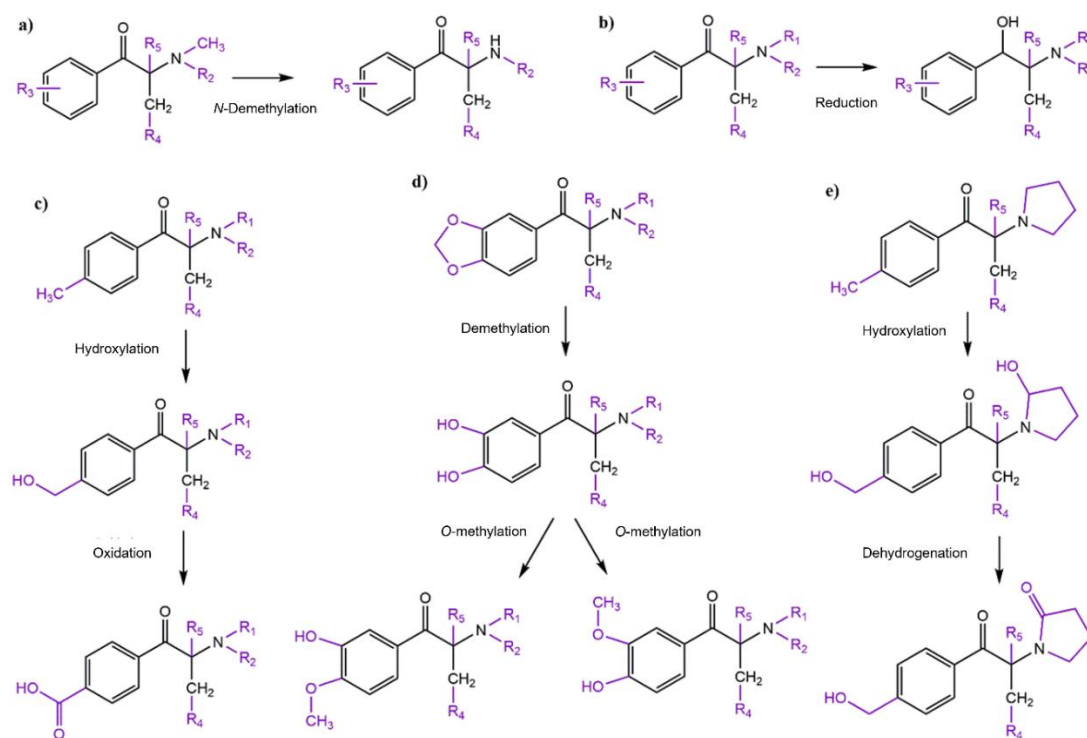


Figure 10 - General metabolic pathways of synthetic cathinones: **a)** main metabolic pathways of *N*-alkylated cathinones; **b)** reduction of the β -keto moiety to an alcohol; **c)** hydroxylation and further oxidation of the methyl substituent of the aromatic ring to a carboxylic acid; **d)** metabolism of the 3,4-methylenedioxy ring; **e)** metabolism of the pyrrolidinyl ring to a lactam.

Considering the phase I reactions, in a general way, each one of the four groups previously described (*N*-alkylated cathinones, pyrrolidinophenone cathinones, 3,4-methylenedioxy cathinones and mixed cathinones) present similar intragroup metabolic pathways [28].

For the first group, the *N*-alkylated cathinone derivatives, *N*-demethylation represents one of the main metabolic pathways (**Figure 10a**). However, since the β -keto group is shared among synthetic cathinones, for most of them, this moiety undergoes reduction to the corresponding alcohol (**Figure 10b**). Additionally, derivatives with a methyl group on the aromatic ring suffer hydroxylation of the methyl substituent, which can be further oxidized to the corresponding carboxylic acid (**Figure 10c**) [62-64]. The 3,4-methylenedioxy cathinones undergo demethylation mediated by CYP2D6 and CYP2C19 followed by *O*-methylation mediated by catechol *O*-methyltransferase (COMT) (**Figure 10d**) [21, 62].

Some general metabolic pathways found for the pyrrolidinyl ring of the pyrrolidinophenone derivatives are hydroxylation followed by dehydrogenation to the

corresponding lactam (**Figure 10e**). However, differences can be found in the main metabolic pathways of the derivatives of this group depending on the alkyl chain length [29, 62-64].

Lastly, the 3,4-methylenedioxy-pyrrolidinophenones share metabolic pathways with the corresponding methylenedioxy and pyrrolidinophenone derivatives [65].

The generated hydroxyl metabolites in the various metabolic pathways can undergo phase II metabolism (glucuronidation or sulfation) being the conjugates excreted in urine as well as the unmetabolized cathinones [21, 62].

2.5. Mechanism of action and effects

In a similar way to other illicit drugs, synthetic cathinones seem to display their psychostimulant properties due to interactions with membrane transporters for monoamines, such as noradrenaline transporters (NAT), serotonin transporters (SERT), and dopamine transporters (DAT) [19, 21, 35]. These interactions can occur through inhibition of the monoamine reuptake from the synaptic cleft (by binding to the NAT, SERT and/or DAT) and/or promotion of the release of monoamines (for instance, by inhibition of the vesicular monoamine transporter-2 (VMAT₂)). Both mechanisms lead to an increase in extracellular concentrations of monoamines amplifying cell-to-cell monoamine signaling (**Figure 11**) [66, 67]. The affinity of this interaction can, however, differ greatly between derivatives. Therefore, besides the previous classification based on the substitution pattern, synthetic cathinones can also be divided according to the type of interaction with monoamine transporters [19, 21, 35].

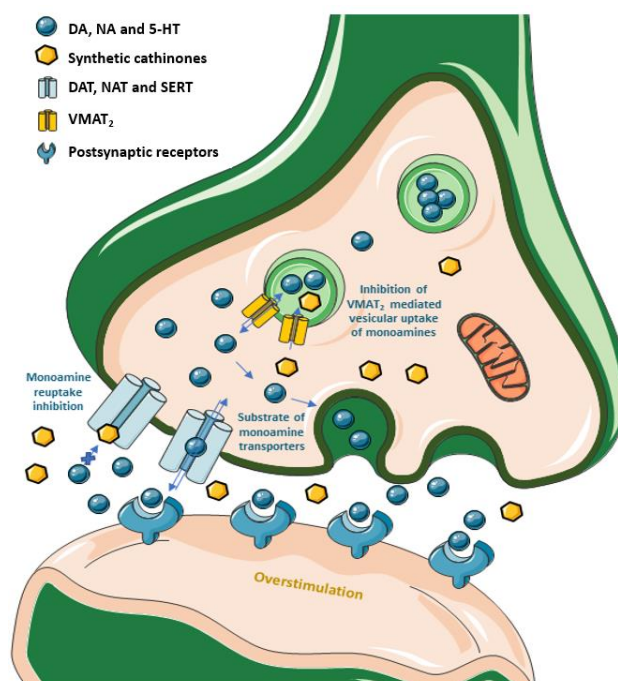


Figure 11 - Modes of action of synthetic cathinones at the central nervous system. (DA: dopamine; NA: noradrenaline; 5-HT: 5-hydroxytryptamine (serotonin); DAT: dopamine transporters; NAT: noradrenaline transporters; SERT: serotonin transporters; VMAT₂: vesicular monoamine transporter-2).

Firstly, some cathinones such as mephedrone (**9**), naphyrone (**21**) and methylone (**24**) have similar chemical structures to cocaine or/and MDMA, being, for that reason, designated as cocaine-MDMA-mixed synthetic cathinones. As cocaine, these cathinones can inhibit monoamine uptake in a nonselective way presenting more affinity to DAT than SERT. Additionally, except naphyrone (**21**), these derivatives promote the release of serotonin in a similar way to MDMA [19, 29, 35]. The second group, the methamphetamine-like synthetic cathinones, have a preferential reuptake inhibition of catecholamines and are dopamine releasers like methamphetamine. Cathinone (**1**), methcathinone (**8**) and 4-FMC (**16**) are part of this group. MDMA-like synthetic cathinones are the third group, which includes methedrone (**18**). This group is characterized by a great potency to inhibit NAT and SERT but low to DAT [19, 29, 35]. Finally, the fourth group is designated as pyrovalerone-cathinones since the synthetic cathinones that compose this group, such as MDPBP (**27**) and MDPV (**28**), present similar properties to pyrovalerone. They have great selectivity and potency to inhibit catecholamine uptake but do not promote the release of monoamines [19, 35].

Synthetic cathinones are widely consumed due to some of their desired effects such as

euphoria, increased sensory senses and sensory perception, promotes sociability, enhanced energy, mental stimulation, openness, empathy connection, decreased inhibition and increased libido [32]. However, along with all these effects, some other negative effects have also been reported by users. Since these compounds are simultaneously consumed with other drugs and might sometimes be clueless about which drug they have taken, it might be hard to relate the effects directly caused by synthetic cathinones [68]. The misunderstanding of the potency of these drugs can result in death being overdose and suicide the two most common causes (due to the psychological effects of the drug, such as loss of impulse control) [69].

Frequent adverse effects of synthetic cathinones are consistent with a sympathomimetic syndrome whose symptoms include delusions, hallucinations, paranoia, tachycardia, hypertension, abdominal pain, hyperthermia, dizziness, tremors, rhabdomyolysis and kidney damage [70]. Moreover, the use of high doses of synthetic cathinones can induce tolerance, dependence, craving and withdrawal syndrome after abrupt cessation. This syndrome might include symptoms such as sleep disorders, fatigue, depression, anxiety and craving [61].

2.6. Enantioselectivity studies

Synthetic cathinones are chiral molecules meaning that they can exist in two enantiomeric forms that, consequently, can differ in their biological and toxicological properties [32]. Although synthetic cathinones are widely studied, few studies about enantioselectivity have been performed [71]. Nonetheless, the number of available studies has been growing and enantioselectivity was found in some cases. Relevant examples of enantioselectivity include a study by Glennon *et al.* [72] with the enantiomers of methcathinone (**8**), which the *S*-enantiomer showed higher stimulating effects in central nervous system than the *R*-enantiomer.

Additionally, a study about the neurochemical effects of the enantiomers of mephedrone (**9**) in rats was performed by Gregg *et al.* [73], in which the enantiomers displayed some differences (**Figure 12**); while the *S*-enantiomer presented a higher serotonergic profile, the *R*-enantiomer showed a dopaminergic profile with locomotor activity and rewarding properties, suggesting higher addiction potential. Moreover, the *R*-enantiomer demonstrated less potency to serotonin transporters than the *S*-enantiomer or the racemate resulting in lower release of serotonin [73].

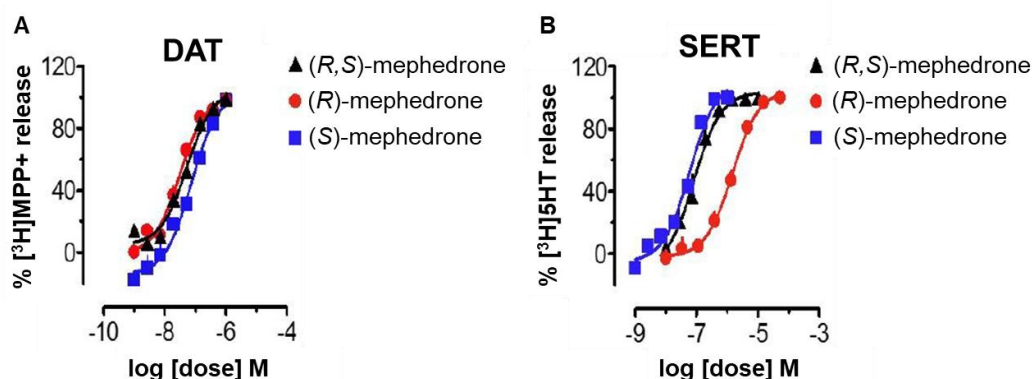


Figure 12 - Effects of the enantiomers and racemate of mephedrone on monoamine release via DAT and SERT [73]. DAT: dopamine transporters; MPP⁺: 1-methyl-4-phenylpyridinium (substrate for DAT); SERT: serotonin transporters; 5HT: 5-hydroxytryptamine (serotonin).

For the enantiomers of 4-methylcathinone (nor-mephedrone), the monoamine release and behavioral effects in rats through the response of electrical brain stimulation by an intracranial self-stimulation (ICSS) procedure were evaluated by Hutsell *et al.* [74] (**Figure 13**). In both *in vitro* and *in vivo* assays, the S-enantiomer showed higher potency than the R-enantiomer. For the *in vitro* assay, the S-enantiomer was able to promote monoamine release to a higher extent. However, the abuse potential of monoamine releasers seems to be related to their DAT vs SERT selectivity meaning that compounds with higher selectivity to DAT present higher abuse potential than non-selective or SERT-selective. In this study, the R-enantiomer displayed a higher DAT vs SERT than the S-enantiomer meaning higher abuse potential. Furthermore, the two enantiomers displayed qualitatively different effects in the ICSS behavioral study. The R-enantiomer facilitated ICSS while the S-enantiomer depressed it [74].

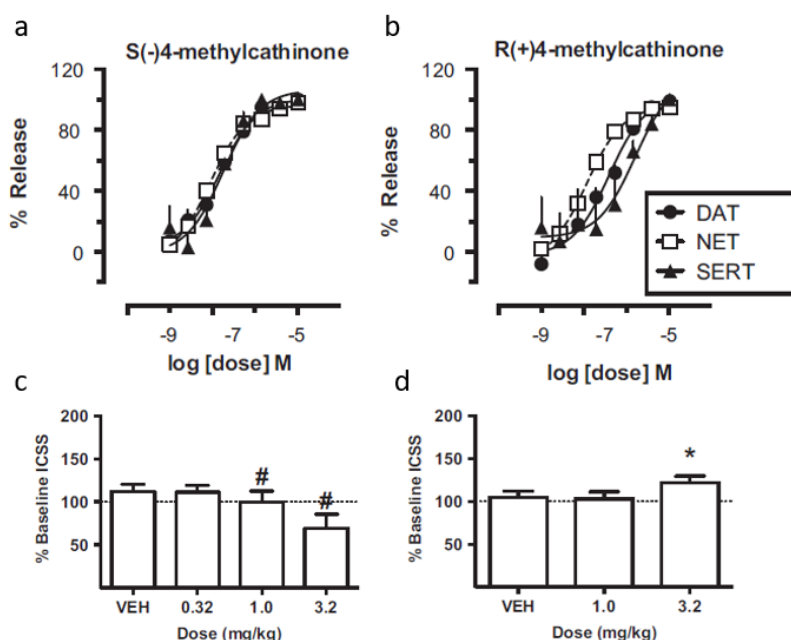


Figure 13 - Effects of S-(-)-4-methylcathinone and R-(+)-4-methylcathinone on monoamine release via DAT, SERT and NET ((a) and (b), respectively) and on ICSS ((c) and (d), respectively) [74]. DAT: dopamine transporters; NET: norepinephrine transporters; SERT: serotonin transporters; ICSS: intracranial self-stimulation. * indicates significant increases and # indicates significant decreases in ICSS rates relative to vehicle for at least one stimulation frequency as determined by analysis of frequency–rate curves in panels (a,b)

Similar studies were performed for the enantiomers of MDPV (**28**) by Kolanos *et al.* [75]. The S-(+)-enantiomer was found to be the most potent one, displaying a greater potency as a reuptake inhibitor of the monoamine transporters of dopamine and norepinephrine and facilitation of ICSS. On the other hand, the R-(-)-enantiomer was unsuccessful to change the ICSS [75]. Moreover, in another study, Gannon *et al.* [76] showed that the S-(+)-enantiomer is predominantly, if not entirely, responsible for the effects of the racemate on locomotor activity and core temperature. Silva *et al.* [77] evaluated the hepatotoxicity *in vitro* for both enantiomers in primary cultures of rat hepatocytes. In this case, no enantioselectivity was found.

Recent examples, include the reinforcing effects of MDPV (**28**) and α -PVP (**22**) enantiomers, studied by Gannon *et al.* [78] in rats, to compare their potency and effectiveness. Although the enantiomers of both synthetic cathinones were found to be highly effective reinforcers, the S-enantiomers displayed greater potency than the R-enantiomers [78].

Since the S-enantiomer of mephedrone (**9**) was previously found to be a potent serotonin releaser with no significant rewarding effects when compared to the R-enantiomer,

Philogene-Khalid *et al.* [79] performed a study of the potential ability to reduce anxiety and depression-like effects from withdrawal following chronic cocaine or MDPV abuse. As anticipated, this study found that *S*-enantiomer, at doses with no rewarding effects, can reduce withdrawal effects [79]. The same research group also investigated rewarding and reinforcing properties of the enantiomers of mephedrone (**9**). The results suggested that *R*-enantiomer was mainly responsible for these properties in the racemate [80].

In another study, Nelson *et al.* [81] assessed the contribution of the enantiomers of α -PVP (**22**) to the aversive effects of this synthetic cathinone using a conditioned taste avoidance (**Figure 14**). For this, a saccharin solution was associated with α -PVP. The racemate and *S*-enantiomer showed avoidance while for the *R*-enantiomer no avoidance was observed. Moreover, it was found that the racemate displayed a greater avoidance than the additive effects of the enantiomers suggesting that the *R*-enantiomer interacts synergistically with the *S*-enantiomer in the racemate [81].

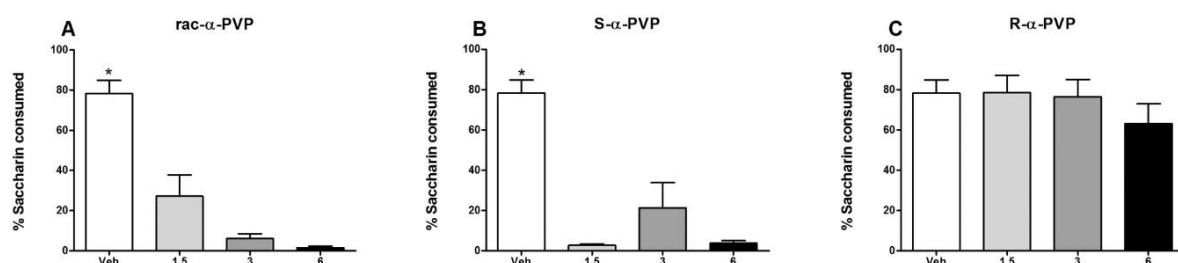


Figure 14 - Avoidance tests with racemic α -PVP (**A**), *S*- α -PVP (**B**) and *R*- α -PVP (**C**) [81].

*Significantly different from 1.5, 3, and 5

More recently, the enantioselectivity of phase-I metabolites of mephedrone (**9**), nor-mephedrone, 4-hydroxytolyl-mephedrone (4-OH-mephedrone) and dihydro-mephedrone, was evaluated by Mayer *et al.* [82]. All the enantiomers were found to be inhibitors of monoamine transporters; however, enantioselectivity was observed at SERT inhibition effect, being the *S*-enantiomers of nor-mephedrone and 4-OH-mephedrone more potent than the *R*-enantiomers. Urine sample analysis also found that the *S*-enantiomer of nor-mephedrone is the predominant form [82].

Schindler *et al.* [83] investigated the neurochemical, behavioral and cardiovascular effects of α -PVP (**22**) enantiomers in rats. Racemic α -PVP was able to inhibit dopamine and norepinephrine uptake, increase extracellular dopamine concentrations in the *nucleus accumbens*, increase locomotor activity, blood pressure and heart rate. It was found that the *S*-enantiomer is most likely to be responsible for these effects since it was found to be 30-fold

more potent than the *R*-enantiomer [83].

To evaluate the influence of chirality on the permeability across the gastrointestinal tract, Silva *et al.* [84] performed an *in vitro* study with the enantiomers of pentedrone (**14**) and methylone (**24**) (**Figure 15**) using the Caco-2 cell line. In this study, enantioselectivity was observed for both synthetic cathinones, being the *R*-(-)-enantiomer of pentedrone and the *S*-(-)-enantiomer of methylone the most permeable compounds [84].

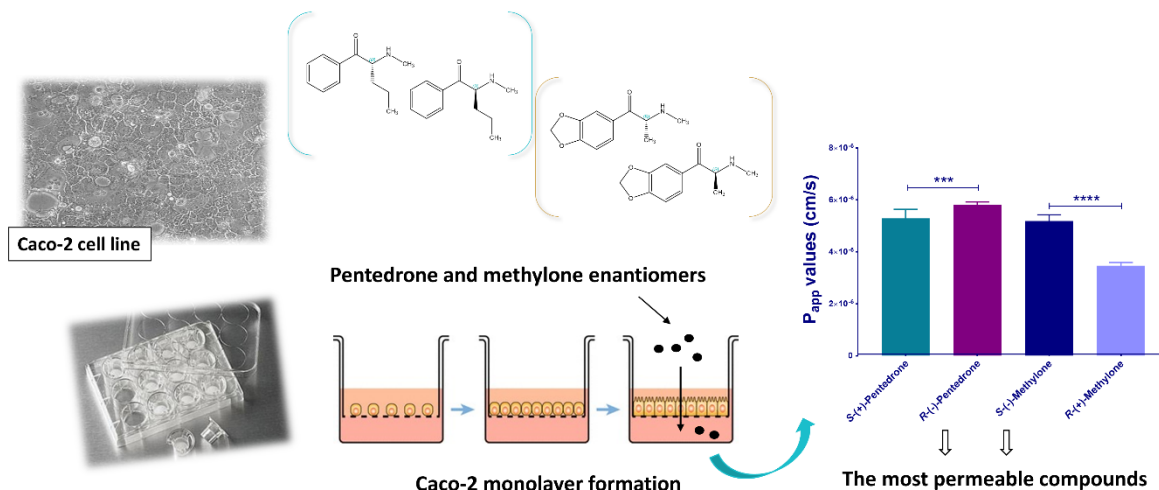


Figure 15 – Enantioselectivity studies on the absorption of methylone (**24**) and pentedrone (**14**) using Caco-2 cell line. *** p < 0.001, **** p < 0.0001.

Davies *et al.* [85] studied the actions of the enantiomers of methcathinone (**8**) at monoamine transporters, discovering that they presented similar inhibition potencies at DAT and NET. At SERT, the *S*-enantiomer displayed a lower potency than at DAT and NET while the *R*-enantiomer was practically inactive. Furthermore, in this study, an ICSS procedure to evaluate abuse-related drug effects in rats showed that the *S*-enantiomer presented almost twice the potency of the *R*-enantiomer [85].

The most recent study about enantioselectivity of cathinone derivatives was performed by Silva *et al.* [86], which evaluated the enantioselective effect of pentedrone (**14**) and methylone (**24**) enantiomers in human neuronal cells. The results showed that the *S*-(+)-enantiomer of pentedrone and the *R*-(+)-enantiomer of methylone were the most oxidative and cytotoxic enantiomers (**Figure 16**). Additionally, *R*-(-)-pentedrone presented higher affinity to the efflux transporter multidrug resistance associated protein 1 (MRP1). It was also observed enantioselectivity in the binding to P-glycoprotein (P-gp) being *R*-(-)-pentedrone and *S*-(-)-methylone the most transported enantiomers, which means a higher affinity to this efflux protein

[86].

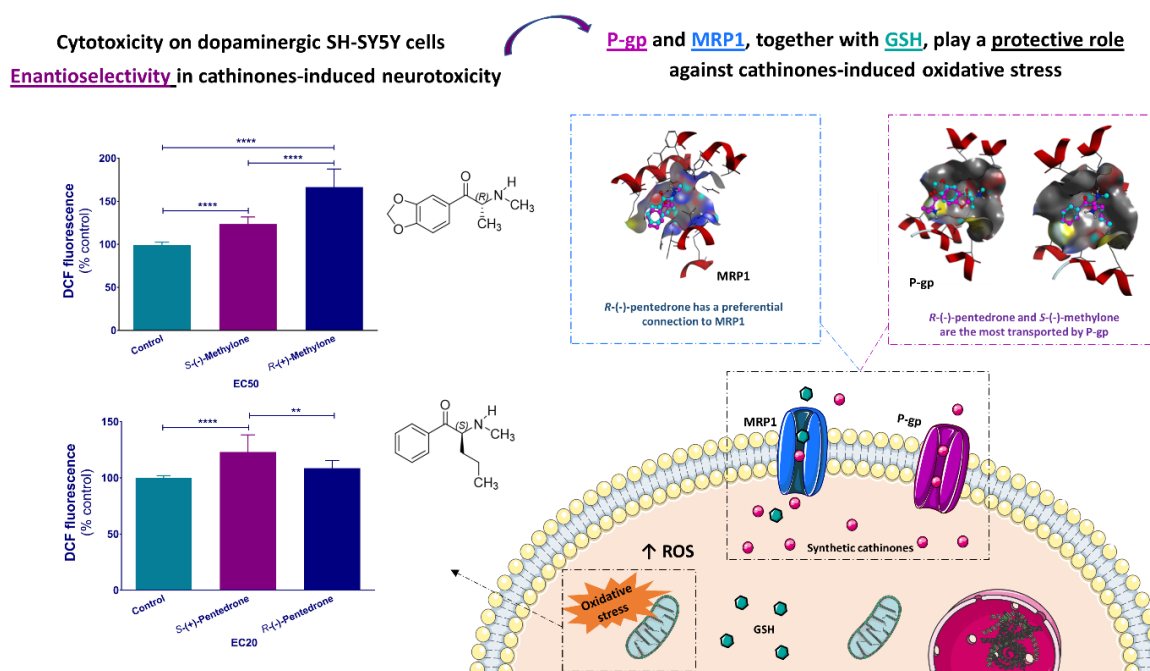


Figure 16 – Enantioselectivity studies on neurocytotoxicity of methylone (**24**) and pentedrone (**14**) towards dopaminergic SH-SY5Y cells and the role of the efflux transporter multidrug resistance associated protein 1 (MRP1) and P-glycoprotein (P-gp). ** $p < 0.01$, **** $p < 0.0001$. (Reprint with permission from [86], Copyright (2021) ELSEVIER).

2.7. Enantiomeric resolution

In order to perform enantioselectivity studies, the enantiomers must be in their enantiomerically pure form [87]. A common way to achieve that is through the resolution of a racemate into the single enantiomers. The chiral separation of the enantiomers is important not only to further evaluate their enantioselectivity by testing the single enantiomers but also to find out whether these drugs are sold as racemates or single enantiomers [88, 89]. Determination of the enantiomeric composition of synthetic cathinones and other NPS may give information about the laboratory where they come from, the starting material used for the synthesis and even help the tracking of these compounds [90]. Thus, the development of analytical methods for the enantioseparation of synthetic cathinones is of great interest [91].

Several methods allow enantiomeric resolution and can be divided into indirect and direct methods [92]. Indirect methods are based on the formation of diastereomers through derivatization of the enantiomers with an enantiomerically pure reagent via covalent bond. The diastereomers are, then, separated under achiral conditions, by crystallization or chromatography methods, for example [2, 93]. On the other hand, direct methods use a chiral selector present in the resolution method. Chromatography is the most used direct resolution method, in which the chiral selector can be a component of the stationary phase or an additive in the mobile phase [93]. The chiral selector binds preferentially with one of the enantiomers resulting in the formation of transient diastereomeric complexes with different stabilities and, consequently, different retention times. The less stable diastereomeric complex is eluted first [92]. The direct approach is frequently preferred over the indirect since there is no need of previous derivatization, less sample manipulation is needed and the results are rapidly obtained after the separation [94].

Chromatographic enantioseparations by gas chromatography (GC) and liquid chromatography (LC) can be performed either through indirect or direct methods. For GC, an indirect approach is the most common since few chiral stationary phases (CSPs) are available. For LC, high performance liquid chromatography (HPLC) using CSPs is the most used method since there are several available CSPs. Moreover, this method can be coupled with different detection methods such as ultra-violet (UV) and mass spectrometry (MS), which is an advantage [15]. Thus, HPLC using CSPs is considered the most versatile and practical method being used for both analytical and preparative purposes [95]. Nonetheless, ultra-high-performance liquid chromatography (UHPLC) has been gaining more attention since it has higher selectivity, efficiency, and a short analysis time than HPLC and there are already available CSPs that can be adapted for this method [96, 97].

Additionally, supercritical fluid chromatography (SFC), a hybrid of GC and LC, is another chromatographic method that can be used in direct chiral separation. Although this method allows a faster separation of the enantiomers than HPLC, it presents higher costs and more complex hardware, and, as result of that only few studies are reported [15]. Moreover, direct enantiomeric separation can also use capillary electromigration techniques such as capillary electrophoresis (CE) and capillary electrochromatography (CEC). These methods are based on the electrophoretic phenomena for the movement of the enantiomers [93]. In CE, chiral selectors, as cyclodextrins (CD) and their derivatives, are usually added to a running buffer [98, 99]. For CEC, although the mobile phase is also driven by electroosmosis like in CE, the separation mechanism is based on the partition between the liquid and stationary phases, like in HPLC, making this technique a hybrid of CE and HPLC [15, 98]. As will be shown, all these resolution techniques have been used for enantioseparation of cathinone derivatives.

Schmid and Hagele [100] reviewed different techniques that have been developed for the enantiomeric separation of chiral NPS comprising drugs such as, cathinones, amphetamines and ketamines. Similarly, Silva *et al.* [71] have focused their studies on chiral separation of synthetic cathinones.

Silva *et al.* [71] found 12 direct HPLC studies using UV detection and different types of CSPs [77, 88, 101-106]. Aboul-Enein and Serignese [101, 102] developed two direct HPLC methods for the separation of the enantiomers of cathinone using protein-based and crown-ether CSPs. Wolrab *et al.* [103] performed the enantioseparation of 14 cathinone derivatives by HPLC using structurally different ion-exchange type CSPs. Moreover, Silva *et al.* [77] successfully separated nine cathinone derivatives using a HPLC method with polysaccharide-based CSPs under normal phase elution conditions.

Additionally, three CE [106-108] and three CEC [88, 109] methods were also described using a direct chiral separation. For instance, CE using CD derivatives as additives for the buffer was used by Merola *et al.* [107] to separate 12 cathinone derivatives. Ten cathinones were separated using β -CD with UV detection and the other two were separated using highly sulfated(HS)- γ -CD with MS detection [107].

Albals *et al.* [88] performed a comparative study between CEC, SFC and three LC modes: polar organic solvent chromatography (POSC), reversed-phase liquid chromatography (RPLC) and normal-phase liquid chromatography (NPLC). Four different polysaccharide-based CSPs were used for chiral separation of ten cathinone and amphetamine derivatives [88].

Besides that, as indirect chiral separations of synthetic cathinones, three GC-MS methods [89-91, 106] were found, using trifluoroacetyl-L-prolyl chloride (L-TPC) as a chiral

derivatization agent, and lastly, one crystallization method for the chiral separation of MDPV [110].

Most studies were performed with solid samples of cathinones bought online, obtained from seized drugs, or some even synthesized in the laboratories apart from one. To emphasize a study by Baciú *et al.* [108], which developed a method for chiral separation of mephedrone (**9**) and MDPV (**28**) in human hair samples using CE combined in-line with solid-phase extraction (SPE).

Recently, since 2018, many other enantioseparation studies of synthetic cathinones were performed which are compiled in **Table 1**.

Table 1 - Development of analytical methods for enantioresolution of synthetic cathinones from 2018 to 2021.

Analyte	Sample	Method	Analytical conditions	Ref.
3-FMC; 4-FEC; Ethcathinone; Buphedrone; 3-MMC; Pentadron; 4-Methylbuphedrone; 3,4-DMMC; Methedrone; 2,3-MDMC; Eutylone; Pentylone	Urine and plasma	GC-MS (indirect method)	Achiral stationary phase: HP-5MS capillary column Derivatization with L-TPC	[111]
2-AIMP; bk-IVP; 4-BMC; 4-CMC; 5-DBFPV; DL-4662; 4-FMC; 4F-PV8; Methedrone; 3-MeOMC; 3-MEC; 4-MEC; 2-MMC; 3-MMC; 4-MMC; 5-PPDi; α -PVP; 4-MeO- α -PVP; TH-PVP	Solid	HPLC-UV (direct method)	Polar organic mode CSP: Lux® Cellulose-2 column Mobile phase: ACN/IPA/DEA/FA (95:5:0.1:0.1) Flow rate: 1 mL/min UV detection: 254 nm	[112]
4-FMC; 4-FEC; Nor-mephedrone; Buphedrone; 3-MMC; 3-Methylbuphedrone; 4-Methylbuphedrone; 3-EMC; 3-EEC; 4-EEC; 3,4-DMEC; 2,3-MDMC; Butylone; Pentylone	Urine and plasma	GC-MS (indirect method)	Achiral stationary phase: HP-5MS Ultra Inert capillary column Derivatization with L-TPC Flow rate: 0.8 mL/min	[113]
4-MMC; 3-MMC; 2-MMC; 3,4-DMMC; 4-MeOMC; 3-MeOMC; 3-CMC; 4-CMC; 4-EMC; Mexedrone; 4-FMC; 3-FMC; 2-FMC; 4-BMC; Buphedrone; 4-Methylbuphedrone; Pentadron; 3-CEC; 4-CEC; N-Ethyl-Buphedrone; N-Ethyl-Hexedrone; Amfepramone; 4-MEC; 3-MEC; Methylone; Dimethylone; Butylone; N,N-Dimethylbutylone; Pentylone; Ethylone; 5-ME; N-Ethylpentylone; MDPV; MD-PHP; bk-IVP; 5-DBFPV; DOMC; 5-PPDi; TH-PVP; 4-MC; α -PPP; M-PPP; 4-MPrC; 4-MeO- α -PVP; 4-Cl-PVP; Naphyrone	Hydrochloride salts	HPLC-UV (direct method)	NPLC mode CSP: Lux® i-cellulose-5 column Mobile phase: Hex/IPA/DEA (95:5:0.1), Flow rate: 1 mL/min UV detection: 254 nm	[114]
Methcathinone;	Solid	CE	BGE: phosphate buffer I	[115]

4-MMC and 3-MMC		(direct method)	(H ₃ PO ₄ /NaH ₂ PO ₄ , pH 3.0), acetic buffer (CH ₃ COOH/CH ₃ COONa, pH 5.0), and phosphate buffer II (NaH ₂ PO ₄ /Na ₂ HPO ₄ , pH 8.7), all of 50 mM ionic strength with different CD additives DAD: set at 200 nm	
Pentedrone	Powder	HPLC-UV (direct method)	NPLC mode CSP: Chiralpak® AS-H column Mobile phase: Hex/IPA (97:3) Flow rate: 2 mL/min UV detection: 254 nm	[116]
Methylone	Powder	HPLC-UV (direct method)	NPLC mode CSP: Chiralpak® AS-H column Mobile phase: Hex/IPA (85:15, v/v) Flow rate: 2 mL/min UV detection: 254 nm	[116]
4-MC; 2-MMC; 3-MMC; 4-MMC; 3,4-DMMC; 3-MeO-MC; Methedrone; 3-CMC; 4-CMC; 4-EMC; Mexedrone; 2-FMC; 3-FMC; 4-FMC; 4-BMC; Buphedrone; 4-Methylbuphedrone; Pentedrone; Amfepramone; 3-CEC; 4-CEC; DL-4662; <i>N</i> -Ethylhexedrone; 3-MEC; 4-MEC; Bupropione; 4-MPD; <i>N</i> -Ethylbuphedrone; <i>N</i> -Ethylpentedrone; Ethylone; <i>N</i> -Ethylpentylone; 5-ME; bk-lvp; 5-DBFPV; DOMC; 5-PPDi; 4-MBC; Methylone; 2-AIMP; Dimethylone; Butylone; <i>N</i> -Benzylnorbutylone; <i>N,N</i> -Dimethylbutylone; Pentylone; PV8; 4-F-PV8; α -PVP; 4-Cl-PVP; 4F-PVP; 4-MeO- α -PVP; PV9; α -PPP; M-PPP; α -PIHP; 4F-PHP; Naphyrone; MDPV; MDPHP	Solid	CE (direct method)	BGE: 10 mM of a β -CD derivative, 10 mM sodium phosphate adjusted with diluted phosphoric acid (pH 2.5) DAD: set at 209 nm	[117]
Dimethylone; α -PPP; <i>N,N</i> -DMC; 2-Methyl- α -PPP; 4-Ethyl- <i>N,N</i> -DMC; 3-Methyl- α -PPP; 3,4-MD- α -PPP; 4'-MeO- α -PPP; 4'-Methyl- α -PHP; Diethylcathinone; 4-Methyl PBP; α -PVP; α -PBP; 4'-Methyl- α -PPP; 3-Methyl PBP; 3,4-MDPBP; <i>N</i> -Ethyl- <i>N</i> -Methylcathinone; 2-Methyl PBP; 4-MeO- <i>N,N</i> -DMC	Blood and urine	HPLC-UV (direct method)	CSP: Astec® Cellulose DMP column Mobile phase: Hex/IPA/TEA (99.0:1.0:0.1) Flow rate: 0.5 mL/min UV detection: 270 nm	[118]
Dimethylone; <i>N,N</i> -DMC; 2-Methyl- α -PPP; 4-Ethyl- <i>N,N</i> -DMC; 4'-MeO- α -PPP; 3,4-MDPBP; 2-Methyl PBP	Blood and urine	HPLC-UV (direct method)	<u>Direct chiral separation:</u> CSP: Amylose-based Chiralpak® AS-H Mobile phase:	[118]

			Hex/IPA/TEA (99.0:1.0:0.1) Flow rate: 0.5 mL/min UV detection: 270 nm	
MDPV; Mephedrone; Methylephedrine	Urine	SPE- CE (direct method)	BGE: aqueous solution of 70 mM of monosodium phosphate, adjusted to pH 2.5 with concentrated phosphoric acid, containing 8 mM 2- hydroxypropyl β -CD and 5 mM β -CD DAD: set at 200 nm	[119]
Cathinone	<i>Catha edulis</i>	GC-MS (indirect method)	Achiral stationary phase: HP-5 MSI capillary column Derivatization with MCF Flow rate: 1 mL/min	[120]
Mephedrone; Butylone; Flephedrone; Methylone; Methedrone	River water	LC- HRMS (direct method)	RPLC mode CSP: Chiralpak® CBH column Mobile phase: 1 mM ammonium acetate buffer/ MeOH (98:2) Flow rate: 0.4 mL/min	[121]
Nor-Mephedrone; 3-MMC; 4-MMC; 3,4-DMMC; 3-MeO-MC; Methedrone; 3-CMC; 4-CMC; 4-EMC; Mexedrone; 2-FMC; 3-FMC; 4-FMC; 4-BMC; Buphedrone; 4-Methylbuphedrone; Pentedrone; 3-CEC; 4-CEC; DL- 4662; 3-MEC; 4-MEC; Ethcathinone; 4-MPD; <i>N</i> -ethylbuphedrone; <i>N</i> - ethylpentedrone; 4-ethylcathinone; Methylone; 2-AIMP; Dimethylone; Butylone; <i>N,N</i> -dimethylbutylone; Pentylone; Ethylone; 5-ME; bk-iVP; 5-DBFPV; DOMC; 5-PPDi	Hydrochloride salts	HPLC- UV (direct method)	CSP: Phenomenex Lux® AMP Mobile phase: ammonium bicarbonate (5 mM) adjusted to pH 11.3 with conc. ammonium hydroxide/ ACN (70:30) Flow rate: 0.5 mL/min UV detection: 230 nm	[122]
4-MC; 4-MMC; 3-MMC; 3,4-DMMC; 3-CMC; 4-CMC; 4-EMC; 4-FMC; 4- BMC; Buphedrone; 4- Methylbuphedrone; Ethcathinone; 4- EEC; 3-CEC; 4-CEC; <i>N</i> - Ethylbuphedrone; <i>N</i> - Ethylpentedrone; DL-4662; 3-MEC; 4-MEC; <i>N</i> -Propcathinone; 4-MPC; 4- CPRC; Dimethylone; 2-AIMP; Butylone; Ethylone; 5-ME; <i>N</i> - Ethylpentylone; 4-MBC; bk-iVP; DOMC; 4-CDC	Hydrochloride salts	HPLC- UV (direct method)	NPLC mode CSP: Trefoil® CEL1 column with cellulose tris- (3,5-dimethylphenyl- carbamate) Mobile phase: Hex/ButOH/DEA (100:0.3:0.2) Flow rate: 1 mL/min UV detection: 230 nm	[123]
4-MMC; 3-MMC; 2-MMC; Methedrone; 3-MeOMC; 4-CMC; 4- BMC; 4-FMC; 4-EMC; Mexedrone; Buphedrone; 4-Methylbuphedrone; Pentedrone; 3-CEC; 4-CEC; 4-MPD; <i>N</i> -Ethyl-pentedrone; DL-4662; 4-	Solid	HPLC- UV (direct method)	NPLC mode CSP: Lux® i-Amylose-1 column Mobile phase: Hex/IPA/DEA (90:10:0.1) Flow rate: 1 mL/min	[124]

EEC; 4-MPC; 4-CPRC; 4-F-PVP; 4-M-PHP; <i>N</i> -Ethylpentylone; MDPV and TH-PVP			UV detection: 254 nm	
4-MC; 3-CMC; 2-FMC; 3-FMC; 3,4-DMMC; <i>N</i> -ethyl-buphedrone; <i>N</i> -ethyl-hexedrone; Amfepramone; 3-MEC; 4-MEC; Ethcathinone; 4-ClC; 4-Chlorbutcathinone; α -PPP; M-PPP; α -PVP; 4-Cl-PVP; 4-MPrC; 4-MeO- α -PVP; Naphyrone; Methylone; Dimethylone; 2-AIMP; Butylone; <i>N,N</i> -Dimethylbutylone; Pentylone; 5-ME; Ethylone; MD-PHP; bk-iVP; 5-DBFPV; DOMC; 5-PPDI; 5-BPDI; 4-MBC	Solid	HPLC-UV (direct method)	NPLC mode CSP: Lux® i-Amylose-1 column Mobile phase: Hex/IPA/DEA (99:10:0.1) Flow rate: 1 mL/min UV detection: 254 nm	[124]
4-MMC; 3,4-DMMC; 4-EMC; 4-MEC; 4-Methylbuphedrone; Buphedrone; <i>N</i> -Ethylbuphedrone; Pentedrone; Pyrovalerone; bk-PMA; bk-PMMA; Methylone; Ethylone; Butylone; Pentylone; MDPV; MDPBP; Naphyrone; 4F-NEB; 4F-MABP; 2-FMC; 4-FMC; 4-CMC; 4-BMC	Solid	SFC-MS (direct method)	CSP: Chiralpak® ZWIX (+) and Chiralpak® ZWIX (-) Mobile phase: MeOH/H ₂ O/FA (90:10:1) using a gradient elution method Flow rate: 1 mL/min	[125]
Methylone and ethylone	Crystals	LC-MS/MS (direct method)	RPLC mode CSP: Lux® AMP polysaccharide-based chiral column Mobile phase: MeOH with a decreasing concentration gradient from 95% to 85% Flow rate: 0.48 mL/min	[126]
2-FMC; 2-FEC; Buphedrone; 3-MMC; 4-MEC; 3-MethylBP; 2,4-DMMC; 4-Methyl- α -ethylaminobutiophenone; 3,4-DMEC; 4-BMC; Butylone	Urine	GC-NCI-MS/MS (indirect method)	Achiral stationary phase: Agilent Ultra Inert capillary column Derivatization with MCF Flow rate: 1 mL/min	[127]
Mephedrone; Methylone; 4-Methylephedrine; MDPV	Urine	EKS-CE (direct method)	BGE: 70 mM of monosodium phosphate, 8 mM of 2-hydroxypropyl β -CD and 5 mM of β -CD (adjusted to pH 2.5 with concentrated phosphoric acid) DAD: set at 220 nm	[128]
Mephedrone and its metabolites	Hydrochloride salts	CE (direct method)	BGE: 50 mmol/L Phosphate buffer; pH 2.75; 7.5 mmol/L CM- β -CD DAD: set at 258; 236 or 214 nm	[129]
Cathinone	Horse plasma and urine	HPLC-MS/MS (indirect method)	RPLC mode Achiral stationary phase: fused core HALO-C18 column Mobile phase: 5 mM	[130]

			ammonium formate/ 0.1 % FA in H ₂ O/ACN, in linear gradient Derivatization with DNFP- L-V Flow rate: 0.3 mL/min
MDPV	Urine	SPE- CE-MS (direct method)	BGE: 10 mM ammonium acetate aqueous solution (pH 7) with 0.5% (m/v) of sulphated- α -CD Sheath liquid: [131] IPA/H ₂ O/FA 60:40:0.25 (v/v) Flow rate: 3.3 μ L/min

ACN: Acetonitrile; **BGE:** Background electrolyte; **ButOH:** Butanol; **CBH:** Cellobiohydrolase I; **CD:** Cyclodextrin; **CE:** Capillary electrophoresis; **CEC:** Capillary electrochromatography; **CM- β -CD:** Carboxymethyl- β -cyclodextrin; **CSP:** Chiral stationary phase; **DAD:** Diode array detection; **DEA:** Diethylamine; **DNFP-L-V:** *N* α -(2,4-Dinitro-5-fluorophenyl)-L-valinamide; **EKS:** Electrokinetic supercharging; **FA:** Formic acid; **GC:** Gas chromatography; **Hex:** Hexane; **HPLC:** High performance liquid chromatography; **HRMS:** High resolution mass spectrometry; **IPA:** Isopropanol; **L-TPC:** Trifluoroacetyl-L-prolyl chloride; **MCF:** (1*R*)-(-)-Menthylchloroformate; **MeOH:** Methanol; **MS:** Mass spectrometry; **NCI:** Negative ion chemical ionization; **NPLC:** Normal-phase liquid chromatography; **RPLC:** Reversed-phase liquid chromatography; **SFC:** Super critical fluid chromatography; **SPE:** Solid phase extraction; **TEA:** Triethylamine; **UV:** Ultra-violet.

As shown in **Table 1**, recently, Alremeithi *et al.* [113] developed a highly sensitive and selective method for the separation of 14 cathinone derivatives in urine and plasma samples using GC-MS and L-TPC as a derivatization agent. Meetani *et al.* [118] were able to detect and quantify, for the first time, the enantiomers of 18 synthetic cathinones with tertiary amine structure in urine and plasma samples using a direct HPLC-UV method with amylose-based and cellulose-based CSPs. Moreover, Loganathan *et al.* [130] developed an indirect HPLC-MS/MS method using *N* α -(2,4-dinitro-5-fluorophenyl)-L-valinamide (DNFP-L-V) as a derivatization agent for the detection, resolution and quantitation of cathinone enantiomers in horse blood plasma and urine samples, which can be useful for equine anti-doping analysis [130]. Silva *et al.* [116] performed, for the first time, the enantioseparation on a semipreparative scale of the enantiomers of pentedrone (**14**) and methylone (**24**) by HPLC-UV using an amylose-based CSP (**Figure 17**).

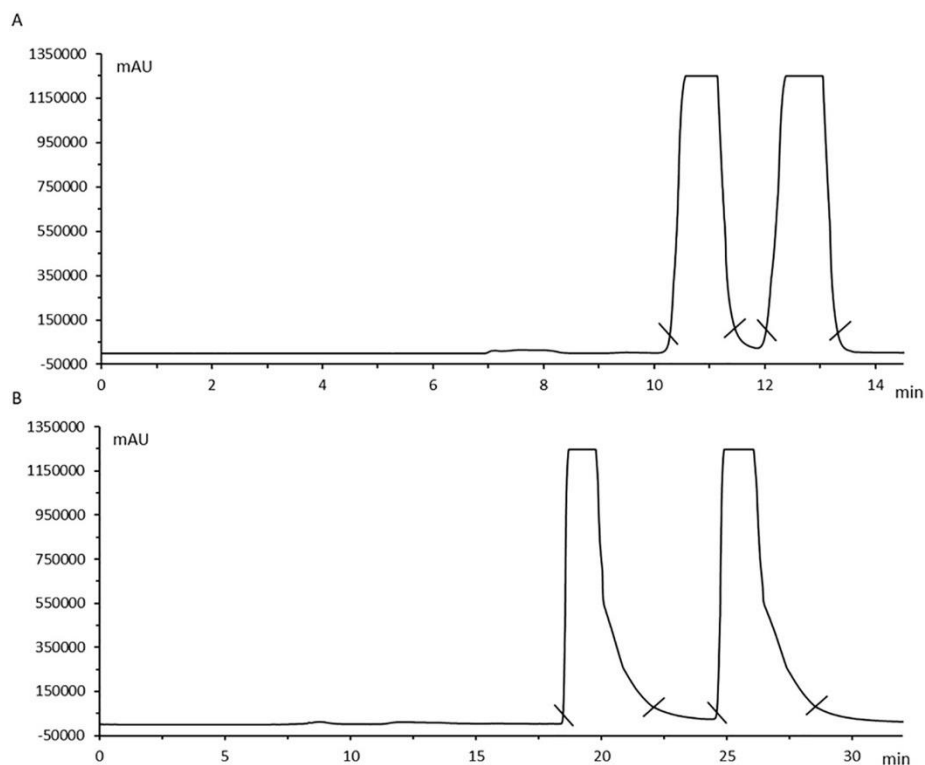


Figure 17 - Chromatograms of the semipreparative enantioseparation of pentedrone (**A**) and methylone (**B**). (Reprint with permission from [116], Copyright (2018) ELSEVIER).

In another study, Fu *et al.* [121] reported a direct method for the chiral separation of cathinones in environmental water samples using LC coupled with high resolution mass spectrometry (HRMS) [121]. Many of the most recent methods were able to successfully separate the enantiomers of a vast number of cathinones derivatives. For instance, Hagele *et al.* [122] used an HPLC-UV method to separate the enantiomers of 39 cathinone derivatives which included ethcathinone, 3-MEC and 4-CEC (**Figure 18**).

Moreover, Kadkhodaei *et al.* [114] developed a direct HPLC-UV method using a cellulose-based CSP, which had the ability to separate 47 synthetic cathinones. Hagele *et al.* [117] used β -CD-assisted CE to separate 58 cathinone derivatives. Kadkhodaei *et al.* [124] separated 62 cathinone derivatives along with some other NPS with a direct HPLC-UV method using an amylose-based CSP. Additionally, these studies found that all the analyzed NPS were purchased as racemic compounds [114, 117, 124].

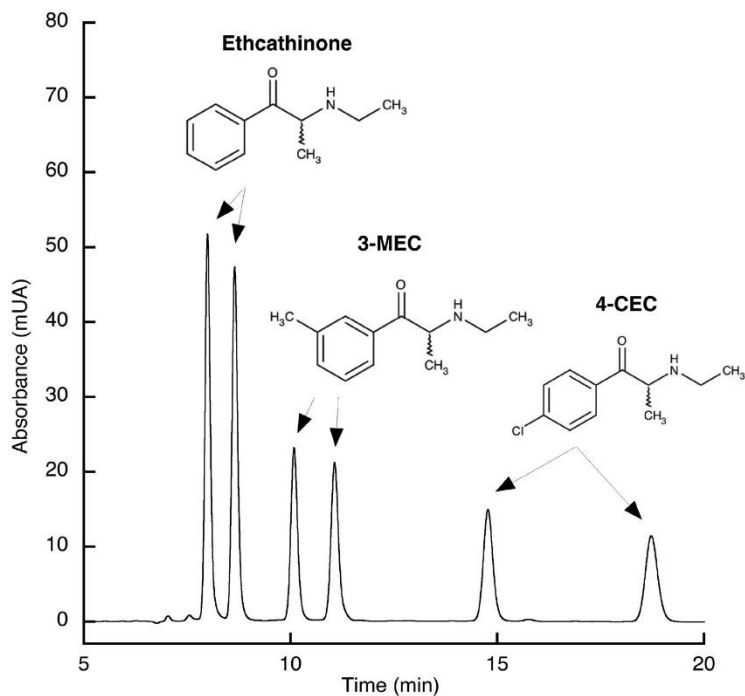


Figure 18 – Chiral separation of three different cathinone derivatives (ethcathinone, 3-MEC and 4-CEC) by HPLC-UV [122].

Figure 19 summarizes all studies previously compiled by Silva *et al.* [71] in addition to more recent studies compiled in **Table 1**, where it is possible to observe that direct methods are preferred over indirect methods. Besides that, HPLC is, undoubtedly, the most used technique. Only one of the 23 HPLC/LC studies reported an indirect chiral separation that used a derivatization step. Moreover, with exception of one study that used a β -CD derivative as a chiral additive for the mobile phase, the rest of them used CSPs. Regarding the detection mode, most HPLC/LC methods used UV detection. Only three studies used MS detection. For UV detection, mobile phases generally contain non-volatile buffers while for MS detection, volatile buffers are necessary. Furthermore, since MS detection needs the formation of ions, the mobile phase should be used to create charged analytes. Thus, the mobile phase pH and the pKa of the analyte are important conditions for this detection. The selection of the pH of the mobile phase can increase sensitivity [132].

For GC-MS, all the methods mentioned were indirect chiral separations as well as the crystallization method previously mentioned. CE, CEC and SFC were all performed as a direct chiral separation. However, while for CE chiral additives were added to the BGE to allow an enantiomeric separation, for the CEC and SFC techniques, CSPs were used. Additionally, NPLC and RPLC modes were the most used.

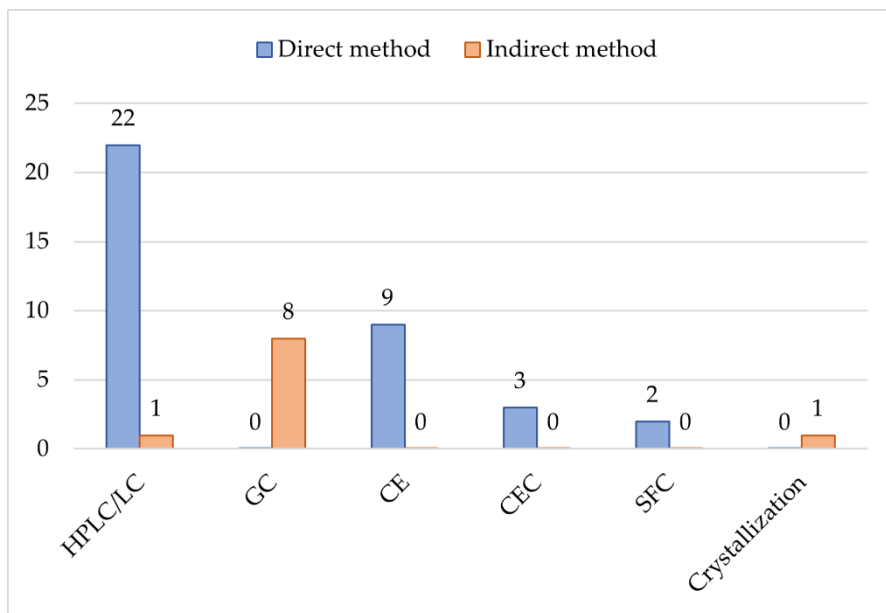


Figure 19 – Methods used for the enantiomeric resolution of cathinones. HPLC: high performance liquid chromatography; LC: liquid chromatography; GC: gas chromatography; CE: capillary electrophoresis; CEC: capillary electrochromatography; SFC: super critical fluid chromatography.

Furthermore, the pie chart in **Figure 20** shows the type of CSPs used in all the HPLC/LC, CEC and SFC methods described above. Clearly, polysaccharide-based are the preferred type of CSPs for the enantioseparation of cathinones since they were chosen in most of the studies. Only 27% of the methods used other type of CSPs.

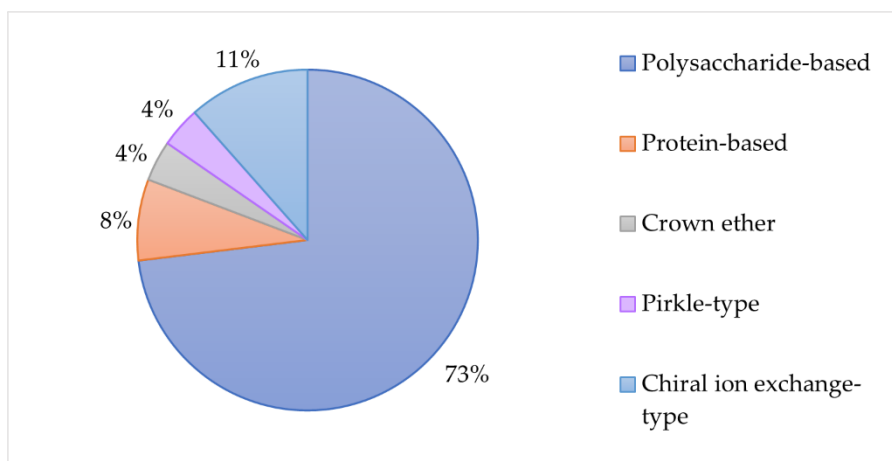


Figure 20 - Types of CSPs used for the enantiomeric resolution of cathinones.

2.8. Conclusions

This review presents an up-to-date report of synthetic cathinones described for the first time since 2014, which include more than 50 derivatives. Synthetic cathinones are still widely abused and novel derivatives keep emerging every year with unknown chemical and biological properties, some of them after minor chemical structure modifications. Consequently, there is still a long way to go to achieve the identification and characterization of all new synthetic cathinones, particularly the properties related to chirality.

Furthermore, this review highlights the relevance of the stereochemistry of synthetic cathinones, which is often overlooked, providing a compilation of the most recent developments in enantioselectivity studies and enantioresolution methods, which will be very useful for everyone working in this research field, and affording innovative perspectives on this topic. Most of the enantioselectivity studies evidenced that the enantiomers of cathinone derivatives displayed different toxicokinetic and/or toxicodynamic properties. These studies are important to determine which enantiomer is responsible for the main biological or toxicological effects and/or potency, presenting a crucial role in cases of cathinone abuse.

Regarding the enantiomeric resolution methods, it was found that HPLC using polysaccharide-based CSPs was the most-used method for the enantioseparation of synthetic cathinones. Even if the number of studies considering stereochemical issues in both biological/toxicological activities and enantioresolution analysis has been growing, it is crucial to go deeper into research regarding the enantioselectivity of these drugs of abuse as the consumption of cathinones continues to increase.

2.9. Updates on novel synthetic cathinones and enantioselectivity studies with synthetic cathinones between February to October 2022

An update of the review article previously presented concerning novel synthetic cathinones that have emerged and studies on enantioselectivity of synthetic cathinones was performed.

Kuropka *et al.* [133] reviewed novel synthetic cathinones emerging from 2019 to 2022 (81-107, Figure 21).

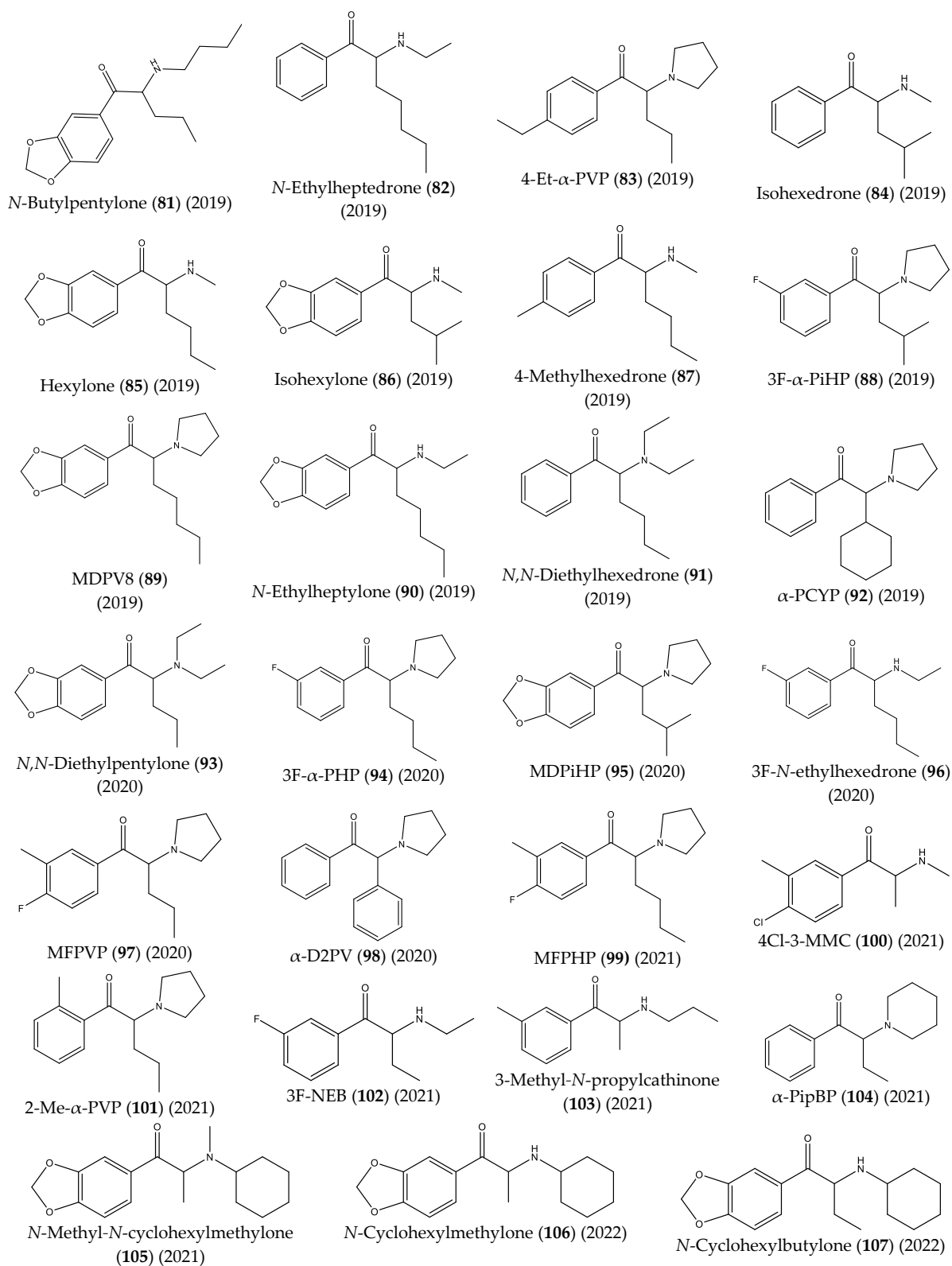


Figure 21 – Structures of the most recent cathinone derivatives (2019-2022) **81-107**.

N-butylpentylone (**81**) was reported in 2019 [134], being one of the most described cathinones in that year in the USA [135]. *N*-Ehtylheptedrone (**82**), 4-ethyl- α -PVP (**83**), isohexedrone (**84**), hexylone (**85**), isohexylone (**86**), 4-methylhexedrone (**87**), 3-F- α -PiHP (**88**), MDPEP (or MDPV8, **89**), *N*-ethylheptylone (**90**) [136] and *N,N*-diethylhexedrone (**91**) [137] were also reported for the first time in 2019. Moreover, although α -PCYP (**92**) was reported for the first time in 2019 [138], it had been previously synthesized in labs in 2015 [139].

Reports of *N,N*-diethylpentylone (**93**), 3F- α -PHP (**94**), MDPiHP (**95**), 3F-*N*-ethylhexedrone (**96**), 4F-3-methyl- α -PVP (or MFPVP, **97**) [136, 140] and α -D2PV (**98**) emerged in 2020 [141, 142].

Furthermore, 4F-3-methyl- α -PHP (or MFPHP, **99**), 4-Cl-3MMC (**100**), 2-Me- α -PVP (**101**), 3F-NEB (**102**), 3-methyl- α -propylaminopropiophenone (or 3-methyl-*N*-propylcathinone, **103**) and α -PipBP (**104**) were described in 2021 [141].

In late 2021 and early 2022, *N*-cyclohexyl-*N*-methylmethylone (**105**) and *N*-cyclohexylmethylone (**106**) were reported for the first time [143, 144]. Lastly, *N*-cyclohexylbutylone (**107**) was reported in 2022 [145].

Relatively to updates on enantioselectivity studies, Silva *et al.* [146] evaluated, for the first time, enantioselectivity in the metabolic pathways of methylone and pentedrone by studies performed human liver microsomes (HLM) and further demonstrated in human stem-cell-derived hepatocyte-like cells (HLCs) cultured under 3D and 2D conditions. The results showed different and preferential metabolic pathways for the enantiomers of these two synthetic cathinones with production of distinct metabolites. *R*-(+)-methylone and *R*-(-)-pentedrone were found to be the most metabolized enantiomers, being *N*-hydroxy-methylone and dihydro-pentedrone, the compounds mostly formed by 3D-HLCs and 2D 2D-HLCs. Additionally, cytotoxic enantioselectivity was found for pentedrone in 3D human hepatocyte-like cells (HLCs) with *R*-(-)-pentedrone displaying the most cytotoxicity [146].

3. BINDING AFFINITY STUDIES ON HUMAN SERUM ALBUMIN

The content presented in this chapter is part of the review article “Enantioresolution and binding affinity studies on human serum albumin: Recent applications and trends” published in Chemosensors in 2021 (<https://doi.org/10.3390/chemosensors9110304>).

3.1. Human Serum Albumin

The binding of drugs and other bioactive compounds to plasma proteins is one of many factors that affect the activity, distribution, excretion and toxicity of such compounds after they enter circulation [147, 148]. Thus, studying this interaction is important to understand how compounds behave in the body and to also determine the unbound portion which reflects the pharmacological/biological effect [149].

Since plasma proteins often present intrinsic chirality, their interaction with chiral molecules can be enantioselective which means that they can display different affinity for the enantiomers, resulting in differences in pharmacodynamic, pharmacokinetic and toxicological properties. As previously mentioned, exploring these differences through enantioselectivity studies is of great importance [3, 150].

The plasma proteins that frequently interact with drugs and other bioactive compounds are HSA and AGP [151]. HSA is the most abundant protein in the human plasma presenting a normal range in adults of 35-50 g/L and a half-life of approximately 20 days [152]. HSA is a 66kDa single polypeptide chain composed by 585 amino acids and a structure of three similar helical domains (I-III), each divided into A and B subdomains [153]. This globular domain structure contributes to a diversity of ligand binding sites [154]. Many frequently used drugs with acidic or electronegative characteristics, such as warfarin and ibuprofen, usually bind to HSA at one of the two main binding sites, Sudlow's site I (located in subdomain IIA) and Sudlow's site II (located in subdomain IIIA) (**Figure 22**) [154, 155].

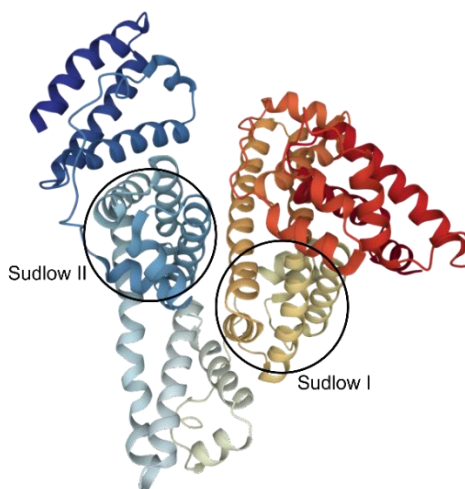


Figure 22 – HSA's structure (from Protein Data Bank:1AO6) with Sudlow's site I and Sudlow's site II, the main binding sites, evidenced.

HSA presents a wide range of physiological roles being often described as a “multifunctional plasma protein”. These roles include the ability to maintain the oncotic pressure and capillary permeability, regulation of acid-base balance, influence in blood coagulation and antioxidant properties. Moreover, HSA is responsible for the transport of various endogenous such as fatty acids, thyroxine and bilirubin and exogenous compounds as well as the protection against xenobiotics such as drugs and toxins [152, 153]. Due to its abundance and participation in many key physiological functions, HSA also displays the highest potential for stereoselectivity among all plasma proteins [156]. Many examples of enantioselective binding to HSA and other plasma proteins have been described for several compounds [157-159]. For instance, Maddi *et al.* [157] studied the binding of the enantiomers of amlodipine to HSA, bovine serum albumin (BSA), AGP, and human plasma (HP). A higher affinity was found for the binding of (*S*)-amlodipine to HSA and HP whereas (*R*)-amlodipine bound to BSA and AGP to a higher extent [157]. Additionally, another study showed that HSA and BSA present a stronger binding affinity for (*R*)-diclazuril than the (*S*)-enantiomer [158]. Similarly, for mexiletine, the (*R*)-enantiomer displayed higher affinity to HSA [159].

3.2. High-Performance Affinity Liquid Chromatography

Being the most abundant protein, HSA is, consequently, the most studied in binding affinity and enantioseparation studies [160]. Over the years, several methods have been described for HSA binding affinity studies which include traditional separative techniques such

as equilibrium dialysis [161], ultrafiltration [162] and ultracentrifugation [163] as well as chromatographic [164, 165] and electrophoretic methods [166, 167]. In fact, in addition to binding affinity studies, several chromatographic and electrophoretic methods using HSA as a chiral selector have been developed for the enantioseparation of chiral compounds [168, 169].

One method that is becoming increasingly popular in the area of binding affinity studies is high-performance affinity liquid chromatography (HPALC). HPALC uses an HPLC column with an immobilized ligand capable of binding the compound or group of compounds of interest [170]. Compounds with more affinity to the immobilized ligand are eluted later than compounds with less or even no affinity [171].

HPALC offers many advantages such as high precision and speed, easy automation, great reproducibility, extensive applicability and the possibility to work under near-physiological conditions [172, 173]. Additionally, the same ligand can be reused for various applications and a small amount of protein is required for a large number of studies [147, 170].

Regarding chiral compounds, by HPALC using an HSA-CSP, the enantioseparation of certain compounds can be achieved allowing the analysis of both enantiomers of racemic or enantiomeric mixtures in the same run [174, 175]. For instance, Do Carmo *et al.* [168] achieved the enantioseparation of 14 chiral derivatives of xanthenes by a HPALC method using an HSA-CSP. Moreover, the chiral derivatives of xanthenes analyzed in this work were found to present, in general, a high affinity to HSA [168]. Andrisano *et al.* [176] studied the stereoselective binding of a series of arylpropionic acids on HSA. The enantioseparation of the racemates was obtained by an HSA column. The retention properties of the compounds were analyzed, and enantioselective differences were found when comparing stereoisomeric forms.

HPALC has been used to study the overall binding of many achiral and chiral compounds to HSA along with information about the equilibrium constants and number of binding sites [172]. Furthermore, in order to obtain information about the binding sites where compounds might bind to HSA, this technique allows the possibility to perform displacement studies using competitive agents known to bind selectively to the main binding sites of HSA [177, 178]. For instance, Pérez *et al.* [179] studied the binding of several quinazoline derivatives to HSA by HPALC. Displacement chromatography was performed to obtain information of the HSA binding site of these compounds by addition of increasing concentrations of sodium salicylate, ibuprofen and sodium valproate (markers for site I, II and bilirubin site on HSA) in the mobile phase of the study. The results showed that both salicylate and ibuprofen present competition with quinazolines which suggests that sites I and II are both involved in the binding of these compounds to HSA [179]. Likewise, a competition study was performed to identify the

binding sites of imipramine on HSA using warfarin and L-tryptophan (known to bind to site I and II) as competitors. The results indicated that imipramine seems to bind to site II due to competition observed with L-tryptophan whereas no competition was observed with warfarin [164].

AIMS

AIMS

The general aim of this work was to optimize the enantioresolution of MDPV by liquid chromatography and to evaluate, *in vitro*, the enantioselectivity of the MDPV enantiomers at some toxicodynamic and toxicokinetic parameters.

The specific aims of this work were:

- The semi-preparative enantioresolution of MDPV by liquid chromatography using an amylose-based CSP;
- A racemization study with the separated MDPV enantiomers by liquid chromatography using an amylose-based CSP to evaluate the effects of different temperatures (room temperature (RT), 37 °C and 70 °C) and basic conditions (diisopropylethylamine (DIIPEA)) over two days;
- Evaluation of the potential enantioselectivity of the enantiomers of MDPV in cytotoxicity by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral red or NR) assays in undifferentiated and differentiated SH-SY5Y cells;
- Evaluation of the potential enantioselectivity of the enantiomers of MDPV in the expression of neuroplasticity-involved proteins: brain-derived neurotrophic factor (BDNF) and cyclin-dependent kinase 5 (Cdk5) in undifferentiated SH-SY5Y cells by western blotting analysis;
- Development and validation of an UHPLC-UV method for the detection and quantification of MDPV in HBSS(+/+) to further evaluate potential enantioselectivity of the enantiomers of MDPV on absorption using the Caco-2 cell line;
- Displacement experiment by HPLAC using an HSA chiral column and (S)-ibuprofen and warfarin as competitors to obtain information about the binding sites of several synthetic cathinones (enantiomers of synthetic cathinones MDPV, bk-DMBDB and pentylone and racemic pentedrone) on HSA.

MATERIAL AND METHODS

1. Semi-preparative enantioresolution of the enantiomers of MDPV
2. Racemization study with the enantiomers of MDPV
3. Enantioselectivity studies with the enantiomers of MDPV in the SH-SY5Y cell line
4. Permeability assay with the enantiomers of MDPV using the Caco-2 cell line
5. Displacement experiment with synthetic cathinones on HSA

1. Semi-preparative enantioresolution of the enantiomers of MDPV

1.1. Reagents and samples

MDPV was obtained online from the Sensearomatics website (www.sensearomatics.eu, website unavailable at the moment). Ethanol (EtOH) and Hex for HPLC, DEA and HCl 2 M in diethyl ether were purchased from Sigma-Aldrich Co (St. Louis, Missouri, USA).

1.2. Sample preparation

Racemic MDPV was dissolved in EtOH to afford a concentration of 10 mg/mL from which injections of 100 μ L were performed.

1.3. Instrumentation and chromatographic conditions

A Thermo® Scientific HPLC with a Thermo® Scientific Spectra System P4000 pump was used, with a Thermo® Scientific Spectra AS3000 automatic injector and a Thermo® Scientific Spectra System UV8000 model DAD. The software used to process the chromatographic data was Chromeleon™ 7.0.

Chromatography analyses were performed on an home-made column of tris-3,5-dimethylphenylcarbamate amylose coated onto aminopropylsilyl Nucleosyl (500 Å, 7 μ m, 20%, w/w) and packed into a stainless-steel column (20 cm $\times$ 7.0 mm ID) [180].

The analyses were performed in normal phase elution mode with Hex:EtOH:DEA (97:3:0.1) as the mobile phase. The mobile phases were degassed in an ultrasonic bath (Sonorex Digitec, Bandelin) for at least 15 min before use. The flow rate used was 1.5 mL/min. Cycles of 2 injections of 100 μ L of racemic MDPV were intercalated with one injection of 50 μ L of EtOH being the duration of each run 25 min. Chromatographic analyses were performed in isocratic mode at 25 ± 2 °C with UV detection at 254 nm.

1.4. Hydrochloride formation

After collecting, the enantiomeric fractions were placed in a round bottom flask and solvent evaporation was performed using a rotary evaporator under reduced pressure. The enantiomers were transferred to smaller vials. Hydrochloride formation was performed by precipitation on ice with HCl (2 M) on diethyl ether.

1.5. Chromatographic parameters determination

The retention factor (k) was obtained using the equation (1):

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

The dead time (t_0) was considered to be the peak of the solvent front. The separation factor (α) was calculated using the equation (2):

$$\alpha = \frac{k_2}{k_1} \quad (2)$$

Furthermore, the following equation was used to calculate the resolution factor (R_s):

$$R_s = 1.18 \frac{t_{R2} - t_{R1}}{W_{10.5} + W_{20.5}} \quad (3)$$

where t_{R1} and t_{R2} are the retention times of the first and second enantiomers, respectively, and $W_{10.5}$ and $W_{20.5}$ are the corresponding peak width measured on half height.

Enantiomers were reinjected to determine the enantiomeric ratio (e.r.) obtained by the relative percentages of the peak areas [181]:

$$e.r. (\%) = 100 \frac{[E_1]}{[E_1] + [E_2]} \text{ or } 100 \frac{[E_2]}{[E_1] + [E_2]} \quad (4)$$

where $[E_1]$ and $[E_2]$ are the area of the peak of each enantiomer.

2. Racemization study with the enantiomers of MDPV

2.1. Reagents and samples

EtOH and Hex for HPLC, and DEA were purchased from Sigma-Aldrich Co.

2.2. Sample preparation

Fractions of each enantiomer collected previously were dissolved in EtOH to afford a concentration of 0.5 mg/mL. Twelve vials, six from each enantiomer, were prepared by diluting the previous solutions to afford a concentration of 50 μ g/mL and, to three vials from each enantiomer, DIPEA (0.1%) was added. A set of vials, one with amine and one without, from each enantiomer, were exposed to different temperatures: RT, 37 °C and 70 °C.

2.3. Instrumentation and chromatographic conditions

A JASCO model 880-PU pump HPLC apparatus with a Rheodyne model 7125 injector equipped with a 20 μ L sample loop, a JASCO model 880-30 solvent mixer and a JASCO model 875-UV detector (Tokyo, Japan) was used. Data was obtained using ChromNAV.

Chromatographic analyses were performed on a Lux Amylose-I ® column (tris-3,5-dimethylphenylcarbamate amylose bonded to 5 μ m particle size; 25 cm, 4.6 mm I.D.) from

Phenomenex, Inc. USA. Injections (10 μ L) were performed every 30 min for 3 h and after 24 h and 48 h for each condition with a flow rate of 1 mL/min. The analyses were performed in normal phase elution mode with a mixture of Hex:EtOH:DEA (97:3:0.1) as mobile phase.

Chromatographic analyses were performed in isocratic mode at 25 ± 2 °C with UV detection at 254 nm.

Each run (20 min) was monitored for the formation of the other enantiomer and e.r. were calculated for each chromatogram (equation 4).

3. Enantioselectivity studies with the enantiomers of MDPV in the SH-SY5Y cell line

3.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, Triton X-100, MTT and NR solutions were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), antibiotic (10 000 U/mL penicillin, 10 000 μ g/mL streptomycin), 0.25% trypsin/1 mM EDTA and Hank's balanced salt solution with or without calcium and magnesium [HBSS (+/+) or HBSS (-/-), respectively] were obtained from Gibco Laboratories (Lenexa, KS, USA). All other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany), unless stated otherwise.

3.2. Cell culture

The SH-SY5Y human neuroblastoma cell line was acquired from ATCC (American Tissue Culture Collection, Manassas, VA, USA), and routinely maintained in DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin solution, at 37 °C in a humidified atmosphere of 5% CO₂ and the medium was changed every 2 days. Subcultures were performed by trypsinization with a 0.25% trypsin/EDTA solution once cultures reached about 70-80% confluence.

Cytotoxicity assays were performed in both undifferentiated and differentiated SH-SY5Y. Cells were seeded in 96-well plates at a density of 25 000 cells/cm². SH-SY5Y cell differentiation was induced with 10 μ M retinoic acid (RA) for 72 h, followed by 80 nM tetradecanoyl phorbol 13-acetate (TPA) for another 72 h, as previously described to obtain a dopaminergic phenotype [182]. Undifferentiated cells were allowed to grow during the same time without the addition of differentiation factors.

For the preparation of total protein extracts for western blot analysis, undifferentiated cells were seeded in 6-well plates at a density of 25 000 cells/cm² and left to adhere overnight

before exposure.

For all assays, cells were used between passages 23 to 31.

3.3. MTT reduction assay

The cells' metabolic activity was assessed using the MTT reduction assay as previously described [183]. This method assesses the reduction of MTT by mitochondrial dehydrogenases and intracellular NADPH-dependent oxidoreductases into formazan crystals. After the differentiation protocol described above, the medium was removed and the cells exposed to different concentrations of each enantiomer of MDPV (0.773; 1.165 and 1.506 mM [182]), in fresh cell culture medium, for 24 h. After the selected time interval, medium was removed and replaced by a 0.5 mg/mL MTT solution in HBSS(+/-) for 90 min, after which the formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and quantified at 550 nm in a multi-well plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA). 1% Triton X-100 was used as the positive control. Data were expressed as the percentage of metabolic activity relative to untreated cells. Data were obtained from four independent experiments, performed in triplicate.

3.4. Neutral Red (NR) assay

Lysosomal integrity was assessed using the NR uptake assay as previously described [183]. This method provides an indication of the ability of lysosomes from viable cells to incorporate the NR. After the differentiation protocol described above, the medium was removed and the cells were exposed to different concentrations of each enantiomer of MDPV (0.773; 1.165 and 1.506 mM [182]), in fresh cell culture medium, for 24 h. After the selected time interval, medium was removed and replaced by a 50 µg/mL NR solution in HBSS(+/-) for 90 min, after which the NR solution was discarded, a lysis solution (50% EtOH/1% glacial acetic acid solution) added and the absorbance read at 540 nm in a 96-well plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA). 1% Triton X-100 was used as the positive control. Data were expressed as the percentage of cell viability relative to untreated cells. Data were obtained from four independent experiments performed in triplicate.

3.5. Preparation of total protein extracts

After 24 h of exposure to 0.773 µM and 0.773 mM and of each enantiomer of MDPV, total protein extracts were prepared. Cells were collected with a Teflon cell scraper and centrifuged at 1000 g for 5 min at 4 °C. The pellets were washed with cold HBSS(-/-) and again centrifuged at 1000 g for 5 min, at 4 °C. The pellets were resuspended in collecting buffer (20

mM HEPES, 250 mM Sucrose, 10 mM KCl, 2 mM MgCl₂, 1mM EDTA, pH 7.5) supplemented with 2 mM dithiothreitol (DTT), 100 μ M phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail containing 1 μ g/ml of leupeptin, antipain, chymostatin, and pepstatin A. The cells were disrupted through sonication with three 10 s pulses, separated by 10 s intervals on ice, and stored at -80 °C until use. The Bradford method was used to obtain the total protein content.

3.6. Western Blotting analysis

Expression of BDNF and Cdk5 was detected through Western Blotting [183]. A total of 25-30 μ g of protein for each sample were denatured in sample loading buffer at 95 °C and loaded onto 10 or 15% acrylamide/bis-acrylamide sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE). After electrophoresis, the proteins were transferred to nitrocellulose membranes. Membranes were, then, blocked in 5% skim milk for 2 h, washed three times in TPBS (phosphate buffered saline, pH 7.4, containing 0.05% Tween 20), and incubated overnight at 4 °C with the following antibodies: anti-Cdk5 antibody (1:200 dilution) (Santa Cruz Biotechnology, CA, USA) and anti-BDNF antibody (1:200) (Santa Cruz Biotechnology, CA, USA). β -actin detection was used as a protein loading control, by incubation with an anti- β -actin antibody (1:5000) (Sigma-Aldrich, St. Louis, MO, USA). In the following day, the membranes were washed in TPBS and incubated for 1 h with a secondary anti-mouse horseradish peroxidase (HRP) conjugate antibody (1:20000). Immunoreactive bands were viewed using the chemiluminescent Clarity™ Western ECL substrate and analyzed using the Bio-Rad ChemiDoc™ XRS + System with Image Lab™ Software (Hercules, CA, USA). Band intensities were quantified using ImageLab 6.1 (Bio-Rad, Berkeley, CA, USA) and normalized against the intensities of β -actin. Results were expressed as fold-change relatively to the control (untreated cells).

3.7. Statistical analysis

All statistical analysis were performed using the GraphPad Prism 9.0 software. Data were presented as mean $\pm$ standard deviation of the mean (SD) of the indicated number of independent experiments. The Shapiro-Wilk and Kolmogorov-Smirnov normality tests were used to evaluate the normality of data distribution. One/two-way analysis of variance (ANOVA) were used to make statistical comparisons, followed by the Holm-Sidak's/Tukey's multiple comparisons test. Statistical significance was considered for p values lower than 0.05.

4. Permeability assay with the enantiomers of MDPV using the Caco-2 cell line

4.1. Reagents

Ammonium acetate and ACN were acquired from Carlo Erba Reagents S.A.S (Val de Reuil, FR). FA, DMEM with 4.5 g/L glucose, Triton X-100, BSA and the NR solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibiotic (10 000 U/mL penicillin, 10 000 µg/mL streptomycin), 0.25% trypsin/1 mM EDTA, FBS and HBSS (+/+) were obtained from Gibco Laboratories (Lenexa, KS, USA).

4.2. Instrumental and chromatographic conditions

A Thermo® Scientific UHPLC with a Thermo® Scientific Spectra System P4000 pump was used, with a Thermo® Scientific Spectra AS3000 automatic injector and a Thermo® Scientific Spectra System UV8000 model DAD. The software used to process the chromatographic data was Chromeleon™ 7.0. A Kinetex® EVO C18 LC column (1.7 µm particle diameter; 2.1 mm × 100 mm ID) connected to an SecurityGuard™ ULTRA pre-column (sub-2 µm; 2 mm × 2.1 mm ID) was used. Chromatography analyses were performed at room temperature at a flow rate of 0.12 mL/min using 25 mM ammonium acetate:ACN:FA (75:25:0.1) as the mobile phase. The sample injection volume was 5 µL. Stock standards of 5 mM of MDPV and all solutions were prepared in HBSS (+/+) and filtered through a 13 mm nylon syringe filter with 0.22 µm pore size from Olimpeak™. Calibrators were prepared by the dilution of the stock standards with filtered HBSS (+/+) to final concentrations of 0.5, 1, 5, 10, 25, 50, 100, 250 and 500 µM. To obtain the optimal wavelength for the detection and quantification of MDPV, UV spectra were determined for MDPV [100 µM in HBSS (+/+)], the selected mobile phase and HBSS (+/+) using a UH5300 spectrophotometer.

4.3. Method validation

The Food Drug Administration (FDA) and the International Conference on Harmonization (ICH) guidelines were followed to validate this method [184, 185]. The parameters evaluated were specificity, linearity, accuracy, inter- and intraday precision, limit of detection (LOD) and limit of quantification (LOQ). The stability of the analytes was also evaluated. Results were analyzed using the GraphPad Prism Software 9.0 and Microsoft Excel.

4.3.1. Specificity

Twenty blank samples [HBSS (+/)] were injected into the UHPLC to detect the presence of potential co-eluting peaks that could affect the analysis by the tested method.

4.3.2. Linearity

To evaluate linearity, calibrators with concentrations ranging from 0.5 to 500 µM of MDPV (0.5, 1, 5, 10, 25, 50, 100, 250 and 500 µM) were independently prepared in five different days and injected into the UHPLC in order to obtain a calibration curve each day. The plot of peak area vs concentration was analyzed by linear regression considering the coefficient of determination (r^2) determined.

4.3.3. Accuracy

The accuracy of an analytical method represents the deviation between the experimental concentration and the expected (nominal) concentration being calculated by the following equation [184]:

$$\text{Accuracy (\%)} = \frac{\text{mean experimental concentration}}{\text{nominal concentration}} \times 100 \quad (5)$$

For the evaluation of this parameter, three different calibrators within the linear concentration range (6 (low), 40 (medium) and 300 (high) µM) were selected. Five independent solutions were prepared and injected in triplicate for each concentration. Furthermore, a calibration curve was injected and used to determine the experimental concentration of each calibrator.

4.3.4. Precision

The precision of the methodology shows the proximity between a series of data acquired from several injections under the same conditions. The repeatability of the method can be determined for a short time interval (intra-day precision), or within different days (inter-day precision). Three calibrators, with concentrations within the linear concentration range (10 (low), 100 (medium) and 500 (high) µM), were selected for the evaluation of this parameter.

The inter-day precision of the equipment was evaluated by the preparation and injection in triplicate of the three calibrators in five consecutive days. For the determination of inter-day precision of the analytical method, the three calibrators were independently prepared and injected in triplicate in five consecutive days.

The intra-day precision of the equipment was evaluated by preparing and injecting five times the three calibrators on the same day. Lastly, for the determination of the intra-day precision of the analytical method, five solutions of the three calibrators were prepared independently and injected in triplicate on the same day.

Precision was expressed as the coefficient of variation of each calibrator (%CV).

4.3.5. LOD and LOQ

LOD and LOQ were calculated using the equations [185]:

$$LOD = \frac{3\sigma}{S} \quad (6)$$

$$LOQ = \frac{10\sigma}{S} \quad (7)$$

where σ is the standard deviation of the analytical signal of 20 blank samples and S the slope of the calibration curve.

4.3.6. Stability

Stability was analyzed through temperature and storage interval. Three different calibrators within the linear concentration range (10 (low), 100 (medium) and 500 (high) μM) were prepared and injected into the UHPLC. The remaining volume of each calibrator was divided into several vials and further stored at different temperatures (room temperature, 4 °C, -20 °C and -80 °C) for 6 weeks (same storage time of samples obtained from the permeability assay with Caco-2 cells). After the storage period, samples were injected in duplicate and the obtained analyte peak area for each calibrator in each temperature was compared with the peak area obtained on day 1. Percentages of peak variation were calculated for each concentration.

4.4. Caco-2 cell culture

The Caco-2 cell line was acquired from the European Collection of Cell Culture (ECACC, UK) and routinely maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin solution and 1% non-essential amino acids, at 37 °C in a humidified atmosphere of 5% CO_2 with the medium changed every two days. Subcultures were obtained by trypsinization with a 0.25% trypsin/EDTA solution. For all assays, cells were used between the 14th and 20th passages.

4.4.1. NR assay

MDPV cytotoxicity for the Caco-2 cell line was evaluated through the NR assay. Caco-2 cells were seeded onto 96-well plates using a density of 60 000 cells/cm², to obtain confluent monolayers at the experimental day. The cells were incubated with the racemate of MDPV (0, 50, 150, 300, 500, 750, 1000, 1250, 1500 μM) in fresh cell culture medium for 24 h. After the selected time interval, the cell culture medium was removed and replaced by a 50 $\mu\text{g/mL}$ NR solution in HBSS(+/-) for 90 min, after which the NR solution was discarded, a lysis solution (50% EtOH/1% glacial acetic acid solution) added and the absorbance read at 540 nm in a 96-

well plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA). 1% Triton X-100 was used as the positive control. Data were expressed as the percentage of cell viability relative to untreated cells. Data were obtained from four independent experiments performed in triplicate.

4.4.2. Permeability assay

For the *in vitro* permeability assay, Caco-2 cells were seeded on polycarbonate transwell inserts (12-well, 0.4 µm pores, Corning) using a density of 120 000 cells/cm² and cultivated for twenty-one days to allow the development of a differentiated monolayer. Transepithelial electrical resistance (TEER) was measured to control the integrity of the monolayers, for twenty-one days with a final average value of 1052 Ω·cm².

After twenty-one days, the cells were incubated with 300 µM of the MDPV enantiomers [in HBSS(+/+)]. The assay was performed for both apical (AP) to basolateral (BL) and BL to AP directions. For AP to BL, exposure to the enantiomers was performed in the AP compartment (450 µL) while for BL to AP, exposure was made in the BL compartment (1250 µL). Right after the exposure (time 0) 50 µL were collected from each donor compartment (where exposure was performed). At suitable time intervals (40, 60, 90, 120, 180, 240, 300 min), 600 µL (for AP to BL) and 200 µL (for BL to AL) of samples were collected from the BL and AP compartments (acceptor compartments), respectively, and the same amount of HBSS (+/+) was added. In the last time point (300 min), 50 µL were also collected from the donor compartment. Collected samples were stored at -80°C until the day of the UHPLC analysis. After 6 weeks, samples were filtered with a 13 mm nylon syringe filter with 0.22 µm pore size from Olimpeak™ and quantification was performed by the validated UHPLC-UV method. Results were obtained for three experiments, performed in duplicate.

The final cumulative quantity calculated at the end of the time interval (nmol cum_{final}) and quantity in the donor compartment at 0 and 300 min were used to calculate the mass balance of MDPV [186]:

$$\% \text{ Mass balance} = 100 \times \frac{\text{nmol cum}_{\text{final}} + \text{donor nmol}_{\text{final}}}{\text{nmol}_{\text{initial}}} \quad (8)$$

The permeability coefficient (P_{app}) was calculated using the following equation [84]:

$$P_{\text{app}} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0} \quad (9)$$

where P_{app} is the permeability coefficient (cm/s), $\Delta Q/\Delta t$ is the amount of compound over time (mol/s), A is the surface area of the monolayer (cm²) and C_0 is the initial drug concentration on the donor side (mol/mL).

The efflux ratio, ratio between the permeability coefficients obtained for each direction,

was calculated using the following equation [186]:

$$Efflux\ ratio = \frac{P_{app}(BL \rightarrow AP)}{P_{app}(AP \rightarrow BL)} \quad (10)$$

4.5. Statistical analysis

All statistical calculations were performed using GraphPad Prism 9.0 for Windows (GraphPad Software, San Diego, CA, USA) and Microsoft Excel. Kolmogorov-Smirnov and Shapiro-Wilk normality tests were used to evaluate the normality of the data distribution. For the cytotoxicity studies, the statistical comparisons were performed using one-way ANOVA, followed by the Holm-Sidak's multiple comparisons test. For the permeability experiment, one/two-way ANOVA were used to make statistical comparisons, followed by the Holm-Sidak's/Tukey's multiple comparisons test. Differences were considered significant for p values lower than 0.05.

5. Displacement experiment with synthetic cathinones on HSA

5.1. Reagents and samples

EtOH, Hex, ACN for HPLC, TEA, DEA, HCl 2 M in diethyl ether, potassium phosphate, phosphoric acid, warfarin, and (S)-ibuprofen were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA) or Carlo Erba (Barcelona, Spain). Ultrapure water was generated by a Milli-Q system (Millipore, Bedford, MA).

5.2. Sample preparation

The enantiomers of MDPV, bk-DMBDB and pentylone, separated previously [187], and racemic pentedrone were selected for this study. The synthetic cathinones were dissolved in EtOH to afford concentrations of 0.5 mg/mL which were further diluted in potassium phosphate buffer (10 mM, pH 7) to 50 µg/mL.

5.3. Instrumentation and chromatographic conditions

A Thermo® Scientific HPLC with a Thermo® Scientific Spectra System P4000 pump was used, with a Thermo® Scientific Spectra AS3000 automatic injector and a Thermo® Scientific Spectra System UV8000 model DAD. The software used to process the chromatographic data was Chromeleon™ 7.0.

The chromatography analyses were performed on a CHIRALPAK® HSA column (150 mm × 40 mm, I.D., 5 µm particle size) from Chiral Technologies Europe, Daicel Chemical Industries, LTD. The sample injections (10 µL) were carried out in duplicate and the flow rate

used was 0.5 mL/min. The analyses were performed in reversed-phase mode with a mixture of potassium phosphate buffer (10 mM, pH 7.0):ACN (99:1) as the mobile phase. The pH of the potassium phosphate buffer was controlled by a Gondo® PL-700PV pH monitor. Warfarin and (S)-ibuprofen were chosen as competitors and were added to the mobile phase in various concentrations (0-20 µM).

Chromatographic analyses were performed in reversed-phase isocratic mode at 25 ± 2°C with UV detection at 254 nm. All mobile phases were filtered through polyamide membrane filters with 0.2 µm pore size from Whatman® GmbH (Dassel, Germany) and degassed in an ultrasonic bath (Sonorex Digitec, Bandelin) for at least 15 min before use. All mobile phases were left conditioning overnight to stabilize.

5.4. Determination of the association equilibrium constant (K_I)

The following equation can be used to describe the effect of the competitor (I) on the retention of each analyte (A) [178]:

$$\frac{1}{k} = \frac{V_M K_I [I]}{K_A m_L} + \frac{V_M}{K_A m_L} \quad (11)$$

where k is the retention factor of A, V_M is the void volume of the column, K_I is the association equilibrium constant of I at the binding site of competition with A, $[I]$ is the concentration of the competitor in the mobile phase, m_L is the moles of binding site involved in the competition, and K_A is the association equilibrium constant of A at the binding site of competition with I. When the analyte competes with the competitor, a straight line with a positive slope is observed in the plot of $1/k$ versus $[I]$, while random variations are observed when there is no competition [164]. If competition is observed, the equilibrium dissociation constant (K_D) of the competitor in the binding site of the analyte can be obtained by [177]:

$$\frac{\text{intercept}}{\text{slope}} = \frac{1}{K_I} = K_D \quad (12)$$

RESULTS AND DISCUSSION

1. Semi-preparative enantioresolution of the enantiomers of MDPV
2. Racemization study with the enantiomers of MDPV
3. Enantioselectivity studies with the enantiomers of MDPV in the SH-SY5Y cell line
4. Permeability assay with the enantiomers of MDPV using the Caco-2 cell line
5. Displacement experiment with synthetic cathinones on HSA

1. Semi-preparative enantioresolution of the enantiomers of MDPV

The semi-preparative enantioresolution of the enantiomers of MDPV was based in a previous work by our group where LC with an amylose-based CSP and Hex:EtOH:TEA (97:3:0.1) as the mobile phase were used [77]. In this work, this separation was optimized. Firstly, the mixture Hex:EtOH (97:3) was selected as the mobile phase and TEA was added to the solutions of MDPV, to avoid further procedures for TEA removal which decreased the recovery rates of the enantiomers in the previous work. However, after 24 h, due to the presence of TEA, the samples of MDPV started to degrade. To avoid degradation of the samples, TEA was no longer added to the samples and DEA was selected as the additive of the mobile phase instead of TEA. DEA was selected due to a lower boiling point than TEA (55.5 °C and 89.3 °C, respectively) so evaporation could be performed more easily and no extra steps for its removal were necessary. Thus, the optimized conditions were Hex:EtOH:DEA (97:3:0.1) as the mobile phase and a flow rate of 1.5 mL/min. A solution of MDPV was prepared in a concentration of 10 mg/mL in EtOH. Cycles of 2 injections of 100 μ L of MDPV were intercalated with one injection of 50 μ L of EtOH being 25 min the duration of each run.

The enantiomers of MDPV were successfully separated by this method with a retention time (t_R) of 12.0 min for the first enantiomer (E1) and 15.0 min for the second enantiomer (E2) and t_0 of 4.4 min corresponding to the solvent front (**Figure 23**). α and R_s values of 1.4 and 1.7 were obtained, respectively.

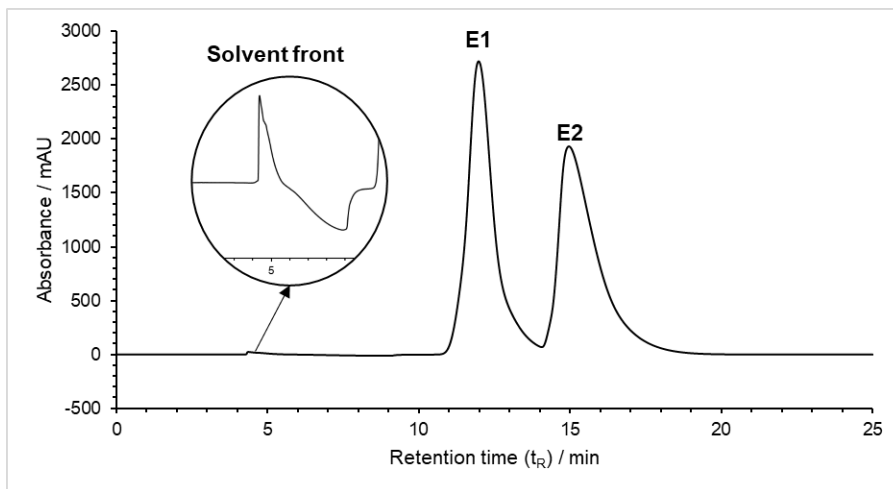


Figure 23 – Chromatogram of semi-preparative separation of the enantiomers of MDPV; Tris-3,5-dimethylphenylcarbamate amylose home-made column; Mobile phase: Hex:EtOH:DEA (97:3:0.1); Flow rate: 1.5 mL/min; UV detection: 254 nm.

In this work, 110 mg of racemic MDPV were injected in total after several cycles of

injections. After collecting both enantiomeric fractions in several injections, the fractions corresponding to the same enantiomer were combined and the solvent was evaporated. Since the enantiomers were collected in their free form which is unstable, hydrochloride formation had to be performed using 2 M HCl on diethyl ether to return the enantiomers to their stable form. Recovery rates were calculated using the mass of racemic MDPV injected and the mass obtained for each enantiomer after hydrochloride formation by considering an initial 50:50 proportion in the injected racemate, leading to a recovery rate of 92% for E1 and 93% for E2.

Obtained enantiomers were reinjected in order to determine the e.r.. For the first enantiomeric fraction (E1) (**Figure 24**), no signs of E2 were observed in the chromatogram so an e.r. of >99% was considered for E1.

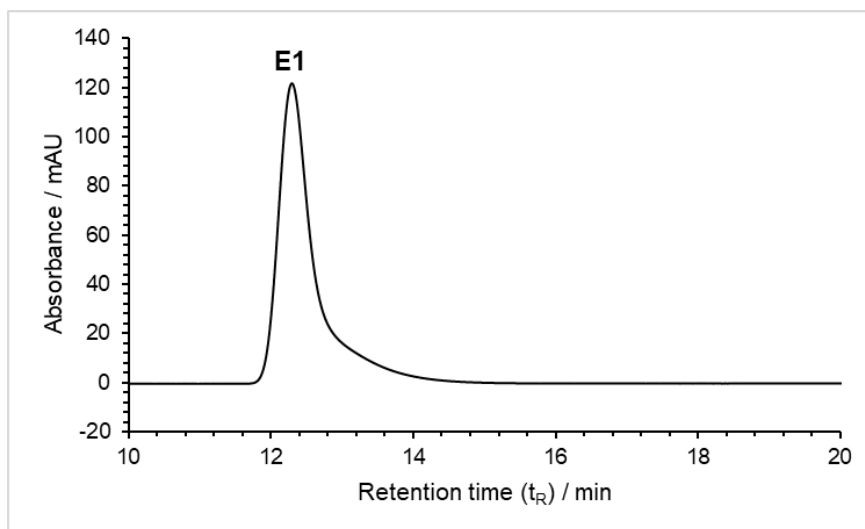


Figure 24 – Chromatogram of the first enantiomeric fraction (E1); Tris-3,5-dimethylphenylcarbamate amylose home-made column; Mobile phase: Hex:EtOH:DEA (97:3:0.1); Flow rate: 1.5 mL/min; UV detection: 254 nm.

For the second enantiomeric fraction (E2) (**Figure 25**), a small peak around 12 min with an area of 3.2 was found corresponding to E1 and a higher peak around 15 min with an area of 61.8 was found corresponding to E2. An e.r. value of 95% was achieved for this enantiomer.

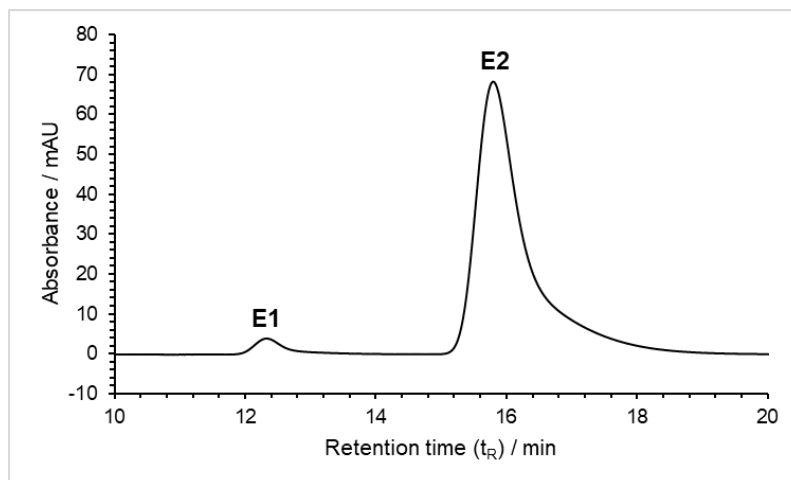


Figure 25 – Chromatograph of the second enantiomeric fraction (E2); Tris-3,5-dimethylphenylcarbamate amylose home-made column; Mobile phase: Hex:EtOH:DEA (97:3:0.1); Flow rate: 1.5 mL/min; UV detection: 254 nm.

2. Racemization study with the enantiomers of MDPV

Enantiomers can undergo racemization when exposed to certain temperatures and/or in acidic/basic environments, so studying their stability in various conditions is of great importance [188]. Many studies have reported the applications of LC methods for racemization studies for a variety of compounds [188-190]. In this work, effects of temperature and the addition of a basic additive, DIPEA, on the racemization of the enantiomers of MDPV was evaluated and analyzed through LC.

Chromatographic conditions were identical to described previously for the separation of the enantiomers, however, since this study used an analytical column, the flow rate was decreased to 1 mL/min and only 10 μ L from each enantiomer in a concentration of 50 μ g/mL were used in each injection.

Firstly, to determinate the retention time of each enantiomer in these conditions, a racemic solution of MDPV was injected in a concentration of 0.5 mg/mL (**Figure 26**). E1 presented a t_R of 9 min while E2 of 12 min.

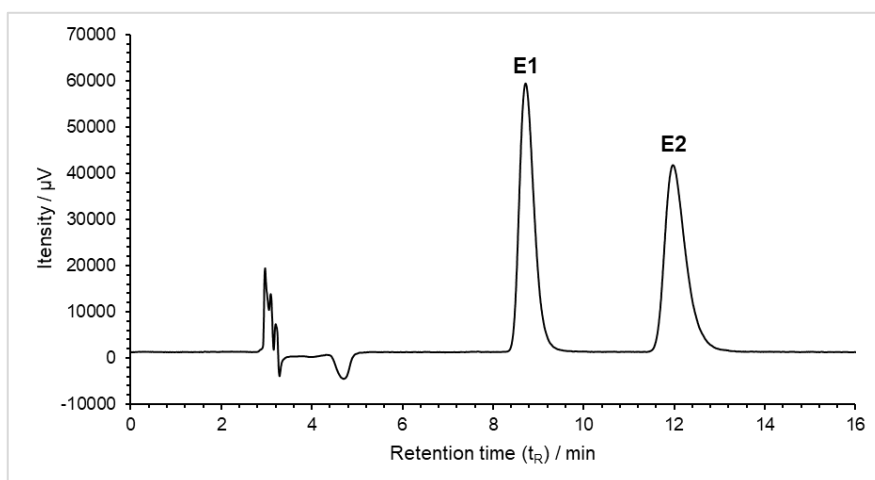


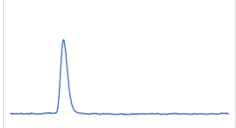
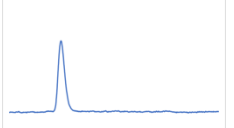
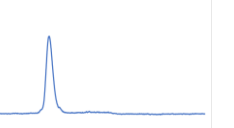
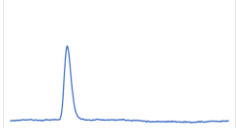
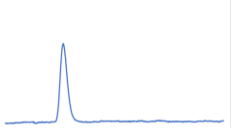
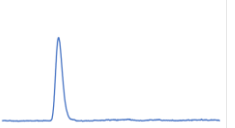
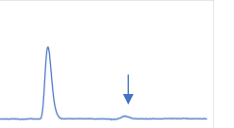
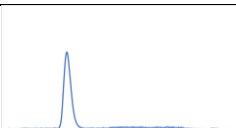
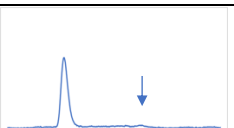
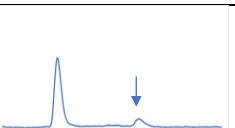
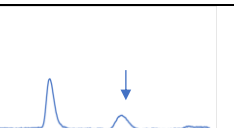
Figure 26 – Chromatogram of the analytical separation of the enantiomers of MDPV; Lux Amylose-I ® column; Mobile phase: Hex:EtOH:DEA (97:3:0.1); Flow rate: 1 mL/min; UV detection: 254 nm.

In order to evaluate the effects of temperature in the racemization of the enantiomers of MDPV, the individual enantiomers were exposed to different temperatures: RT which was the work temperature for all the separation process, 37 °C which is the physiological temperature (also used for biological assays), and 70 °C as a more extreme temperature. Due to the short half-life of 77.8–97.9 min reported for MDPV (in rats) [191], injections were firstly performed closer to each other (every 30 min) for the first 3 h to see the stability of the enantiomers during that time. Moreover, injections were performed after 24 h and 48 h, since for biological assays, exposure to compounds for 24/48 h is common.

Synthetic cathinones are less stable in their base forms so increasing the pH could lead to a higher rate of racemization for the enantiomers [192]. To evaluate the effects of a basic additive in the racemization, the study was also repeated in the presence of DIPEA. Results are found in **Tables 2-5**. These results are expressed as the proportion of E1:E2 for each chromatogram to better display the changes in the e.r..

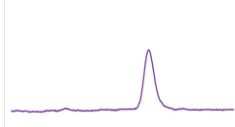
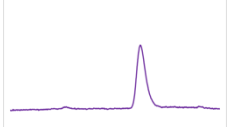
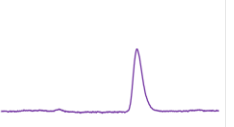
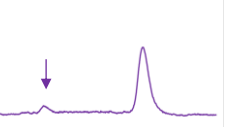
After analyzing the chromatograms corresponding to E1 and calculating the e.r. values after exposure to different temperatures for 48 h (**Table 2**), no signs of racemization were observed for 48 h at RT and 24 h at 37 °C. After exposing E1 for 48 h to 37 °C, a small peak shows up in the chromatogram with the same retention time as E2, suggesting that racemization is starting to occur. A change in the e.r. from 100:0 to 96:4 was observed. After 3 h at 70 °C, a change in the e.r. from 100:0 to 97:3 was also observed. The area of E2 increases after 24 h at 70 °C (e.r. of 88:12) and even more after 48 h (69:31).

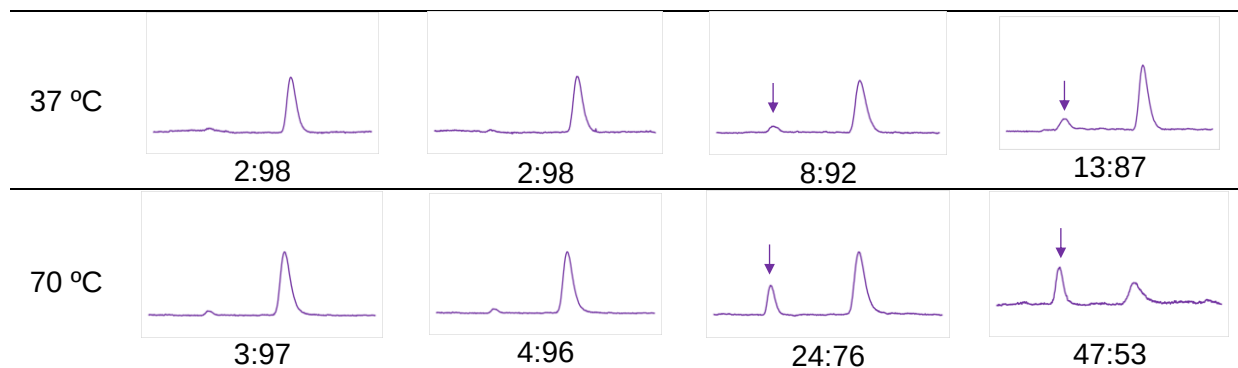
Table 2 - Chromatograms from the racemization study after exposing E1 to different temperatures (RT, 37 °C and 70 °C) for 48 h, and respective e.r. values.

Temp.	Time			
	30 min	3 h	24 h	48 h
RT	 100:0	 100:0	 100:0	 100:0
37 °C	 100:0	 100:0	 100:0	 96:4
70 °C	 100:0	 97:3	 88:12	 69:31

Regarding the results of E2 after exposure to different temperatures for 48 h (**Table 3**), it is important to mention that a small peak corresponding to E1 is observed in every chromatogram since E2 presented a minor contamination from E1 after separation (**Figure 25**). Thus, e.r. of 4:96, 3:97, 2:98 were not considered as signs of racemization for E2. For E2, after 48 h at RT and 24 h at 37 °C there were already changes in the e.r., from 2:98 to 6:94 and 8:92, respectively, which were not observed for E1. Although, no significant change was observed after 3 h at 70 °C, after 24 h the e.r. changed to 24:76 and after 48 h almost full racemization had occurred (47:53). These results suggest that E2 undergoes racemization to a greater extent and quicker than E1 in the studied conditions. This could be explained by the presence of E1 in the samples of E2 prior to the start of the racemization studies.

Table 3 – Chromatograms from the racemization study after exposing E2 to different temperatures (RT, 37 °C and 70 °C) for 48 h, and respective e.r. values.

Temp.	Time			
	30 min	3 h	24 h	48 h
RT	 3:97	 3:97	 2:98	 6:94

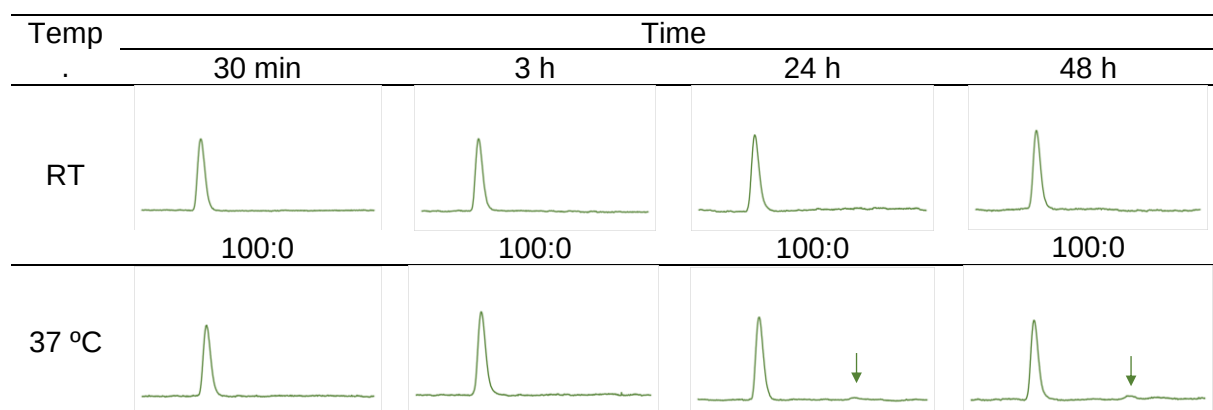


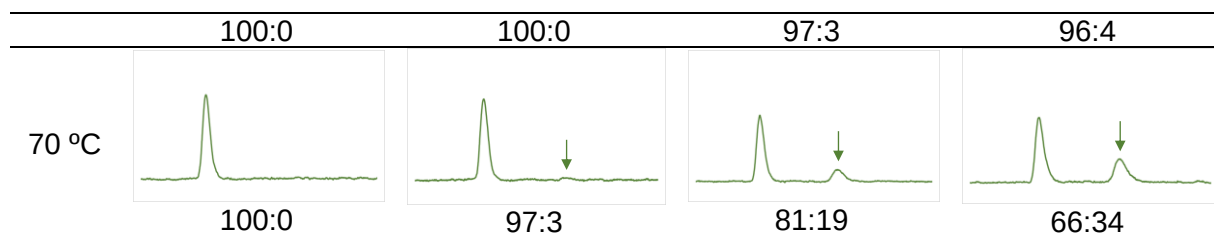
These results show that the racemization of the enantiomers of MDPV is affected by higher temperatures. For 48 h at RT and 24 h at 37 °C, the enantiomers can be considered to be stable since minor or no changes were observed in the e.r..

To evaluate the effect of basic additives in the racemization of the enantiomers of MDPV, 0.1% of DIIPEA was added to the samples and the studies were repeated in the same conditions of temperature as previously.

For E1, results after the addition of DIIPEA (**Table 4**) were similar to the previous results in the absence of DIIPEA with a few exceptions. For instance, after 24 h at 37 °C changes in the e.r. were already observed (from 100:0 to 97:3). However, after 48 h, the ratio had no significant change (from 97:3 to 96:4) and was identical to the one observed in the absence of DIIPEA (96:4). Moreover, after 24 h at 70 °C in the presence of DIIPEA, the increase in the area of E2 was higher (81:19) than in the absence (88:12) suggesting that racemization happened to a greater extent. Nonetheless, after 48 h, the difference between the ratio in the presence (66:34) and absence (69:31) of DIIPEA is smaller.

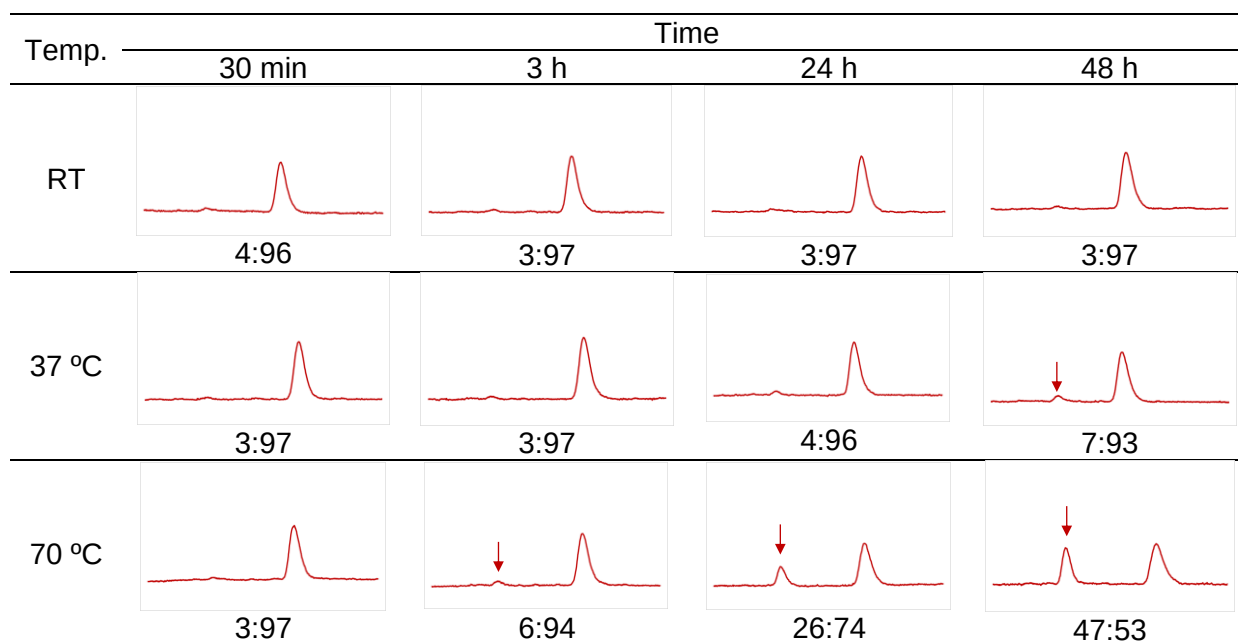
Table 4 – Chromatograms from the racemization study after exposing E1 to different temperatures (RT, 37 °C and 70 °C), in the presence of DIIPEA for 48 h, and respective e.r. values.





For the results of E2 after exposure to 0.1% DIPEA (**Table 5**), no signs of racemization were observed for 48 h at RT and 24 h at 37 °C unlike what happened in the absence of DIPEA. On the other hand, signs of racemization were observed in this case after 3 h at 70 °C with a change in the e.r. from 3:97 to 6:94. The remaining results were similar to the previous in the absence of DIPEA.

Table 5 - Chromatograms from the racemization study after exposing E2 to different temperatures (RT, 37 °C and 70 °C), in the presence of DIPEA for 48 h, and respective e.r. values.



These results suggest that DIPEA has no considerable effect on the racemization of the enantiomers of MDPV since only minor/no differences were observed when comparing e.r. obtained in the presence of DIPEA with the e.r. obtained in the absence of DIPEA. The main difference was found for E1 after 24 h at 70 °C.

3. Enantioselectivity studies with the enantiomers of MDPV in the SH-SY5Y cell line

3.1. Effects of the enantiomers of MDPV in cytotoxicity

To evaluate the potential enantioselectivity of the enantiomers of MDPV in cytotoxicity, the MTT [193] and NR [194] assays were performed in the SH-SY5Y human neuroblastoma cell line.

The SH-SY5Y cell line is a widely used *in vitro* model system for a variety of neurotoxicity assays. This cell line has many advantages such as being rapidly cultured in large quantities with inexpensive maintenance when compared to other models. Additionally, its human origin helps to avoid inter-species differences [195]. A major advantage of SH-SY5Y cells for this work is their catecholaminergic characteristics expressing DA and NE transporters, the main targets of MDPV in neurotoxicity [27, 196].

Both undifferentiated and dopaminergic SH-SY5Y cells were selected for this study. Valente *et al.* [182] previously reported that when SH-SY5Y cells are differentiated into a dopaminergic phenotype, more susceptibility to the cytotoxicity effects of β -keto amphetamines, including racemic MDPV, is displayed than when compared to undifferentiated SH-SY5Y cells. Moreover, in another study, enantioselective differences have been found in cytotoxicity for the enantiomers of two synthetic cathinones, pentedrone and methyllone, in dopaminergic SH-SY5Y cells [86].

In this work, three concentrations of MDPV were selected based on Valente *et al.*'s study [182]: 0.773; 1.165 and 1.506 mM (estimated EC10, EC30 and EC50 in differentiated SH-SY5Y cells). Undifferentiated and dopaminergic SH-SY5Y cells were exposed to the enantiomers of MDPV in these concentrations for 24 h. These assays were used not only to compare if the enantiomers displayed different effects (enantioselectivity) in the same cell type but also to compare effects of the same enantiomer between undifferentiated and differentiated cells, previously reported to be different for racemic MDPV [182].

The results (**Figure 27**) showed that both enantiomers caused a decrease in metabolic activity (**Figure 27A**) and lysosome integrity (**Figure 27B**) in a concentration-dependent manner. Significant cytotoxicity with decreases in cell viability of approximately 50% were observed for the highest concentration (1.506 mM) when compared with control (cells without treatment). Significant decreases were also observed for 1.165 mM in some cases. Moreover, the percentages obtained for each concentration were consistent with percentages expected for EC10, EC30 and EC50 (similar to 90%, 70% and 50% of cell viability, respectively). No

statistically significant differences were found between the enantiomers in both assays for both cell types leading to the conclusion that there is no enantioselectivity for the enantiomers of MDPV in cytotoxicity for the SH-SY5Y cell line. Additionally, although Valente *et al.* [182] reported differences in the effects of racemic MDPV between undifferentiated and differentiated cells, in this work, no statistically significant difference was found between undifferentiated and differentiated cells when exposed to the enantiomers of MDPV.

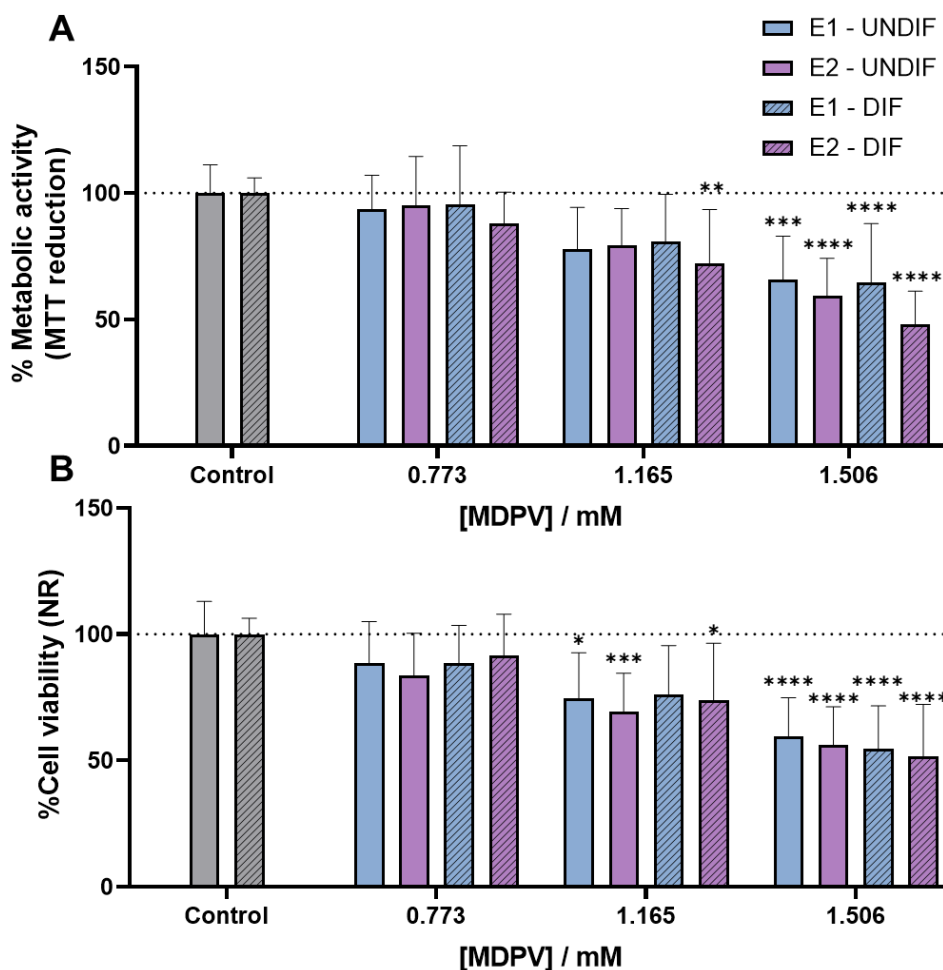


Figure 27 - Reduction of MTT (A) and NR uptake (B) in undifferentiated and dopaminergic SH-SY5Y cells exposed to the individual enantiomers of MDPV in three concentrations (0.773; 1.165 and 1.506 mM) for 24 h. Results were expressed as mean $\pm$ SD obtained from four independent experiments, run in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. respective control (undifferentiated or differentiated cells without treatment)

3.2. Effects of MDPV enantiomers in the expression of proteins involved in neuroplasticity

Neuroplasticity is the ability of the nervous system to modify its neuronal structure or

function in response to the external and internal changes that emerge from, for instance, development or learning. Since drugs of abuse produce long-lasting memories, neuronal plasticity has been involved in their formation and maintenance [197, 198].

BDNF is part of the neurotrophin family of growth factors and displays multiple roles in the central nervous system (CNS) such as the neurons' survival during development, regulation of axons and dendrites growth and cell differentiation [199].

Furthermore, it has been reported that BDNF is a key regulator of synaptic plasticity [200]. Due to its key role in neuroplasticity, BDNF was also found to be involved in the actions of drugs of abuse, primarily psychostimulants. Several studies reported an alteration (generally an increase) in BDNF expression in different brain regions, mostly reward-related areas, for example, after acute or repeated cocaine exposure in rats [201-204].

Synthetic cathinones present psychostimulant properties and MDPV has been reported to display cocaine-like effects by blocking DAT and NET, but, unlike cocaine, MDPV has no effect on SERT [205]. Thus, investigating the potential role of BDNF in the effects of synthetic cathinones could provide some information about their mechanism of action.

Previous studies reported the effects of MDPV in the expression of BDNF *in vivo*. Caffino *et al.* [205] studied the effects of single exposure to MDPV and α -PVP in the expression of molecular markers of neuroplasticity (NPAS4 and BDNF) in the brain of the adult mouse. In the frontal lobe, MDPV, but not α -PVP, increased total BDNF mRNA levels at early time points while after 24 h both drugs upregulated BDNF mRNA levels. On the other hand, in the hippocampus, both cathinones increased BDNF mRNA levels 30 min and 2 h after exposure but this effect vanished after 24 h. The authors concluded that MDPV and α -PVP displayed different effects on BDNF expression dependent on the brain region. Moreover, Duart-Castells *et al.* [206] found that an acute or a repeated exposure to MDPV increased cortical BDNF mRNA in mice, while mBDNF protein levels were decreased in the nucleus accumbens 2 h after repeated exposure.

More recently, Nadal-Gratacós *et al.* [207] investigated the mRNA expression of BDNF and other genes involved in neuroplasticity in several brain areas after acute administration of six synthetic cathinones. After an acute administration, all cathinones displayed a tendency to increase BDNF expression in mice but only *N*-ethyl-pentylone (NEP) promoted a significant increase of BDNF mRNA levels [207].

Although few studies have been performed so far, a potential change in the expression of BDNF after MDPV exposure could be expected. Thus, we investigated the effects of the enantiomers of MDPV in BDNF expression in undifferentiated SH-SY5Y cells through western

blot. Undifferentiated SH-SY5Y were selected for this assay since there is no need for extra differentiation steps, so MDPV exposure could be performed in the following day to seeding, allowing a quicker data acquisition. Moreover, 0.773 mM was selected as one of the concentrations since low cytotoxicity was observed for this concentration in SH-SY5Y cells. A much lower concentration of 0.773 μ M was also selected.

The results are shown in **Figure 28**. Although a decrease was observed in the expression of BDNF after exposing SH-SY5Y cells to 0.773 mM of the enantiomers of MDPV, no statistically significant difference was found between MDPV and control (cells with no treatment) meaning that MDPV, in the experimental conditions, causes no change in the expression of BDNF in undifferentiated SH-SY5Y cells. Additionally, no enantioselectivity was observed for effects of MDPV in the expression of BDNF since there was no statistically significant difference between the enantiomers for both concentrations.

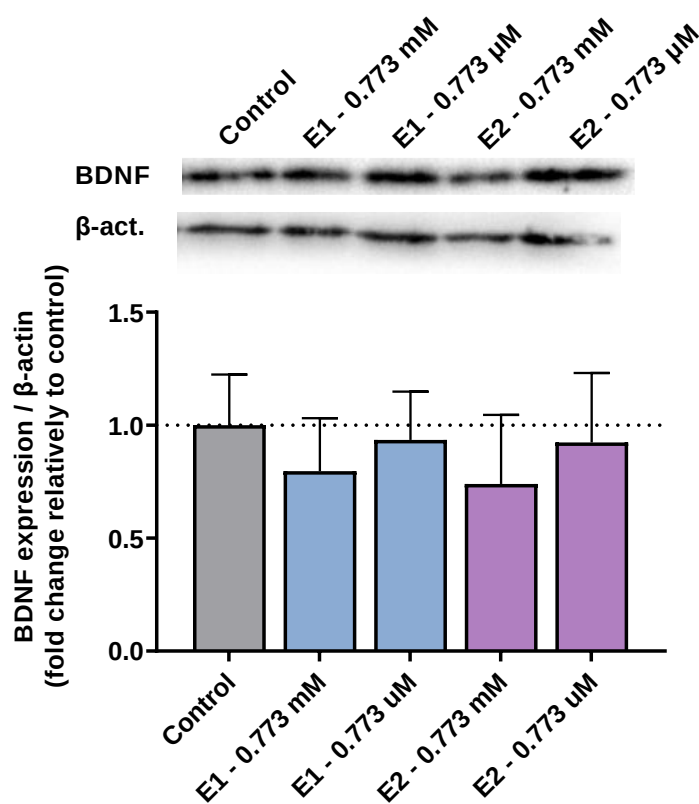


Figure 28 – Effects of MDPV enantiomers (E1 and E2) in BDNF expression in undifferentiated SH-SY5Y cells. The expression was normalized by β -actin. Results are expressed as mean $\pm$ SD for the fold change relatively to control for at least four independent experiments (n=4) performed in duplicate.

Furthermore, the effects of the enantiomers of MDPV in the expression of Cdk5 in the SH-SY5Y cell line were also evaluated. Cdk5 is a serine threonine kinase protein that has been found to be involved in numerous crucial processes for normal brain function such neuronal migration, axonal guidance, and several aspects of structural and functional neuroplasticity [208]. Cdk5 has been linked to cellular and physiological effects of drug addiction playing a potential key role in the action of drugs of abuse [209].

Many studies reported changes in the expression of Cdk5 after cocaine exposure in different brain areas [210-212]. For instance, Bibb *et al.* reported an upregulation of Cdk5 after chronic cocaine administration in transgenic mice [210]. Moreover, another study reported an involvement of Cdk5 in drug reward system being upregulated in the ventral tegmental area of rats after cocaine abuse and downregulated in other areas [211, 212].

Although the investigation of the potential role of Cdk5 in the action of cocaine has been widely studied, this is not the case for synthetic cathinones whose abuse has been incredibly significant in the last few decades [28]. Up to now, only recently, two studies related to the effects of MDPV in Cdk5 expression have been reported [213, 214]. Duarte-Castells *et al.* [214] reported a significant increase in Cdk5 expression in MDPV-treated mice and this overexpression was still apparent after 3 weeks of withdrawal. However, more recently, another study showed that, although an increase in the expression of Δ FosB, a transcription factor for which Cdk5 has been identified as a target, was observed in MDPV treated mice, the expression Cdk5 was not altered 24 h after a conditioned place preference experiment with MDPV [213]. Since different conditions (doses and mouse strain) were used in both studies, the effect of MDPV on CDK5 expression could be dose-dependent or even mouse strain-dependent leading to different results in these two studies [213]. Thus, there is a need for a more comprehensive investigation of the effects of MDPV in Cdk5 expression.

The effects of the enantiomers of MDPV in the expression of Cdk5 in undifferentiated SH-SY5Y cells were analyzed through Western-blot. The results, shown in **Figure 29**, were similar to the results reported for BDNF. No statistically significant difference was observed between MDPV and control conditions meaning that MDPV causes no change in the expression of Cdk5 in undifferentiated SH-SY5Y cells in the experimental conditions. In addition, no statistically significant difference between the enantiomers meaning that there is no enantioselectivity for the expression of Cdk5 in SH-SY5Y cells after exposure to the enantiomers of MDPV.

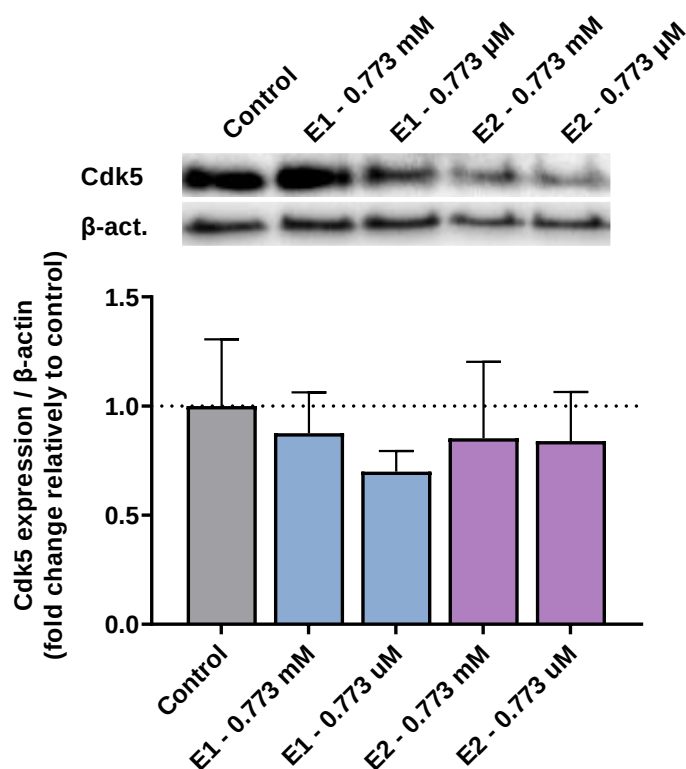


Figure 29 - Effects of MDPV in Cdk5 expression in undifferentiated SH-SY5Y cells. The expression was normalized by β -actin. Results are expressed as mean $\pm$ SD for the fold change relatively to control for at least four independent experiments (n=4) performed in duplicate.

4. Permeability assay with the enantiomers of MDPV using the Caco-2 cell line

To study the potential enantioselective intestinal permeability of MDPV, a permeability *in vitro* assay was performed using the Caco-2 cell line and an UHPLC-UV method was validated to allow the quantification of MDPV in the transporter buffer of this study. This study was based on a previous one by our group with the enantiomers of pentedrone and methylone [84]. In this study, differences were found between the enantiomers of both cathinones for the passage across the monolayer being the *R*-(-)-pentedrone and *S*-(-)-methylone the most permeable compounds [84].

Apart from Silva *et al.* [84] work, no other studies with the Caco-2 cell line have been reported for synthetic cathinones. Nonetheless, some other studies have reported differences between the behavior of enantiomers of other compounds in the permeability across the Caco-2 cell line [215, 216]. For instance, Wang *et al.* [215] studied the permeability of the enantiomers

of propranolol, a nonselective β -adrenoceptor blocker for the treatment of hypertension and cardiovascular disorders. In this study, *S*-propranolol was the most transported in the AP to BL direction while *R*-propranolol was the most transported in the BL to AP direction [215].

4.1. Chromatographic method

The samples from the permeability assay and calibration curves [all in HBSS (+/+)] were injected into the UHPLC after being filtered, no previous extraction and other treatment was needed. To obtain the optimal wavelength for the detection and quantification of MDPV, the UV spectrum of MDPV was determined (**Figure 30**). Four peaks with high absorption were found in the tested wavelength interval. Moreover, UV spectra were determined for the mobile phase and HBSS(+/+) to detect potential interferences with MDPV absorption. The optimum wavelength selected for the detection and quantification of MDPV was 236 nm.

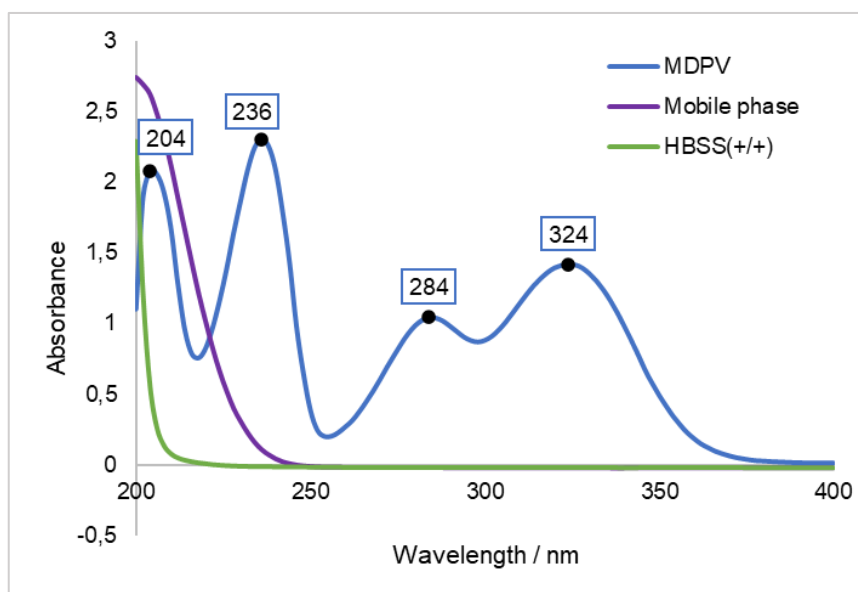


Figure 30 - UV spectra of MDPV [100 μ M in HBSS (+/+)], mobile phase [25 mM ammonium acetate:ACN:FA (75:25:0.1)] and HBSS(+/+).

The chromatographic conditions were based on Silva *et al.*'s work [84] with some changes since a different cathinone and column were used. Flow rate was adjusted from 0.15 mL/min to 0.12 mL/min to allow a lower pressure and the mobile phase was adapted from 25 mM ammonium acetate:ACN:FA (80:20:0.1) to 25 mM ammonium acetate:ACN:FA (75:25:0.1). In the optimized chromatographic conditions, good resolution was obtained for the peak of MDPV being eluted in less than 5 min, as shown in **Figure 31**.

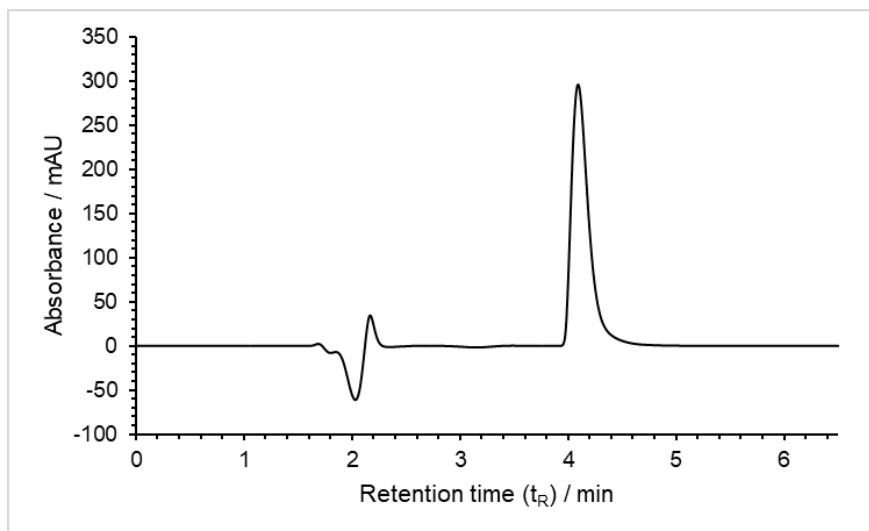


Figure 31 - Chromatogram of MDPV [100 μ M in HBSS(+/+)]. Chromatographic conditions: Kinetex® EVO C18 column, 25 mM ammonium acetate:ACN:FA (75:25:0.1) as the mobile phase, flow rate: 0.12 mL/min, UV detection 236 nm.

4.2. Method validation

4.2.1. Specificity

To evaluate specificity, twenty blank samples containing only an HBSS (+/+) solution were injected and analyzed to detect potential chromatographic interferences. No interference was observed in the retention time corresponding to MDPV (**Figure 32**), meaning that the developed method is specific for MDPV analysis and quantification.

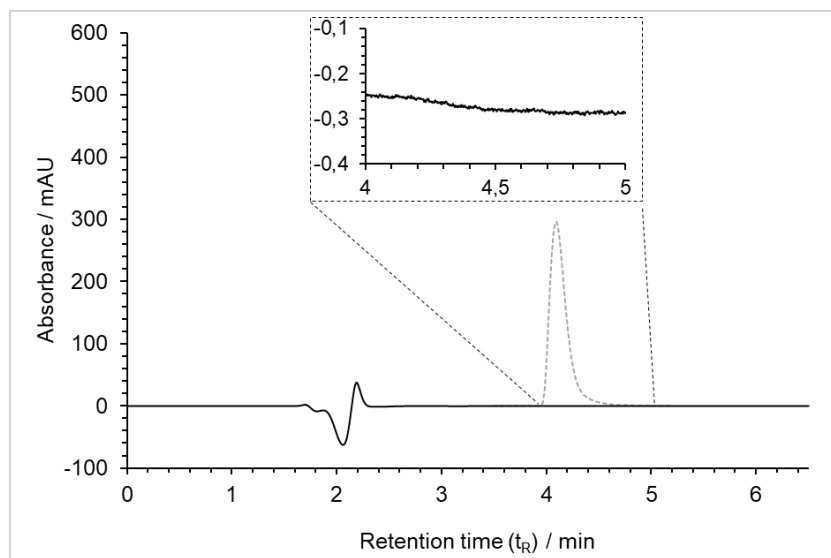


Figure 32 – Chromatogram of blank sample [HBSS (+/+)]. Chromatographic conditions: Kinetex® EVO C18 column, 25 mM ammonium acetate:ACN:FA (75:25:0.1) as the mobile phase, flow rate: 0.12 mL/min, UV detection 236 nm.

4.2.2. Linearity

For the evaluation of linearity, five curves, independently prepared in five different days, were used to obtain the average linear regression equation and r^2 . Acceptable linearity was found for the analyzed concentration interval (0.5 to 500 μM), proved by the values of r^2 higher than 0.999. **Figure 33** shows the average curve obtained along with the equation and r^2 coefficient. Additionally, the linearity data is summarized in **Table 6**.

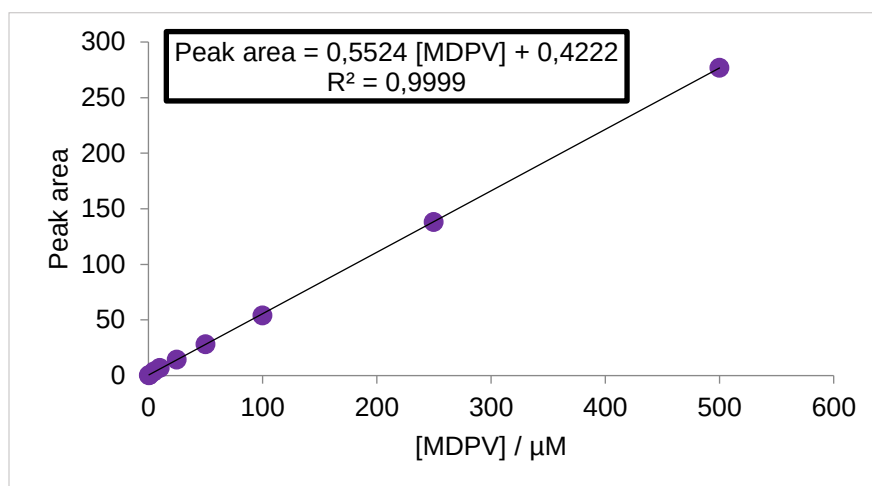


Figure 33 – Average calibration curve (plot of concentration of MDPV vs peak area).

Table 6 – Linear regression data for evaluation of linearity.

Linear equation	r^2	95% confidence intervals		
		Slope	Y-intercept	X-intercept
0.5524x + 0.4222	0.9999	0.5486 to 0.5562	-0.2965 to 1.1409	-2.073 to 0.5348

4.2.3. Accuracy

After injecting three selected calibrators contained in the linear concentration interval (6 (low), 40 (medium) and 300 (high) μM) along with a calibration curve, the experimental concentrations of each calibrator were calculated through the linear regression equation of the curve and accuracy was measured through equation 5. Percentages between 102 and 109% were obtained (**Table 7**), which are included in the recommended limits for this parameter (100

± 15%) [184, 185].

Table 7 – Accuracy data.

Nominal concentration (µM)	Experimental concentration (µM)	Accuracy (%)
6	6.1 ± 0.6	102 ± 10
40	44 ± 2	109 ± 6
300	315 ± 22	105 ± 7

4.2.4. Precision

The results obtained for the evaluation of precision (**Table 8**) showed an acceptable inter-day and intra-day precisions for both equipment and method since all CV% values were below the proposed limit of 15% [184, 185].

Table 8 – Inter-day and intra-day precision data.

Concentration (µM)	Inter-day (CV %)		Intra-day (CV %)	
	Equipment	Method	Equipment	Method
10	6.04	13.87	4.75	10.18
100	9.10	4.74	3.96	10.56
500	9.19	2.88	3.21	8.27

4.2.5. LOD and LOQ

Through **Equations 6** and **7**, a LOD of 0.063 µM and a LOQ of 0.190 µM were calculated for this method. These results show that the developed method displays sensitivity for the detection and quantification of MDPV at a concentration range of low micromolar.

4.2.6. Stability

Table 9 contains the values of % peak area variation between the initial injection and final injection for each temperature tested. The calculated variation in peak area was in general low for every condition tested meaning that the samples remained fairly stable during the 6 weeks of storage. Nonetheless, in order to reduce peak area variation that could affect the results, the solutions should be analyzed in a shorter period after preparation.

Table 9 – Sample stability after 6 weeks of storage expressed as the %peak area variation relative to day 1.

Concentration (µM)	Storage temperature			
	RT	4 °C	-20 °C	- 80 °C
10	11.0	10.9	12.5	7.1
100	11.9	9.1	10.4	8.6

500	10.2	3.4	5.3	6.8
-----	------	-----	-----	-----

4.3. Cell viability assay

In order to find a non-cytotoxic concentration to use for the permeability assay with the Caco-2 cell line, the cells were exposed to racemic MDPV in a concentration range up to 1500 μM for 24 h and the NR assay was performed. The results, in **Figure 34**, show no statistically significant differences in cell viability up to the concentration of 750 μM . The three highest concentrations tested resulted in a decrease of cell viability in a concentration dependent manner, so they were not considered for the assay. The concentration of 300 μM was selected for the permeability assay.

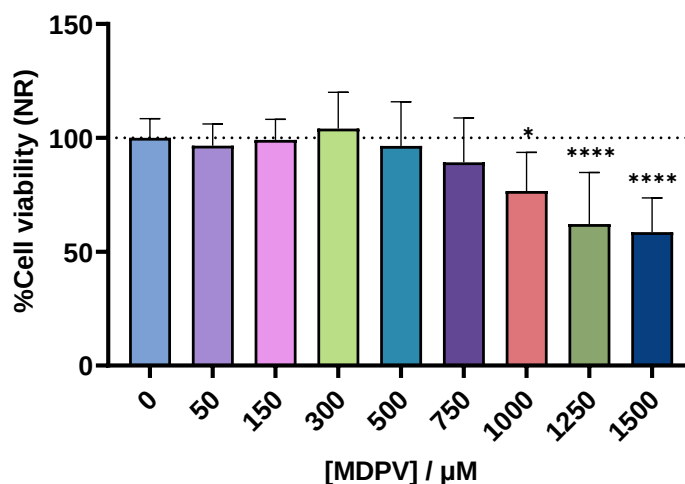


Figure 34 – Cytotoxicity evaluation in Caco-2 cells exposed to racemic MDPV (0-1500 μM) for 24 h performed by the NR assay. Results are expressed as mean $\pm$ SD from 4 independent experiments (performed in triplicate). * $p < 0.05$, **** $p < 0.0001$ vs. control (0 μM).

4.4. Permeability assay

The Caco-2 cell line is a cell line derived from human colorectal adenocarcinoma, which is widely used as *in vitro* model for drug intestinal permeability studies [217]. This cell line can spontaneously differentiate into a polarized epithelial monolayer of cells with tight junctions, microvilli, and a number of enzymes and transporters expressing several morphological and functional properties of enterocytes [218, 219]. Thus, these characteristics provide this cell line the ability to mimic the small intestine.

To better resemblance *in vivo* permeability conditions, the formation of a Caco-2 monolayer with good integrity is highly important. To check potential problems in the monolayers' integrity, TEER values were monitored for 21 days after seeding (**Figure 35**).

Significant TEER values were observed from day 6 (over $500 \Omega \cdot \text{cm}^2$). They gradually increased to approximately $1000 \Omega \cdot \text{cm}^2$ until day 18 after which they remained constant until day 21.

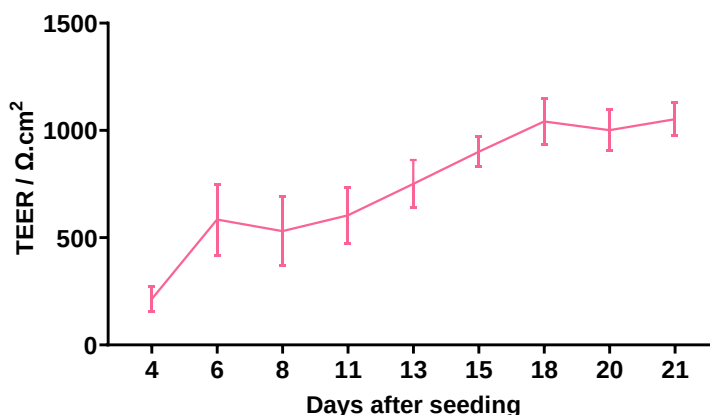


Figure 35 – Monitoring of TEER values from day 4 to day 21 after seeding of Caco-2 cells. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments.

Cells were exposed to $300 \mu\text{M}$ of each enantiomer on day 22 after seeding. Samples were collected in the chosen time points (40, 60, 90, 120, 180, 240, 300 min) from the acceptor compartment. After 6 weeks of storage, the validated UHPLC method was used to quantify MDPV present in the samples. Cumulative quantity transported from the donor compartment to the acceptor compartment was calculated for each time point. Results, in **Figure 36**, were expressed as the percentage of cumulative quantity in the initial quantity. A considerable percentage of passage was observed from the first time point (40 min) in both enantiomers for both directions increasing significantly during the rest of the interval. No statistically significant difference was found between the enantiomers in any of the time points for both directions meaning that there is no enantioselectivity in the transport of MDPV through the Caco-2 monolayer.

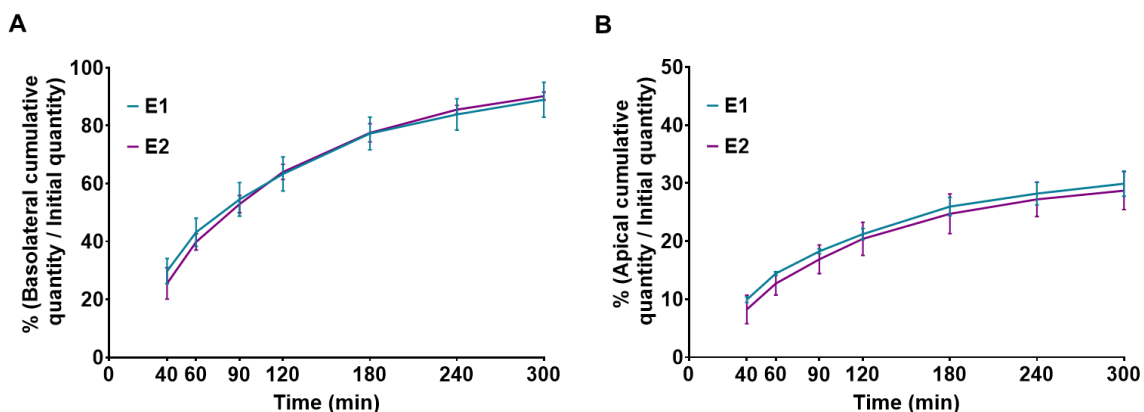


Figure 36 – Percentage of cumulative quantity of the enantiomers of MDPV transported through the Caco-2 monolayer in each time point. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments (performed in duplicate).

Mass balance was calculated using **Equation 8**. A low mass balance can be caused by adsorption of the compound to the experimental material (plate or filter, for instance), metabolism or retention of the compound inside cells or cell membranes, which can, consequently, lead to errors in quantification of the compound and calculation of permeability coefficients [186]. In this work, the mass balances obtained for both enantiomers in both directions (**Figure 37**) were approximately 100% suggesting that there were no compound losses during the assay.

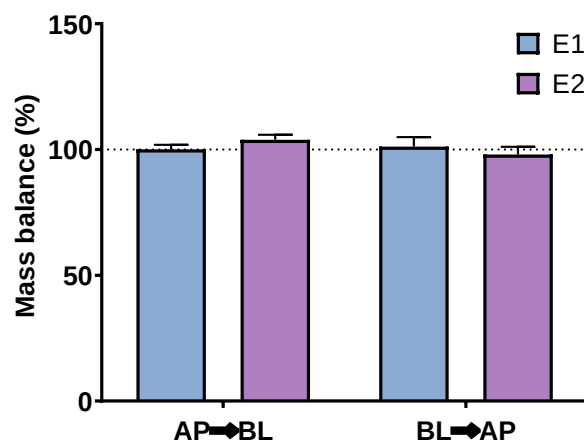


Figure 37 – Mass balance obtained for the enantiomers of MDPV after the permeability assay with Caco-2 cells. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments (performed in duplicate).

P_{app} values were calculated using **Equation 9**. When P_{app} values are higher than 1×10^{-6} cm/s, compounds are described as highly permeable substances while when P_{app} values are lower than 1×10^{-6} cm/s, compounds are considered weakly permeable substances [220]. In this work, P_{app} values obtained for the enantiomers in both directions (**Figure 38**) were higher than 1×10^{-6} cm/s suggesting that the enantiomers of MDPV are highly permeable across the Caco-2 monolayer. Statistically significant differences were found in the P_{app} values between the enantiomers for the BL to AP direction. Additionally, for both enantiomers statistically significant differences were found between directions being the difference more significant for E2.

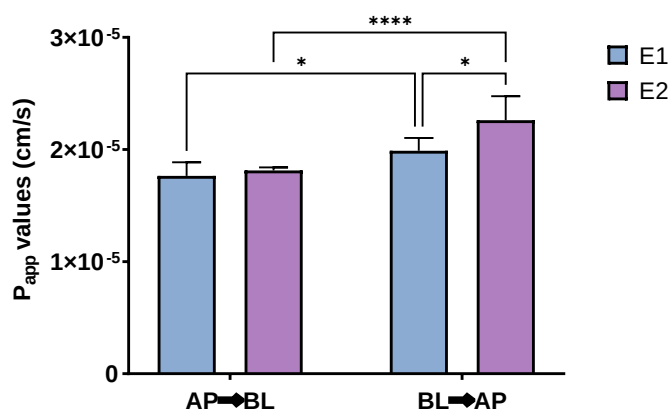


Figure 38 – P_{app} values obtained for the enantiomers of MDPV after the permeability assay with Caco-2 cells. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments (performed in duplicate). * $p < 0.05$, **** $p < 0.0001$

Efflux ratios were calculated for each enantiomer using **Equation 10 (Figure 39)**. The efflux ratio can be used as a first indicator of the potential involvement of an active transport process. Efflux ratios lower than 2 could indicate a passive transport with no active efflux [186]. In this study, although a statistically significant difference was found in the P_{app} values between directions for both enantiomers, efflux ratios of 1.1 for E1 and 1.2 for E2 were obtained, lower than 2. Furthermore, no statistically significant difference was found between the efflux ratios of the enantiomers. These results suggest that there might be no involvement of an active process for the transport of MDPV. Nonetheless, some other reasons could explain the low ratio, for instance, the transport protein could depend on a proton gradient or might not even be expressed in the Caco-2 monolayers [186].

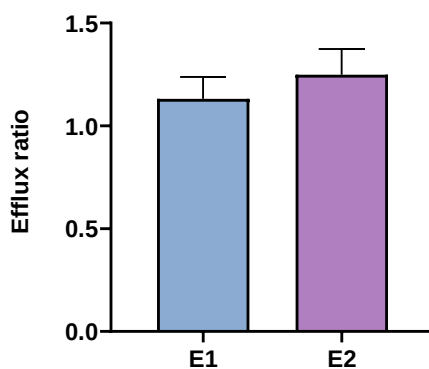


Figure 39 – Efflux ratios obtained for the enantiomers of MDPV after the permeability assay with Caco-2 cells. Results are expressed as mean $\pm$ SD obtained from 3 independent experiments (performed in duplicate).

The values obtained for mass balance, P_{app} values and efflux ratios were compiled in **Table 10**.

Table 10 – Data obtained for mass balance, P_{app} values and efflux ratios expressed as mean $\pm$ SD.

		Mass balance (%)	P_{app}	Efflux ratio
E1	AP $\rightarrow$ BL	100 ± 2	$1.7 \times 10^{-5} \pm 1 \times 10^{-6}$	1.1 ± 0.1
	BL $\rightarrow$ AP	101 ± 4	$2.0 \times 10^{-5} \pm 1 \times 10^{-6}$	
E2	AP $\rightarrow$ BL	104 ± 2	$1.81 \times 10^{-5} \pm 3 \times 10^{-7}$	1.2 ± 0.1
	BL $\rightarrow$ AP	98 ± 3	$2.3 \times 10^{-5} \pm 2 \times 10^{-6}$	

5. Displacement experiment with synthetic cathinones on HSA

Our group recently performed a binding affinity study of 15 synthetic cathinones to HSA by HPLAC using a chiral HSA column [187]. The analytical enantioseparation of 9 synthetic cathinones was successfully performed by the optimized method. Binding percentages were calculated for all synthetic cathinones and values ranging from 56 to 89% were found. In addition, enantiomers of MDPV, bk-DMBDB and pentylone were separated on a semipreparative scale using a polysaccharide-based CSP.

In a continuation of this work, herein, synthetic cathinones with different degrees of affinity to HSA were selected: the enantiomers of MDPV and pentylone with high affinity (higher than 80%), bk-DMBDB with medium affinity ($\pm 80\%$) and racemic pentedrone (enantiomers were not separated) with lower affinity (less than 80%). Displacement studies by LC were performed to increase the knowledge about the binding sites of these selected synthetic cathinones to HSA. Zonal displacement chromatography was chosen [160], by the addition to the mobile phase of increasing concentrations of the competitors warfarin and (S)-ibuprofen, that are known to bind to specific binding sites on HSA, Sudlow's site I and Sudlow's site II, respectively [164, 177]. The values of retention factor (k) for all the compounds in each condition are summarized in **Table 11**. There was a slight decrease in k with the increase of the concentration of warfarin for all the analytes except racemic pentedrone where only random variations were observed. For (S)-ibuprofen, all analytes including racemic pentedrone had a decrease in k with the increase of the concentration of the competitor until the concentration of 10 μ M. For 15 and 20 μ M, no significant changes were found for the k values when compared

to the values obtained for 10 μM which might suggest that there was a saturation of site II on HSA. Thus, the values obtained for 15 and 20 μM were not considered for the following analysis.

Table 11 – k values obtained for selected synthetic cathinones in presence of the competitors warfarin and (S)-ibuprofen (0-20 μM).

Competitor		Warfarin					(S)-ibuprofen				
Concentration / μM		0	5	10	15	20	0	5	10	15	20
k	MDPV (E1)	4.64	4.38	4.42	4.35	4.13	11.84	8.44	7.35	7.70	7.79
	MDPV (E2)	4.23	4.21	3.98	3.94	3.80	11.70	8.64	7.41	7.66	7.90
	Bk-DMBDB (E1)	3.82	3.65	3.41	3.44	3.25	10.32	6.38	5.51	5.70	5.89
	Bk-DMBDB (E2)	5.56	5.20	4.74	4.77	4.34	10.60	6.36	6.00	5.49	5.72
	Pentylone (E1)	13.22	12.12	11.67	11.11	9.87	32.17	7.36	6.20	6.30	6.48
	Pentylone (E2)	5.47	5.15	4.78	4.90	4.48	14.15	6.98	5.96	6.17	6.34
	Pentedrone	1.96	1.98	1.88	1.94	1.91	5.74	5.22	4.56	4.68	5.00

The k values obtained of each analyte in the absence and presence of the different concentrations of the competitors were expressed as the plot of $1/k$ versus the concentration of the competitor [164] (**Figure 40**). A linear relation with a positive slope for the plot of $1/k$ versus concentration was observed in all analytes, except pentedrone in the presence of warfarin. These results suggest that the studied compounds compete with both warfarin and (S)-ibuprofen for their binding sites which, consequently, means that they bind to both site I and II on HSA. The exception is racemic pentedrone, that only seems to compete with (S)-ibuprofen, thus, only binding to site II.

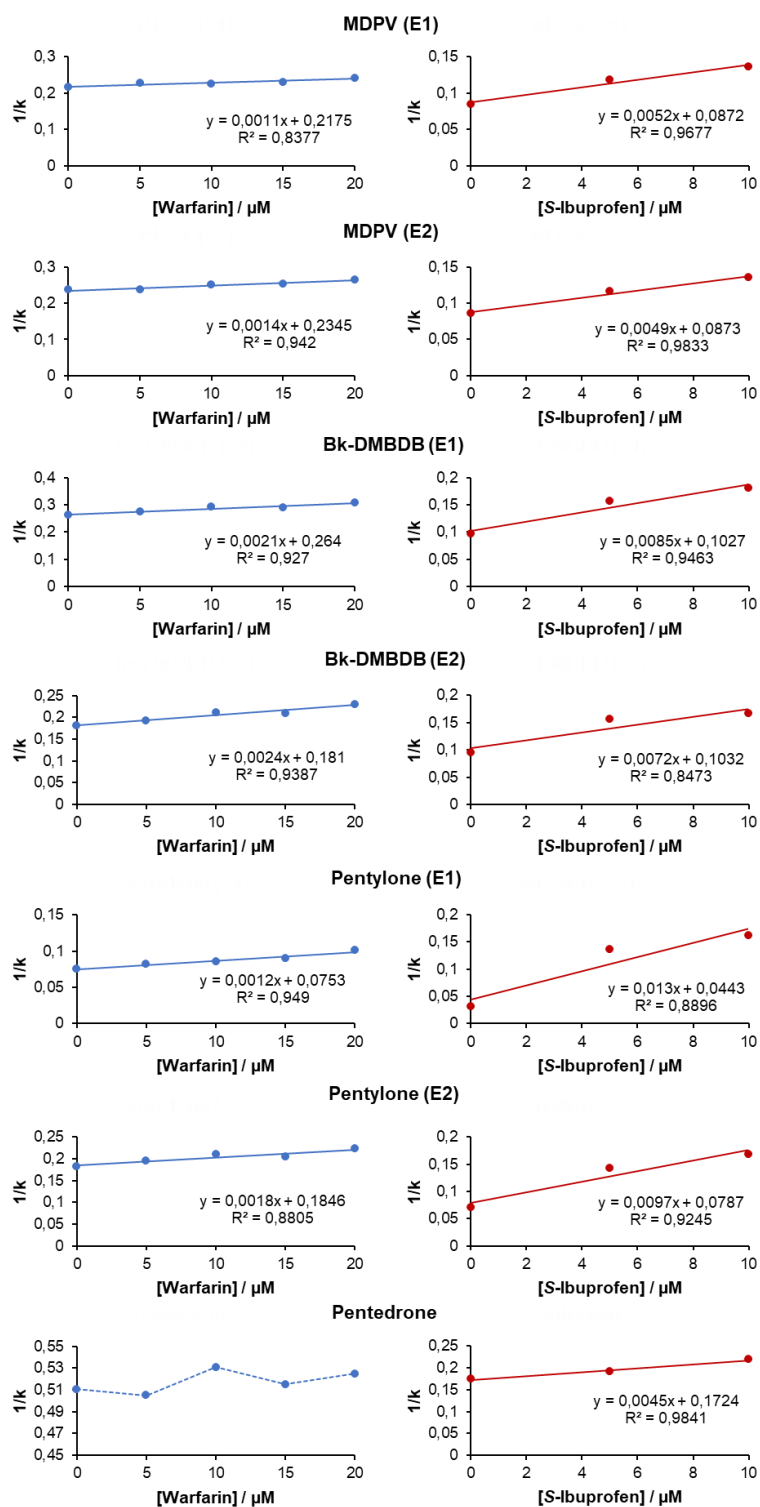


Figure 40 - Results from displacement chromatography experiments with synthetic cathinones in the absence and presence of increasing concentrations of the competitors expressed by the plot of $1/k$ of the analyte versus concentration of competitor.

Moreover, the values of K_i (**Table 12**) were calculated according to equation 12 and were further compared with the reported affinity constants found in literature for warfarin and ibuprofen, $3.3 \times 10^5 \text{ M}^{-1}$ and $2.7 \times 10^6 \text{ M}^{-1}$, respectively [221]. K_i can be used to predict the type of competition analytes have with the competitor and the type of binding mechanism on the site. If K_i values were similar to the competitors' affinity constants found in literature, direct competition should be considered between the analytes and competitors [179]. In this work, the calculated K_i values were significantly lower than the reported in literature for the competitors, suggesting an allosteric competition between the analytes and competitors with a non-cooperative binding mechanism to the respective sites on HSA [177].

Table 12 – K_i values obtained by displacement chromatography analysis. Values were determined by the slope and intercept of the plot of $1/k$ of the analyte vs increasing concentrations of the competitor.

Compound	K_i Warfarin (M^{-1}) (Lit. $3.3 \times 10^5 \text{ M}^{-1}$) ^a	K_i (S)-ibuprofen (M^{-1}) (Lit. $2.7 \times 10^6 \text{ M}^{-1}$) ^a
MDPV (E1)	5.07×10^3	5.92×10^4
MDPV (E2)	5.90×10^3	5.62×10^4
Bk-DMBDB (E1)	8.10×10^3	8.24×10^4
Bk-DMBDB (E2)	1.30×10^4	7.02×10^4
Pentylone (E1)	1.56×10^4	2.94×10^5
Pentylone (E2)	9.80×10^3	1.23×10^5
Pentedrone	-	2.62×10^4

^a [221]

CONCLUSIONS AND FUTURE WORK

In this work, the semipreparative separation of enantiomers of MDPV was successfully performed with good resolution and enantioselectivity being the enantiomers collected with high e.r. and recovery rates.

For 48 h at RT and 24 h at 37 °C, the enantiomers can be considered to be stable since minor/no changes were observed in the e.r.. Nevertheless, racemization is affected by higher temperatures. DIIPEA had no considerable effect on the racemization of the enantiomers of MDPV.

Similar cytotoxicity results were obtained for the enantiomers of MDPV in the SH-SY5Y cell line for both undifferentiated and differentiated (dopaminergic) cells (no enantioselectivity was observed). No statistically significant difference was found between undifferentiated and differentiated cells in each condition.

The enantiomers of MDPV had no effect on the expression of the neuroplasticity involved proteins BDNF and Cdk5 in SH-SY5Y cells and no statistically significant differences were found between enantiomers.

An UHPLC-UV method was successfully validated for the detection and quantification of MDPV in HBSS(+/-). The enantiomers of MDPV were highly permeable across the Caco-2 monolayer, however no enantioselectivity was found between them. Statistically significant differences between the enantiomers were found only in the P_{app} values obtained in the basolateral to apical direction.

In the displacement experiment, competition was observed between the examined cathinones and both competitors, except for racemic pentedrone with warfarin, consistent with an allosteric competition with a non-cooperative binding mechanism. The results suggest that the enantiomers of the studied synthetic cathinones bind to both site I and II on HSA and racemic pentedrone to site II.

Although no enantioselectivity was found in the performed assays, for future work, other enantioselectivity studies must be performed with the enantiomers of MDPV such as cytotoxicity in different cell lines (for instance the HepG2 cell line) and studies related to other toxicokinetic parameters (for instance, investigation of metabolism profiles).

REFERENCES

1. Blaser H-U. Chirality and its implications for the pharmaceutical industry. *Rendiconti Lincei*. 2013;24(3):213-6.
2. Nguyen LA, He H, Pham-Huy C. Chiral drugs: an overview. *Int J Biomed Sci*. 2006;2(2):85-100.
3. Smith SW. Chiral toxicology: it's the same thing...only different. *Toxicol Sci*. 2009;110(1):4-30.
4. Evans AM. Enantioselective pharmacodynamics and pharmacokinetics of chiral non-steroidal anti-inflammatory drugs. *Eur J Clin Pharmacol*. 1992;42(3):237-56.
5. Brocks DR. Drug disposition in three dimensions: an update on stereoselectivity in pharmacokinetics. *Biopharm Drug Dispos*. 2006;27(8):387-406.
6. Lees P, Hunter RP, Reeves PT, Toutain PL. Pharmacokinetics and pharmacodynamics of stereoisomeric drugs with particular reference to bioequivalence determination. *J Vet Pharmacol Ther*. 2012;35 Suppl 1:17-29.
7. Jamali F, Mehvar R, Pasutto FM. Enantioselective aspects of drug action and disposition: therapeutic pitfalls. *J Pharm Sci*. 1989;78(9):695-715.
8. Chhabra N, Aseri ML, Padmanabhan D. A review of drug isomerism and its significance. *Int J Appl Basic Med Res*. 2013;3(1):16-8.
9. Drayer DE. Pharmacodynamic and pharmacokinetic differences between drug enantiomers in humans: an overview. *Clin Pharmacol Ther*. 1986;40(2):125-33.
10. Sekhon BS. Exploiting the Power of Stereochemistry in Drugs: An Overview of Racemic and Enantiopure Drugs. *J Mod Med Chem*. 2013;1:10-36.
11. Maier NM, Franco P, Lindner W. Separation of enantiomers: needs, challenges, perspectives. *J Chromatogr A*. 2001;906(1-2):3-33.
12. Mehvar R, Brocks DR, Vakily M. Impact of stereoselectivity on the pharmacokinetics and pharmacodynamics of antiarrhythmic drugs. *Clin Pharmacokinet*. 2002;41(8):533-58.
13. Barrett AM, Cullum VA. The biological properties of the optical isomers of propranolol and their effects on cardiac arrhythmias. *Br J Pharmacol*. 1968;34(1):43-55.
14. Mannschreck A, Kiesswetter R, von Angerer E. Unequal Activities of Enantiomers via Biological Receptors: Examples of Chiral Drug, Pesticide, and Fragrance Molecules. *J Chem Educ*. 2007;84(12):2012.
15. Ribeiro AR, Castro PML, Tiritan ME. Chiral pharmaceuticals in the environment. *Environ Chem Lett*. 2012;10(3):239-53.

16. UNODC. World drugs report 2014 [Available from: https://www.unodc.org/documents/data-and-analysis/WDR2014/World_Drug_Report_2014_web.pdf]; Accessed: 18.03.2021
17. Zanda MT, Fattore L. Chapter 29 - Novel Psychoactive Substances: A New Behavioral and Mental Health Threat. In: Watson RR, Zibadi S, editors. Addictive Substances and Neurological Disease: Academic Press; 2017. p. 341-53.
18. Coppola M, Mondola R, Oliva F, Picci RL, Ascheri D, Trivelli F. Chapter 63 - Treating the Phenomenon of New Psychoactive Substances: Synthetic Cannabinoids and Synthetic Cathinones. In: Preedy VR, editor. Neuropathology of Drug Addictions and Substance Misuse. San Diego: Academic Press; 2016. p. 679-86.
19. Zawilska JB. Chapter Thirteen - "Legal Highs" – An Emerging Epidemic of Novel Psychoactive Substances. In: Taba P, Lees A, Sikk K, editors. Int Rev Neurobiol. 120: Academic Press; 2015. p. 273-300.
20. EMCDDA. New psychoactive substances: global markets, glocal threats and the COVID-19 pandemic - an update from the EU Early Warning System 2020 [Available from: https://www.emcdda.europa.eu/publications/rapid-communication/new-psychoactive-substances-global-markets-glocal-threats-and-covid-19-pandemic_en]; Accessed: 18.03.2021
21. Valente MJ, Guedes de Pinho P, de Lourdes Bastos M, Carvalho F, Carvalho M. Khat and synthetic cathinones: a review. Arch Toxicol. 2014;88(1):15-45.
22. German CL, Fleckenstein AE, Hanson GR. Bath salts and synthetic cathinones: An emerging designer drug phenomenon. Life Sci. 2014;97(1):2-8.
23. Getasetegn M. Chemical composition of Catha edulis (khat): a review. Phytochem Rev. 2016;15(5):907-20.
24. World Health O. WHO Expert Committee on Drug Dependence : thirty-fourth report. Geneva: World Health Organization; 2006:36 (Technical Report Series No. 942).
25. Bretteville-Jensen AL, Tuv SS, Bilgrei OR, Fjeld B, Bachs L. Synthetic cannabinoids and cathinones: prevalence and markets. Forensic Sci Rev. 2013; 25(1-2):7-26.
26. EMCDDA. European Drug Report 2015. Publications Office of the European Union, L; 2015. p. 86.
27. Baumann MH, Partilla JS, Lehner KR, Thorndike EB, Hoffman AF, Holy M, et al. Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive 'bath salts' products. Neuropsychopharmacol. 2013;38(4):552-62.
28. Gonçalves JL, Alves VL, Aguiar J, Teixeira HM, Câmara JS. Synthetic cathinones: an evolving class of new psychoactive substances. Crit Rev Toxicol. 2019;49(7):549-66.

29. Majchrzak M, Celiński R, Kuś P, Kowalska T, Sajewicz M. The newest cathinone derivatives as designer drugs: an analytical and toxicological review. *Forensic Toxicol.* 2018;36(1):33-50.
30. Soares J, Costa VM, Bastos MdL, Carvalho F, Capela JP. An updated review on synthetic cathinones. *Arch Toxicol.* 2021.
31. Zuba D, Byrska B. Prevalence and co-existence of active components of 'legal highs'. *Drug Test Anal.* 2013;5(6):420-9.
32. Paillet-Loilier M, Cesbron A, Boisselier R, Bourguine J, Debruyne D. Emerging drugs of abuse: Current perspectives on substituted cathinones. *Subst Abuse Rehabil.* 2014;5:37-52.
33. Young R, Glennon RA. Cocaine-stimulus generalization to two new designer drugs: Methcathinone and 4-methylaminorex. *Pharmacol Biochem Behav.* 1993;45(1):229-31.
34. Dal Cason TA, Young R, Glennon RA. Cathinone: an investigation of several N-alkyl and methylenedioxy-substituted analogs. *Pharmacol Biochem Behav.* 1997;58(4):1109-16.
35. Dasgupta A. 3 - Designer drugs including bath salts and spices. In: Dasgupta A, editor. *Alcohol, Drugs, Genes and the Clinical Laboratory*: Academic Press; 2017. p. 53-73.
36. Government. Decreto-Lei n.º 54/2013. *Diário da República* 75. 2013.
37. Uchiyama N, Matsuda S, Kawamura M, Kikura-Hanajiri R, Goda Y. Identification of two new-type designer drugs, piperazine derivative MT-45 (I-C6) and synthetic peptide Noopept (GVS-111), with synthetic cannabinoid A-834735, cathinone derivative 4-methoxy- α -PVP, and phenethylamine derivative 4-methylbuphedrine from illegal products. *Forensic Toxicol.* 2014;32(1):9-18.
38. Uchiyama N, Matsuda S, Kawamura M, Shimokawa Y, Kikura-Hanajiri R, Aritake K, et al. Characterization of four new designer drugs, 5-chloro-NNEI, NNEI indazole analog, α -PHPP and α -POP, with 11 newly distributed designer drugs in illegal products. *Forensic Sci Int.* 2014;243:1-13.
39. Uchiyama N, Shimokawa Y, Kawamura M, Kikura-Hanajiri R, Hakamatsuka T. Chemical analysis of a benzofuran derivative, 2-(2-ethylaminopropyl)benzofuran (2-EAPB), eight synthetic cannabinoids, five cathinone derivatives, and five other designer drugs newly detected in illegal products. *Forensic Toxicol.* 2014;32(2):266-81.
40. Gaspar H, Bronze S, Ciríaco S, Queirós CR, Matias A, Rodrigues J, et al. 4F-PBP (4'-fluoro- α -pyrrolidinobutyrophenone), a new substance of abuse: Structural characterization and purity NMR profiling. *Forensic Sci Int.* 2015;252:168-76.

41. Doi T, Asada A, Takeda A, Tagami T, Katagi M, Matsuta S, et al. Identification and characterization of α -PVT, α -PBT, and their bromothienyl analogs found in illicit drug products. *Forensic Toxicol.* 2016;34(1):76-93.
42. Gambaro V, Casagni E, Dell'Acqua L, Roda G, Tamborini L, Visconti GL, et al. Identification and characterization of a new designer drug thiothinone in seized products. *Forensic Toxicol.* 2016;34(1):174-8.
43. 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.* 2017;9(8):1162-71.
44. Machado Y, Coelho Neto J, Barbosa PEN, Lordeiro RA, Alves RB. Brepheдрone: A new psychoactive substance seized in Brazil. *Forensic Sci Int.* 2017;275:302-7.
45. Bijlsma L, Miserez B, Ibáñez M, Vicent C, Guillamón E, Ramsey J, et al. 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 Toxicol.* 2016;34(1):144-50.
46. Błażewicz A, Bednarek E, Sitkowski J, Popławska M, Stypułkowska K, Bocian W, et al. Identification and structural characterization of four novel synthetic cathinones: α -methylaminohexanophenone (hexedrone, HEX), 4-bromoethcathinone (4-BEC), 4-chloro- α -pyrrolidinopropiophenone (4-Cl-PPP), and 4-bromo- α -pyrrolidinopentiophenone (4-Br-PVP) after their seizures. *Forensic Toxicol.* 2017;35(2):317-32.
47. Apirakkan O, Frinculescu A, Shine T, Parkin MC, Cilibrizzi A, Frascione N, 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.* 2018;10(2):372-8.
48. Fabregat-Safont D, Carbón X, Gil C, Ventura M, Sancho JV, Hernández F, 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):447-57.
49. Błażewicz A, Bednarek E, Popławska M, Olech N, Sitkowski J, Kozerski L. Identification and structural characterization of synthetic cathinones: N-propylcathinone, 2,4-dimethylmethcathinone, 2,4-dimethylethcathinone, 2,4-dimethyl- α -pyrrolidinopropiophenone, 4-bromo- α -pyrrolidinopropiophenone, 1-(2,3-dihydro-1H-inden-5-yl)-2-(pyrrolidin-1-yl)hexan-1-one and 2,4-dimethylisocathinone. *Forensic Toxicol.* 2019;37.
50. Shevyrin V, Eltsov O, Shafran Y. Identification and analytical characterization of the synthetic cathinone N-butylhexedrone. *Drug Test Anal.* 2020;12(1):159-63.

51. Rojkiewicz M, Kuś P, Kusz J, Książek M, Sochanik A. Spectroscopic and crystallographic characterization of a new cathinone derivative: 1-phenyl-2-(butylamino)hexan-1-one hydrochloride (N-butylhexedrone). *Forensic Toxicol.* 2020;38(2):481-9.
52. Botanas CJ, Yoon SS, de la Peña JB, dela Peña IJ, Kim M, Woo T, et al. A novel synthetic cathinone, 2-(methylamino)-1-(naphthalen-2-yl) propan-1-one (BMAPN), produced rewarding effects and altered striatal dopamine-related gene expression in mice. *Behav Brain Res.* 2017;317:494-501.
53. Carlsson A, Sandgren V, Svensson S, Konradsson P, Dunne S, Josefsson M, et al. Prediction of designer drugs: Synthesis and spectroscopic analysis of synthetic cathinone analogs that may appear on the Swedish drug market. *Drug Test Anal.* 2018;10(7):1076-98.
54. Gaspar H, Bronze S, Oliveira C, Victor BL, Machuqueiro M, Pacheco R, et al. Proactive response to tackle the threat of emerging drugs: Synthesis and toxicity evaluation of new cathinones. *Forensic Sci Int.* 2018;290:146-56.
55. Hasegawa K, Wurita A, Minakata K, Gonmori K, Nozawa H, Yamagishi I, et al. Identification and quantitation of a new cathinone designer drug PV9 in an "aroma liquid" product, antemortem whole blood and urine specimens, and a postmortem whole blood specimen in a fatal poisoning case. *Forensic Toxicol.* 2014;32(2):243-50.
56. Majchrzak M, Celiński R, Kowalska T, Sajewicz M. Fatal case of poisoning with a new cathinone derivative: α -propylaminopentiophenone (N-PP). *Forensic Toxicol.* 2018;36(2):525-33.
57. Pieprzyca E, Skowronek R, Korczyńska M, Kulikowska J, Chowaniec M. A two fatal cases of poisoning involving new cathinone derivative PV8. *Leg Med.* 2018;33:42-7.
58. Adamowicz P, Jurczyk A, Gil D, Szustowski S. A case of intoxication with a new cathinone derivative α -PiHP – A presentation of concentrations in biological specimens. *Leg Med.* 2020;42:101626.
59. Weinstein AM, Rosca P, Fattore L, London ED. Synthetic Cathinone and Cannabinoid Designer Drugs Pose a Major Risk for Public Health. *Front Psychiatry.* 2017;8:156-.
60. Karila L, Megarbane B, Cottencin O, Lejoyeux M. Synthetic cathinones: a new public health problem. *Curr Neuropharmacol.* 2015;13(1):12-20.
61. 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):144-9.
62. Zaitzu K. Metabolism of Synthetic Cathinones. 2018. p. 71-96.

63. Kelly JP. Cathinone derivatives: a review of their chemistry, pharmacology and toxicology. *Drug Test Anal.* 2011;3(7-8):439-53.
64. Meyer MR, Maurer HH. Metabolism of designer drugs of abuse: an updated review. *Curr Drug Metab.* 2010;11(5):468-82.
65. Tyrkkö E, Andersson M, Kronstrand R. The Toxicology of New Psychoactive Substances: Synthetic Cathinones and Phenylethylamines. *Ther Drug Monit.* 2016;38(2):190-216.
66. Baumann MH, Walters HM, Niello M, Sitte HH. Neuropharmacology of Synthetic Cathinones. *Handb Exp Pharmacol.* 2018;252:113-42.
67. Altun B, Çok İ. Psychoactive Bath Salts and Neurotoxicity Risk. *Turk J Pharma Sci.* 2020;17(2):235-41.
68. Prosser JM, Nelson LS. The toxicology of bath salts: a review of synthetic cathinones. *J Med Toxicol.* 2012;8(1):33-42.
69. Schifano F, Corkery J, Ghodse AH. Suspected and Confirmed Fatalities Associated With Mephedrone (4-Methylmethcathinone, “Meow Meow”) in the United Kingdom. *J Clin Psychopharmacol.* 2012;32(5):710-4.
70. Herrmann ES, Johnson PS, Johnson M, Vandrey R. Chapter 88 - Novel Drugs of Abuse: Cannabinoids, Stimulants, and Hallucinogens. *Neuropathology of Drug Addictions and Substance Misuse*, Academic Press, 2016;p893-902.
71. Silva B, Fernandes C, Guedes de Pinho P, Remião F. Chiral Resolution and Enantioselectivity of Synthetic Cathinones: A Brief Review. *J Anal Toxicol.* 2018;42(1):17-24.
72. Glennon RA, Martin BR, Dal Cason TA, Young R. Methcathinone (“cat”): An enantiomeric potency comparison. *Pharmacol Biochem Behav.* 1995;50(4):601-6.
73. Gregg RA, Baumann MH, Partilla JS, Bonano JS, Vouga A, Tallarida CS, et al. Stereochemistry of mephedrone neuropharmacology: enantiomer-specific behavioural and neurochemical effects in rats. *Br J Pharmacol.* 2015;172(3):883-94.
74. Hutsell BA, Baumann MH, Partilla JS, Banks ML, Vekariya R, Glennon RA, et al. Abuse-related neurochemical and behavioral effects of cathinone and 4-methylcathinone stereoisomers in rats. *Eur Neuropsychopharmacol.* 2016;26(2):288-97.
75. Kolanos R, Partilla JS, Baumann MH, Hutsell BA, Banks ML, Negus SS, et al. Stereoselective Actions of Methylenedioxypyrovalerone (MDPV) To Inhibit Dopamine and Norepinephrine Transporters and Facilitate Intracranial Self-Stimulation in Rats. *ACS Chem Neurosci.* 2015;6(5):771-7.

76. Gannon BM, Williamson A, Suzuki M, Rice KC, Fantegrossi WE. Stereoselective Effects of Abused "Bath Salt" Constituent 3,4-Methylenedioxypyrovalerone in Mice: Drug Discrimination, Locomotor Activity, and Thermoregulation. *J Pharmacol Exp Ther*. 2016;356(3):615-23.
77. Silva B, Fernandes C, Tiritan ME, Pinto MM, Valente MJ, Carvalho M, et al. Chiral enantioresolution of cathinone derivatives present in "legal highs", and enantioselectivity evaluation on cytotoxicity of 3,4-methylenedioxypyrovalerone (MDPV). *Forensic Toxicol*. 2016;34:372-85.
78. Gannon BM, Rice KC, Collins GT. Reinforcing effects of abused 'bath salts' constituents 3,4-methylenedioxypyrovalerone and α -pyrrolidinopentiophenone and their enantiomers. *Behav Pharmacol*. 2017;28(7):578-81.
79. Philogene-Khalid HL, Hicks C, Reitz AB, Liu-Chen LY, Rawls SM. Synthetic cathinones and stereochemistry: S enantiomer of mephedrone reduces anxiety- and depressant-like effects in cocaine- or MDPV-abstinent rats. *Drug Alcohol Depend*. 2017-a;178:119-25.
80. Philogene-Khalid HL, Simmons SJ, Nayak S, Martorana RM, Su SH, Caro Y, et al. Stereoselective Differences between the Reinforcing and Motivational Effects of Cathinone-Derived 4-Methylmethcathinone (Mephedrone) In Self-Administering Rats. *ACS Chem Neurosci*. 2017-b;8(12):2648-54.
81. Nelson KH, López-Arnau R, Hempel BJ, To P, Manke HN, Crissman ME, et al. Stereoselective effects of the second-generation synthetic cathinone α -pyrrolidinopentiophenone (α -PVP): assessments of conditioned taste avoidance in rats. *Psychopharmacology (Berl.)*. 2019;236(3):1067-77.
82. Mayer FP, Cintulova D, Pittrich DA, Wimmer L, Luethi D, Holy M, et al. Stereochemistry of phase-1 metabolites of mephedrone determines their effectiveness as releasers at the serotonin transporter. *Neuropharmacology*. 2019;148:199-209.
83. Schindler CW, Thorndike EB, Walters HM, Walther D, Rice KC, Baumann MH. Stereoselective neurochemical, behavioral, and cardiovascular effects of α -pyrrolidinovalerophenone enantiomers in male rats. *Addict Biol*. 2020;25(6):e12842.
84. Silva B, Silva R, Fernandes C, Guedes de Pinho P, Remião F. Enantioselectivity on the absorption of methylone and pentedrone using Caco-2 cell line: Development and validation of an UHPLC method for cathinones quantification. *Toxicol Appl Pharmacol*. 2020;395:114970.
85. Davies RA, Baird TR, Nguyen VT, Ruiz B, Sakloth F, Eltit JM, et al. Investigation of the Optical Isomers of Methcathinone, and Two Achiral Analogs, at Monoamine Transporters and in Intracranial Self-Stimulation Studies in Rats. *ACS Chem Neurosci*. 2020;11(12):1762-9.

86. Silva B, Palmeira A, Silva R, Fernandes C, Guedes de Pinho P, Remião F. S-(+)-Pentedrone and R-(+)-methylone as the most oxidative and cytotoxic enantiomers to dopaminergic SH-SY5Y cells: Role of MRP1 and P-gp in cathinones enantioselectivity. *Toxicol Appl Pharmacol.* 2021;416:115442.
87. Francotte ER. Enantioselective chromatography as a powerful alternative for the preparation of drug enantiomers. *J Chromatogr A.* 2001;906(1):379-97.
88. Albals D, Heyden YV, Schmid MG, Chankvetadze B, Mangelings D. Chiral separations of cathinone and amphetamine-derivatives: Comparative study between capillary electrochromatography, supercritical fluid chromatography and three liquid chromatographic modes. *J Pharm Biomed Anal.* 2016;121:232-43.
89. Taschwer M, Weiß JA, Kunert O, Schmid MG. Analysis and characterization of the novel psychoactive drug 4-chloromethcathinone (clephedrone). *Forensic Sci Int.* 2014;244:e56-9.
90. Mohr S, Weiß JA, Spreitz J, Schmid MG. Chiral separation of new cathinone- and amphetamine-related designer drugs by gas chromatography-mass spectrometry using trifluoroacetyl-L-prolyl chloride as chiral derivatization reagent. *J Chromatogr A.* 2012;1269:352-9.
91. Weiß JA, Mohr S, Schmid MG. Indirect chiral separation of new recreational drugs by gas chromatography-mass spectrometry using trifluoroacetyl-L-prolyl chloride as chiral derivatization reagent. *Chirality.* 2015;27(3):211-5.
92. Fernandes C, Tiritan ME, Pinto M. Small Molecules as Chromatographic Tools for HPLC Enantiomeric Resolution: Pirkle-Type Chiral Stationary Phases Evolution. *Chromatographia.* 2013;76(15):871-97.
93. Scriba GKE. Chiral Recognition Mechanisms in Analytical Separation Sciences. *Chromatographia.* 2012;75(15):815-38.
94. Tiritan ME, Ribeiro AR, Fernandes C, Pinto MMM. Chiral Pharmaceuticals. *Kirk-Othmer Encyclopedia of Chemical Technology: John Wiley & Sons, Inc.* 2016:1-28.
95. Okamoto Y, Ikai T. Chiral HPLC for efficient resolution of enantiomers. *Chem Soc Rev.* 2008;37(12):2593-608.
96. Teixeira J, Tiritan ME, Pinto MMM, Fernandes C. Chiral Stationary Phases for Liquid Chromatography: Recent Developments. *Molecules.* 2019;24(5):865.
97. Ismail OH, Ciogli A, Villani C, De Martino M, Pierini M, Cavazzini A, et al. Ultra-fast high-efficiency enantioseparations by means of a teicoplanin-based chiral stationary phase made

on sub-2 μm totally porous silica particles of narrow size distribution. *J Chromatogr A*. 2016;1427:55-68.

98. Ward TJ, Ward KD. Chiral Separations: A Review of Current Topics and Trends. *Anal Chem*. 2012;84(2):626-35.

99. Zhang Y, Wu DR, Wang-Iverson DB, Tymiak AA. Enantioselective chromatography in drug discovery. *Drug Discov Today*. 2005;10(8):571-7.

100. Schmid MG, Hägele JS. Separation of enantiomers and positional isomers of novel psychoactive substances in solid samples by chromatographic and electrophoretic techniques – A selective review. *J Chromatogr A*. 2020;1624:461256.

101. Aboul-Enein HY, Serignese V. Direct enantiomeric separation of cathinone and one major metabolite on cellobiohydrolase (CBH-I) chiral stationary phase. *Biomed Chromatogr*. 1997;11(1):47-9.

102. Aboul-Enein HY, Serignese V. Direct chiral resolution of phenylalkylamines using a crown ether chiral stationary phase. *Biomed Chromatogr*. 1997;11(1):7-10.

103. Wolrab D, Frühauf P, Moulisová A, Kuchař M, Gerner C, Lindner W, et al. Chiral separation of new designer drugs (Cathinones) on chiral ion-exchange type stationary phases. *J Pharm Biomed Anal*. 2016;120:306-15.

104. Perera RW, Abraham I, Gupta S, Kowalska P, Lightsey D, Marathaki C, et al. Screening approach, optimisation and scale-up for chiral liquid chromatography of cathinones. *J Chromatogr A*. 2012;1269:189-97.

105. Taschwer M, Seidl Y, Mohr S, Schmid MG. Chiral separation of cathinone and amphetamine derivatives by HPLC/UV using sulfated β -cyclodextrin as chiral mobile phase additive. *Chirality*. 2014;26(8):411-8.

106. Weiß JA, Taschwer M, Kunert O, Schmid MG. Analysis of a new drug of abuse: cathinone derivative 1-(3,4-dimethoxyphenyl)-2-(ethylamino)pentan-1-one. *J Sep Sci*. 2015;38(5):825-8.

107. Merola G, Fu H, Tagliaro F, Macchia T, McCord BR. Chiral separation of 12 cathinone analogs by cyclodextrin-assisted capillary electrophoresis with UV and mass spectrometry detection. *Electrophoresis*. 2014;35(21-22):3231-41.

108. Baciú T, Borrull F, Calull M, Aguilar C. Enantioselective determination of cathinone derivatives in human hair by capillary electrophoresis combined in-line with solid-phase extraction. *Electrophoresis*. 2016;37(17-18):2352-62.

109. Aturki Z, Schmid MG, Chankvetadze B, Fanali S. Enantiomeric separation of new cathinone derivatives designer drugs by capillary electrochromatography using a chiral

stationary phase, based on amylose tris(5-chloro-2-methylphenylcarbamate). Electrophoresis. 2014;35(21-22):3242-9.

110. Suzuki M, Deschamps JR, Jacobson AE, Rice KC. Chiral Resolution and Absolute Configuration of the Enantiomers of the Psychoactive “Designer Drug” 3,4-Methylenedioxypyrovalerone. Chirality. 2015;27(4):287-93.

111. Alremeithi RH, Meetani MA, Khalil SA. A validated gas chromatography mass spectrometry method for simultaneous determination of cathinone related drug enantiomers in urine and plasma. RSC Advances. 2016;6(84):80576-84.

112. Taschwer M, Grascher J, Schmid MG. Development of an enantioseparation method for novel psychoactive drugs by HPLC using a Lux(®) Cellulose-2 column in polar organic phase mode. Forensic Sci Int. 2017;270:232-40.

113. Alremeithi R, Meetani MA, Alaidaros AA, Lanjawi A, Alsumaiti K. Simultaneous Quantitative Determination of Synthetic Cathinone Enantiomers in Urine and Plasma Using GC-NCI-MS. J Anal Methods Chem. 2018;2018:4396043.

114. Kadkhodaei K, Forcher L, Schmid MG. Separation of enantiomers of new psychoactive substances by high-performance liquid chromatography. J Sep Sci. 2018;41(6):1274-86.

115. Nowak PM, Olesek K, Woźniakiewicz M, Kościelniak P. Simultaneous enantioseparation of methcathinone and two isomeric methylmethcathinones using capillary electrophoresis assisted by 2-hydroxyethyl- β -cyclodextrin. Electrophoresis. 2018;39(19):2406-9.

116. Silva B, Pereira JA, Cravo S, Araújo AM, Fernandes C, Pinto MMM, et al. Multi-milligram resolution and determination of absolute configuration of pentedrone and methylone enantiomers. J Chromatogr B. 2018;1100-1101:158-64.

117. Hägele JS, Hubner EM, Schmid MG. Chiral separation of cathinone derivatives using β -cyclodextrin-assisted capillary electrophoresis-Comparison of four different β -cyclodextrin derivatives used as chiral selectors. Electrophoresis. 2019;40(14):1787-94.

118. Meetani MA, Alremeithi RH, Mousa MK. Enantioseparation of Synthetic Cathinones Enantiomers with Tertiary Amine Structure in Urine and Plasma. J Chromatogr Sci. 2019;57(4):361-8.

119. Pérez-Alcaraz A, Borrull F, Aguilar C, Calull M. Enantioselective determination of cathinones in urine by high pressure in-line SPE-CE. Electrophoresis. 2019;40(14):1762-70.

120. Dhabbah AM. Determination of chiral cathinone in fresh samples of Catha edulis. Forensic Sci Int. 2020;307:110105.

121. Fu Y, Borrull F, Marcé RM, Fontanals N. Enantiomeric determination of cathinones in environmental water samples by liquid chromatography-high resolution mass spectrometry. *J Chromatogr A*. 2020;1626:461359.
122. Hägele JS, Basrak M, Schmid MG. Enantioselective separation of Novel Psychoactive Substances using a Lux® AMP 3 µm column and HPLC-UV. *J Pharm Biomed Anal*. 2020;179:112967.
123. Hägele JS, Seibert E, Schmid MG. A Simple HPLC–UV Approach for Rapid Enantioseparation of Cathinones, Pyrovalerones and Other Novel Psychoactive Substances on a 2.5-µm Cellulose Tris-(3,5-dimethylphenyl-carbamate) Column. *Chromatographia*. 2020;83(3):321-9.
124. Kadkhodaei K, Kadisch M, Schmid MG. Successful use of a novel lux® i-Amylose-1 chiral column for enantioseparation of “legal highs” by HPLC. *Chirality*. 2020;32(1):42-52.
125. Kolderová N, Jurásek B, Kuchař M, Lindner W, Kohout M. Gradient supercritical fluid chromatography coupled to mass spectrometry with a gradient flow of make-up solvent for enantioseparation of cathinones. *J Chromatogr A*. 2020;1625:461286.
126. Lin HR, Kuo FW. Determination of the R- and S-enantiomers of methylone and ethylone in seized drugs by enantioselective liquid chromatography tandem mass spectrometry analysis. *Forensic Sci Int*. 2020;317:110528.
127. Meetani M, Ja A, Mk A. Simultaneous Quantitative Determination of Synthetic Cathinone Enantiomers in Urine by GC-NCI-MS/MS using Menthylchloroformate as Chiral Derivatization Reagent. *J Chromatogr Sep Tech*. 2020;11:436.
128. Pérez-Alcaraz A, Borrull F, Aguilar C, Calull M. An electrokinetic supercharging approach for the enantiodetermination of cathinones in urine samples by capillary electrophoresis. *Microchem J*. 2020;158:105300.
129. Řezanka P, Macková D, Jurok R, Himl M, Kuchař M. Enantioseparation and Determination of Mephedrone and Its Metabolites by Capillary Electrophoresis Using Cyclodextrins as Chiral Selectors. *Molecules*. 2020;25(12).
130. Loganathan D, Yi R, Patel B, Zhang J, Kong N. A sensitive HPLC-MS/MS method for the detection, resolution and quantitation of cathinone enantiomers in horse blood plasma and urine. *Anal Bioanal Chem*. 2021;413(8):2147-61.
131. Pérez-Alcaraz A, Borrull F, Aguilar C, Calull M, Benavente F. Enantiodetermination of R,S-3,4-methylenedioxypyrovalerone in urine samples by high pressure in-line solid-phase extraction capillary electrophoresis-mass spectrometry. *Talanta*. 2021;225:121994.

132. Naegele E. Making your LC method compatible with mass spectrometry. Technical overview. Agilent Technologies. Inc 2011 [Available from: <http://www.chem.agilent.com/Library/technicaloverviews/Public/5990--7413EN.pdf>]; Accessed: 19.06.2021
133. Kuropka P, Zawadzki M, Szpot P. A review of synthetic cathinones emerging in recent years (2019-2022). Forensic Toxicol. 2022:1-22.
134. NMSLabs. N-Butyl pentylone. 2019 [Available from: https://www.npsdiscovery.org/wp-content/uploads/2019/06/N-butyl-Pentylone_051619_NMSLabs_Report.pdf]; Accessed: 23.09.2022
135. DEA. Emerging threat report 2019 annual. 2019 [Available from: <https://cesar.umd.edu/publicationprofile/170>]; Accessed: 23.09.2022
136. EMCDDA. New psychoactive substances : global markets, global threats and the COVID-19 pandemic : an update from the EU early warning system: Publications Office; 2020.
137. NMSLabs. N,N-Diethyl hexedrone. 2019 [Available from: https://www.npsdiscovery.org/wp-content/uploads/2019/05/NN-Diethyl-Hexedrone_031819_NMSLabs_Report.pdf]; Accessed: 23.09.2022
138. NMSLabs. alpha-PCYP 2020 [Available from: https://www.npsdiscovery.org/wp-content/uploads/2020/05/alpha-PCYP_052720_NMSLabs_Report.pdf]; Accessed: 23.09.2022
139. Kolanos R, Sakloth F, Jain AD, Partilla JS, Baumann MH, Glennon RA. Structural Modification of the Designer Stimulant α -Pyrrolidinovalerophenone (α -PVP) Influences Potency at Dopamine Transporters. ACS Chem Neurosci. 2015;6(10):1726-31.
140. NMSLabs. 4-Fluoro-3-methyl-alpha-PVP 2020 [Available from: https://www.npsdiscovery.org/wp-content/uploads/2020/12/4-Fluoro-3-Methyl-Alpha-PVP_120120_NMSLabs_Report.pdf]; Accessed: 23.09.2022
141. EMCDDA. New psychoactive substances: 25 years of early warning and response in Europe : an update from the EU Early Warning System: Publications Office of the European Union; 2022.
142. National Forensic Laboratory of Slovenia. α -D2PV. 2020 [Available from: https://www.policija.si/apps/nfl_response_web/0_Analytical_Reports_final/A-D2PV-ID-2200-20_report.pdf]; Accessed: 23.09.2022
143. CFSRE. Recommended scope for NPS testing in the United States Q4. 2021 [Available from: https://www.npsdiscovery.org/wp-content/uploads/2022/02/Q4-2021-NPS-Scope-Recommendations_NPS-Discovery_021722.pdf]; Accessed: 23.09.2022

144. CFSRE. N-Cyclohexyl methylone. 2022 [Available from: https://www.npsdiscovery.org/wp-content/uploads/2022/05/N-Cyclohexyl-Methylone_050922_CFSRE-Chemistry_Report.pdf]; Accessed: 23.09.2022
145. CFSRE. N-Cyclohexyl buthylone. 2022 [Available from: https://www.npsdiscovery.org/wp-content/uploads/2022/06/N-Cyclohexyl-Butylone_060922_CFSRE-Chemistry_Report.pdf]; Accessed: 23.09.2022
146. Silva B, Rodrigues JS, Almeida AS, Lima AR, Fernandes C, Guedes de Pinho P, et al. Enantioselectivity of Pentedrone and Methylone on Metabolic Profiling in 2D and 3D Human Hepatocyte-like Cells. *Pharmaceuticals*. 2022;15(3).
147. Hage DS, Jackson A, Sobansky MR, Schiel JE, Yoo MJ, Joseph KS. Characterization of drug-protein interactions in blood using high-performance affinity chromatography. *J Sep Sci*. 2009;32(5-6):835-53.
148. Zhang F, Xue J, Shao J, Jia L. Compilation of 222 drugs' plasma protein binding data and guidance for study designs. *Drug Discov Today*. 2012;17(9-10):475-85.
149. Bohnert T, Gan LS. Plasma protein binding: from discovery to development. *J Pharm Sci*. 2013;102(9):2953-94.
150. Sekhon BS, editor *Exploiting the Power of Stereochemistry in Drugs: An Overview of Racemic and Enantiopure Drugs*. 2013; p. 10-36.
151. Fournier T, Medjoubi-n N, Porquet D. Alpha-1-acid glycoprotein. *Biochim Biophys Acta*. 2000;1482(1-2):157-71.
152. Dos Santos R, Figueiredo C, Viecinski AC, Pina AS, Barbosa AJM, Roque ACA. Designed affinity ligands to capture human serum albumin. *J Chromatogr A*. 2019;1583:88-97.
153. More J, Bulmer M. Human Serum Albumin: A Multifunctional Plasma Protein. *Production of Plasma Proteins for Therapeutic Use*. 2013; p. 159-83.
154. Ascenzi P, Bocedi A, Notari S, Fanali G, Fesce R, Fasano M. Allosteric modulation of drug binding to human serum albumin. *Mini Rev Med Chem*. 2006;6(4):483-9.
155. Ghuman J, Zunszain PA, Petitpas I, Bhattacharya AA, Otagiri M, Curry S. Structural basis of the drug-binding specificity of human serum albumin. *J Mol Biol*. 2005;353(1):38-52.
156. Shen Q, Wang L, Zhou H, Jiang HD, Yu LS, Zeng S. Stereoselective binding of chiral drugs to plasma proteins. *Acta Pharmacol Sin*. 2013;34(8):998-1006.
157. Maddi S, Yamsani MR, Seeling A, Scriba GK. Stereoselective plasma protein binding of amlodipine. *Chirality*. 2010;22(2):262-6.

158. Chen R, He RJ, Guo D, Zhang ZF, Zhang WG, Fan J. Interactions of diclazuril enantiomers with serum albumins: Multi-spectroscopic and molecular docking approaches. *J Mol Recognit*. 2022;35(4):e2948.
159. Yu L, Hong Y, Li L, Jin Y, Zheng M, Jiang H, et al. Enantioselective drug-protein interaction between mexiletine and plasma protein. *J Pharm Pharmacol*. 2012;64(6):792-801.
160. Cardoso T, Almeida AS, Remião F, Fernandes C. Enantioresolution and Binding Affinity Studies on Human Serum Albumin: Recent Applications and Trends. *Chemosensors*. 2021;9(11):304.
161. Sakurama K, Kawai A, Tuan Giam Chuang V, Kanamori Y, Osa M, Taguchi K, et al. Analysis of the Binding of Aripiprazole to Human Serum Albumin: The Importance of a Chloro-Group in the Chemical Structure. *ACS Omega*. 2018;3(10):13790-7.
162. Tan H, Chen L, Ma L, Liu S, Zhou H, Zhang Y, et al. Fluorescence Spectroscopic Investigation of Competitive Interactions between Quercetin and Aflatoxin B₁ for Binding to Human Serum Albumin. *Toxins*. 2019;11(4).
163. Chen J, Hou L, Zheng K, Wang J, Chen N, Huang J, et al. Blood distribution and plasma protein binding of PHOTOCYANINE: a promising phthalocyanine photosensitizer inphase II clinical trials. *Eur J Pharm Sci*. 2020;153:105491.
164. Yoo MJ, Smith QR, Hage DS. Studies of imipramine binding to human serum albumin by high-performance affinity chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877(11-12):1149-54.
165. Tong Z, Hage D. Characterization of interaction kinetics between chiral solutes and human serum albumin by using high-performance affinity chromatography and peak profiling. *J Chromatogr A*. 2011;1218:6892-7.
166. Liu X, Song Y, Yue Y, Zhang J, Chen X. Study of interaction between drug enantiomers and human serum albumin by flow injection-capillary electrophoresis frontal analysis. *Electrophoresis*. 2008;29(13):2876-83.
167. Ratih R, Wätzig H, Stein M, El Deeb S. Investigation of the enantioselective interaction between selected drug enantiomers and human serum albumin by mobility shift-affinity capillary electrophoresis. *J Sep Sci*. 2020;43(20):3960-8.
168. do Carmo JP, Phyo YZ, Palmeira A, Tiritan ME, Afonso C, Kijjoa A, et al. Enantioseparation, recognition mechanisms and binding of xanthenes on human serum albumin by liquid chromatography. *Bioanalysis*. 2019;11(13):1255-74.

169. Martínez-Gómez MA, Villanueva-Camañas RM, Sagrado S, Medina-Hernández MJ. Evaluation of enantioselective binding of antihistamines to human serum albumin by ACE. Electrophoresis. 2007;28(15):2635-43.
170. Kim HS, Kye YS, Hage DS. Development and evaluation of N-hydroxysuccinimide-activated silica for immobilizing human serum albumin in liquid chromatography columns. J Chromatogr A. 2004;1049(1-2):51-61.
171. Vuignier K, Guillarme D, Veuthey J-L, Carrupt P-A, Schappler J. High performance affinity chromatography (HPAC) as a high-throughput screening tool in drug discovery to study drug-plasma protein interactions. J Pharm Biomed Anal. 2013;74:205-12.
172. Li Z, Hage DS. Analysis of stereoselective drug interactions with serum proteins by high-performance affinity chromatography: A historical perspective. J Pharm Biomed Anal. 2017;144:12-24.
173. Kim HS, Wainer IW. Rapid analysis of the interactions between drugs and human serum albumin (HSA) using high-performance affinity chromatography (HPAC). J Chromatogr B. 2008;870(1):22-6.
174. Loun B, Hage DS. Chiral separation mechanisms in protein-based HPLC columns. 1. Thermodynamic studies of (R)- and (S)-warfarin binding to immobilized human serum albumin. Anal Chem. 1994;66(21):3814-22.
175. Loun B, Hage DS. Chiral Separation Mechanisms in Protein-Based HPLC Columns. 2. Kinetic Studies of (R)- and (S)-Warfarin Binding to Immobilized Human Serum Albumin. Anal Chem. 1996;68(7):1218-25.
176. Andrisano V, Gotti R, Recanatini M, Cavalli A, Varoli L, Bertucci C. Stereoselective binding of 2-(4-biphenyl)-3-substituted-3-hydroxypropionic acids on an immobilised human serum albumin chiral stationary phase. J Chromatogr B Analyt Technol Biomed Life Sci. 2002;768(1):137-45.
177. Bertucci C, Pistolozzi M, Felix G, Danielson UH. HSA binding of HIV protease inhibitors: a high-performance affinity chromatography study. J Sep Sci. 2009;32(10):1625-31.
178. Pistolozzi M, Fortugno C, Franchini C, Corbo F, Muraglia M, Roy M, et al. Species-dependent binding of tocainide analogues to albumin: affinity chromatography and circular dichroism study. J Chromatogr B Analyt Technol Biomed Life Sci. 2014;968:69-78.
179. Pérez DI, Pistolozzi M, Palomo V, Redondo M, Fortugno C, Gil C, et al. 5-Imino-1,2,4-thiadiazoles and quinazolines derivatives as glycogen synthase kinase 3 β (GSK-3 β) and phosphodiesterase 7 (PDE7) inhibitors: determination of blood-brain barrier penetration and binding to human serum albumin. Eur J Pharm Sci. 2012;45(5):677-84.

180. Matlin SA, Tiritan ME, Crawford AJ, Cass QB, Boyd DR. HPLC with carbohydrate carbamate chiral phases: Influence of chiral phase structure on enantioselectivity. *Chirality*. 1994;6(2):135-40.
181. Tiritan ME, Fernandes C, Maia AS, Pinto M, Cass QB. Enantiomeric ratios: Why so many notations? *J Chromatogr A*. 2018;1569:1-7.
182. Valente MJ, Bastos MdL, Fernandes E, Carvalho F, Guedes de Pinho P, Carvalho M. Neurotoxicity of β -Keto Amphetamines: Deathly Mechanisms Elicited by Methylenedioxymethamphetamine and MDPV in Human Dopaminergic SH-SY5Y Cells. *ACS Chemical Neuroscience*. 2017;8(4):850-9.
183. Silva JP, Carmo H, Carvalho F. The synthetic cannabinoid XLR-11 induces in vitro nephrotoxicity by impairment of endocannabinoid-mediated regulation of mitochondrial function homeostasis and triggering of apoptosis. *Toxicol Lett*. 2018;287:59-69.
184. FDA. Bioanalytical method validation guidance for industry. US Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research and Center for Veterinary Medicine. 2018.
185. Harmonization I. Q2(R1) Validation of Analytical Procedures: Text and Methodology Guidance for Industry. 2021.
186. Hubatsch I, Ragnarsson EGE, Artursson P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat Protoc*. 2007;2(9):2111-9.
187. Cardoso T. Mecanismos de reconhecimento molecular e determinação da afinidade de ligação de catinonas sintéticas à albumina sérica humana, por cromatografia de afinidade de alta eficiência [Master's Dissertation]: Faculty of Pharmacy of University of Porto. <https://hdl.handle.net/10216/131743>; 2020.
188. Huang Z, Guo D, Fan J, Zhong Y, Zhang M, He L, et al. HPLC semi-preparative separation of diclazuril enantiomers and racemization in solution. *J Sep Sci*. 2020;43(7):1240-7.
189. Steber SE, Pham ANDL, Nelson E, Wolf C. Enantioseparation and racemization of α -aryl- α -fluoroacetonitriles. *Chirality*. 2021;33(12):891-8.
190. Hofmann J, Faye S, Scheiner M, Hoffmann M, Oerter S, Appelt-Menzel A, et al. Sterubin: Enantioresolution and Configurational Stability, Enantiomeric Purity in Nature, and Neuroprotective Activity in Vitro and in Vivo. *Chem Eur J*. 2020;26(32):7299-308.
191. Anizan S, Concheiro M, Lehner KR, Bukhari MO, Suzuki M, Rice KC, et al. Linear pharmacokinetics of 3,4-methylenedioxypyrovalerone (MDPV) and its metabolites in the rat: relationship to pharmacodynamic effects. *Addict Biol*. 2016;21(2):339-47.

192. Tsujikawa K, Mikuma T, Kuwayama K, Miyaguchi H, Kanamori T, Iwata YT, et al. Degradation pathways of 4-methylmethcathinone in alkaline solution and stability of methcathinone analogs in various pH solutions. *Forensic Sci Int.* 2012;220(1):103-10.
193. Präbst K, Engelhardt H, Ringgeler S, Hübner H. Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin. *Methods Mol Biol.* 2017;1601:1-17.
194. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc.* 2008;3(7):1125-31.
195. Strother L, Miles GB, Holiday AR, Cheng Y, Doherty GH. Long-term culture of SH-SY5Y neuroblastoma cells in the absence of neurotrophins: A novel model of neuronal ageing. *J Neurosci Methods.* 2021;362:109301.
196. Kovalevich J, Langford D. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods Mol Biol.* 2013;1078:9-21.
197. Castrén E, Antila H. Neuronal plasticity and neurotrophic factors in drug responses. *Mol Psychiatry.* 2017;22(8):1085-95.
198. von Bernhardt R, Bernhardt LE, Eugenín J. What Is Neural Plasticity? *Adv Exp Med Biol.* 2017;1015:1-15.
199. Poo MM. Neurotrophins as synaptic modulators. *Nat Rev Neurosci.* 2001;2(1):24-32.
200. Bramham CR, Messaoudi E. BDNF function in adult synaptic plasticity: The synaptic consolidation hypothesis. *Prog Neurobiol.* 2005;76(2):99-125.
201. Filip M, Faron-Górecka A, Kuśmider M, Gołda A, Frankowska M, Dziedzicka-Wasylewska M. Alterations in BDNF and trkB mRNAs following acute or sensitizing cocaine treatments and withdrawal. *Brain Res.* 2006;1071(1):218-25.
202. Fumagalli F, Caffino L, Racagni G, Riva MA. Repeated stress prevents cocaine-induced activation of BDNF signaling in rat prefrontal cortex. *Eur Neuropsychopharmacol.* 2009;19(6):402-8.
203. Fumagalli F, Moro F, Caffino L, Orrù A, Cassina C, Giannotti G, et al. Region-specific effects on BDNF expression after contingent or non-contingent cocaine i.v. self-administration in rats. *Int J Neuropsychopharmacol.* 2013;16(4):913-8.
204. Li X, Wolf ME. Multiple faces of BDNF in cocaine addiction. *Behav Brain Res.* 2015;279:240-54.
205. Caffino L, Mottarlini F, Bilel S, Targa G, Tirri M, Maggi C, et al. Single Exposure to the Cathinones MDPV and α -PVP Alters Molecular Markers of Neuroplasticity in the Adult Mouse Brain. *Int J Mol Sci.* 2021;22(14):7397.

206. Duart-Castells L, López-Arnau R, Vizcaíno S, Camarasa J, Pubill D, Escubedo E. 7,8-Dihydroxyflavone blocks the development of behavioral sensitization to MDPV, but not to cocaine: Differential role of the BDNF-TrkB pathway. *Biochem Pharmacol.* 2019;163:84-93.
207. Nadal-Gratacós N, Alberto-Silva AS, Rodríguez-Soler M, Urquizu E, Espinosa-Velasco M, Jäntschi K, et al. Structure-Activity Relationship of Novel Second-Generation Synthetic Cathinones: Mechanism of Action, Locomotion, Reward, and Immediate-Early Genes. *Front Pharmacol.* 2021;12:749429.
208. Cortés N, Guzmán-Martínez L, Andrade V, González A, Maccioni RB. CDK5: A Unique CDK and Its Multiple Roles in the Nervous System. *J Alzheimers Dis.* 2019;68(3):843-55.
209. Benavides DR, Bibb JA. Role of Cdk5 in drug abuse and plasticity. *Ann N Y Acad Sci.* 2004;1025:335-44.
210. Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, et al. Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature.* 2001;410(6826):376-80.
211. Lu L, Grimm JW, Shaham Y, Hope BT. Molecular neuroadaptations in the accumbens and ventral tegmental area during the first 90 days of forced abstinence from cocaine self-administration in rats. *J Neurochem.* 2003;85(6):1604-13.
212. Wedzony K, Markowicz-Kula K, Chocyk A, Fijał K, Maćkowiak M. The effect of 'binge' cocaine administration on the expression of cyclin-dependent kinase 5 and its activator p35 in various regions of rat brain. *Brain Res.* 2005;1063(2):195-200.
213. Duart-Castells L, Blanco-Gandía MC, Ferrer-Pérez C, Puster B, Pubill D, Miñarro J, et al. Cross-reinstatement between 3,4-methylenedioxypyrovalerone (MDPV) and cocaine using conditioned place preference. *Prog Neuropsychopharmacol Biol Psychiatry.* 2020;100:109876.
214. Duart-Castells L, López-Arnau R, Buenrostro-Jáuregui M, Muñoz-Villegas P, Valverde O, Camarasa J, et al. Neuroadaptive changes and behavioral effects after a sensitization regime of MDPV. *Neuropharmacology.* 2019;144:271-81.
215. Wang Y, Cao J, Wang X, Zeng S. Stereoselective transport and uptake of propranolol across human intestinal Caco-2 cell monolayers. *Chirality.* 2010;22(3):361-8.
216. He Y, Liu Y, Zeng S. Stereoselective and multiple carrier-mediated transport of cetirizine across Caco-2 cell monolayers with potential drug interaction. *Chirality.* 2010;22(7):684-92.
217. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stamatii A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol.* 2005;21(1):1-26.

218. Yang Y, Zhao Y, Yu A, Sun D, Yu LX. Chapter 12 - Oral Drug Absorption: Evaluation and Prediction. In: Qiu Y, Chen Y, Zhang GGZ, Yu L, Mantri RV, editors. *Developing Solid Oral Dosage Forms (Second Edition)*. Boston: Academic Press; 2017. p. 331-54.
219. Lea T. Caco-2 Cell Line. In: Verhoeckx K, Cotter P, López-Expósito I, Kleiveland C, Lea T, Mackie A, et al., editors. *The Impact of Food Bioactives on Health: in vitro and ex vivo models*. Cham: Springer International Publishing; 2015. p. 103-11.
220. Artursson P, Karlsson J. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem Biophys Res Commun*. 1991;175(3):880-5.
221. Peters Jr T. *All about albumin: biochemistry, genetics, and medical applications*: Academic press; 1995.

ANNEXS

Annex 1

Enantioseparation and racemization of MDPV by liquid chromatography

Ana Sofia Almeida^{1,2,3,4}, Fernando Remião^{3,4}, Carla Fernandes^{1,2}

¹ Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto

² Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões

³ UCIBIO – Applied Molecular Biosciences Unit, REQUIMTE, laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto

⁴ Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto

Introduction: Synthetic cathinones are a vast group of new psychoactive substances, similar to amphetamines and widely abused due to their psychostimulant effects [1]. Although they are chiral, few studies report the influence of the stereochemistry in their biological/toxicological properties [2, 3]. To study the enantiomers, the development of enantiomeric resolution methods is crucial, being liquid chromatography the technique of choice [4]. Furthermore, under certain conditions such as high temperature and acidic/basic environments, pure enantiomers in solution can undergo racemization. Thus, racemization studies are of great importance [5].

Aims: The aims of this work were the semi-preparative enantiomeric resolution of the synthetic cathinone MDPV along with a racemization study with the collected enantiomers. The potential enantioselectivity of MDPV in neurotoxicity will be further evaluated.

Material and methods: The semi-preparative enantiomeric resolution and racemization study were performed by liquid chromatography using polysaccharide-based chiral stationary phases. For the racemization study, the enantiomers were exposed to different temperatures (room temperature, 37°C and 70°C) in the presence/absence of diisopropylethylamine for 48 h.

Results: The enantiomers of MDPV were separated with a resolution factor (R_s) of 1.7 and selectivity factor (α) of 1.4. The enantiomeric ratio values for the collected enantiomers were >99% for the first enantiomer and 90% for the second with recovery rates of 96% found for both enantiomers. While no racemization occurred after 3 hours for all tested conditions, signs of racemization started to appear for both enantiomers after 48 h at 37°C and after 24 h at 70°C. **Conclusions:** The enantiomers of MDPV were successfully separated with a good resolution and enantioselectivity and collected with high enantiomeric ratio and recovery rates. Racemization starts to occur after 48 h at 37°C and 24 h at 70°C.

Key words: Synthetic cathinones; Enantioselectivity; Liquid chromatography; Racemization

Acknowledgements: This research was supported by national funds by FCT (UIDB/04423/2020, UIDB/MULTI/04378/2020, UIDP/04423/2020 LA/P/0140/2020, PTDC/CTA-AMB/6686/2020 projects) and BYT-Plus 2021/22 scholarship from CIIMAR and Amadeu Dias Foundation.

References:

1. Valente MJ, *et al.*, Arch Toxicol. 2014;88(1):15-45.
2. Silva B, *et al.*, Forensic Toxicol. 2016;34:372-85.
3. Silva B, *et al.*, Toxicol Appl Pharmacol. 2020;395:114970.
4. Silva B, *et al.*, J Anal Toxicol. 2018;42(1):17-24.
5. Huang Z, *et al.*, J Sep Sci. 2020;43(7):1240-7.

Enantioseparation and racemization of MDPV by liquid chromatography

Ana Sofia Almeida^{1,2,3,4}, Fernando Remião^{3,4}, Carla Fernandes^{1,2*}*Email: cfernandes@ff.up.pt

¹ Laboratório de Química Orgânica e Farmacéutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
² Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, s/n, 4450-208 Matosinhos, Portugal
³ UCIBIO – Applied Molecular Biosciences Unit, REQUIMTE, lab. of Toxicology, Dep. of Biological Sciences, Faculty of Pharmacy, Univ. of Porto, Rua de Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal
⁴ Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal

Introduction

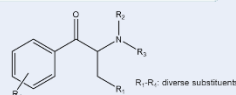
New psychoactive substances (NPS)

- Sold as legal alternatives to illicit drugs
- Easily accessible online and in smartshops
- Limited information about properties [1]

Public health threat

Synthetic cathinones

- New derivatives emerge every year with unknown properties [1]



Chirality

Enantioselectivity studies

- To study the influence of stereochemistry in biological/toxicological properties [2-3]

Racemization studies

- Enantiomers undergo racemization in certain conditions (temperature, acidic/basic environments) [4]

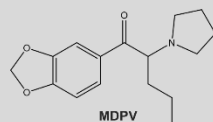
Enantioresolution methods

Liquid chromatography

- Technique of choice for synthetic cathinones [5]

Aims

- Semi-preparative enantioresolution of MDPV
- Racemization study with the collected enantiomers



Methods

Semi-preparative enantioseparation of MDPV

- LC**
- Column: amylose-based (20 cm, 7 mm i.d.)
 - Mobile phase: Hexane:EtOH:DEA (97:3:0.1)
 - Sample: MDPV 10 mg/mL
 - Injection volume: 100 µL
 - Flow rate: 1.5 mL/min
 - UV detection: 254 nm
- Collected enantiomers**
- Evaporation of mobile phase
 - Addition of hydrogen chloride solution to form hydrochlorides

Racemization study

- LC**
- Column: amylose-based (15 cm, 4.6 mm i.d.)
 - Mobile phase: Hexane:EtOH:DEA (97:3:0.1)
 - Samples: 50 µg/mL
 - Injection volume: 10 µL
 - Flow rate: 1 mL/min
 - UV detection: 254 nm
- Conditions:**
- Temperature: r.t., 37°C and 70°C
 - Basic environment: addition of diisopropylethylamine (0.1%)
 - Injections every 30 min for 3 h, after 24 h and 48 h

Results

Semi-preparative enantioseparation of MDPV



E1

t = 12 min
e.r. = 99%
Recovery rate = 92%

E2

t = 15 min
e.r. = 95%
Recovery rate = 93%

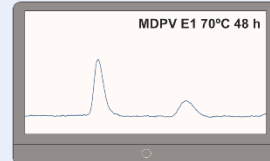
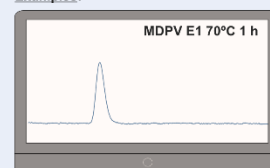
Racemization study

		0.5	1	1.5	2	2.5	3	24	48h
E1	r.t.	x	x	x	x	x	x	x	x
	37°C	x	x	x	x	x	x	x	x
	70°C	x	x	x	x	x	x	✓	✓
E2	r.t.	x	x	x	x	x	x	x	x
	37°C	x	x	x	x	x	x	x	✓
	70°C	x	x	x	x	x	x	✓	✓
Diisopropylethylamine (0.1%)									
E1	r.t.	x	x	x	x	x	x	x	x
	37°C	x	x	x	x	x	x	x	✓
	70°C	x	x	x	x	x	x	✓	✓
E2	r.t.	x	x	x	x	x	x	x	x
	37°C	x	x	x	x	x	x	x	✓
	70°C	x	x	x	x	x	x	✓	✓

x No racemization

✓ Racemization starting

Examples:



Conclusions

Enantioresolution

- Enantioseparation of MDPV with good enantioselectivity and resolution
- Enantiomers collected with high e.r. and recovery rates

Racemization study

- No racemization at room temperature for 48 h
- Racemization starts after 48 h at 37°C and 24 h at 70°C
- Diisopropylethylamine has no effect on racemization

Future work

- Evaluation of potential enantioselectivity in neurotoxicity using the SH-SY5Y neuroblastoma cell line

References

- Valente MJ, et al., Arch Toxicol. 2014;88(1):15-45.
- Silva B, et al., Toxicol Appl Pharmacol. 2021;416:115442.
- Silva B, et al., Toxicol Appl Pharmacol. 2020;395:114970.
- Huang Z, et al., J Sep Sci. 2020;43(7):1240-7.
- Silva B, et al., J Anal Toxicol. 2018;42(1):17-24.

Annex 2

Semi-preparative enantioresolution of MDPV enantiomers and racemization study by liquid chromatography

Ana Sofia Almeida^{1,2,3,4}, Fernando Remião^{3,4}, Carla Fernandes^{1,2}

¹ Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto

² Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões

³ UCIBIO – Applied Molecular Biosciences Unit, REQUIMTE, laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto

⁴ Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto

New psychoactive substances (NPS) represent a public health threat since they are not controlled by international conventions, are easily accessible online and are sold as a legal alternative to illicit drugs. Among them, synthetic cathinones, such as MDPV, are widely abused due to their psychostimulant effects [1]. Being chiral compounds, studies reporting the influence of stereochemistry in their biological/toxicological properties [2, 3], as well as the conditions of temperature or acidic/basic environments by which pure enantiomers undergo racemization [4] are necessary. To this, many methods of enantioresolution have been developed over the years being liquid chromatography using chiral stationary phases (CSPs) the technique of choice [5].

In this work, the semi-preparative enantioresolution of MDPV and a racemization study were performed by liquid chromatography using polysaccharide-based CSPs. The enantiomers were separated with a good resolution and enantioselectivity, being collected with high enantiomeric ratio and recovery rates. While no racemization occurred after 3 hours for all tested conditions, signs of racemization started to appear after 48h at 37°C and after 24h at 70°C. The potential enantioselectivity in neurotoxicity will be further evaluated.

Acknowledgements

This research was supported by national funds by FCT (UIDB/04423/2020, UIDB/MULTI/04378/2020, UIDP/04423/2020 LA/P/0140/2020, PTDC/CTA-AMB/6686/2020 projects) and BYT-Plus 2021/22 scholarship from CIIMAR and Amadeu Dias Foundation.

References

1. Valente MJ, *et al.*, Arch Toxicol. 2014;88(1):15-45.
2. Silva B, *et al.*, Toxicol Appl Pharmacol. 2021;416:115442.
3. Silva B, *et al.*, Toxicol Appl Pharmacol. 2020;395:114970.
4. Huang Z, *et al.*, J Sep Sci. 2020;43(7):1240-7.
5. Silva B, *et al.*, J Anal Toxicol. 2018;42(1):17-24.



Semi-preparative enantioresolution of MDPV enantiomers and racemization study by liquid chromatography

Ana Sofia Almeida^{1,2,3,4}, Fernando Remião^{3,4}, Carla Fernandes^{1,2*}

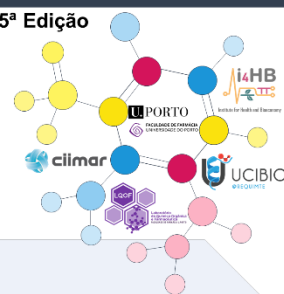
*Email: cfernandes@ff.up.pt

¹ Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

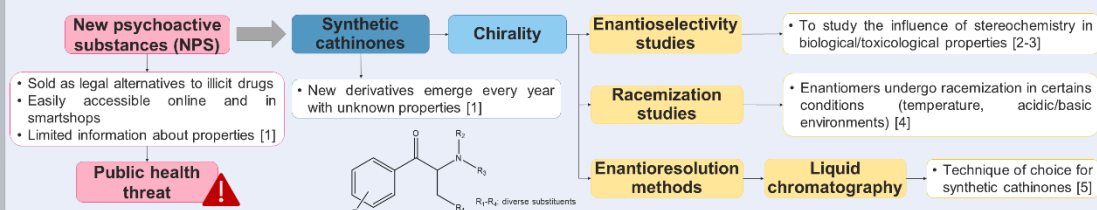
² Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, s/n, 4450-208 Matosinhos, Portugal

³ UCIBIO-Applied Molecular Biosciences Unit, REQUIMTE, lab. of Toxicology, Dep. of Biological Sciences, Faculty of Pharmacy, Univ. of Porto, Rua de Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal

⁴ Associate Laboratory I4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal

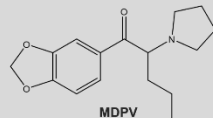


Introduction



Aims

- Semi-preparative enantioresolution of MDPV
- Racemization study with the collected enantiomers



Semipreparative enantioresolution of MDPV

- LC**
- Column: amylose-based (20 cm, 7 mm i.d.)
 - Mobile phase: Hexane:EtOH:DEA (97:3:0.1)
 - Sample: MDPV 10 mg/mL
 - Injection volume: 100 µL
 - Flow rate: 1.5 mL/min
 - UV detection: 254 nm
- Collected enantiomers**
- Evaporation of mobile phase
 - Addition of hydrogen chloride solution to form hydrochlorides

Racemization study

- LC**
- Column: amylose-based (15 cm, 4.6 mm i.d.)
 - Mobile phase: Hexane:EtOH:DEA (97:3:0.1)
 - Samples: 50 µg/mL
 - Injection volume: 10 µL
 - Flow rate: 1 mL/min
 - UV detection: 254 nm
- Conditions:**
- Temperature: r.t., 37°C and 70°C
 - Basic environment: addition of diisopropylethylamine (0.1%)
 - Injections every 30 min for 3 h, after 24 h and 48 h

Results

Semipreparative enantioresolution of MDPV



E1

t = 12 min
e.r. = >99%
Recovery rate = 92%

E2

t = 15 min
e.r. = 95%
Recovery rate = 93%

Racemization study

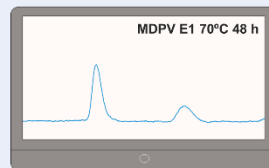
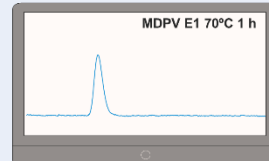
		0.5	1	1.5	2	2.5	3	24	48h
E1	r.t.	x	x	x	x	x	x	x	x
	37°C	x	x	x	x	x	x	x	x
	70°C	x	x	x	x	x	x	✓	✓
E2	r.t.	x	x	x	x	x	x	x	x
	37°C	x	x	x	x	x	x	x	x
	70°C	x	x	x	x	x	x	✓	✓

Diisopropylethylamine (0.1%)

E1	r.t.	x	x	x	x	x	x	x	x
	37°C	x	x	x	x	x	x	x	x
	70°C	x	x	x	x	x	x	✓	✓
E2	r.t.	x	x	x	x	x	x	x	x
	37°C	x	x	x	x	x	x	x	x
	70°C	x	x	x	x	x	x	✓	✓

x No racemization ✓ Racemization starting

Examples:



Conclusions

Enantioresolution

- Enantioresolution of MDPV with good enantioselectivity and resolution
- Enantiomers collected with high e.r. and recovery rates

Racemization study

- No racemization at room temperature for 48 h
- Racemization starts after 48 h at 37°C and 24 h at 70°C
- Diisopropylethylamine has no effect on racemization

Future work

- Evaluation of potential enantioselectivity in neurotoxicity using the SH-SY5Y neuroblastoma cell line

References

- Valente MJ, et al., Arch Toxicol. 2014;88(1):15-45.
- Silva B, et al., Toxicol Appl Pharmacol. 2021;416:115442.
- Silva B, et al., Toxicol Appl Pharmacol. 2020;395:114970.
- Huang Z, et al., J Sep Sci. 2020;43(7):1240-7.
- Silva B, et al., J Anal Toxicol. 2018;42(1):17-24.

Annex 3

Semi-preparative enantioresolution, racemization and cytotoxicity studies with MDPV enantiomers

Ana Sofia Almeida^{1,2,3,4}, Fernando Remião^{3,4}, Carla Fernandes^{1,2}

¹ Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto

² Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões

³ UCIBIO – Applied Molecular Biosciences Unit, REQUIMTE, laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto

⁴ Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto

New psychoactive substances represent a public health threat since they are sold as legal alternatives to illicit drugs, are easily accessible online and there is limited information about their properties. Among them, synthetic cathinones, such as 3,4-methylenedioxypyrovalerone (MDPV), are widely abused due to their psychostimulant effects¹. Being chiral compounds, although scarce, the study of both enantiomers is of great importance, including the influence of stereochemistry in their biological/toxicological properties^{2,3}, and the racemization phenomena since enantiomers can undergo racemization in certain temperature and acidic/basic environments⁴. Moreover, enantioresolution methods that allow the separation of the enantiomers of synthetic cathinones have been reported, being liquid chromatography (LC) using chiral stationary phases (CSPs) the main technique of choice⁵.

In this work, first, the semi-preparative enantioresolution of MDPV and a racemization study were performed by LC using polysaccharide-based CSPs. The enantiomers were separated with a good resolution and enantioselectivity and collected with high enantiomeric ratio and recovery rates. While no racemization occurred after 3 h for all tested conditions, signs of racemization started to appear after 48 h at 37°C and after 24 h at 70°C. Moreover, to evaluate the potential enantioselectivity of MDPV in cytotoxicity, MTT and NR assays were performed using the SH-SY5Y cell line. The cells were exposed to each MDPV enantiomer, in a concentration of 1.506 mM, for 24 h and no enantioselectivity was found.

Acknowledgements

This research was supported by national funds by FCT (UIDB/04423/2020, UIDB/MULTI/04378/2020, UIDP/04423/2020 LA/P/0140/2020, PTDC/CTA-AMB/6686/2020 projects) and BYT-Plus 2021/22 scholarship from CIIMAR and Amadeu Dias Foundation.

References

- [1] Valente MJ, *et al.*, Arch Toxicol. 2014;88(1):15-45.
- [2] Silva B, *et al.*, Toxicol Appl Pharmacol. 2021;416:115442.
- [3] Silva B, *et al.*, Toxicol Appl Pharmacol. 2020;395:114970.
- [4] Huang Z, *et al.*, J Sep Sci. 2020;43(7):1240-7.
- [5] Almeida AS, *et al.*, Molecules. 2022;27(7).

Semi-preparative enantioresolution, racemization and cytotoxicity studies with MDPV enantiomers

Ana Sofia Almeida^{1,2,3,4*}, Fernando Remião^{3,4}, Carla Fernandes^{1,2}

*Email: anasofiaalmeida1998@gmail.com

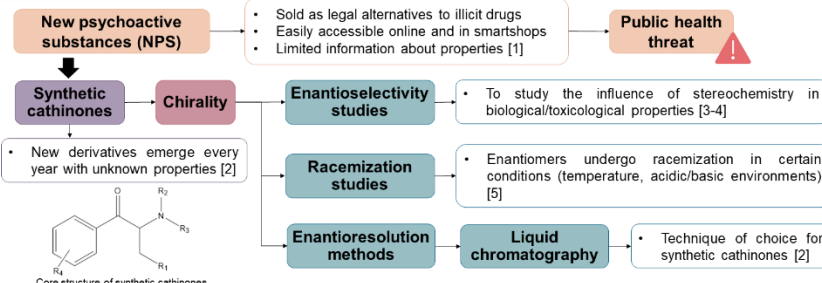
¹ Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto

² Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto

³ UCIBIO – Applied Molecular Biosciences Unit, REQUIMTE, laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto

⁴ Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto

INTRODUCTION



AIMS

- Semi-preparative enantioresolution of MDPV
- Racemization study with the collected enantiomers
- Cytotoxicity studies to evaluate potential enantioselectivity in undifferentiated and dopaminergic SH-SY5Y cells

Semi-preparative enantioresolution

Chromatographic conditions

- Column: amylose-based (20 cm, 7 mm i.d.)
- Mobile phase: Hexane:Ethanol:Diethylamine (Hex:EtOH:DEA; 97:3:0.1)
- Sample: MDPV 10 mg/mL
- Injection volume: 100 μ L
- Flow rate: 1.5 mL/min
- UV detection: 254 nm

Formation of hydrochlorides (stable form)

- Addition of hydrogen chloride solution to the collected enantiomers



Racemization study

Chromatographic conditions

- Column: amylose-based (25 cm, 4.6 mm i.d.)
- Mobile phase: Hex:EtOH:DEA (97:3:0.1)
- Samples: 50 μ g/mL of each enantiomer
- Injection volume: 10 μ L
- Flow rate: 1 mL/min
- UV detection: 254 nm
- Conditions:
 - Temperature: r.t., 37°C and 70°C
 - Basic environment: addition of diisopropylethylamine (0.1%)
- Injections every 30 min for 3 h, after 24 h and 48 h

Evaluation of enantioselectivity

Cell culture of SH-SY5Y

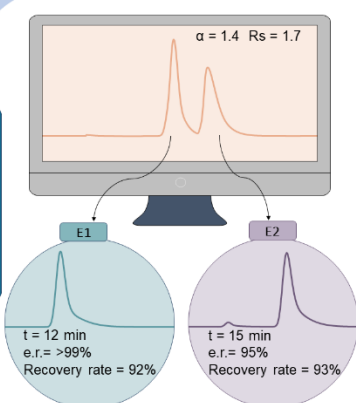
- Seeded in 96-well plates
- Density: 2.5×10^4 cells/cm²
- Concentrations: 0.773; 1.165 and 1.506 mM [6]
- Differentiation into dopaminergic phenotype: 10 μ M RA for 72 h followed by 80 nM TPA for another 72 h.

Cytotoxicity assays

- MTT: incubation with a 0.5 mg/mL MTT solution for 90 min, dissolution of formazan crystals in DMSO and quantification at 550 nm
- NR: incubation with a 50 μ g/mL NR solution for 90 min, addition of lysis solution and quantification at 540 nm

METHODS

RESULTS

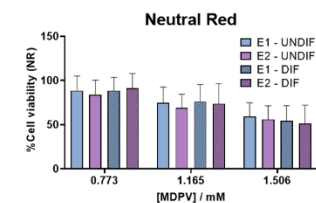
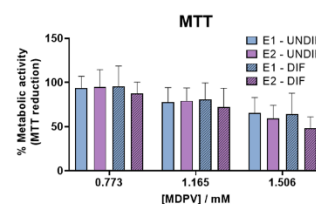


		0.5	3	24	48 h
E1	r.t.	100:0	100:0	100:0	100:0
	37 °C	100:0	100:0	100:0	96:4
	70 °C	100:0	97:3	88:12	69:31
E2	r.t.	3:97	3:97	2:98	6:94
	37 °C	2:98	2:98	8:92	13:87
	70 °C	3:97	4:96	24:76	47:53

Diisopropylethylamine (0.1%)

E1	r.t.	100:0	100:0	100:0	100:0
	37 °C	100:0	100:0	97:3	96:4
	70 °C	100:0	97:3	81:19	66:34
E2	r.t.	4:96	3:97	3:97	3:97
	37 °C	3:97	3:97	4:96	7:93
	70 °C	3:97	6:94	26:74	47:53

Examples:



CONCLUSIONS

- MDPV was successfully enantioresolved with good enantioselectivity and resolution.
- Enantiomers were collected with high enantiomeric ratios and recovery rates.

- For 48 h at RT and 24 h at 37 °C, the enantiomers were stable (no/minor changes in the e.r. values).
- Racemization was affected by higher temperatures.
- Racemization of E2 happened to a greater extent.
- DIPEA had no considerable effect on the racemization.

- Similar cytotoxicity results were obtained for the enantiomers of MDPV in the SH-SY5Y cell line for both undifferentiated and differentiated (dopaminergic) cells (no enantioselectivity was observed).
- Additionally, no statistically significant difference was found between undifferentiated and differentiated cells in each condition.

References:

- [1] Valente MJ, et al., *Arch Toxicol*, 2014;88(1):15-45.
- [2] Almeida AS, et al., *Molecules*, 2022;27(7).
- [3] Silva B, et al., *Toxicol Appl Pharmacol*, 2021;416:115442.
- [4] Silva B, et al., *Toxicol Appl Pharmacol*, 2020;395:114970.
- [5] Huang Z, et al., *J Sep Sci*, 2020;43(7):1240-7.
- [6] Valente MJ, et al., *ACS Chem Neurosci*, 2017;8(4):850-9

Acknowledgments:

This research was supported by national funds by FCT (UIDB/04423/2020, UIDB/MULTI/04378/2020, UIDP/04423/2020, LA/P/140/2020, PTDC/CTA-AMB/6686/2020 projects) and BYT-Plus 2021/22 scholarship from CIIMAR and Amadeu Dias Foundation.

Annex 4

Development and validation of an UHPLC-UV method for MDPV quantification to evaluate enantioselectivity on absorption using the Caco-2 cell line

Ana Sofia Almeida^{1,2,3,4}, Bárbara Silva^{3,4}, Sara Cravo^{1,2}, Fernando Remião^{3,4}, Carla Fernandes^{1,2}

¹ Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto

² Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões

³ UCIBIO – Applied Molecular Biosciences Unit, REQUIMTE, laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto

⁴ Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto

Synthetic cathinones, such as 3,4-methylenedioxypyrovalerone (MDPV), are psychoactive substances well absorbed by the oral mucosa¹. However, the level at which these compounds and their enantiomers cross the intestinal barrier has only been determined for methylone and pentedrone². Actually, literature on enantioselectivity studies with synthetic cathinones is still scarce³. Thus, the present study aimed to analyze the potential enantioselectivity of the enantiomers of MDPV in the passage across the Caco-2 monolayer, a widely used in vitro model for intestinal permeability studies. The Caco-2 monolayer was exposed to 300 µM of each enantiomer of MDPV in both apical (AP) to basolateral (BL) and BL to AP directions and samples were collected for 5 hours. To detect and quantify MDPV in the transport buffer of the study, HBSS(+/-), an UHPLC-UV method was developed and validated. The parameters studied were specificity, linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). Additionally, evaluation of samples' stability was also performed.

High selectivity for MDPV and good linearity were observed in the tested concentration range (0.5-500 µM) with correlation coefficients always higher than 0.999. An accuracy ranging between 102 and 109%, inter-day and intra-day precisions with coefficients of variation below 15%, LOD and LOQ of 0.063 µM and 0.19 µM, respectively, were also observed. Samples were stable for 6 weeks of storage in different temperatures (room temperature, 4 °C, -20 °C and -80 °C). No statistically significant differences were found between the enantiomers in the passage across the Caco-2 monolayer (no enantioselectivity).

Acknowledgements: This research was supported by national funds by FCT (UIDB/04423/2020, UIDB/MULTI/04378/2020, UIDP/04423/2020 LA/P/0140/2020, PTDC/CTA-AMB/6686/2020 projects) and BYT-Plus 2021/22 scholarship from CIIMAR and Amadeu Dias Foundation.

References:

1. M.J. Valente, P.G. Pinho, M.L. Bastos, F. Carvalho, M. Carvalho. Arch Toxicol. (2014) 88(1):15-45.
2. B. Silva, R. Silva, C. Fernandes, P.G. Pinho, F. Toxicol Appl Pharmacol. (2020) 395:114970.
3. A.S. Almeida, B. Silva, P.G. Pinho, F. Remião, C. Fernandes, Molecules. (2022) 27(7).

Annex 5

Semi-preparative separation and racemization study of MDPV enantiomers by liquid chromatography and absorption study on Caco-2 model

Ana Sofia Almeida^{1,2,3,4}, Bárbara Silva^{3,4}, Sara Cravo^{1,2}, Fernando Remião^{3,4}, Carla Fernandes^{1,2}

¹ Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto

² Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões

³ UCIBIO – Applied Molecular Biosciences Unit, REQUIMTE, laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto

⁴ Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto

Synthetic cathinones, such as 3,4-methylenedioxypyrovalerone (MDPV), are widely abused due to their psychostimulant effects [1]. Being chiral compounds, studying the influence of stereochemistry in their biological/toxicological properties (enantioselectivity) is of great importance [2,3] as well as the racemization phenomena since enantiomers can undergo racemization in certain temperature and acidic/basic environments [4]. Moreover, enantioresolution methods that allow the separation of the enantiomers of synthetic cathinones have been reported, being liquid chromatography (LC) using chiral stationary phases (CSPs) the technique of choice [5].

In this work, the semi-preparative enantioresolution of MDPV and a racemization study were performed by LC using amylose-derived CSPs. The enantiomers were successfully separated with a selectivity factor of 1.4 and a resolution factor of 1.7, collected with enantiomeric ratios (e.r.) higher than 95% and recovery rates of 92% and 93%. The racemization of the enantiomers of MDPV was affected by higher temperatures. Nonetheless, for 48 h at room temperature and 24 h at 37 °C, the enantiomers were stable since minor/no changes were observed in the e.r. values.

Additionally, a permeability study was performed to evaluate the potential enantioselectivity of MDPV in the passage across the Caco-2 monolayer, a widely used in vitro model for intestinal permeability studies. To detect and quantify MDPV, an UHPLC method was developed and validated. High selectivity for MDPV and good linearity were observed in the tested concentration range (0.5-500 µM) with correlation coefficients always higher than 0.999. An accuracy ranging between 102 and 109%, inter-day and intra-day precisions with coefficients of variation below 15%, limits of detection and quantification of 0.063 µM and 0.19 µM, respectively, were also found. No statistically significant differences were found between the enantiomers in the passage across the Caco-2 monolayer (no enantioselectivity).

Acknowledgements

This research was supported by national funds by FCT (UIDB/04423/2020, UIDB/MULTI/04378/2020, UIDP/04423/2020 LA/P/0140/2020, PTDC/CTA-AMB/6686/2020 projects) and BYT-Plus 2021/22 scholarship from CIIMAR and Amadeu Dias Foundation.

References

- [1] Valente MJ, et al., Arch Toxicol. 2014;88(1):15-45.
- [2] Silva B, et al., Toxicol Appl Pharmacol. 2021;416:115442.
- [3] Silva B, et al., Toxicol Appl Pharmacol. 2020;395:114970.
- [4] Huang Z, et al., J Sep Sci. 2020;43(7):1240-7.
- [5] Almeida AS, et al., Molecules. 2022;27(7).